

## SPOTLIGHT

# Can laboratory model systems instruct human limb regeneration?

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## ABSTRACT

Regeneration has fascinated scientists since well before the 20th century revolutions in genetics and molecular biology. The field of regenerative biology has grown steadily over the past decade, incorporating advances in imaging, genomics and genome editing to identify key cell types and molecules involved across many model organisms. Yet for many or most tissues, it can be difficult to predict when and how findings from these studies will advance regenerative medicine. Establishing technologies to stimulate regrowth of a lost or amputated limb with a patterned replicate, as salamanders do routinely, is one of the most challenging directives of tissue regeneration research. Here, we speculate upon what research avenues the field must explore to move closer to this capstone achievement.

## Limb regeneration starts with a blastema

Traditional laboratory model systems and atypical models alike have informed key concepts and mechanisms of limb regeneration. Human limb regeneration remains a dream of medical science, but it is a pressing and growing need – it has been estimated that 1.6 million people in the USA alone are living with limb loss, with the potential of that number to more than double by the mid-century point (Ziegler-Graham et al., 2008). In considering the possibility of human limb regeneration, we choose to focus our speculative piece on vertebrate models that have regeneration-competent major appendages (e.g. limbs, fins), as well as vertebrates with scant regenerative capacity resembling that of humans. The former can be interrogated for information on the molecular underpinnings of successful innate events, while the latter represent a canvas upon which manipulations, often informed by studies of the former, can push the limits of regenerative capacity and open doors to medical applications. We will primarily consider regeneration of hindlimbs, forelimbs and their equivalents. A striking example of mammalian appendage regeneration, deer antler renewal, does not restore a replicate of the original appendage and responds to hormone levels rather than to injury, and is reviewed elsewhere (Kierdorf et al., 2007). Regeneration of internal tissues such as liver and intestinal lining, and attempts to augment regeneration in poorly regenerative tissues other than limbs, have been well summarized in other reviews (Iismaa et al., 2018; Tzahor and Poss, 2017).

Teleost fish like zebrafish and urodele salamanders such as newts and axolotls (Box 1) are capable of complete regeneration after fin or limb amputation. Vertebrate appendages are complex structures composed of interdependent tissues. Thus, faithful regeneration

involves not only replenishing the mass of large structures such as bone and skin, but also precise vascularization and innervation to achieve proper metabolism and function. Adult *Xenopus* limb amputation partially restores limb mass in the form of a cartilaginous spike, but fails to reconstitute morphology and function (Dent, 1962). These failures elevate the virtues of models like teleosts and newts, the impressive regenerative capabilities of which depend on a structure called a blastema: a transient mass of proliferative cells that forms at the site of injury and provides the source material for eventual regeneration of lost structures. The blastema has been referred to as the link between healing, a universal response to damage, and morphogenesis of a new appendage, which is the exclusive domain of a subset of species with elevated regenerative abilities (Seifert and Muneoka, 2018).

A wound epidermis forms after injury and acts as a signaling hub for blastema formation; without these signals, mesenchymal cells at the injury site fail to assemble the blastema (Tassava and Garling, 1979; Thornton, 1957). The structure and composition of the wound epidermis are particularly important for successful regeneration, as apico-basal polarization of wound epidermal cells is essential for proper expression and delivery of regenerative signaling factors (Chablais and Jazwinska, 2010; Chen et al., 2015; Shibata et al., 2016; Stoick-Cooper et al., 2007). Molecules distinguishing an activated regeneration epidermis have been identified through diverse experimental methods, including yeast two-hybrid assay screens, forward genetic screens and candidate gene approaches. These approaches have identified factors ranging from signaling pathway members such as Fgf20a to extracellular matrix components and regulators such as laminin  $\beta$ 1a and newt anterior gradient protein (Chen et al., 2015; Kumar et al., 2007; Shibata et al., 2016; Whitehead et al., 2005). Yet we are far from fully comprehending the breadth, dynamics and relative importance of regeneration factors. Moving forward, recent technologies such as single cell transcriptome sequencing will help refine the genetic makeup of the wound epidermis and elaborate its crucial role as a structural and signaling center for the blastema (Aztekin et al., 2019).

## Advocating developmental biology

This article is part of Development's advocacy collection – a series of review articles that make compelling arguments for the field's importance. The series is split into two: one set of articles, including this one, addresses the question 'What are the big open questions in the field?' We would argue that there has never been a more exciting time to get involved in developmental biology: incredible new tools mean making fundamental problems are increasingly within reach. A complementary set of articles will ask 'What has developmental biology ever done for us?' Together, the articles will provide a collection of case studies looking backwards to the field's achievements and forwards to its potential, and a resource for students, educators, advocates and researchers alike. To see the full collection as it grows, go to <http://dev.biologists.org/content/advocating-developmental-biology>.

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Understanding the origins and fates of blastemal progenitor cells is a key step toward achieving limb regeneration. No vertebrate has yet been shown to engage a true pluripotent stem cell during regeneration; rather, fate-mapping experiments indicate that appendage blastemas are composed of restricted progenitor cell populations, each responsible for one or a small number of cell types (Ando et al., 2017; Fei et al., 2017; Gerber et al., 2018; Kragl et al., 2009; Lehoczy et al., 2011; Rinkevich et al., 2011; Shibata et al., 2018; Tu and Johnson, 2011). Notably, recent studies also indicate that when one progenitor population is experimentally eliminated, other progenitors can be recruited or repurposed as an ancillary source. For example, bone-forming osteoblasts normally dedifferentiate – that is, reduce their characteristic functional properties – and divide to contribute new osteoblasts in zebrafish fins, but if osteoblasts are ablated, joint-associated progenitors ramp up osteoblast production (Ando et al., 2017; Singh et al., 2012). Like osteoblasts, cardiomyocytes of zebrafish can and do re-enter the cell cycle to serve as progenitors (Knopf et al., 2011; Singh et al., 2012; Sousa et al., 2011). This strategy is also found among salamanders: satellite cells serve as progenitors in axolotl blastemas, whereas myofibers dedifferentiate and fragment in adult newt limb stumps to facilitate muscle regeneration (Sandoval-Guzmán et al., 2014; Tanaka et al., 2016). In summary, appendage regeneration does not employ a pluripotent stem cell, but system plasticity can enable multiple origins for mature tissues – a concept relevant to human limb regeneration.

Understanding how individual blastema cells behave in concert to create population-level morphogenetic changes is a technically challenging obligation of the field. Recent studies have improved the spatiotemporal resolution of regeneration by combining longitudinal imaging platforms with techniques to permanently label cells, before or during blastema formation. For example, genetic cell labeling has been used to detail the recruitment of connective tissue cells to form the blastema, as well as the extent to which these cells build different proximodistal skeletal elements (Currie et al., 2016). Real-time live imaging is being extended to follow differentiated cell populations and catalog cell shape changes, proliferation dynamics and cell death rates. Improvements in platforms that maintain animal health and stability for long periods during imaging, as well as longitudinal studies using permanent lineage tracing, have enabled capture of novel biology during appendage regeneration in fish and axolotls (Cox et al., 2018; Currie et al., 2016; Tornini et al., 2016; Xu et al., 2015). Community initiatives will be key to efficiently generate knock-in fluorescent fusion proteins, thereby allowing visualization of endogenous activity of molecular regulators. Mapping spatiotemporal changes in cell behavior in tandem with assessment of dynamic gene expression can illuminate the blueprints for appendage regeneration.

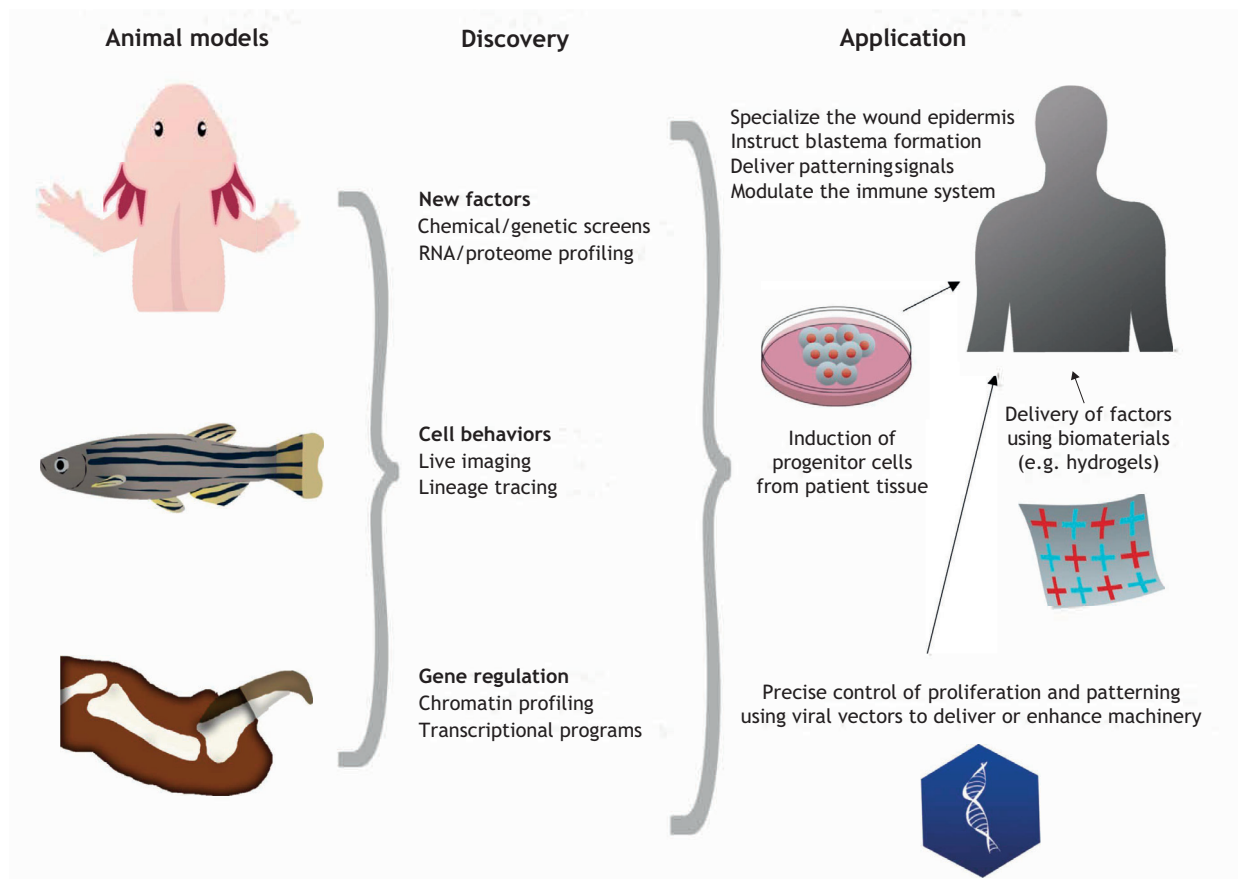
Induction of a mammalian limb blastema is a major challenge to consider at present, requiring methods to activate latent progenitor populations (if they exist) to stimulate dedifferentiation of cells in spared tissue to increase proliferative capacity or modify fate, and/or to deliver exogenous progenitor cells that will colonize the limb stump and form a functional structure. There is evidence that transplantation of embryonic limb cells enhances regeneration from adult *Xenopus laevis* limb stumps; thus, it is conceivable that transplantation of induced pluripotent stem cell-derived limb progenitors could facilitate limb regeneration (Lin et al., 2013). Alternatively, one can envisage clinical regeneration initiated by removing skin from the limb stump and targeting delivery of factors unique to a functional regeneration epidermis (Dawson et al., 2017; Seyedhassantehrani et al., 2017). Blastema formation could be motivated in turn by delivery of additional factors that might

induce dedifferentiation and proliferation in the remaining tissue, and/or by transplantation of the proper distribution of patient-derived cells representing the precursors of all necessary cell types (Fig. 1). Given that it is possible for a blastema to form in certain mammalian contexts like mouse digit tips (discussed in the following section), the prospect of inducing blastema formation in other weakly or non-regenerative mammalian tissues seems not so far-fetched.

### Instructing regeneration with pro-regenerative factors

Salamander blastemas contain all of the positional information necessary to pattern new limbs; i.e. a shoulder blastema transplanted to a limb will generate a full limb, and a transplanted wrist blastema will form distal components (Maden, 1980; Pescitelli and Stocum, 1980; Roensch et al., 2013). It is possible that a perfected therapeutic blastema will self-pattern, although it seems more likely that this striking feature would have to be replicated through additional manipulations. For example, salamander limb regeneration depends on re-innervation of axons severed during amputation; without nerves, the stump generates a blastema with reduced proliferation and differentiation capacities (Mescher and Tassava, 1975; Singer and Craven, 1948). Molecular genetic experiments have identified molecular factors accounting for the nerve dependence of regeneration in salamanders and *Xenopus*; hyperinnervation has been shown to have small positive effects on regeneration in adult *Xenopus* (Kumar et al., 2007; Zhang et al., 2018). Additionally, an ectopic blastema – and eventual limb – can be induced to sprout from an unamputated limb by creating a small skin wound, deviating a nerve to the site and applying a skin graft to provide positional information (Endo et al., 2004; Seyedhassantehrani et al., 2017). This accessory limb model has been used to model how factors encode positional information or substitute for innervation (Makanee et al., 2014; Nacu et al., 2016; Vieira et al., 2019). It has also highlighted the role of extracellular factors such as heparan sulfate in patterning (Phan et al., 2015). These discoveries offer proof of concept for the hypothesis that the environment necessary for regeneration can be recreated by manipulation of sources of signaling molecules.

Many classic developmental signaling pathways are known to play roles in patterning regenerating appendages (Currie et al., 2016; Dawson et al., 2017; Grotek et al., 2013; Kujawski et al., 2014; Whitehead et al., 2005). Broad similarities between ontogenetic and regenerative development led to the initial compilation of molecular factors that influence regeneration. The more recent generation of experiments has incorporated unbiased surveys of regenerating adult tissues or proxies for these tissues. Lineage tracing and single cell sequencing studies have highlighted that it is an oversimplification to assume that adult tissue regeneration merely recapitulates a genetic program of earlier development (Gerber et al., 2018; Tsai et al., 2019). These surveys have broadened the scope of factor discovery, e.g. implicating melanocortin 4 receptor (Mc4r) signaling, which is classically studied in energy metabolism, as a factor that can augment limb regeneration in *Xenopus* during stages in which it is typically defective (Zhang et al., 2018). Transcriptomic analysis of entire tissues and single cells has been employed in appendage regeneration in a variety of species to reveal candidates based on gene expression dynamics (Leigh et al., 2018; Nachtrab et al., 2013). Libraries of transcripts can in many cases be functionally assayed in cell culture; e.g. successive subfractionation of pooled cDNAs capable of inducing cell cycle entry in cultured salamander cells recently identified MARCKS-like protein as a factor with *in vivo* mitogenic properties in limb tissues (Sugiura et al., 2016).



**Fig. 1. From highly regenerative model systems to regenerative therapies.** A possible pipeline for how discovery in lab model systems can influence applications for regenerative therapies of complex structures like limbs.

Contrasting the effects of factors on poorly versus effectively regenerating tissues or species can yield insights into how genetic responses to injury may differ among vertebrates. For example, cell culture experiments have demonstrated that regeneration-competent myotubes from newts increase ERK signaling and re-enter the cell cycle upon serum stimulation, whereas mouse myotubes are unable to sustain ERK signaling or cell cycle entry, even when harboring mutations that constitutively activate the ERK pathway (Yun et al., 2014). These contrasting responses imply that there is complex genetic and epigenetic regulation of regeneration genes, which we discuss in the next section; as a result, individual molecules may be potent, but not sufficient to induce complete regeneration. Relief of cell cycle inhibition is also closely tied to regeneration competence: p53 activity is dampened in the salamander blastema (Yun et al., 2013). Likewise, the mouse *Acomys cahirinus* is able to regenerate hole punches in ear tissue, and cell cycle re-entry correlates with a lack of nuclear localization of the cell cycle inhibitors p21 and p27 in the ear hole blastema. By contrast, common mice (*Mus musculus*) maintain each of these inhibitors in muscle nuclei, and studies in cultured mouse myoblasts have found that knockdown of cell cycle regulators can modulate proliferation and indicators of regenerative capacity (Gawriluk et al., 2016; Pajcini et al., 2010; Wang et al., 2015). Thus, it is not just the presence and activity of pro-regenerative factors that determine regenerative competence, but also the attenuation of inhibitory factors. Correspondingly, a balance of triggers and breaks, produced by drug or gene therapy cocktails, is likely to be needed for consideration of human limb regeneration therapies.

The capacity of mouse limb structures to regenerate upon amputation is limited to the digits, specifically the distal half of the third phalangeal element (P3), which consists of bone surrounded by connective tissue and a nail (Seifert and Muneoka, 2018). Human fingertips also have some regenerative capacity, especially in children, so the mouse digit tip model could aid in identifying methods for augmenting limited human regeneration (Dolan et al., 2018; Illingworth, 1974). Immediately anterior to P3 in the mouse is the second phalangeal element (P2), which does not regenerate but instead truncates bone and heals skin upon amputation. Treatment with bone morphogenetic protein (Bmp) family members has been shown to induce regeneration of the P2 bone to its original length after amputation, but the regenerated region lacked hair follicles and articular cartilage, and did not initiate regeneration of the distal P3 segment (Dawson et al., 2017). Furthermore, P2 digits that were amputated and failed to regenerate could be induced to undergo modest regeneration by re-amputation and Bmp pathway activation. Similarly, while *Acomys* species are able to regenerate small full-thickness skin wounds, including hair follicles, common mice normally fail to regenerate hair follicles after this injury but can be induced to do so by Hedgehog signaling activation (Seifert et al., 2012). Thus, a limited number of molecules can potentially initiate an otherwise failed regenerative response, and the regeneration window can be reopened through a second injury and properly timed application of regeneration factors. The latter is an important practical consideration, as it might not be feasible to perform a clinical limb regeneration procedure immediately upon injury.

### Box 1. The salamander limb: the right model at the right time – a personal case study by Maximina Yun

While finishing my graduate studies at Cambridge University, I was invited to teach undergraduates in cell and developmental biology. This somewhat serendipitous turn took me on a journey of discoveries in the field of regeneration, and exposed me for the first time to salamanders, the vertebrates evolutionarily closest to us that are able to regenerate full limbs as adults. Ever since then I have shared with many of my scientific muses, from Thomas H. Morgan to Victor C. Twitty, a fascination for this animal and the will to uncover the molecular and cellular basis of regeneration. The latter became my primary scientific aim, driving my subsequent studies and career moves – something that, at the time, some considered a commendable yet daunting prospect. As it was bluntly put to me by a well-respected scientist during my first interview for a group leader position, ‘salamanders have been used in regeneration studies for many decades; however, significant translational progress based on this model is yet to be seen’.

Perhaps he was right then, given the ingeniously crafted yet limited tools available in the system. But a mere 5 years later, and the tide has turned. Transgenic techniques have become established, allowing crucial functional studies; advanced imaging approaches are being used for *in vivo* analysis of progenitor behaviors; and the giant genomes for two experimental salamander models, the axolotl and the Spanish ribbed newt, have recently become available, with all the wealth of experimental and theoretical approaches this implies (Elawa et al., 2017; Nowoshilow et al., 2018). Thus, the salamanders are finally catching up with more conventional research models such as zebrafish. And while they do so, they bring a great deal of interesting biology – besides regeneration – to the table.

And now, every day I spend in my lab I marvel at how these techniques are revolutionizing salamander studies and speeding up our understanding of the choreography of molecular and cellular events that result in the regeneration of a complex structure such as a limb. In particular, they are enabling our investigations into important issues such as the nature of the mechanisms controlling cellular plasticity in regeneration, the role of the immune-senescent cell interplay in this process and the basis for interspecies differences in regenerative capacity (Czarkwiani and Yun, 2018; Yun et al., 2015). Thus, I believe we are at an inflexion point in regeneration research, as technical advances have finally emerged to allow us to unlock the mysteries of the extreme regenerative abilities found in these captivating animals.

Recent innovative approaches combining molecular factors with bioengineering methodology have improved *Xenopus* limb regeneration beyond a cartilaginous spike. Slack and colleagues regenerated a forelimb containing multiple digits with partial mineralization by application of a fibrin patch containing larval limb progenitor cells and supplemented with additional growth factors (Lin et al., 2013). Levin and colleagues developed a wearable bioreactor that delivered progesterone in the 24 h after amputation: this induced regeneration of an appendage that lacked bone and digits but was thicker, contained more nerve bundles and vasculature than controls, and had an improved range of motion (Herrera-Rincon et al., 2018). Other engineering methods of drug and factor delivery, including the use of hydrogels, present interesting possibilities, as their protean composition could allow the creation of microenvironments for multiple cell types, thereby reproducing the different conditions amenable to formation of cartilage, bone and other tissue types over the course of regeneration (Moreira Teixeira et al., 2014). Hydrogels made from either naturally occurring molecules or synthetic polymers engineered for breakdown by naturally occurring enzymes could possibly be supplemented with regeneration-promoting factors and seeded with cells to mimic a regeneration blastema (Fig. 1). Furthermore, the immune system is a target of many current studies in tissue

regeneration research, and it has become clear that there is important interplay between cells like macrophages and regulatory T cells with the regeneration machinery, ostensibly via both phagocytotic and paracrine mechanisms (Godwin et al., 2013; Hui et al., 2017; Mescher, 2017; Simkin et al., 2017). Immunomodulatory and immunoengineering approaches are thus of significant interest for the field of appendage regeneration, as they are for other fields.

Engineering and manipulation of pro-regenerative factors have been shown to significantly stimulate regeneration in multiple contexts, and CRISPR/Cas9 will likely accelerate the discovery of new pro-regenerative factors. However, the appendages regenerated in these studies typically fall short of the originals in form and function. Although a perfect replicate of a lost limb is the gold standard for regenerative therapy, this will not be achieved in the earliest cases of clinical limb regeneration. Thus, as an aside, there are likely to be many years during which patients balance the tradeoffs of mini-limbs or missing digits with prosthetics, or during which hybrid alternatives may be favored.

### Regeneration is an epigenomic phenomenon

A two-step formula for creating a blastema and instructing its pattern underestimates the complexity of the epigenetic landscape for regeneration. The central questions might be: is the regenerative capacity of a tissue like the limb hard-wired, and have hundreds of millions of years of evolution permanently turned off or removed the machinery in mammals? Or, is the machinery there to be awakened? Recent work indicates that regeneration contexts are associated with large-scale changes in the expression of broad regulators of DNA and histone methylation, as well as histone acetylation (Hirose et al., 2013; Pfefferli et al., 2014; Takayama et al., 2014). These chromatin-level changes are the cause and/or consequence of changes in the expression levels of many hundreds of genes that have been cataloged in various models of appendage regeneration (Bryant et al., 2017; Johnston et al., 2016; Leigh et al., 2018; Rabinowitz et al., 2017). Disrupting the expression dynamics of individual or combinations of genes to find a cocktail capable of inducing regeneration would be a gargantuan undertaking. Alternatively, hacking into the epigenetic regulation to reboot the choreography of gene expression and interactions might represent an efficient shortcut to regeneration, with a large potential payout.

Epigenetic profiling has led to discovery of short DNA elements capable of regulating one or more genes in response to injury and/or regeneration. Studies in amputated zebrafish fins and hearts, fractured mouse bones, and injured *Drosophila* wing discs have identified sequences that preferentially direct gene expression upon injury and during regeneration, in some cases maintaining activated expression until regeneration is completed (Goldman et al., 2017; Guenther et al., 2015; Harris et al., 2016; Kang et al., 2016). Interestingly, a DNA region near the *Drosophila wingless (wg)* gene contains a silencing element that increases methylation with development and decreases induction of *wg/wnt*, supporting a hypothesis that regenerative capacity can be controlled through changes at gene regulatory regions (Harris et al., 2016). Developmental stage-related changes in regeneration competence have been linked to changes in epigenetic regulation of signaling factors in other contexts, such as *Shh* signaling in *Xenopus* limb regeneration (Yakushiji et al., 2007). Discovering how these elements are regulated by injury, and how their regulation changes upon recovery of regenerated structures, can ultimately reveal master upstream control mechanisms for tissue regeneration events. For example, a recent study of the acoel worm *Hofstenia miamia* identified regeneration-responsive chromatin regions that are enriched with binding sites for the early growth response (Egr) gene,

implicating Egr as a pioneer factor that regulates gene expression during head or tail regeneration (Gehrke et al., 2019). Advances in genome assembly have paralleled epigenetic profiling and have highlighted features in the genomes of regenerative amphibians that may account for their enhanced capacity, e.g. expansion of transposable elements and genes encoding microRNAs (Elewa et al., 2017; Nowoshilow et al., 2018). These findings emphasize the importance of non-coding elements, some of them likely to be unique to highly regenerative animals, in the orchestration of large-scale changes in gene expression during regeneration.

Understanding the epigenetic control of regeneration may also reveal applications that reduce off-target effects of gene delivery mechanisms to induce regeneration. Gene therapy using non-integrating, non-replicative vectors like adeno-associated viruses has received recent attention as a means to deliver wild-type gene products in models or actual cases of human genetic disease (Amoasii et al., 2018; Mendell et al., 2017). This is effective in particular when the system can tolerate variable doses and tissue location of the therapeutic factor. Gene therapy for a broad application like regeneration is conceivable, although in this case potent developmental factors would be employed to reprogram tissue to a regenerative state. A major concern is therefore restricting the intended effect to the desired tissue for the appropriate length of time. This issue also exists when considering the idea of drugging limb regeneration: it is hard to imagine how one could target regenerative growth and avoid large-scale havoc wreaked by systemic application of potent developmentally interventional compounds.

Regulatory regions that activate exclusively during regeneration suggest methods to resolve this issue. For example, the activity of an enhancer linked to the zebrafish *leptin b* gene tracks closely with regeneration, with little or no transcriptional activation during animal development or in uninjured adult tissue, but sustained activation of gene expression in regenerating hearts or fins (Kang et al., 2016). This genetic element was used in proof of principle experiments to boost growth factor expression and local tissue regeneration in zebrafish. Conceivably, injury- and regeneration-specific enhancers could be used in adeno-associated viruses to target expression of therapeutic factors to the correct tissues and contexts when attempting to induce regeneration (Fig. 1).

### Concluding remarks

Regenerating a human limb is a monumental challenge. The path to this endpoint will be dogged, and success may lie several decades away or more. Discovery science in the field of appendage regeneration has merged classic experimental methods like tissue grafting and genetic screens with modern technologies that deconstruct regeneration to context-dependent behaviors of individual cells, gene products and DNA regulatory sequences. Holistic ideas are also revealing insights into how communication between distant tissues can influence regeneration (Busse et al., 2018; Kang et al., 2013; Rodgers et al., 2014). The new mechanistic vantage points reached by contemporary studies, coupled with the evolution of delivery methods with better-than-surgical precision, will provide beacons for the journey ahead.

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