Review

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Signaling networks organizing regenerative growth of the zebrafish fin

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In contrast to mammals, adult salamanders and fish can completely regenerate their appendages after amputation. The cellular and molecular mechanisms underlying this fascinating phenomenon are beginning to emerge, including substantial progress in the identification of signals that control regenerative growth of the zebrafish caudal fin. Despite the fairly simple architecture of the fin, the regulation of its regeneration is complex. Many signals, including fibroblast growth factor (FGF), Wnt, Hedgehog (Hh), retinoic acid (RA), Notch, bone morphogenic protein (BMP), activin, and insulin-like growth factor (IGF), are required for regeneration. Much work needs to be done to dissect tissue-specific functions of these pathways and how they interact, but Wnt/β-catenin signaling is already emerging as a central player. Surprisingly, Wnt/β-catenin signaling appears to largely indirectly control epidermal patterning, progenitor cell proliferation, and osteoblast maturation via regulation of a multitude of secondary signals.

Mechanisms of regenerative growth

The ability to completely regenerate damaged organs and appendages seen in many adult invertebrates and some vertebrates, like salamanders and fish, has fascinated biologists for centuries. Clearly, these phenomena not only pose intriguing biological problems but also raise the question of why humans and other mammals largely lack such regenerative abilities. Despite this fascination, it is only recently that considerable progress in elucidating the cellular and molecular mechanisms underlying tissue regeneration in fish and salamanders has been made, largely due to the development of transgenic tools for cell lineage tracing and manipulation of gene expression (Box 1). The cellular regenerative mechanisms of salamander and zebrafish limbs and fins, the zebrafish heart, and the zebrafish central nervous system are slowly emerging and genetic lineage tracing experiments have surprisingly challenged some long-held beliefs about the role of cellular plasticity in regeneration [1–6]. It has become clear that regeneration of vertebrate appendages can involve intriguing cases of mature cells undergoing cellular dedifferentiation [2,6,7]. However, models postulating the reprogramming of adult cells to multipotent stem cells during vertebrate regeneration could not be confirmed [2,5,8,9]. In addition, the cellular source of regenerating tissue can surprisingly vary between fairly closely related species, as seen in limb skeletal muscle, which regenerates via dedifferentiation of mature muscle fibers in newts but from muscle stem cells in axolotls [6].

Considering that cellular mechanisms of regeneration can dramatically differ between species and cellular lineages, it is difficult to predict to what extent molecular principles regulating regeneration are conserved. Yet, regeneration of any complex structure requires molecular solutions to similar problems. These include: (i) which molecular cues sense and initiate the regenerative program in response to injury; (ii) how regenerative growth is controlled (that is, how cell proliferation, differentiation, and pattern formation are coordinated); and (iii) by which mechanisms the regenerative program is terminated when the structure has reached its pre-injury size.

Although some progress has been made in defining early wound-sensing signals (for example, from the immune system) that are required to initiate vertebrate regeneration [10–13], the issue of regeneration termination at the right time and size (i.e., positional memory) remains poorly understood [14]. In this review we focus on the molecular control of regenerative fin growth, where the most progress has been made. Other fascinating facets of fin regeneration have been recently discussed in excellent reviews and are not covered here [15,16].

Zebrafish fin regeneration

Zebrafish combine a high regenerative capacity with the availability of fairly well developed genetic and genomic methods [17] and their fins are easily accessible to surgery and regenerate rapidly and robustly [18]. These experimental advantages have arguably made the zebrafish caudal (tail) fin model the vertebrate regenerative system best understood on the molecular mechanistic level to date.

The caudal fin comprises 16–18 bony rays, the lepidotrichia, that extend along the whole length of the fin and are separated by soft inter-ray tissue. Ray bones are segmented, covered by a multilayered epidermis, and enclose blood vessels, nerves, pigment cells, and

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Box 1. Genetic tools for manipulation of gene expression in the regenerating fin

Because the vast majority of signaling pathways regulating zebrafish fin regeneration have essential roles during embryonic development, tools for conditional pathway overactivation or suppression are required to study their function during regeneration. Techniques allowing targeted, conditional gene inactivation in zebrafish are currently not established but will most likely be available soon considering the rapid development of nuclease-mediated genome editing, particularly using the CRISPR/Cas9 system [60]. To date, most studies have used transgenic fish lines allowing inducible overexpression of pathway activators or inhibitors, in addition to pharmacological manipulations (Table I). The simplest, most robust, and thus most widely used system utilizes regulatory sequences of the *heat-shock protein 70-like* gene to induce expression of a gene of interest after shifting fish from their standard temperature of 28.5°C to 37°C [61]. The main limitation of this technique is that it drives systemic expression in the entire fish and thus cannot be used to address tissue-specific functions. Creation of chimeric fish carrying the transgene in only a subset of cells using random transposon-based transgene integration in early embryos has been used to avoid this limitation [62].

Two transgenic systems are currently being used in zebrafish for inducible and tissue-specific overexpression of transgenes in regeneration studies. Conditional overexpression using Cre–Lox is achieved by Cre-mediated removal of a STOP cassette preceding the gene of interest, thus bringing it under the control of a ubiquitous or tissue-specific promoter. Tamoxifen-inducible CreERT2 lines provide temporal control. Such systems have to date successfully been used for cell lineage tracing and cell ablation studies in the adult fin, but not yet for manipulation of gene function [2,5,23]. Limitations are the irreversible nature of induction and the rather poor recombination efficiency achieved by many CreERT2 lines, which can result in mosaic expression or can necessitate repeated rounds of recombination, making rapid regenerative processes inaccessible for study. The TetON system by contrast uses tissue-specific expression of a doxycycline-inducible transcriptional activator (TetA) that controls transcription of a responder transgene carrying the gene of interest under control of tetracycline-responsive regulatory sequences [47,63]. Here leakiness poses a problem, which can be overcome by dually inducible TetActivator variants (requiring dexamethasone or tebufenozide in addition to doxycycline). All zebrafish transgenic systems used in adults are also susceptible to silencing during ontogeny, which can result in mosaic expression and stochastic variations of expression strength. Development of knock-in strategies into a yet-to-be-identified non-silenced locus (similar to Rosa26 in mouse [64]) will hopefully overcome this problem in the future.

Table I. Characteristics of currently available transgenic systems for gene overexpression in adult zebrafish fins

System	Inducible	Tissue-specific	Tight regulation	Reversible
Heat shock	+++	–	+	++
CreERT2–LoxP	+	+++	++	–
TetON	++	+++	±	+

fibroblast-like cells [9,19]. Lepidotrichia bone is formed through direct ossification (in the absence of a cartilage template) by a monolayer of osteoblasts that cover the bone on the inner and outer surface [2,20]. In addition to the lepidotrichia bone, rigid, non-calcified, collagenous skeletal elements termed actinotrichia provide further support to the fin edge but do not have a homologous structure in tetrapod limbs [21,22]. Following amputation, the fin completely regenerates through the establishment of blastemas – populations of lineage-restricted mesenchymal progenitor cells that form via dedifferentiation of mature stump cells – distally to each fin ray [2,9]. While differentiated osteoblasts appear to be a major source of regenerating bone during unperturbed regeneration, stem or progenitors cells might also be involved, because genetic ablation of committed osteoblasts does not result in bone regeneration defects, indicating that osteoblasts can be replenished from an unidentified cellular source under certain experimental conditions [23]. Once the blastema has formed (48 h after amputation when fish are kept at 28°C), the regenerative outgrowth phase is initiated, which restores the fin in size and shape within 2–3 weeks.

The fin blastema: a highly organized tissue

Once formed, the blastemal mesenchyme matures into a highly organized structure that can be divided into at least four domains with distinct proliferative potential and gene expression (Figure 1). (i) The very distal tip of the blastema is composed of a small group of cells that is largely non-proliferative during regenerative growth [24]. (ii) Highly proliferative osteoblasts are located in lateral regions of the blastema, which undergo maturation along the proximal–distal axis. *runx2*-positive osteoblast progenitors are located distally, followed by *sp7* (*osterix*)-positive committed osteoblasts and further proximally differentiated, *osteocalcin*-positive cells [25]. (iii) Cells located directly medial to the osteoblast progenitors are likewise proliferative and are thought to give rise to actinotrichia because they are positive for the actinotrichia-specific *and1* and *and2* transcripts [22]. (iv) The remainder of the mesenchymal cells of the blastema, which are located medially, are thought to represent fibroblasts [9].

The blastema is surrounded by a multilayered epidermis whose basal layer is formed by characteristic cuboidally

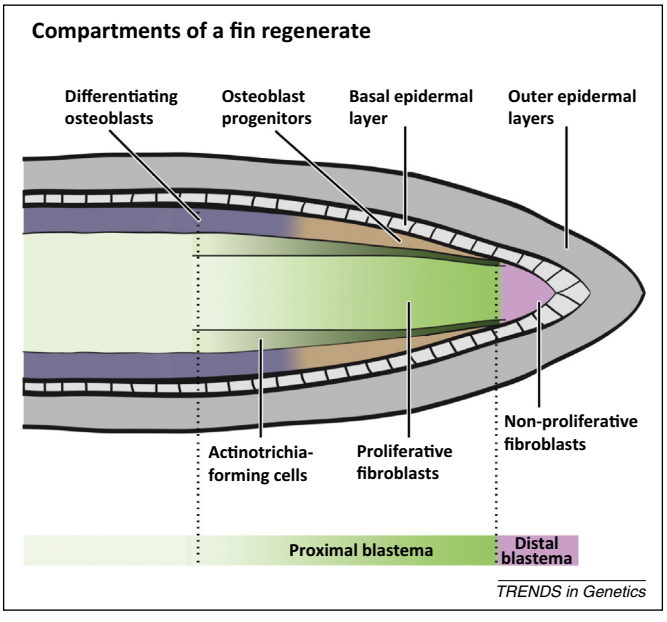

Figure 1. Starting at about 2 days post-amputation, several tissue domains can be distinguished in the zebrafish fin regenerate. Shown is a schematic representation of the distal tip of a regenerating fin ray in a longitudinal section view.