Review

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The zebrafish as a model for complex tissue regeneration

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For centuries, philosophers and scientists have been fascinated by the principles and implications of regeneration in lower vertebrate species. Two features have made zebrafish an informative model system for determining mechanisms of regenerative events. First, they are highly regenerative, able to regrow amputated fins, as well as a lesioned brain, retina, spinal cord, heart, and other tissues. Second, they are amenable to both forward and reverse genetic approaches, with a research toolset regularly updated by an expanding community of zebrafish researchers. Zebrafish studies have helped identify new mechanistic underpinnings of regeneration in multiple tissues and, in some cases, have served as a guide for contemplating regenerative strategies in mammals. Here, we review the recent history of zebrafish as a genetic model system for understanding how and why tissue regeneration occurs.

A versatile model system

Zebrafish are native to river basins in and surrounding East India and were established as a laboratory model system first by Streisinger and colleagues during the 1970s, as a potential means to apply genetic analysis to vertebrate development [1,2]. Over the decades that have followed, zebrafish have become a valuable tool to dissect embryogenesis. Experimental advantages of zebrafish for this use include large clutches, rapid external development, amenability to mutagenesis, a relatively small genome, and a reasonably short generation time. By utilizing these advantages, researchers have uncovered key factors in myriad developmental events, from early germ layer patterning to how tissues derived from these layers acquire form and function [3,4]. Recently, zebrafish have been used increasingly to investigate additional aspects of biology, including behavior, stem cells, and disease [5–9].

In this review, we provide an overview of how, over the past decade, zebrafish have become a primary model system for vertebrate tissue regeneration (see Glossary). We have focused on their remarkable regeneration of fins, heart, and central nervous system structures, although they also regenerate jaw, hair cells (lateral line), pancreas, liver, and kidney [10–20]. We summarize what is known about mechanisms of regeneration in different tissues and contexts, and describe how new discoveries and approaches in zebrafish are impacting the field of tissue regeneration.

Zebrafish fin regeneration

Zebrafish fins are complex appendages that quickly and reliably regenerate after amputation, restoring both size and shape. The key regenerative units are their many rays of dermal bone, which are segmented and lined by osteoblasts. Rays are cylindrical and hollowed, with two concave hemirays surrounding an inner mesenchymal tissue that is innervated, vascularized, and comprised primarily of fibroblasts. An amputated fin ray is covered within the first several hours by epidermis, and within 1–2 days, a regeneration blastema forms. The blastema is a proliferative mass of morphologically similar cells, formed through disorganization and distal migration of fibroblasts and osteoblasts (or scleroblasts) proximal to the amputation plane. As with blastemas in other classical regenerating

Glossary

Blastema: a proliferative mass of morphologically similar cells that accumulates in certain tissues after trauma and develops into the lost structures.
CRISPR-Cas: the Cas9 protein can be targeted through a CRISPR guide RNA to induce site-specific double-stranded DNA breaks for targeting genome editing.
Dedifferentiation: process by which a differentiated cell reverts to a less differentiated state to enable proliferation or differentiation.
Epicardium: mesothelial cell type that covers the periphery of the heart and can act as progenitor tissue for fibroblasts, vascular support cells, and possibly other cells.
Fate mapping: permanent labeling of a cell type to determine the contribution of these cells and their progeny during developmental and regenerative events.
Genetic ablation: selective killing of a specific cell type by the expression of a toxin, pro-apoptotic factor, or pro-drug converting enzyme.
Müller glia: specialized glial cells found in the retina that act as neuronal support cells and resident stem cells after injury.
Myocardial infarction (MI): massive cardiac muscle cell death and a leading cause of morbidity and mortality in humans, typically caused by coronary artery occlusion and ischemia.
Osteoblasts: bone-depositing cells.
Positional memory: the process by which spared adult cells retain positional information to recover only those structures lost by injury, of correct size and pattern.
Radial glial cell: glial cells in the brain and spinal cord that act as neuronal progenitors during development and after injury.
Regeneration: events by which lost or damaged tissue is replaced through endogenous mechanisms, restoring organ form and function.
Telencephalon: the most rostral of two subdivisions of the developing forebrain, the caudal subdivision being the diencephalon.
Transdifferentiation: conversion from one differentiated cell type to another.
Transection: a precise transverse cut into the tissue that leaves much of the surrounding tissue undisturbed.

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systems, such as the salamander limb and planarian head, the fin ray blastema is the major source of new structures.

The ability of teleost fish to regenerate amputated fins was first reported in 1786, in pectoral fins of goldfish, by Broussonet [21]. Although Thomas Hunt Morgan was fascinated by fin regeneration at the turn of the 20th century [22], it took nearly an additional century for fin regeneration to reach the genetic era. In 1995, Johnson and Weston described a screen for mutations that disrupt regeneration of tailfins in adult zebrafish [23], arguably the first experiments to demonstrate a technical advantage of studying regeneration in zebrafish. This screen was novel not only in its application of genetics to vertebrate regeneration, but also in its use of temperature-sensitive (TS) mutations in zebrafish. Given that regeneration is expected in most cases to re-employ genes used during early development, a TS screen enables identification of mutations in adults that would be lethal during early development. Over the next decade, genetic screening uncovered a handful of mutations that inhibited fin regeneration and could be localized to specific molecular defects by positional cloning [24–27]. These discoveries have contributed to the molecular models described below; yet, there has been a large time gap since the most recent identification of a regeneration gene by mutagenesis. New advances in high-throughput genome, exome, and transcriptome sequencing are likely to reboot forward genetic approaches to studying regeneration [28–33].

To best understand regeneration in any system, one must conclusively know the sources of the different cell types that are restored after injury. Only recently have modern genetic fate-mapping approaches been applied to address this question, including Cre recombinase-based technology used routinely in mice for lineage analysis. Multiple recent studies used transgenic Cre lines to focus on bone-forming osteoblasts. Their results indicated that differentiated osteoblasts transiently downregulate the osteogenic program, or dedifferentiate, as they contribute to the blastema. After this, resident osteoblasts contribute only osteoblasts to new regenerated structures [34–37]. This idea of lineage restriction was extended to other cell types, such as endothelium, epidermis, and fibroblasts, by other studies [38]. These findings agree with similar lineage restriction observed in axolotl (Mexican salamander) limbs and mouse digit tips [39–41]. However, these studies could not exclude rare transdifferentiation events; neither do most of them address possible ancillary mechanisms under different injury contexts. For instance, osteoblasts form and bone regenerates efficiently even when resident osteoblasts are potently ablated, indicating that other cell types are capable of differentiating into osteoblasts and supporting bone regeneration [35].

Many groups have examined the molecular mechanisms underlying the formation and proliferation of the blastema. In response to injury, increased expression of key signaling components of the Wnt/β-catenin and Activin-βA pathways are detectable by 3-h post amputation (hpa) [42,43], followed by upregulation of retinoic acid (RA), insulin-like growth factor (Igf), and fibroblast-like growth factor (Fgf) signaling pathway components by 6 hpa [24,44,45]. Although more complete functional testing is needed, one model for blastema formation is that increases in RA synthesis in response to injury induce expression of *igf2b* and *wnt10b*. These ligands then signal through canonical Wnt and Igf pathways to induce expression of *fgf20a*, a marker and critical regulator of blastema formation [43,44]. Independent of this signaling cascade, *activin-βA* is upregulated in the inter-ray region and is involved in reorganization of the underlying mesenchyme during blastema formation [42]. Blockade of these signaling pathways results in improper wound healing and blastema formation, implicating them in initiation of the blastema.

Blastema formation is only one step in zebrafish fin regeneration, and fins must then grow to the appropriate size. Regenerative outgrowth occurs by two processes: maintenance of a proliferative compartment at the distal end of the regenerate, and differentiation of more proximal cells. The proliferative compartment is maintained by signaling interactions between the mesenchyme and basal epidermis [46]. In addition to regulating blastema formation, RA, Fgf, and canonical Wnt signaling positively regulate blastemal proliferation and outgrowth, whereas noncanonical Wnt signaling inhibits these events [43,45,47]. Inhibition of Igf receptors or the Tgf-β receptor *alk4* also blocks blastemal proliferation during outgrowth, further indicating continued requirements for these pathways [42,44]. Interestingly, inhibition or ectopic activation of the Notch signaling pathway results in a regenerative block, leading authors to propose models in which Notch signaling, through an unknown mechanism, enhances blastemal proliferation while suppressing osteoblast differentiation during regeneration [48,49].

In addition to Notch signaling, other pathways have been examined for their ability to influence differentiation within the blastema. Bmp and Hedgehog signaling induce bone formation in the regenerate when ectopically activated, suggesting that the normal function of these molecules is to drive redifferentiation of osteoblasts in the proximal blastema [50,51].

Finally, fins provide a potentially useful model for considering the mechanisms by which an appendage regains its original shape and size after amputation. This phenomenon of positional memory, in which adult cells in the stump somehow retain and recall the correct developmental coordinates and instructions, remains a mystery in many ways. Regeneration occurs at different rates depending on the proximodistal amputation plane, regulation that involves position-dependent control of amounts of Fgf signaling [47]. Signals responsible for this, and factors that retain coordinates in adult fins and enact precise recovery, remain to be found and are likely to be broadly relevant to regeneration in other systems.

Heart regeneration

There is no significant regeneration of adult mammalian cardiac muscle after experimental injury paradigms. This deficiency is highly relevant to human disease, given that ischemic myocardial infarction (MI) and scarring is a primary cause of morbidity and mortality. Zebrafish have a high natural ability for heart regeneration and, thus, can inform as to how this process occurs or might be induced [52]. There are currently several injury models that

stimulate heart regeneration in zebrafish, including surgical resection of the ventricular apex, the first and most-used injury method, cryoinjury, and inducible genetic ablation [52–56]. Each of these models offers experimental advantages. For instance, whereas cryoinjury mimics aspects of MI, genetic ablation produces massive injuries, removing 60% or more of cardiomyocytes and inducing signs of end-stage heart failure. Unlike severe heart failure in humans, these signs regress within weeks and the animals typically make a full recovery concomitant with muscle regeneration [56]. Studies in zebrafish have revealed that heart regeneration involves two fundamental components: (i) proliferation of existing cardiomyocytes as the primary cellular source; and (ii) an environment that stimulates muscle generation from this source. In theory, regenerated cardiomyocytes could derive directly from a progenitor pool akin to the embryonic heart fields that first create the cardiac chambers, from stem cells that populate the adult heart, or from circulating progenitor cells. However, genetic fate-mapping experiments in zebrafish have made it clear that the regenerative ability of the zebrafish heart relies mainly or exclusively on proliferation of existing cardiomyocytes [57,58]. These source cardiomyocytes show characteristics of dedifferentiation, including a reduction in contractile structure, and can be identified after apical resection by activation of regulatory sequences of the *gata4* transcription factor gene [57]. There is as of yet no definitive lineage-tracing evidence that indicates an undifferentiated progenitor cell could be anything but a minor source of heart muscle.

Cardiomyocyte proliferation occurs at a low rate in the adult zebrafish heart, but is sharply increased in response to tissue damage [52]. There is considerable evidence that nonmuscle cells create an environment that enables this response. Injury to the zebrafish heart initiates an organ-wide reaction detectable as induced expression of *raldh2* (a RA-synthesizing enzyme) as early as 1-h post-injury in the endocardium, the endothelial lining of the lumen (Figure 1) [59]. The endocardium remains activated in the area of injury for several days adjacent to regenerating cardiomyocytes, and requires further study as a player in heart regeneration. Within a day or two of injury, the epicardium, the outer lining of the heart, shows an analogous organ-wide response of *raldh2* induction [60]. Then, epicardial cells proliferate and surround the regenerating muscle, where they release signals that facilitate cardiomyocyte proliferation. RA, Tgf-β ligands, Igf2, Shh, and platelet-derived growth factor (Pdgf) ligands all are released in the vicinity of proliferating cardiomyocytes, and have positive influences on muscle regeneration [59–64]. Epicardial cells have been fate mapped and act as a source of vascular support cells for regeneration, just as they do during initial heart development [65]. Fgf signaling is important for vascularizing the regenerate, which ultimately aids muscle regeneration [60]. Recently developed culture techniques may enable better characterization of

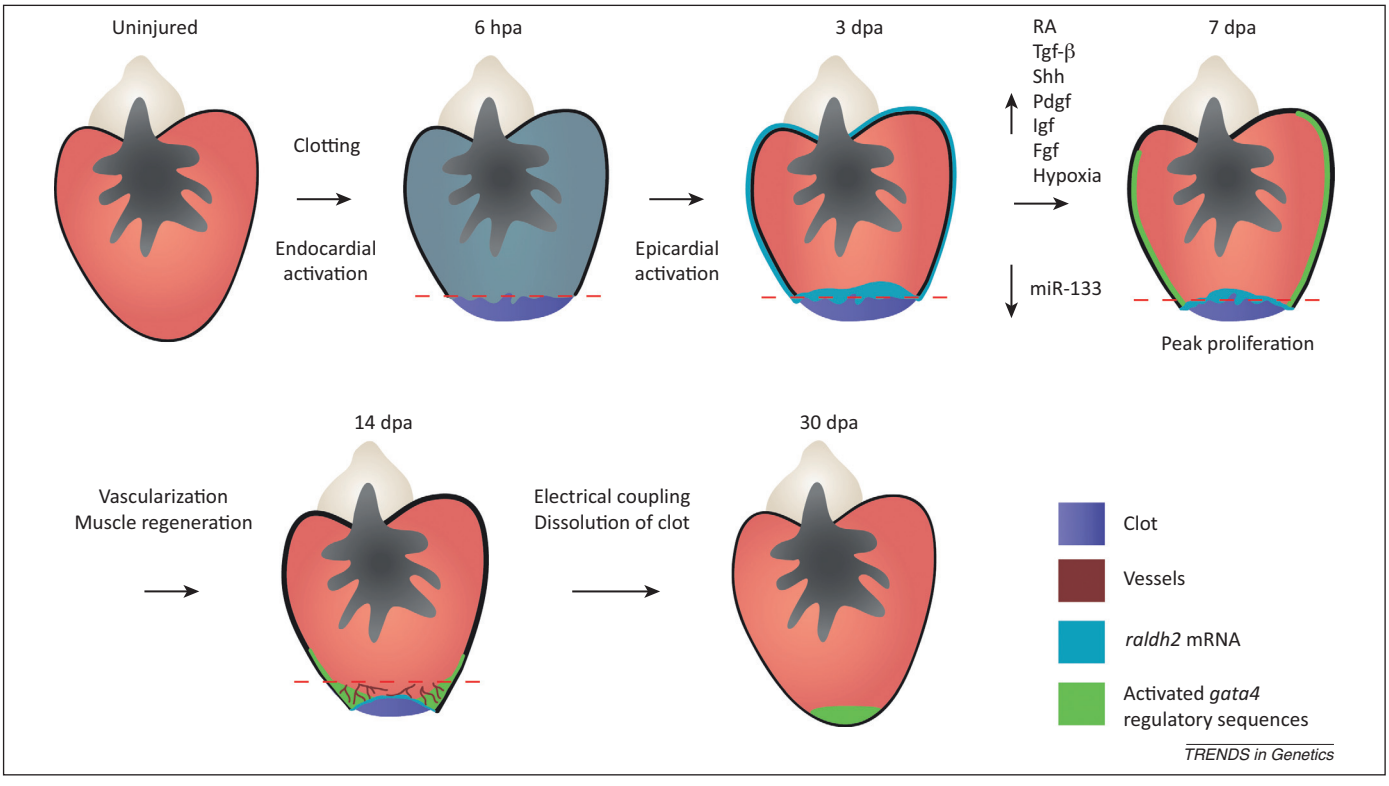

Figure 1. Model for regeneration after partial resection of the cardiac ventricle. After injury, the retinoic acid (RA)-synthesizing enzyme, *raldh2*, is induced throughout the endocardium within a few hours of amputation (hpa) and later the epicardium, before these responses localize to the wound. By 7 days post amputation (dpa), *gata4* regulatory sequences are activated throughout the cortical muscle layer of the ventricle. At this point, cardiomyocyte proliferation is stimulated, under the influences of hypoxia and signaling pathways as described in the main text, and epicardial cells have begun to integrate into the wound. By 14 dpa, vascularization of the regenerating muscle begins, aided by fibroblast-like growth factor (Fgf) and platelet-derived growth factor (Pdgf) signaling. By 30 dpa, a new wall of cardiac muscle is typically formed, in large part by the progeny of early cardiomyocytes that activate *gata4* sequences. At this point, the myocardium is vascularized and electrically coupled with the existing muscle.

epicardial cells and a greater understanding of the dynamic nature of this cell population [66]. Other potential influences on zebrafish heart regeneration have recently been examined. Hypoxia is a general factor that appears to have a positive role in cardiomyocyte proliferation, whereas hyperoxia and the miRNA miR-133 have negative roles [67,68]. In addition to cardiomyocyte proliferation, it has been reported that chemokine-mediated cardiomyocyte migration to the injury site is a critical step in the regenerative process [69]. New tools for manipulating gene expression in muscle, epicardium, endocardium, and other cell types, such as inflammatory cells, will be critical for a higher resolution view of the mechanisms of heart regeneration.

Studies of zebrafish heart regeneration have helped in considering approaches to cardiac regeneration in mammals. Because division of mature cardiomyocytes has been difficult to observe definitively in injured mammalian hearts, muscle cells have until recently received somewhat limited attention as an endogenous target cell that could be expanded for regeneration. Arguably more consideration has instead gone to several potential cardiac stem cells [70], or to fibroblasts that can be experimentally reprogrammed into cardiomyocytes by cardiac transcription factors [71,72]. However, recent lineage-tracing approaches have supported the idea that endogenous cardiomyocytes are a potential regenerative target. There is now evidence that adult murine cardiomyocytes proliferate at low levels into adulthood. Some cardiomyocyte proliferation does occur after injury [73,74], although not nearly to the extent as in the injured zebrafish or neonatal mammalian heart [75]. Of potential major significance was an expression screen that reported identification of several miRNAs enhancing proliferation of adult mammalian cardiomyocytes *in vitro*, with some able to stimulate significant regeneration after myocardial infarction in adult mice [76]. Given that cardiomyocyte proliferation as well as epicardial activation [77,78] appear to be shared components for cardiac repair, discoveries of natural regulators of cardiac regeneration in zebrafish should continue to relate directly to mammals. The pace of the field of heart regeneration has markedly accelerated over the past few years, a development that forecasts new regenerative therapies for the injured human heart in the near future.

Neural stem cell-based regeneration

Neural regeneration

Neuronal cell loss causes visual, motor, or mental impairment in humans. This neuronal cell death often leads to glial cell hypertrophy, limited proliferation, and gliotic scarring, which prevents neuronal regeneration. Zebrafish, by contrast, have the capacity to regenerate neurons within the retina, spinal cord, and brain from resident radial glial cells. New genetic approaches have facilitated the investigation of commonalities and distinctions in the pathways necessary for regeneration of different neuronal tissues and cell types.

Retina

Because of the relative ease of manipulating the retina, numerous damage strategies have been used to either destroy all or a restricted type of retinal neurons [79–90]. All of these damage models induce some Müller glia to dedifferentiate and re-enter the cell cycle to produce multipotent neuronal progenitor cells (NPCs, Figure 2),

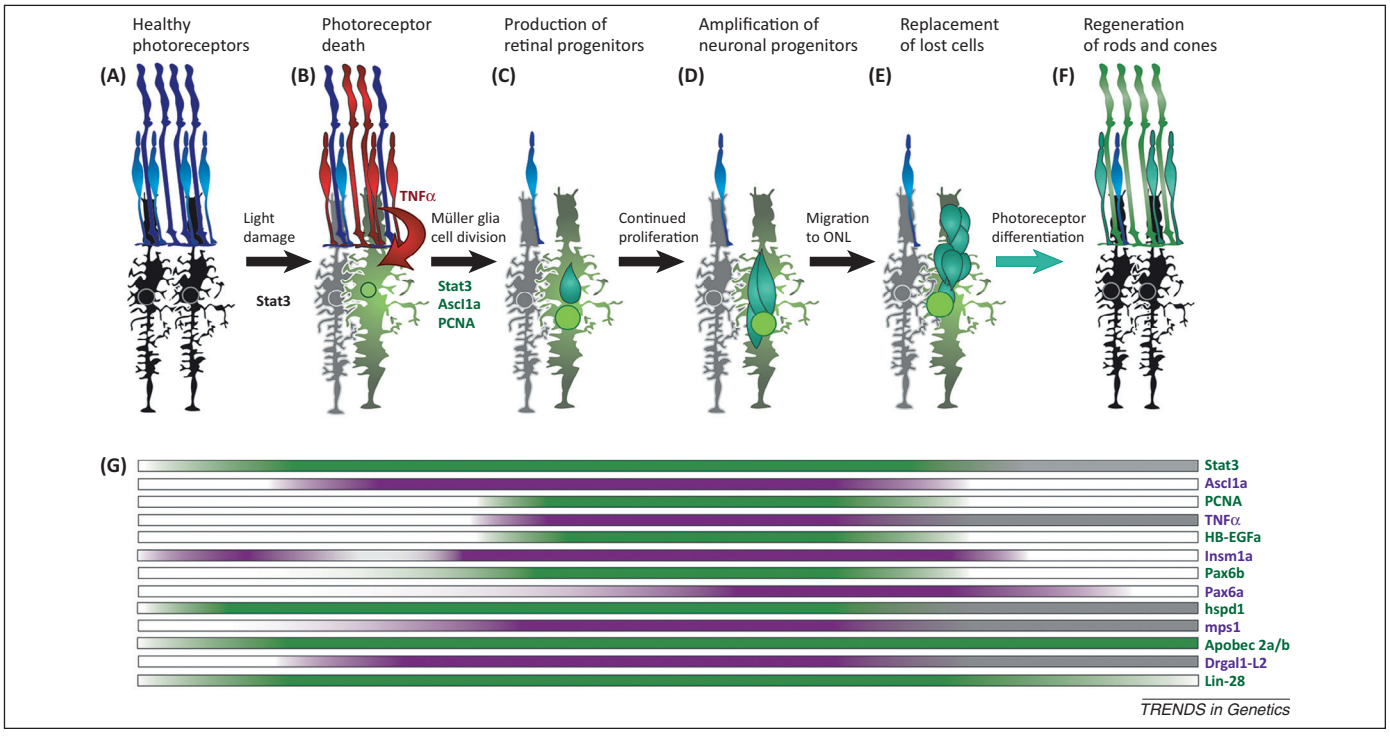

Figure 2. Model for regeneration after light damage to photoreceptors. **(A)** Before light damage, the retina comprises healthy photoreceptors (blue) and quiescent Müller glia (black). **(B)** Soon after intense light exposure, dying photoreceptors (red) produce TNFα and all the Müller glia express Stat3. The responding Müller glia (green), but not the bystander Müller glia (gray), upregulate Ascl1a and PCNA as they enter S phase of the cell cycle. **(C)** This first cell division produces neuronal progenitors (dark green), which further proliferate **(D)**. **(E)** The new retinal progenitor cells migrate to the site of damage. **(F)** Neuronal progenitor cells (NPCs) differentiate into rod and cone cells (green). **(G)** Expression of genes throughout the phases of regeneration described in (A–F) are indicated schematically directly below the hallmark images. Colored shading represents increased expression relative to undamaged retinas. Gray shading represents times not assessed for gene expression. Abbreviation: ONL, Outer nuclear layer.

which express many of the genes known to direct retinal development [91]. These NPCs proliferate, migrate to the region of damage, and differentiate into the appropriate neuronal cell type. Cre-lox-mediated lineage tracing, BrdU incorporation, and PCNA immunolabeling all demonstrated that Müller glia are the source of the new neurons in a damaged retina [79,84,92–94] (Figure 2B). The number of dedifferentiating and proliferating Müller glia varies according to the damage models and increases relative to the extent of damage [92,95]. Within hours of retinal damage, all Müller glia upregulate expression of Stat3 [96], rapidly followed by Ascl1a in a subset of Müller glia [94,96–99], both of which are required for Müller glia to re-enter the cell cycle. Responder Müller glia are distinguished from nonproliferating Müller glia by their expression of Ascl1a [97].

Microarray studies [87,100–104] and 2D differential protein gel analysis [99] identified several candidate genes and proteins (Figure 2G) that may be required for aspects of regeneration. Recently, a technique to electroporate morpholinos into the adult retina to knockdown the expression of specific target proteins was developed to validate functionally the role of these proteins in regeneration [93,97,105]. This work has been complemented by the development of heat shock-inducible transgenes and TS zebrafish mutants, which have revealed requirements for specific genes in regeneration [103,106].

A variety of experiments have demonstrated that the canonical Wnt pathway is required for zebrafish retinal regeneration [98,107]. Wnt repression by heat-shock-induced dickkopf 1 (*dkk1)* misexpression [98], small-molecule drug inhibition with XAV939, which indirectly stabilizes Axin activity, or misexpression of a dominant-negative version of the Wnt target gene, T cell transcription factor 3 (*tcf3)* [107] reduces the number of proliferating Müller glia in the damaged retina. Wnt activation, using intravitreally injected lithium chloride or GSK3-β inhibitor I, results in the stimulation of Müller glial proliferation in the absence of detectable retinal damage [98], but incubation of undamaged fish with the GSK3-β inhibitor, 1-azakenpaullone, does not [107]. This suggests that Wnt is sufficient to induce Müller glia proliferation in the absence of significant cell death, but only when the retina is stressed through either blunt force trauma or increased intraocular pressure caused by intravitreal injections of large volumes. Wnt signaling, as well as EGF and Shh, can also increase the number of proliferating Müller glia in the damaged rodent retina [108–111]. In the transgenic rat S334ter model, which expresses a prematurely truncated mouse rhodopsin protein at serine 334 and results in retinal degeneration similar to rapid-onset retinitis pigmentosa in humans with an analogous rhodopsin variant, overexpression of Notch and Wnt activation followed by a regimen of Shh and the Notch inhibitor DAPT was able to spare the rats from vision loss seen in the S334ter control littermates [112].

Other candidate molecules that appear to stimulate Müller glia proliferation in the zebrafish retina include heparin-binding epidermal growth factor (HB-EGF) [113], insulinoma associated 1a (Insm1a) [104], ADP [114], and free radicals [98], suggesting a complicated process for full activation of regeneration. Additionally, TNFα expression is markedly increased specifically in dying photoreceptors following light damage, as well as in dying ganglion and amacrine cells following ouabain treatment [99]. This TNFα expression is required for Ascl1a and Stat3 expression in Müller glia [99]. Thus, several proteins have been shown to be required for Müller glia to dedifferentiate and re-enter the cell cycle in the damaged retina, but only TNFα has been shown to be expressed in the dying neurons, seemingly a requirement of a *de novo* signal to initiate neuronal regeneration.

Zebrafish retinal regeneration is highly tuned to regenerate specifically the cell types that are damaged. For example, light-induced photoreceptor cell death results in the regeneration of only photoreceptors [86,92]. This specificity in cell regeneration led to the identification of the molecular pathways required to regenerate rods and cones (Figure 2F). For example, Fgf receptor 1 (Fgfr1) signaling [106] and lectin, galactoside-binding, soluble, 2a (Drgal1-L2 or Lgals2a) [115] are necessary for rod regeneration, whereas paired-box gene 6b (Pax6b) [116] and Ttk protein kinase (Ttk or Mps1) [103] are required for cone regeneration. To facilitate these studies, new transgenic tools were created, including transgenes that express the *Escherichia coli* nitroreductase enzyme in specific neuronal cell subtypes. Treating the different transgenic lines with the prodrug metronidazole specifically ablates the neuronal cell subtype expressing the nitroreductase enzyme, including rods [89], cones [90], or bipolar cells [88]. Although much still remains to be learned about how cell regeneration is targeted, these genetic tools make zebrafish an ideal model system to address the questions.

Spinal cord

Damage to the human spinal cord results in irreversible loss of neurons and impaired sensory and motor functions. By contrast, zebrafish have the ability to regrow new axonal projections from viable brain neurons across the severed spinal cord [117]. In addition to axonal growth, zebrafish can produce new neurons and interneurons at the region of damage [118,119]. Similar to development, the type of regenerated neuron depends on dorsoventral location of its corresponding progenitor radial glial cell in the spinal cord. For example, motor neurons, characterized by Hb9, Islet-1/2, and ChAT expression, are regenerated from Olig2 radial glia cells located in the spinal cord ependyma, which surrounds the spinal ventricle [118]. The subset of Olig2-positive cells that gives rise to motor neurons also co-expresses the developmental transcription factors, *pax6a* and *nkx6.1* [120]. Additionally, motor neuron regeneration is inhibited by transgenic overexpression of the Notch intracellular domain (NICD), resulting in the increased expression of hairy-related 4.1 (*her4.1)* [121]. By contrast, regenerated V2 interneurons derive from more dorsally located Nkx6.1^{+}, Pax6^{+}, and Olig2^{-} p2 glia, whereas serotonergic neurons derive from the ventral glia [119]. The more dorsally derived neurons show limited regeneration in zebrafish, which may be due to the lack of the expression of genes that determine dorsal biases in development, such as *pax3, pax7, bmp2, bmp4*, and *tcf7* [119]. Inhibition of the Hh pathway reduces neurogenesis at the

lesion site; interestingly, this phenotype does not impair functional recovery as assessed by swimming activity [120], suggesting that the surviving neurons exhibit synaptic plasticity.

Axonal regeneration across the lesion site is dependent on proliferating radial glial cells that infiltrate the site. The responding glia divide soon after lesion and assume bipolar morphology. They migrate into the damaged site and connect the two sides of the lesion guiding the new axons, a process termed a 'glial bridge' [122]. The early proliferation and migration of responding glia is dependent on Fgf signaling and was reduced by either global overexpression of dominant-negative Fgfr1 or by injecting SU5402, a small molecule inhibitor of Fgfr1 tyrosine kinase activity. These events were enhanced in the *sprouty 4* (*spry4*) mutant fish [122], which is a downstream target and inhibitor of Fgf signaling. Spry4 expression is increased in activated radial glia following transection of the mouse spinal cord, and human FGF2 induces marmoset astrocytes to adopt a bipolar morphology in culture, suggesting conservation in mammals [122].

Brain

Studies of regeneration in the teleost brain were recently extensively reviewed [123]. Surgical lesion of the telencephalon causes neuronal cell death and induces radial glia to proliferate and new neurons to regenerate. Similar to the spinal cord, regeneration originates from radial glia that line the ventricles and express GFAP and Olig2 [124]. These glia proliferate to yield progenitor cells that then express the transcription factor Eomesa, which regulates glutamatergic neuron differentiation [124]. Recent advances using Vivo-Morpholinos to knockdown protein expression and transgenesis have allowed for functional studies of specific proteins during brain regeneration. Vivo-Morpholinos, which contain eight guanidium residues covalently attached to a trizine residue in the standard morpholino antisense-oligonucleotides [125], penetrate into the most proximal cells of the zebrafish telencephalon ventricle without electroporation or intracellular injection, after cerebroventricular microinjection (CVMI) [126]. CVMI studies indicated that Fgf signaling, the chemokine receptor, Cxcr5, and the zinc finger transcription factor Gata3, are necessary for regeneration in damaged brains, but are insufficient to stimulate proliferation in undamaged brains [127,128]. Although Notch signaling is inhibitory in the retina [113] or motoneurons [121], it is required for brain regeneration. Her4.1+ ventricular radial glia divide in response to distal surgical injury [129], whereas DAPT-mediated inhibition of Notch signaling decreases the number of neurogenin 1 (Ngn1) and T-box brain 1 (Tbr1)-expressing neuronal progenitors [130], which give rise to the telencephalic cells during development. It is unclear why Notch seems to have contradictory roles in these systems.

The inflammation stimulator, leukotriene C4, signals through the cysteinyl leukotriene receptor 1 (CysLT1) to induce radial glial proliferation in the undamaged zebrafish brain, whereas inhibition of CysLT1, specifically by pranlukast or generally by dexamethasone, reduces the number of dividing radial glia in the damaged telencephalon [131]. This inflammatory response might be necessary for the regeneration of multiple zebrafish tissues, because dexamethasone-induced immunosuppression disrupts caudal fin regeneration after amputation [131]. The source of this inflammatory response, the underlying regulatory targets, and their impact in less regenerative mammalian tissues will be important to explore.

Future advances

Zebrafish have advantages over other regenerative vertebrate model systems in regards to the relative ease and diversity by which potential factors can be manipulated (Table 1). Full utilization of emerging technologies in zebrafish will strengthen the foundation for regeneration studies.

One drawback of the zebrafish model system has been the inability to generate conditional loss-of-function alleles. Over the past few years, zinc finger nucleases (ZFNs) and, more recently, transcription activator-like effector nucleases (TALENs) and the CRISPR-Cas system have aided directed mutagenesis [132–136]. Recently, a system was described for inducing site-specific homologous recombination in zebrafish embryos utilizing TALENs [137]. Double-stranded breaks could enable the incorporation of sequences from co-injected short single-stranded DNA oligos at a low frequency, in both somatic and germline cells. Adapting this technology, one can envision creating conditional knockout alleles through the insertion of two compatible *loxP* sites targeting a gene of interest. This technology will enable the study of individual gene products in a tissue-specific manner during regeneration, and provide potential upgrades over current dominant-negative, pharmacologic, and antisense morpholino-based approaches for loss-of-function studies. These current strategies are less specific than genetic mutants, and the ranges in treatment conditions or phenotypic penetrance can make it difficult to connect multiple pathways and synthesize a coherent blueprint for regeneration. Conditional loss-of-function alleles when standardized will remove a key element of speed, but can provide clarity by eliminating some of the drawbacks of other approaches. In addition to loss-of-function, the ability to perform homologous recombination downstream of endogenous regulatory sequences may help circumvent transgenic silencing in adult zebrafish, a current challenge in the field [138].

In addition to using TALENs for genome editing, there is evidence that the TALE architecture can be used as a transcriptional activator or repressor to alter expression of specific target genes in living cells [139]. Combining TALEs with Cre-Lox technology could also provide another method for altering gene expression in a conditional and tissue-specific manner.

High-throughput screening using zebrafish embryos is a powerful method to identify small molecules with the potential to enhance regeneration. For instance, transgenic zebrafish expressing cell cycle indicators specifically in cardiomyocytes were used to identify several small molecules capable of enhancing or blocking cardiomyocyte proliferation in growing embryonic or injured adult hearts [64]. In the best-case scenario, promising candidates can be directly applied to mammalian systems to assess their impact on regeneration. A successful series of studies

Table 1. A brief list of tools available for studying regeneration in zebrafish

Technique	Pros	Cons
Gene manipulation		
Gal4-UAS	Modular reagents	Requires multiple transgenic lines
	Tissue specificity	Nonreversible
		Noninducible
		Generational or stage-specific silencing
hsp70 (heat-inducible promoter)	Reversible	No tissue specificity
	Tunable	Elevated temperature may affect regenerative events
	Single transgenic line	
	Inducible	
Cre-mediated recombination	Tissue specificity	Nonreversible
	Inducible	Requires multiple transgenic lines
	Modular	
ENU mutagenesis	Unbiased screening technique	Requires much time and animal space
	Can identify temperature-sensitive alleles	
Morpholinos	Rapid	Nonspecific effects
	Versatile loss-of-function approach	
ZFNs	Site-directed mutagenesis	Low efficiency
TALENs	Site-specific mutations	
	High efficiency	
	Can facilitate homologous recombination in zebrafish	
CRISPR-Cas system	Site-specific mutations	Potential off-target effects
	Easy to produce guide RNAs	
	High efficiency	
Visualization strategies		
Reporter lines	Ease of visualization	Lengthy generation time
	BACs enable inclusion of enhancers	Potential insertional effects on expression
		Generational or stage-specific silencing
Multicolor clonal analysis	High-resolution clonal analysis	Requires specific inducible Cre lines
Photoconvertible proteins	Can be used to trace regionalized cell subsets	Trace is neither genetic nor permanent

toward this end came from a small molecule screen for effects on hematopoietic stem cell markers in zebrafish embryos. It was found that inducers of prostaglandin E2 synthesis and prostaglandin E2 itself are capable of expanding hematopoietic stem cell (HSC) numbers in zebrafish, and that they have similar effects on the adult HSCs of mice and nonhuman primates [140]. Thus, studying stem cell and regenerative biology in the zebrafish system might lead to potential new therapies in humans.

Concluding remarks

Adult mammals are naturally incapable of regrowing amputated limbs, significant amounts of cardiac muscle, or recovering from traumatic injury to the brain or spinal cord. Current studies address two main options for functional recovery after these injuries: (i) introduction of an exogenous cell source, which could engraft and integrate with existing tissue; or (ii) stimulation of endogenous cell populations to induce regeneration. By pairing model organism genetics with remarkable regenerative abilities, the zebrafish has a strong track record and high potential to inform methodology to activate endogenous cell populations for regeneration. Recent studies with zebrafish have used an evolving toolset to identify the cellular sources activated for regeneration, an important first step in understanding the complex events of organ regeneration. Continued insights into the molecular mechanisms regulating regeneration will provide guidance for understanding and augmenting the regenerative abilities of less naturally capable vertebrate species like humans. Advances in gene targeting, chemical screening, and visualization techniques in zebrafish should facilitate the next generation of insights and discoveries.

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