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# Zebrafish *Danio rerio* as a Model for Understanding the Process of Caudal Fin Regeneration

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## Abstract

After its introduction for scientific investigation in the 1950s, the cypriniform zebrafish, *Danio rerio*, has become a valuable model for the study of regenerative processes and mechanisms. Zebrafish exhibit epimorphic regeneration, in which a nondifferentiated cell mass formed after amputation is able to fully regenerate lost tissue such as limbs, heart muscle, brain, retina, and spinal cord. The process of limb regeneration in zebrafish comprises several stages characterized by the activation of specific signaling pathways and gene expression. We review current research on key factors in limb regeneration using zebrafish as a model.

**Keywords:** biomedicine, *Danio rerio*, model organism, regeneration, zebrafish

## Introduction

The zebrafish, *Danio rerio,* is a small tropical fish of the family Cyprinidae that inhabits freshwater rivers and ponds in India, Bangladesh, Myanmar, and Nepal.[1] It was introduced into science as a model organism in the early 1930s. Owing to external fertilization and the ability to fully regrow most damaged tissue, zebrafish are widely used to reveal mechanisms involved in embryo development and regeneration in vertebrates.[2–4]

Zebrafish characteristics such as small size, high fertility, ease, and low cost of rearing contribute to its extensive use in major fields of biological and biomedical science.[5] In 1951, the number of publications in PubMed related to zebrafish was 7, increasing to 3,911 in 2019. Although zebrafish does not replace mammalian domestic mice, *Mus musculus,* and gray rat, *Rattus norvegicus*, the leaders among model organisms, it has supplanted the clawed frog, *Xenopus laevis,* in studies of regenerative processes, with 269 articles compared with 29 published in 2019.

Cancer, obesity, stress, regeneration, diabetes, cardiovascular pathology, and other common disorders might be studied through the use of zebrafish.[6] The zebrafish serves not only as a model organism, but as a test subject, widely used to detect toxicity of chemical agents and pollution in soil and water.[7,8] A major feature of zebrafish is its ability to fully restore damaged organs and tissues, such as fins, cardiac muscle, retina, and muscle, allowing their use in exploration of intracellular mechanisms for hypothetical and practical purposes.[9]

## History

The first scientific interest in zebrafish was expressed by the Scottish naturalist, zoologist, and voyager, Francis Hamilton in 1822. While working for the British East India Company, Hamilton found *D. rerio* in the state of Bihar in northeastern India and described it as ''beautiful fish'' with several blue and silver stripes on each side.[10]

In the early 1900s, the zebrafish was successfully introduced into Europe as an aquarium fish.[11] It became highly popular among hobbyists because of its diverse pigment patterns, high fertility, low mortality, and ease of rearing.

Beginning in the 1930s, the American embryologist Jane Oppenheimer used zebrafish as a model organism for study of the basics of vertebrate embryology. She demonstrated that the genes responsible for body formation, are identical in both lower and upper vertebrates.[12]

The success story of zebrafish as a model organism began at the end of the 1960s, when George Streisinger of the University of Oregon obtained a ''golden'' zebrafish, one of the first mutant strains. The golden recessive homozygote zebrafish produces pigment similar to the normal pink zebrafish, but at much lower intensity.[13–15]

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In 1995, scientists from the University of Oregon, Charles Kimmel and William Ballard, and their colleagues from Dartmouth College, described seven phases of zebrafish early embryogenesis: zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching, revealing patterns of early development and organ formation. Three stages of postembryonic growth were identified: larva, juvenile, and adult. This research allowed use of zebrafish for studying, not only embryonic development of fish, but also of humans. It was the beginning of the era of zebrafish as a model for studying human biology.

In 2003, scientists from the Sanger Institute, comparing 26,000 zebrafish genes to the human genome, reported that almost 70% of human genes have an ortholog in zebrafish.[16] Thus, the genes responsible for human myodystrophy, Peters plus syndrome, Alzheimer's disease, and others might be studied on zebrafish.[17–19] The same group of researchers later described the function of 10,000 zebrafish genes. A German biologist and Nobel Prize winner from the University of Tübingen, Nüsslein-Volhard, and her colleagues published the results of the first large-scale screening of the zebrafish genome, which became known as The Big Screen. They examined more than 4,000 mutant specimens and studied cell migration from the site of origin to destination, facilitating identification of genes involved in human embryo development.[20–25]

## Early and Current Practice

The complex process and mechanisms involved in tissue regeneration has been an important ongoing topic in Biology.[26] In the 8th century BC, the Greek poet Hesiod introduced Prometheus, whose liver was pecked out by the eagle Ethon every day, but was fully regenerated the following morning. Aristotle, in his articles dedicated to natural history, mentioned that animal embryos regenerate more efficiently than adults.[27] Several centuries later, in 1744, a Genevan naturalist and zoologist, Abraham Trembley, published a book on the process of regeneration in *Hydra*. With serial sectioning, he demonstrated that *Hydra* can regenerate from a small fragment of its body.[28]

It was not until the 20th century, when Thomas Morgan presented his *Regeneration* that the nature of organ and tissue regeneration was revealed. Morgan described observations of *Hydra* and worms and formulated the basic principle of two types of regeneration: morphallaxis and epimorphosis.[29,30] Morphallaxis is the common form exhibited by polyps, in which pre-existing cells are reorganized immediately after injury to reform a lost part of the body. Regeneration of limbs in fish, amphibians, and reptiles is epimorphic, a process in which an undifferentiated cell mass migrates to the site of injury, forms a blastema, and rapidly proliferates to restore an amputated organ.[31]

Later experiments allowed detection of the role of stem cells in this process and defined stages of regeneration.[32,33] Two key processes, differentiation and dedifferentiation, regulate tissue and organ regrowth. Initially, following tissue injury or an amputation, multipotent stem cells are activated, and differentiated somatic cells near the site of injury are transformed into unipotent stem cells and begin division. These unipotent stem cells subsequently dedifferentiate into somatic cells, replacing damaged structures.[34]

Currently, scientists, as in the past, are focused on identifying mechanisms responsible for re-establishment of normal function in damaged organs in mammals, mainly humans, organisms with low regenerative potential.[9]

In humans, organs can be categorized according to their regenerative capacity as those with high capacity (blood and skin); medium capacity, with regeneration after physical trauma (bone, liver, and skeletal muscle); and with low capacity (neural system, cardiac muscle, and limbs) that cannot regenerate the lost cell mass.[35] Because of the lack of regenerative potential, any degenerative process in the third group of organs results in serious disability or death. The effects of a heart attack, limb amputation (although human and mouse fetuses can spontaneously regenerate tips of digits), or neuron degradation are permanent.[9] Despite knowledge of the molecular mechanisms, regulation of the process of regeneration is still not fully understood, but it is known that fibrotic tissue formation is a critical factor limiting restoration of organs.

It was traditionally believed that only invertebrates and lower vertebrates could regrow injured organs, whereas higher vertebrates, with the exception of reptiles and amphibians, did not have this ability. Neotenic larvae of the salamander axolotl *Ambystoma mexicanum*, the clawed frog *X. laevis* tadpole, and some species of lizard, including the ornate crevice dragon, *Ctenophorus ornatus*, and Tenerife lizard, *Gallotia galloti*, can regenerate the tail, limb buds, eye lens, and neurons.[36–40] Even among the organisms demonstrating extraordinary regenerative potential, zebrafish exhibits a remarkable capacity for the restoration of lost structures and shows high potential as a model for study of regenerative medicine related to heart, retina, skin, and brain.[9,41,42]

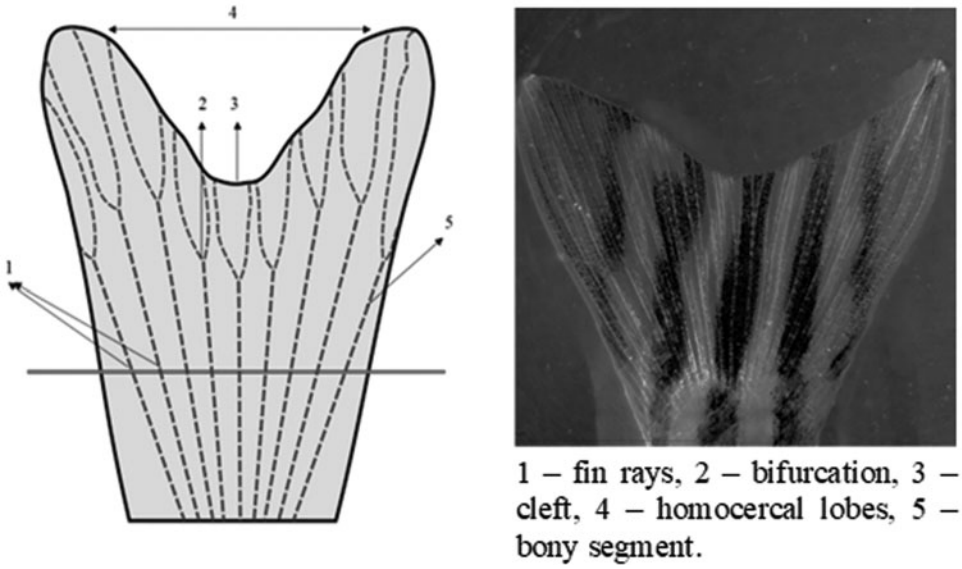
In both embryonic and adult zebrafish, in contrast to the majority of amniotes, including birds and mammals, the process of regeneration occurs without replacing the injured site by fibrous tissue. The absence of this process allows development of organs *de novo* and total restoration of lost function of heart muscle, eyes, fins, and other essential organs.[43–45]

## Caudal Fin Regeneration

Mammals, unlike fish, amphibians, such as axolotl that remain at the larval stage for their entire lifespan, and reptiles, cannot regenerate limbs. Although the key genes responsible for limb regeneration and inducing dedifferentiation, proliferation, and differentiation of somatic and stem cells are similar in anamniotes and amniotes, in mammals these genes are not activated after amputation.[46]

In anamniotes, the site of amputation is usually covered by a scar, which cannot replace a lost limb or protect underlying vital organs as well as normally functioning skin.[47] In contrast to anamniotes, the amniote zebrafish presents an ideal model to study epimorphic regeneration, in which progenitor cells mobilize, migrate, form an undifferentiated cell mass, proliferate, differentiate and eventually fully restore size, form, and function of the amputated organ. A series of repeated amputations does not reduce the regenerative capacity of zebrafish.[48]

The caudal fin is chosen for investigation by many researchers because of characteristics that allow study of the process of regeneration clearly. It is the largest of zebrafish fins, simplifying manipulation and observation. The study of fin growth does not require reagents or equipment. The




1 – fin rays, 2 – bifurcation, 3 – cleft, 4 – homocercal lobes, 5 – bony segment.

**FIG. 1.** Caudal fin structure.



caudal fin has two lobes that can be either heterocercal and homocercal, making it possible to monitor its growth along the medial–lateral axis, independent of the fish body. Compared with amphibian or mammalian limbs, the anatomy of the fin is simpler.[49] The process of regeneration is complete in 14–28 days, depending on water quality, temperature, diet, pathogenic organism invasion, and stocking density.[50]

Caudal fins in zebrafish, similar to the anal, dorsal, and pectoral fins, are of teleost type, formed by osteoblasts and fibroblasts. The main structural component of the fin, providing its shape, is a segmented, calcified, cylindrical ray, hollow at the distal end, produced and lined on the inside and outside by osteoblasts, as opposed to connective tissue, which makes up the outer layer of fin rays in most fish. The fin ray has an unsegmented region at the proximal end.[4] Each fin segment (lepidotrichium) is composed of two concave segments called hemitrichia connected by flexible fibers.[51] Lepidotrichia comprises fibroblasts, blood vessels, pigmented cells, and neurons. They are covered by the epidermis and connected by joints.[52] The majority of proximal segments are supported by a mineralized matrix, but three to four segments forming the distal tip of each ray have no bony matrix, but are supported by noncalcified spicules called actinotrichia. Due to the presence of collagenous and noncollagenous elements in elastoidin, the main element of actinotrichia, the fin is simultaneously rigid and flexible.[53] The actinotrichia provide a membrane to support the bone matrix, forming the locomotor apparatus. Although the formation of lepidotrichia lags behind that of actinotrichia, the latter is an essential element for migration and differentiation of osteoblasts.[54] A thin skin fold, produced by fibroblasts, covers all fin rays and allows them to contract and release, thereby decreasing and increasing the fin area. Muscles essential for movement and locomotion are attached at the base of the hemitrichia. The number of rays, segments, and muscles depends on the type of fin.[55] The zebrafish caudal fin consists of 16–18 rays. The difference between the lateral rays in the lobes and the medial rays of the cleft is approximately four segments. As a fish grows throughout its life, the rays grow by addition of new segments to the distal ends (Fig. 1).[56]

In zebrafish, the process of fin regeneration comprises five stages: wound epidermis formation, mesenchymal disorganization, blastema formation, regenerative growth, and termination

After caudal fin loss, to minimize negative effect, it is essential to oppress bleeding at the site of injury and prevent the expansion of microorganisms, many of them might be pathogenic for fish. To attract immune cells to the wound, the first danger signal is send by the reactive oxygen species (ROS), working as a chemoattractant at the place of amputation. Then the series of overlapping signaling molecules, including chemokines, attract immune cells, basically neutrophils, which arrive within several minutes. If blood vessels are destroyed, neutrophils form blood clot to stop bleeding.[57] Besides, neutrophils accumulate at the site of injury and remove debris of antigens (Fig. 2).

The roles of myeloid cells in fin regeneration are not fully understood, however, a recent study demonstrated a time-

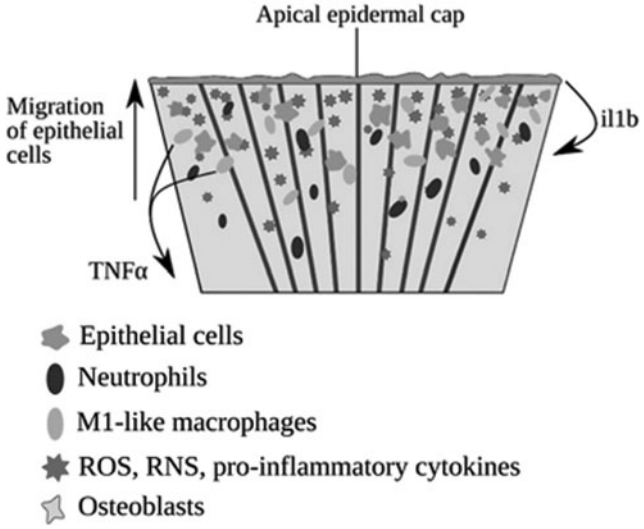



**FIG. 2.** Wound epithelium formation process.

dependent manner of macrophage and neutrophil migration to damaged tissue.[58] In contrast to previous studies showing that neutrophil ablation led to an elevated regeneration rate, inhibition of neutrophil recruitment to the site of injury by diphenyliodonium chloride did not affect the rate of tissue restoration. On the other hand, macrophage depletion resulted in significant decrease of caudal fin outgrowth and changes in fin morphology, including areas of aberrant tissue growth, defects in bony ray patterning, and heterogenic bone calcification.[59]

The second key player of the immune system, macrophages regulate tissue homeostasis, organ repair, and angiogenesis, migrate to the wound and secrete growth factor and cytokines that in turn activate extracellular matrix (ECM) production,[60,61] which presents in all tissues and regulates wound healing, providing a patch to protect the damaged area.

In contrast to zebrafish and other fully regenerating species, mammals form scar tissue after amputation through deposition of fibronectin and collagens, the major components of the ECM, leading to fibrosis. In zebrafish, when bleeding following caudal fin amputation is stopped, the amount of fibrin and fibronectin decreases, what makes prevent scarring,[62] because when a wound is closed, proteolytic enzymes called matrix metalloproteinases (MMPs) remodel matrix proteins to protect the healing organ or tissue and migrating cells to replace the lost tissue.[63]

Although MMPs participate in tissue remodeling, cell migration and proliferation, angiogenesis and apoptosis, and degradation of ECM proteins, and are essential for normal embryo development, reports of their role in limb regeneration are scarce. It has been shown that MMP genes are expressed in both the blastema and the basal epithelium layer in regenerating caudal fins, and that the presence of GM6001, an enzyme that inhibits MMP activity, results in fin growth restriction.[64] Research also suggests that MMP enzymes positively influence activation of chemokines that stimulate migration of immune cells to the injury site, maintain normal homeostasis, and control cell migration during embryogenesis and regeneration.[65] Increasing GM6001 concentration results in a reduction of leukocytes, negatively regulating immune response to injury.

Hasegawa *et al.* studied effects of proinflammatory cytokine interleukin-1 beta (*il1b*) on larval fin-fold regeneration.[66] and found caudal fin amputation to result in increased expression of *il1b* in epithelial cells, triggering regeneration-inducing genes. However, in the absence of macrophages, overexpression of this cytokine caused apoptosis of regenerative cells. Thus, macrophages play an important role in the progression to subsequent stages of the regeneration process. Another proinflammatory cytokine, TNF$\alpha$, was identified as a crucial component of caudal fin regeneration. This anti-inflammatory cytokine is synthesized by M1-like macrophages recruited to an injury region and exhibits dual action of enhancing accumulation of macrophages and participating in blastema formation, as a downstream element of the Wnt/$\beta$-catenin signaling pathway.[67]

Hyaluronic acid has been shown to be highly abundant in ECM of a regenerating fin. The *hyaluronan synthase 3* (*has3*) gene is overexpressed within 6 h after caudal fin amputation and increases its maximum concentrations within 24 h. Proteins encoded by this gene produce hyaluronic acids differing in molecular weight that perform different biological functions. Short hyaluronic acid polymers are associated with fin regeneration. Inhibition of hyaluronic acid synthesis during the first 24 h postamputation (hpa) produces suppression of blastema formation and regeneration, whereas inhibition at later stages does not significantly affect the regeneration process. Research findings suggest that hyaluronic acid molecules activate signaling cascades required for blastema establishment, as inhibition of hyaluronic acid synthesis eliminates some blastema-specific markers.[68]

Within 1–3 hpa, the site is covered by multilayer wound epidermis formed by nonproliferating epithelial cells distally migrating to the site and creating an apical epidermal cap.[69] Over time, the wound epidermis increases in size and builds apical epidermal cap, which works as a physical barrier between the body and the environment.[70] At 18–24 hpa, the mesenchymal cells underlying the wound epidermis, mainly osteoblasts and fibroblasts, lose their organization and turn into a highly proliferative whitish mass of undifferentiated cells called a blastema that can be detected under the apical cap. The blastema increases in size over the course of 2 days.[71]

The establishment of the appropriate blastema is essential for normal regeneration, including the presence of blood vessels, nerves, bony elements, and connective tissue, so first 48 h are critical, and all primary mechanisms initiating regeneration must be properly activated[72] (Fig. 3).

Blastema, which have two functions: to support outgrowth and to form specific structural elements essential for fin restoration, is mainly regulated by Wnt/$\beta$-catenin,[73] sonic hedgehog (Shh),[74] fibroblast growth factor (FGF),[75] bone morphogenic proteins (BMP),[76] Activin,[77] insulin-like growth factor (IGF),[78] Notch,[79] and RA[80] (retinoic acid) pathways.

Hedgehog, a key regulator of embryo development in vertebrates, initiates appropriate osteoblast progenitor (pObs) cell differentiation, and fin ray bifurcation, regulating cell-to-cell interactions between basal epithelial cells and pObs. Activation of Shh allows temporary divided clusters of epidermal cells

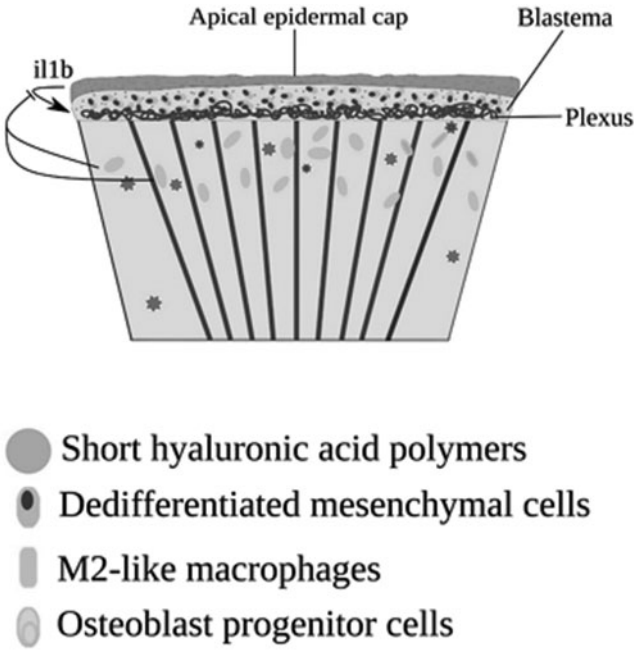



**FIG. 3.** Blastema formation process.

to escort progenitor osteoblast cells (pObs) regenerating hollow cylindrical bony rays with stereotypical branched structure or bifurcation.[81] Under impact of the steroidal alkaloid cyclopamine, Shh is blocked, which may result in both teratomas and tumor reduction. If Shh is overexpressed, it causes tumors, inappropriate cell proliferation, and excessive bone sedimentation in regenerating fins.[82,83] Inhibition of Shh signaling in injured tissue by cyclopamine leads to suppressed expression of Wnt/$\beta$-catenin, FGF, and RA genes, and loss of regeneration. However, activation of the Wnt pathway results in restoration of the regenerative process. Thus, Shh is an upstream regulator of this pathway.[84]

At the level of blastema establishment, the canonical signaling pathway, Wnt/$\beta$-catenin, regulating stem cell pluripotency, is critical to blastema formation, which in turn is essential for further fin ray development. Wnt/$\beta$-catenin is only active in a limited number of nonproliferating cells at the distal region of blastema and directly regulates osteoblast differentiation and, consequently, bone formation. It has been suggested that along with the functions of Wnt/$\beta$-catenin signaling during the process of regeneration is to establish organizing centers within the blastema to indirectly regulate the activity of other signaling pathways, such as Fgf and Bmp, which control cellular proliferation, differentiation, and tissue restoration.[85] Long-term oppression of Wnt/$\beta$-catenin signaling pathway blocks regenerative growth of the amputated fin.

The superexpression of the Wnt/$\beta$-catenin inhibitor glycoprotein Dkk1, which suppresses tumorigenesis and cell proliferation, causes cessation of blastema formation and, usually, the process of regeneration.[86] This antagonist has been reported to regulate cardiac tissue formation in the clawed frog *X. laevis*.[87] Knocking out the *Dkk1* gene results in impaired limb and brain development during embryogenesis in mice.[88] Thus, normal Wnt/$\beta$-catenin/Dkk1 interaction seems to be required for successful initiation of fin regeneration. With temporary overexpression of Dkk1, repeat amputation reboots the process of normal regeneration and leads to normal structure restoration. Interestingly, distal blastema cells produce a gradient of Wnt proteins in regenerating tissue, directly and indirectly regulating various cell populations.[89]

*FGF* is expressed at the early stages of fin regeneration and controls blastema formation, osteoblasts dedifferentiation and proliferation, actinotrichia regeneration, and, subsequently, fin restoration.[90,91] Errors in regulation of the FGF pathway result in severe abnormalities such as tumors. Fibroblast growth factor receptors (FGFRs) make a significant contribution to effective regeneration. FGFR1 is essential to maintaining subpopulations of epidermal and blastemal cells to initiate blastema proliferation.[92] In mutant zebrafish in which the *fgf20a* gene was temporarily knocked down, epithelial cells covered the site of injury as seen in wild-type fish, but were not replaced by blastema cells.[93] This modification halted the process of regeneration at 48 hpa. The wound was healed completely without restoring the missing fin. Temporary inhibition of fgfrs results in pathology at intersegmental joints and bony elements. Inhibition of FGF signaling pathways using heat-shock or chemical reagents 2 days post amputation (dpa) resulted in rudimentary blastema, what proves that FGF is essential not only for *de novo* synthesis, but to maintain existing protein structures. After activation of this pathway all structures, including blastema and actinotrichia can restart regenerative process and reestablish their structure and functions.[90]

The Notch signaling pathway is a universal regulator of cellular homeostasis in embryogenesis and important to maintaining the integrity of adult tissue. Through cellular interactions, the Notch pathway exercises control over the development of neighboring cells and also regulates their ability to self-renew, grow, survive, differentiate, and die through apoptosis. Recent studies have shown that the control of regenerative processes depends on maintaining the proliferative state of blastemal cells. It was found that Notch is activated in response to caudal fin amputation, but is not involved in cell migration or dedifferentiation after the blastema is formed.

Bmp comprise a large subgroup of ligands from the transforming growth factor-$\beta$ family. In humans, BMP signaling is initiated by BMP dimers that bind to a cell surface multimeric complex consisting of homodimers of Bmp receptors type 1 and 2 that exhibit serine/threonine kinase activity. Upon ligand binding, the BMP receptor 1 dimer phosphorylates the BMP receptor 2 dimer and initiates its kinase activity, stimulating action of downstream mediators. The main targets of BMP receptor phosphorylation are SMAD proteins, regulators of fibrosis that translocate from the cytoplasm to the nucleus, where they bind to DNA at SMAD-binding motifs, causing tissue-specific gene expression.[76] Different homologs of type 1 Bmp receptors are expressed in almost all types of regenerating fin tissue.

BMP actives in the distal blastema, wound epidermis, osteoblasts, and blood vessels of the regenerating tissues. Initially Bmp is essential to remodel plexus into blood vessels. Besides, it promotes deposition of collagen fibers into the basement membrane, while closing the wound, and induces actinotrichia formation. Pharmacological inhibition of type 1 receptors resulted in the arrest of regeneration at 3 dpa after blastema formation, affecting wound epidermis structure. Impairment of Bmp pathway in wound epidermis arrested actinotrichia formation. Inhibition of Bmp signaling leads to abnormal formation of capillaries, resulting in impaired blood circulation.[94]

RA is an important effector molecule of osteoblast re- and dedifferentiation during bone regeneration in zebrafish. During growth, RA positively regulates bone matrix synthesis by osteoblasts. Equilibrium between bone matrix synthesis and degradation is established within a caudal fin to provide an optimal combination of flexibility and stiffness of hemirays. In an uninjured caudal fin, this equilibrium is primarily controlled by two proteins: Aldh1a2 in fibroblasts that synthesizes RA, and Cyp26b1 in osteoblasts that degrades it. After amputation, RA signaling should be suppressed by Cyp26b1 to provide conditions required for osteoblast dedifferentiation. However, proliferation of preosteoblasts is positively regulated by RA, preventing their redifferentiation and possibly interfering with BMP signaling and upregulating the Wnt/$\beta$-catenin pathway. Redifferentiation of preosteoblasts occurs due to a proximodistal RA concentration gradient produced by blastema cells that produce Cyp26b1 in the proximal region, while RA is mainly secreted in the distal region.[66] A recent study demonstrated that NF-$\kappa$B is an upstream regulator of RA signaling during osteoblast de- and redifferentiation. Mishra *et al.* suggested a model according to which NF-$\kappa$B is downregulated after amputation by the action of unknown injury-induced signals. NF-$\kappa$B downregulation induces *cyp26b1* expression in osteoblasts, stimulating their dedifferentiation (Fig. 4).[95]

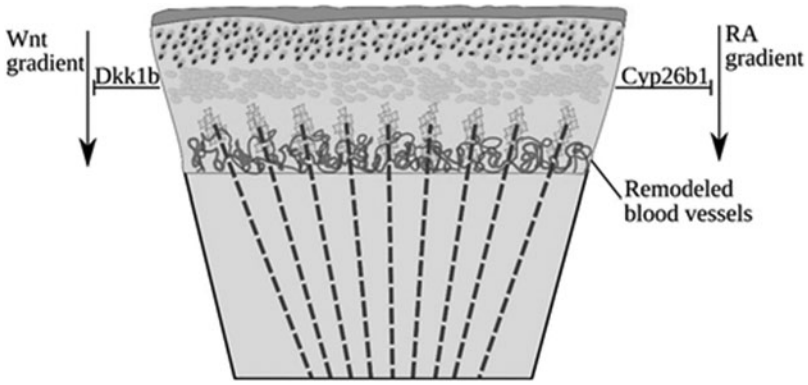



**FIG. 4.** The process of regenerative growth.



IGFs are evolutionary conservative proteins, regulating cellular processes involved in growth, survival, and metabolism. Effects of IGF signaling pathway are predominantly mediated through IGF-1 receptor, which possesses tyrosin kinase activity and phosphorylates downstream targets upon binding to a ligand. Downstream cascades of IGF signaling include Ras-MAPK and PI3-kinase pathways.[96] Chablais and Jazwinska demonstrated that establishment of proper IGF signaling is required for fin regeneration. In particular, insulin-like growth factor 2b (igf2b) expression showed fivefold increase in blastema at 72 hpa compared with amputated fin tissue. Interestingly, activation of IGF type 1 tyrosine kinase receptor (IGF1R) was detected not in blastema but in wound epidermis. Inhibition of IGF signaling using morpholino oligonucleotides and pharmacological blockers of IGF1's ATP-binding site resulted in aberrant regeneration and the 42%–48% smaller regenerate at 24 hours post injury or amputation (hpi).[97] IGF signaling is possibly important for the establishment of wound epidermis due to antiapoptotic action of IGF1R activation.[98] For instance, inhibition of IGF signaling during formation of wound epidermis was associated with 55- to 95-fold enhanced apoptosis. However, massive apoptosis did not occur if fish were treated with igf1r inhibitor after wound epithelium formation. Despite the absence of upregulation of IGF1R in blastema, inhibition of this signaling pathway resulted in defective cell proliferation and lack of expression of blastemal markers msxB and fgf20a.[97]

Activins are ligands, belonging to TGF-$\beta$ family. Binding of these ligands to activin type 1 and type 2 receptors tirggers activation of Smad proteins due to serine/threonine kinase activity of intracellular domains of the receptors. After activation, Smad complexes alter expression of multiple genes in the nucleus.[77] Activin signaling was shown to be important for zebrafish regeneration. Jaźwińska *et al.* demonstrated significant upregulation of activin-$\beta$A transcription in fins after amputation compared with several genes of TGF-$\beta$ ligands (TGF$\beta$1, TGF$\beta$2, TGF$\beta$3) and nodal-related genes. A peak of act$\beta$A expression was detected 18 hpa (18-fold increase) with slight decline at 24 hpa. Pharmacological inhibition of Activin type 1 receptor before blastema formation leads to impaired restoration of wound margins due to defective mesenchymal remodeling, arising from $\sim$75% reduction of cell proliferation compared with uninhibited controls. Treatment of truncated zebrafish with activin type 1 receptor inhibitors after blastema formation resulted in significantly reduced proliferation of blastemal cells at 12 hpi, and decreased expression of blastemal marker msxB after 24 hpi. Using act$\beta$A- and alk4-morpholino oligonucleotides for impairment of activin signaling, Jaźwińska and colleagues identified 50%–80% reduction of regeneration. Thus, activin signaling impairment during fin outgrowth results in defective regeneration even after normal initiation of the process.[99]

To dedifferentiate mesenchymal cells, strongly methylated genes must be demethylated[100] through action of DNA methyltransferase (*dnmt*), which removes methyl groups from cytosine, transforming heterochromatin into its active form, euchromatin.[101] Investigation has shown that the levels of 5-methylcytosine and 5-hydroxymethylcytosine decrease immediately after amputation and during the ensuing 36 h, and return to normal levels when the regenerative process is completed. This suggests increased expression of one or more *dnmt* genes and of the protein(s) encoded by those genes. Mutations in these genes can arrest normal dedifferentiation of somatic and stem cells and impair the process of regeneration.[102] Results similar to those in fish have been obtained in axolotl, confirming that the level of dnmt3a and dnmt1 is higher in blastema than in other cells.[103]

In zebrafish, the stage of mesenchymal cell proliferation is preceded by dedifferentiation of mature osteoblasts, which transform into osteogenic precursors, providing a source of newly formed bony structure. The concentration of osteocalcin, a bone Gla protein that is the hallmark of a mature osteoblast, is reduced significantly 24 hpa at the site of damage and 48 hpa in the nearest fin segments. In mammals, by contrast, mesenchymal stem cells produce new osteoblasts that, in turn, synthesize osteocalcin in large quantities. Because bone is a core element of limbs, this might be one of the limiting factors in human limb regeneration.[104,105]

During dedifferentiation, changes occur in both the cell nucleus and in the cytoplasm, causing synthesis of new proteins and degradation through autophagy of those no longer needed. When the blastema is being formed, the level of autophagy is high, and deactivation of autophagy results in apoptosis.[106] According to some reports, zebrafish treated with the autophagy inhibitor bafilomycin demonstrated reduced length of caudal fin compared with controls. Bafilomycin is not the only antibiotic suppressing activation of autophagy. Drugs negatively regulating autophagy include chloroquine.[107]

To initiate epimorphic regeneration, it is necessary to significantly increase the proliferation of normal mitotic cells and reactivate genes, which are responsible for embryo development. One of the target genes *fam53b/simplet* (*smp*) is highly expressed in the distal mesenchyme, what leads to rapid proliferation at the early stages of regeneration and

increases the outgrowth of regenerative tissues. Temporary arrest of this gene results in decreased number of cells, which cannot restore the primary structure of caudal fin.[108]

At 24 hpa *smp* expression is oppressed, and the cells comprising the blastema are separated into distal blastema, expressing *msxb*, *msxc*, and *sly1* with slow proliferation in which the G2 phase of mitosis is generally longer than 6 h, and proximal blastema, expressing *mps1*, *hsp60*, and *pcna*, proliferating rapidly. Marker genes of the distal blastema, such as *sly1* and *msxc*, do not lead to cell proliferation, but support homeostasis of the number of cells within a tissue; whereas *mps1*, a mitotic serine/threonine-protein kinase, affects controlled cell division.[109] The distal blastema makes a critical contribution to the proximal blastema when newly produced cells migrate to new positions, differentiate, and replace the amputated rays.

In fin ray architecture, as actinotrichia are formed before lepidotrichia, the gene expression responsible for collagen production is significant.[110] Actinotrichia are constructed of elastoidin and serve as skeletal support for the skin fold and as a substrate for the mesenchymal cells migrating toward the site of injury. All collagenous elements are encoded by the *col1a1a*, *col1a1b*, and *col1a2* genes and type II collagen. Noncollagenous elements are encoded by the actinodin gene family. Zebrafish, as a representative of teleost fish, and the elephant shark *Callorhinchus milii*, a species of cartilaginous fish, possess the specific proteins, actinodin 1 (And1) and actinodin 2 (And2), required for the formation of actinotrichia. Double knockout of And1 and And2 genes in zebrafish embryos leads to the absence of actinotrichia and, consequently, fin formation *de novo*.[56]

In addition to normal regeneration of caudal fin rays, it is important to establish the initial size of the fin, which must grow proportionally to the total body length.[111] This regulation is under control of calcineurin (also called PP2B), a protein phosphatase that is responsible for growth.[112,113] With suppression of calcineurin by the inhibitors FK506 and cyclosporin A, the caudal fin demonstrates excessive regenerative growth.[114]

The duration of regeneration has been demonstrated to be dependent on the length of the amputated structure. Proximally amputated fin rays take a longer time to regenerate than distally amputated. All fin rays regenerate at the same rate, regardless of their position along the dorsal/ventral axis.[115] Termination is finished with structurally and functionally regenerated caudal fin (Fig. 5).

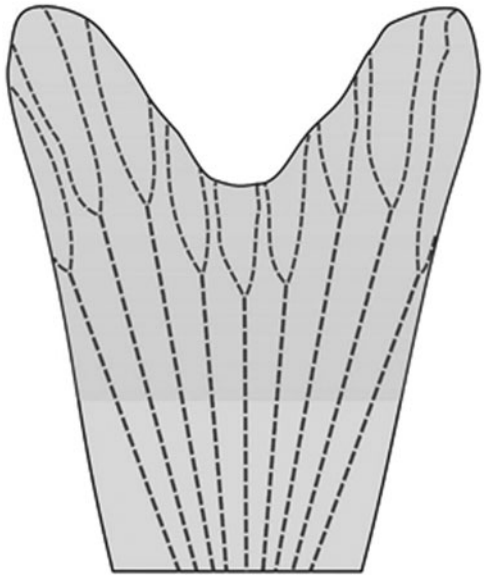


**FIG. 5.** Fully regenerated caudal fin.

## Factors Influencing Fin Regeneration

Fin regeneration as any processes taking place in living organisms, depends on many environmental factors, which must be taken into account during the planning the experiments. Stress is one of the most critical factors affecting processes, such as inflammation, regeneration, and the immune response, producing anxiety and depression, which suppress physiological functions.[116–118] In vertebrates, elevated glucocorticoid plasma levels are considered an indication of stress. Stressors activate the hypothalamic/pituitary/interrenal axis, leading to modulation of diverse physiological processes through glucocorticoid receptors such as stress hormones and heat-shock protein production. Metabolic changes increase levels of blood glycogen and decrease tissue glycogen, initiating immune system activation and osmoregulatory changes.[119] Stressed fish might be detected by physiological alterations or behaviors such as aggression, decreased movement, and changes in feeding behavior.[120]

Acute and chronic stress induced by chemical, physical, or psychological stressors such as temperature, high stocking density, diet, chemical agents, the presence of predators, novel tanks, and capture and handling can increase concentration of whole-body cortisol. Short-term stress acts as an enhancer of immune processes[121]; whereas long-term chronic stress inhibits the regenerative processes.[122] It has been demonstrated that high corticosteroid levels initiate metabolic dysfunction, tissue degradation, and programmed cell death.[123–125]

### Temperature

Temperature is an important regulator of zebrafish physical condition and behavior.[121] To maximize growth, feed intake, feed conversion, and stomach evacuation it is essential to maintain a consistent optimal water temperature.[126] Optimal temperature for zebrafish embryos is reported to be 27°C, with 22°C and 32°C representing lower and upper limits for normal growth and development. In a study of short-term exposure to one of the two extreme temperatures, embryos were raised to maturity under normal temperature. Both extreme groups demonstrated reduced thermal sensitivity according to swimming performance tests, due to difference in composition of muscle fibers responsible for swimming and active movements. Transfer from the thermal environment at which the embryo and early life stages were reared to a nonadapted temperature regime provokes chronic stress, which will negatively regulate tissue and organ regeneration.[127]

In a study of regeneration at permissive (25°C) and restrictive (33°C) temperatures, 60% regeneration was attained at 8 dpa at the higher temperature, while, at 25° C, this point was reached at 15 days.[128]

Other authors demonstrated that keeping fish at 37°C for 1 h increased concentration of cortisol to 10–40 times that of

a control group. Even a short exposure of fish to extremely high temperatures might lead to death. Although a single case of acute stress has not been shown to critically affect regeneration, repetitive stress significantly decreases full regeneration of an amputated structure. After regular exposure to heat shock resulted in damaged heart muscle, only 25% of fish successfully replaced collagenous tissue and regenerated damaged site.[129]

## Diet

### *Food deprivation*

Autophagy is recognized as a key mechanism to maintain cellular homeostasis. It induces removal of aggregated and misfolded proteins; damaged ribosomes, mitochondria and other organelles; prevents necrosis; and regulates recycling of cellular wastes and nonessential elements.[130] In zebrafish, normally functioning autophagy is important for maintaining the regenerative capacity of stem cells.[131] Fish, in which autophagy is inhibited by mutations, knockout or knockdown of genes, or chemical or pharmacological agents, exhibit increased levels of cell death,[132] as well as suppression of dedifferentiation and proliferation of blastema, impairing caudal fin regeneration.[107]

Short-term starvation rapidly activates the autophagic response, which in turn participates in ATP production and *de novo* protein synthesis.[133] Many species of fish tolerate lack of food during migration, seasonal changes, spawning periods, oxygen and water deficit, and temperature fluctuations.[134] Food deprivation for a limited period of time does not necessarily exert a negative effect on physical activity or cellular processes, but accelerates regeneration in its critical stages.[135,136]

### *Diet and feed supplements*

Different commercial feeds differentially impact growth, age of sexual maturity, fertility, viability, and regeneration. For normal zebrafish tissue regeneration, it is necessary to use feed with an optimal ratio of amino and fatty acids, vitamins, carbohydrates, and minerals and to establish an adequate feeding regime.[137]

The polysaccharides 1,3–1,6 $\beta$-glucans, usually extracted from bacteria, fungi, yeast, algae, or plants, have been evaluated as feed supplements for zebrafish. A supplementary dose of 0.35 g of 1,3–1,6 $\beta$-glucans/kg feed for 14 days was associated with decreased mortality and an increase in the regenerated area of caudal fin.[138] The 1,3–1,6 $\beta$-glucans are known as immune system modulators, enhancing immune response through different pathways.[139,140] Although specific 1,3–1,6 $\beta$-glucan targets have not been identified in teleosts, homologs of the mammalian C-type lectin receptor family are believed to be responsible for activation of macrophages. Several candidate genes for $\beta$-glucan receptors have been identified in carp macrophages.[141] Similar research shows that they induce immune resistance against both bacterial and viral invasions because of stimulating humoral and cellular processes. In carp, trout, and salmon, food additives with $\beta$-glucans protect the immune system by the activation of macrophages.[142] Vitamin A (RA) is an important signaling molecule. In *Oreochromis niloticus* treated with vitamin A at concentrations of 2, 4, and 8 IU/mL, no signs of improved regeneration was detected. However, RA affected morphology of regenerated fins: the number of ray segments regenerated was higher in the treated groups compared with controls[142] Vitamin A suppressed growth of connective tissue between bony segments, causing fusion of adjacent rays. Normally a newly regenerated single fin ray is divided into two daughter rays. In fish exposed to RA, a metabolite of vitamin A, dissolved in dimethyl sulfoxide (DMSO) at a concentration of $10^{-6}$ M for 72 hpa, these daughter rays fused into a single ray.[143]

Vitamin C, ascorbic acid, participates in multiple enzyme reactions and is a powerful antioxidant. Vitamin C protects the body against free radicals and oxidative stress, consuming ROS. Zebrafish, similar to humans, cannot produce this vitamin and must obtain it from the diet. In vitamin C deficiency, fish undergo oxidative stress and exhibit an increased level of metabolites in the purine nucleotide cycle, resulting in changes to cell energy metabolism.[144] Fish experiencing severe oxidative stress show critical abnormalities in the function of liver, brain, muscle, gut, and other vital organs, without which regeneration cannot successfully progress.[145]

### *Overfeeding*

Contrary to earlier published articles, some authors have reported that consistent overfeeding and a high-fat diet over the course of several weeks did not result in differences in body weight, but impaired cognitive function in zebrafish that might be compared with cognitive disorders in humans. Compared with those provided 3.79 kcal/g, fish consuming 4.90 kcal/g showed lower expression of 97 genes in the telencephalon, the brain area responsible for recall of information.[146] This may be speculated to have a long-term negative effect on regeneration, because effective regeneration depends on the ability to maintain vitality for normal physiological and biological function, which is supported by the brain. Overfeeding was shown to alter zebrafish gut microbiota in a sex-specific manner, which can be associated with obesity and related disease.[147]

Overfeeding has been shown to impact osteogenesis, a major factor in fin regeneration, essential for fin ray formation in zebrafish. A high-fat diet has been shown to alter concentrations of blood glucose and insulin in zebrafish, increase weight by 23%, and result in resorbed borders of the scales, indicating a failure in bone mineralization. Activity of tartrate-resistant acid phosphatase, an osteoclastic enzyme, showed increase, whereas alkaline phosphatase, which initiates bone mineralization, decreased in fish consuming a high-fat diet on a regular basis. The imbalance results in osteoporosis and impaired bone formation.[148]

### *Stocking density*

Although zebrafish importance as a model organism is increasing, optimal stocking density for its culture has still not been identified. Several studies report the optimal density to be 12–15 fish/L with water aeration.[149,150] Overpopulation negatively influences not only regeneration, but oppresses all physiological processes.[151,152] It has been reported that zebrafish are sensitive to crowding (>10 fish/0.25 L) as a biological stressor.[129] In a study of fish stress, the concentration of whole-body cortisol with crowding was twice that recorded with heat shock, approaching 80 ng/g fish. Only

33% of fish exposed to crowding successfully regenerated cardiac muscle.[129] In similar research, fish held at density of 40 fish/L for 3 h to induce acute stress and for 5 days for chronic stress. In both categories, whole-body cortisol concentration was approximately fourfold that in control fish maintained at a 0.25 fish/L.[153] Although the zebrafish is a highly social species, low stocking densities of 0.13, 0.30, and 1.2 fish/L did not result in significant differences from concentrations of cortisol dissolved in the tank water.[136]

### Aquarium Equipment

Aquarium equipment makes an important contribution to the process of cell proliferation. Authors measured the number of actively dividing neurons in zebrafish held individually in tanks enriched with gravel and artificial plants or in barren aquariums.[154] Fish kept in the enriched tanks showed slightly elevated cortisol levels compared with those in the barren tanks. This could be assumed to negatively affect cell proliferation, but short-term exposure (6 and 30 min) of fish to novel conditions containing unfamiliar elements not only activates a stress response, but demonstrates stimulating effect on regenerative processes in brain.[154] Environmental changes may alter ability of fish to move, take in food, and initiate schooling, in turn impacting regeneration. Differences in artificial lightning (6500 K, imitating daylight; 7100 K, typical of aquariums; and 4100 K, cool white fluorescent lighting) was not shown to result in differences in fish behavior or cortisol levels.[136]

### Chemical Agents

Chemical agents such as caffeine, nicotine, ethanol, and cocaine added to tank water may be depressors or enhancers of regeneration. Caffeine increases heart rate and accelerates oxygen transport to tissues and organs, which can, hypothetically impact regeneration. The research[155] presents the results of behavior assessment of fish under the impact of caffeine. Zebrafish exposed to caffeine spent more time at the bottom of the tank indicating anxiety. Stressed behavior signals suppressed reactions of the organism, which results in retarded regeneration. To analyze effects of caffeine, zebrafish were exposed to caffeine in concentrations of 0.5–150 mg/L for 60 min. At concentrations >50 mg/L the distance fish traveled was reduced compared with concentrations from 0 to 25 mg/L. Exposure to caffeine at concentrations >50 mL/L increased freezing behavior and time spent at the bottom of the tank.

In many animals, leukocytes are attracted to an injury site by proinflammatory substances such as cytokines and ROS. Production of ROS is initiated immediately after caudal fin amputation, reaching peak concentrations after $\sim$20 min. The $H_2O_2$ concentration has been identified as a key mediator of leukocyte recruitment to the wound.[156] Recent research has demonstrated that silver nanoparticles, which are widely used in biomedical applications such as antibacterial wound dressings, suppress the regeneration process in zebrafish. Impairment of the wound healing process is stage-dependent, affecting wound epithelialization and early blastema formation. Silver nanoparticle treatment was reported to lead to reduction of ROS generation during the period from 18 to 36 hpa and significantly increase the number of recruited neutrophils.[157] Reactive nitrogen species (RNS) represent another group of inflammatory mediators. Study of nitrite and nitric oxide on zebrafish regeneration showed that RNS are required for proper neutrophil recruitment to an injured site under hypoxic conditions. Nitrite treatment of fish with cryoinjured heart tissue promoted cardiomyocyte proliferation and increased angiogenesis.[158]

Exposure of zebrafish with an amputated fin to the glucocorticoid prednisolone impairs bone formation and osteoblast differentiation and increases length of time of regeneration. Glucocorticoid treatment alters protein expression patterns in regenerating tissues. Prednisolone exposure in particular affects expression of transporters involved in lysosomal acidification as well as those involved in vesicular transport and secretion. Hence, calcium-transporting ATPases are also overexpressed, resulting in altered bone mineralization.[159]

## Conclusions

This is only the beginning of zebrafish studies in regenerative medicine. This review does not include all aspects of regeneration of caudal fin, and there are many mechanisms regulating this process that are unknown. Multiple environmental factors facilitating or inhibiting tissue regeneration must to be taken into account during planning of experiments.

## Author Contributions

L.L., B.Z., and Z.A. designed the study. I.K. and T.G. wrote the ''Factors Influencing Fin Regeneration'' section. All authors participated in writing the article. All authors read and approved the final article.

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