

Review

Getting to the heart of regeneration in zebrafish

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Abstract

A scientific and clinical prerogative of the 21st century is to stimulate the regenerative ability of the human heart. While the mammalian heart shows little or no natural regeneration in response to injury, certain non-mammalian vertebrates possess an elevated capacity for cardiac regeneration. Adult zebrafish restore ventricular muscle removed by surgical resection, events that involve little or no scarring. Recent studies have begun to reveal cellular and molecular mechanisms of this regenerative process that have exciting implications for human cardiac biology and disease.

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Keywords: Cardiac muscle; Zebrafish; Progenitor cells; Epicardium; Neovascularization; Regeneration

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1. Introduction

The promise of stem cells and their potential applications has rejuvenated a research movement toward understanding and promoting regeneration, the replacement of lost organs or portions of the body. Stem and progenitor cells have been identified from most human organs, including brain, skeletal muscle, liver, lung, skin, kidney, blood, bone, reproductive tissues, intestine, and heart. These cells ostensibly function to maintain homeostasis, the replacement of cells damaged and lost during day-to-day performance of organ functions. Some progenitor cells also have

the potential to renew tissue after minor injury, or even after major damage or removal of structures. Robust examples of mammalian organ regeneration are demonstrated by blood and liver. Transplanted hematopoietic stem cells can reconstitute the hematopoietic system of an irradiated mouse with remarkable efficiency, and the liver is able to renew its organ mass within 2 weeks after removal of 70% of hepatic tissue [1–3].

Unfortunately, not all of our organs are equally competent to regenerate (Fig. 1). The heart and central nervous system are particularly resistant to regeneration after injury. In the CNS, the inability to regenerate may contribute to morbidity caused by neurodegenerative disease or stroke. With regard to cardiac tissue, acute myocardial infarction (MI) is a leading cause of death worldwide. MI is typically caused by coronary artery occlusion, and in many cases leads to immediate death. For those fortunate

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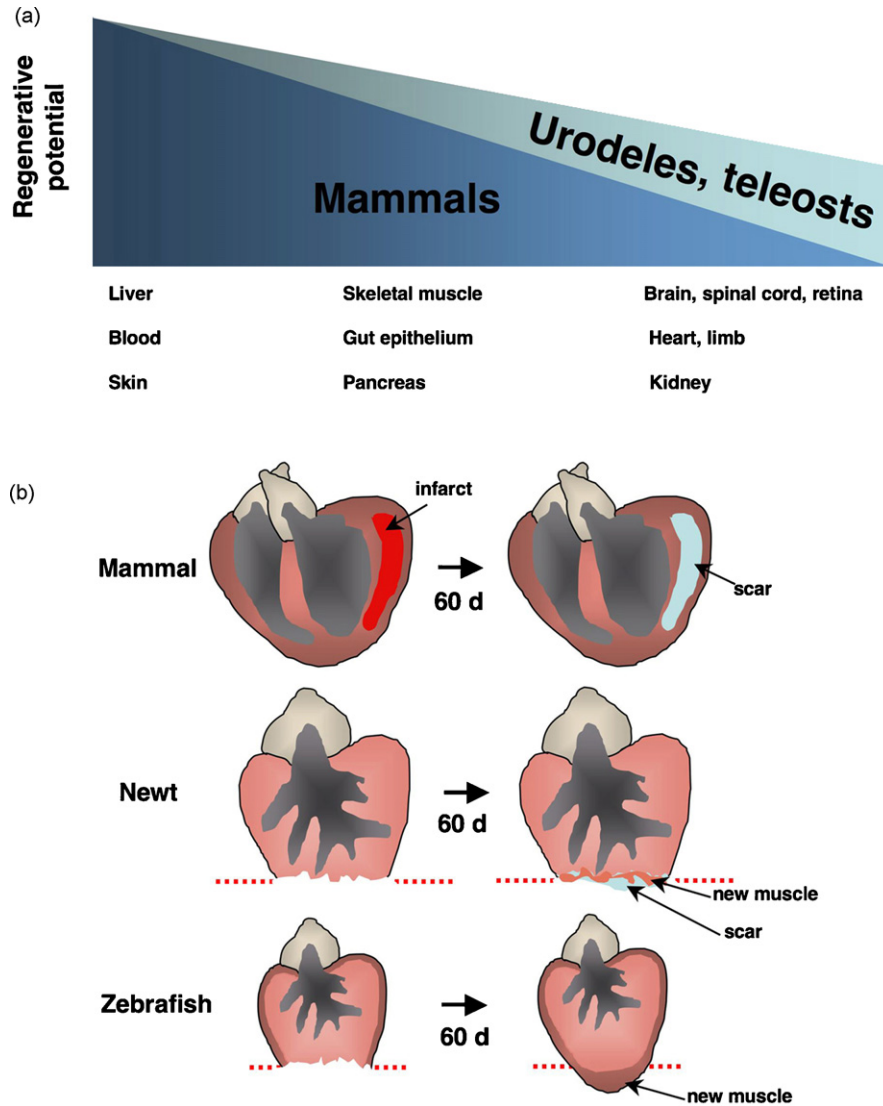


Fig. 1. Non-mammalian vertebrates have an elevated regenerative spectrum. (a) Many mammalian tissues like liver, blood, skin, skeletal muscle, gut, and pancreas possess a significant capacity for regeneration. However, mammalian CNS structures like brain, spinal cord, and retina fail to regenerate, as do heart, kidney, and limb. These latter structures can regenerate after severe injury in certain urodeles and/or teleosts. How and why the regenerative capacity of these tissues has been unequally retained during evolution is unclear. (b) The typical mammalian injury model (top) involves an ischemic myocardial infarct, here producing a focal injury in the left ventricular wall. This injury kills cardiac muscle, which is replaced by scar tissue (blue) over the course of several weeks. The newt heart (middle) mounts a partial regenerative response after apical resection, healing the injury with new muscle (orange) and scar tissue. The zebrafish heart (bottom) can regenerate a similar apical resection injury with little or no scarring, replacing a substantial portion of lost muscle in several weeks.

enough to survive MI, necrotic myocardium is replaced in the next few weeks by non-contractile scar tissue. Cardiac fibrosis provides a short-term solution in replacing necrotic muscle with a stable, though irreversible, patch. However, the spared cardiac muscle typically undergoes pathologic hypertrophy to recover contractile force. Without optimal cardiac performance, quality of life usually suffers, while the heart is rendered more susceptible to organ failure and future MI events [4]. Therefore, development of therapies that can facilitate survival or regenerative replacement of destroyed myocardium would be of enormous social and economic impact.

One possible approach to effecting cardiac regeneration in mammals is to establish a means to activate and mobilize progenitor cells. This new and exciting pursuit is the subject of an accompanying review in this issue [5]. A complementary method

is to identify successful examples of cardiac regeneration among vertebrates, and to dissect how this success is achieved. Indeed, the capacity for organ regeneration is remarkably elevated in certain lower vertebrates like urodele amphibians and teleost fish (Fig. 1a), as well as invertebrates like starfish, hydra, and planarians. The study of regeneration in these champions has fascinated scientists for over 300 years. In this review, I will focus on cardiac regeneration in non-mammalian vertebrates, paying particular attention to a relatively new laboratory model system, the zebrafish.

2. Cardiac regenerative capacity in mammals

Planarians and hydra fully replace large portions of their bodies, regenerating in an essentially perfect manner [6,7]. Sim-

ilarly, limb or fin amputation in many lower vertebrates results in rapid recovery of their former size and patterning [8–10]. By contrast, the definition of regeneration in cardiac tissue has generally been more lenient and inclusive, involving any replacement of lost myocardium by production of new cardiomyocytes (CMs), or hyperplasia. Even with this relaxed definition, significant cardiac regeneration of any form has not been reported in mammals after multiple models of injury, including ischemic infarction, burning, freezing, mechanical injury, chemical injury, etc. [11].

While it is generally accepted that mature mammalian CMs have little or no proliferative capacity [12], recent studies have demonstrated that existing CMs need not divide in order for potential regeneration to occur. Several groups have identified multiple types of undifferentiated or poorly differentiated cardiac progenitor cells in the postnatal heart that have the potential to mature into contractile cells [13–15]. The presence of cardiac progenitor cells is not necessarily surprising, given findings over the years that most mammalian organs are equipped with one or more progenitor cell types. Indeed, a progenitor cell mechanism could explain how cardiac growth occurs postnatally despite the resistance of mature CMs to division. However, it is not at all clear why the potential of progenitor cells for creating new CMs is not harnessed after injury.

3. Cardiac regenerative capacity in amphibians

Urodele amphibians like newts and axolotls can regenerate limbs, tail, spinal cord, retina, lens, jaws, portions of intestine, and brain tissue (Fig. 1a) [16]. The possibility of cardiac regeneration in amphibians has been examined in frogs, newts, and axolotls, with much of the original literature reporting work of Soviet scientists in the 1960s [11]. This body of work indicated that amphibians survive massive mechanical injury to the ventricle, including removal of as much as one-quarter of the chamber. This resiliency is a feat in itself, and likely reflects a lesser reliance on vigorous circulation. While there is some variability in reports and reviews on the extent of regeneration after injury, the prevailing view is that some cardiac regeneration occurs after resection [17–19]. There is definitive evidence for proliferative activity, including the presence of mitotic figures in CM nuclei as visualized by standard histology and transmission electron microscopy. Furthermore, a high frequency of ³H-thymidine incorporation, and more recently, BrdU incorporation, was reported in CMs of the injured ventricle [20]. Thus, there are fundamental differences in the extent to which mammals and amphibians respond to cardiac injury.

The idea that these *in vivo* findings represent CM cytokinesis are bolstered by studies of adult amphibian CMs in cell culture. Historically, adult mammalian CMs are highly resistant to cytokinesis in culture, although a recent finding indicated that experimental manipulation can increase their division. In that study, Fgf application combined with p38 MAP kinase inhibition could induce BrdU incorporation and the formation of contractile rings [21]. By contrast, CMs isolated from the adult newt ventricle readily divide in cell culture medium [22]. It is not clear what molecules stimulate CM division; however, newts

are reported to have a heterogeneous population of CMs, some of which may be better suited to divide [23].

Despite evidence for *in vitro* cytokinesis and *in vivo* cell cycle entry and mitosis in the newt, the predominant response to resection injury is scarring (Fig. 1b). That is, while it appears that adult newt CMs have the potential to regenerate and do so to some degree, there is only minor replacement of cardiac muscle. Explanations for this disconnect are few. It has been proposed that there exist barriers for reorganization of proliferating CMs. Supporting this notion, CMs within minced myocardium grafted to an injured ventricle will show proliferation and can form a contiguous, contractile mass [24]. Another idea is that, following resection, growth of scar tissue overcomes that of myocardium. This idea of competition between regeneration and scarring has recently reemerged and been addressed to some extent through use of zebrafish [25], as described later in this review.

4. Zebrafish and organ regeneration

Zebrafish have been a popular model for embryologists over the past 10–15 years. Clear advantages of zebrafish include ease and relatively low cost of maintenance, regular mating and large clutch sizes of 50–300 embryos, and transparent development outside of the mother. One of the most productive fields employing zebrafish examines development of their cardiovascular system, which is easily visualized and highly amenable to mutant analysis and live imaging. This topic is reviewed extensively in this issue by Schoenebeck and Yelon [26]. The diligence and productivity of these embryologists has generated strains, reagents, tools, and ideas for the study of organ regeneration in adult zebrafish.

More and more groups are becoming interested in the fact that adult zebrafish possess a high capacity for regeneration. The three organs most studied for their regenerative prowess are the fins, retina, and spinal cord. Zebrafish have five fin types, two of which are paired. Fins are comprised of several segmented, bony fin rays. Each ray has two concave, facing hemirays, surrounding nerves, blood vessels, and connective tissue. All seven fins can regenerate after as much as 95% of the tissue is amputated, although the caudal fin is most often used in regeneration experiments. The process of fin regeneration is epimorphic, involving the formation and proliferation of an undifferentiated and presumably multipotent mesenchymal structure called a blastema [8,9]. Teleost retinæ also regenerate, producing new rods and cones after an acute injury or after complete ablation by phototoxicity [27]. Retinal regeneration appears to involve a progenitor cell-based mechanism; here, stem cells in the inner nuclear layer give rise to new photoreceptor neurons after injury [28]. Amazingly, spinal cord tissue can regenerate after a complete transection. In a process that takes about 6 weeks, ~80% of animals given a posterior injury achieve functional recovery [29]. This phenomenon is based on axonal regeneration, the striking ability of CNS neurons to recover, traverse the lesion, and reestablish functional connections. Thus, adult zebrafish, like newts and axolotls, are capable of regenerating many structures that mammals cannot. As discussed later, zebrafish also bring certain practical advantages to the study of

regeneration not available in other non-mammalian vertebrate model systems.

5. Cardiac regeneration in zebrafish

Because zebrafish effectively regenerate multiple adult structures, it is natural to be curious about their capacity for heart regeneration. Ischemic myocardial injury to the zebrafish ventricle would be highly representative of human disease, and MI models are routinely used in small mammals like mouse and rat. However, the zebrafish ventricle is tiny ($\sim 1 \text{ mm}^3$) and the coronary vasculature relatively unexplored, making coronary artery occlusion a daunting task. Two groups recently examined the effects of removing approximately 20% of the ventricle by surgical resection with iridectomy scissors (Fig. 2a) [25,30]. By mechanical removal of ventricular myocardium, these studies examined what would occur after the strongest possible stimu-

lus for regeneration. Resection injury penetrates the ventricular lumen, releasing a large amount of blood. As in the amphibian heart, the injury clots quickly, and the organ sustains sufficient contractile force to drive circulation.

Over the next month, a remarkable series of events occurs in response to ventricular resection. First, the clot that seals the apex matures within several days into fibrin, a complex milieu containing serum factors and degenerated erythrocytes. In the infarcted mammalian ventricle, fibrin deposition attracts fibroblasts and inflammatory cells, and is a precursor to scarring. Strikingly, fibrin is not replaced by scar tissue during cardiac repair in zebrafish; in fact, little or no collagen is retained by 1–2 months after injury [25]. Instead, new cardiomyocytes are created that supplant the fibrin and seal the severed ventricle with a new wall of muscle (Fig. 2b). It is possible that the fibrin has a critical role in regeneration, perhaps acting as a scaffold or even a paracrine signaling center. Peak proliferative activity of car-

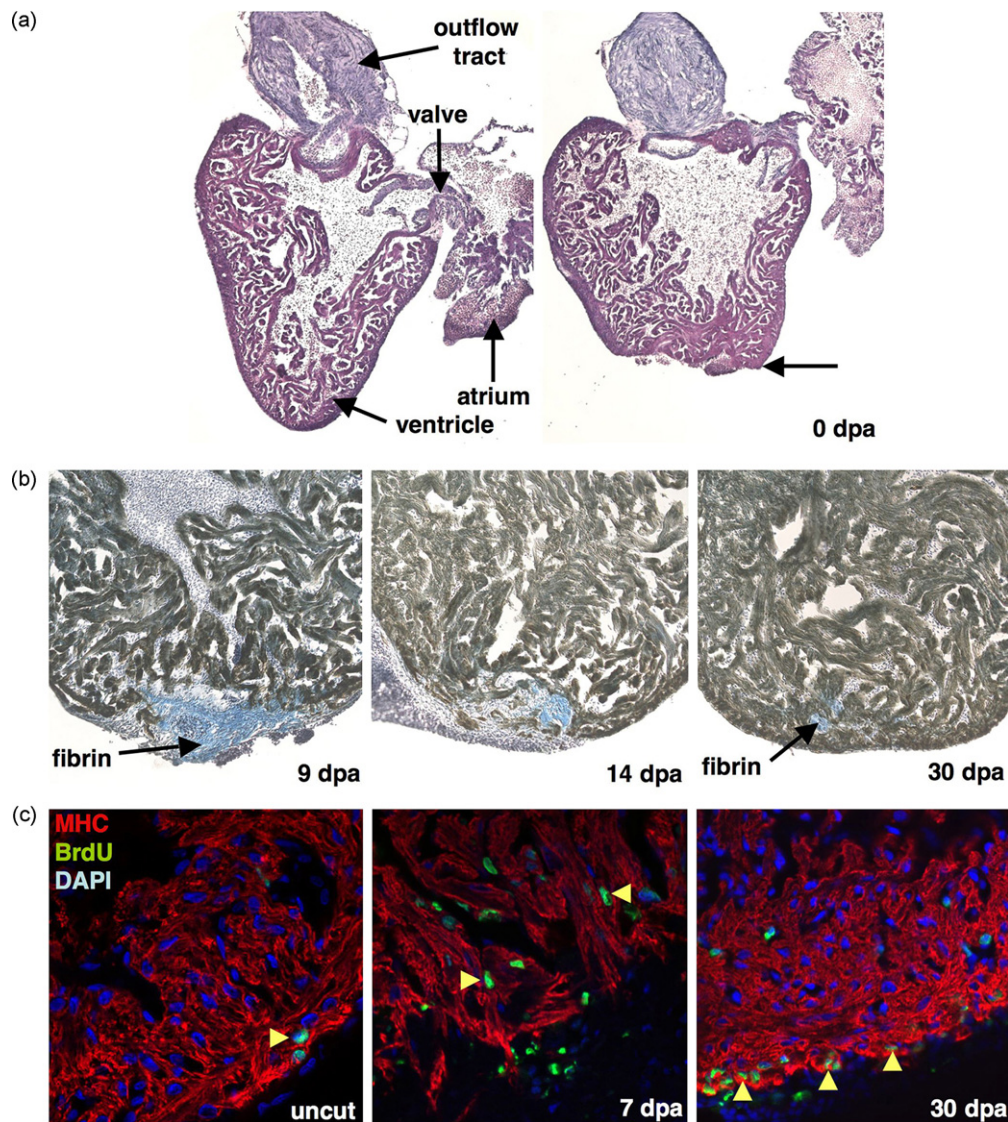


Fig. 2. Cardiac regeneration in zebrafish. (a) Hematoxylin and eosin stain of the adult zebrafish heart before and after 20% ventricular resection. (b) Ventricular sections stained for myosin heavy chain to identify cardiac muscle (brown) and aniline blue to identify fibrin (blue). Mature fibrin seals the wound by 7 dpa, and is gradually replaced by cardiac muscle. The wall is typically restored by 30 dpa. (c) BrdU incorporation, a marker of DNA synthesis and cellular proliferation, is activated in CMs by 7 dpa. The ventricular wall is restored by proliferation at the leading edge of CMs. Figure adapted with permission from Ref. [25].

diomyocytes as assessed by BrdU incorporation is observed at 14 days post-amputation (dpa), and the wall is typically restored by 30 dpa [25].

What are the cellular mechanisms of regeneration? The initial published studies revealed some details. First, regenerative proliferation events are enhanced at the distal or apical edge of the regenerate. Extensive pulse-chase labeling with BrdU indicated a mechanism in which muscle is restored by CM generation at this gradually shifting proliferative edge (Fig. 2c) [25]. This finding implies that heart regeneration may share some mechanisms with a process like fin regeneration, where a proliferative domain is maintained in a specific region of the blastema as regeneration proceeds distally [31,32].

That differentiated zebrafish CMs can actively enter mitosis seems clear, as histological studies have found rare CMs positive for phosphorylated histone-3 or showing mitotic figures during regeneration [25]. How CM proliferation is initiated and then sustained at the apical edge of the regenerate is a critical question that has only recently been addressed. There are several possible mechanisms that might explain the initial BrdU labeling results. First, differentiated, contracting CMs in existing myofibers could be stimulated to enter the cell cycle, divide, and reform the apex. Second, regeneration could proceed through the recruitment of undifferentiated progenitor cells that form new, proliferative CMs. A third conceivable mechanism for the origin of regenerative muscle is a chimera of these two mechanisms called “dedifferentiation”, in which existing muscle would downregulate contractile genes like *cardiac myosin light chain 2* (*cmlc2*) and *ventricular myosin heavy chain* toward creation of undifferentiated or poorly differentiated cells. There is evidence that dedifferentiation assists in creating blastemal cells after amputation of the urodele limb [33].

Recently, Lepilina and colleagues critically examined these potential mechanisms of heart regeneration [34]. In that study, transgenic strains reporting expression of *cmlc2*

by EGFP or nuclear-localized DsRed2 were used [35,36]. The DsRed moiety is known to mature very slowly [37,38], and, consequently, employment of the *cmlc2:nuc-DsRed2* transgenic strain allowed visualization of many individual CMs undergoing transitions in contractile gene expression in the regenerate (Fig. 3a). Because EGFP and DsRed2 have very different maturation and stability characteristics, a double transgenic *cmlc2:nuc-DsRed2; cmlc2:EGFP* strain was used to characterize activity at the *cmlc2* promoter in developmental timing assays. For example, the emergence of tissue positive for EGFP (fast-fluorescing) and negative for DsRed (slow-fluorescing) at the apical edge of the regenerate indicated that the transitions in *cmlc2* expression represent increases in differentiation from an undifferentiated state. By contrast, these assays revealed no evidence that reductions in *cmlc2* expression occur during regeneration. Coincident with these differentiation events was the emergence of cells at the resection plane expressing multiple markers, including *nkx2.5*, *tbx20*, and *hand2*, characteristic of cardiac progenitors during embryonic heart development [34] (Fig. 3b). These and other findings from the study argued that undifferentiated progenitor cells similar to those that make up the embryonic heart field are responsible for generating new, proliferative CMs during regeneration.

The source of these progenitors prior to their acquisition of cardiac fate is unknown. They might be derived from populations similar to adult mammalian cardiac progenitors, or from other cell types in the heart. While there is no evidence that dedifferentiation is important for zebrafish heart regeneration, a recent investigation of the injured newt heart suggested that injured CMs lose contractile gene expression and may be able to create progenitor-like cells [39]. Cre recombinase-mediated genetic lineage tracing technologies used in mice should be helpful if applied in zebrafish to experimentally resolve this question [40–42]. In any case, that zebrafish effectively influence progenitor cells to achieve cardiac regeneration is a

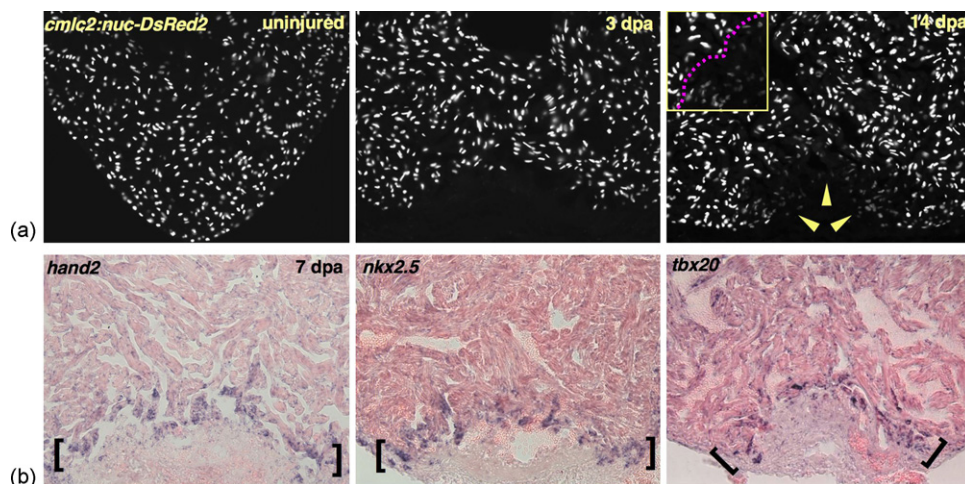


Fig. 3. Progenitor cells mediate heart regeneration. (a) Sections through uninjured and injured *cmlc2:nuc-DsRed2* ventricles. CM nuclear fluorescence is uniform in uninjured and 3 dpa ventricles. A subpopulation of weakly fluorescing nuclei manifests by 14 dpa (arrowheads), indicating CMs differentiating from undifferentiated progenitor cells. Magenta line in high magnification insets delineates high fluorescing CMs (above line) from low fluorescing CMs. (b) The embryonic heart field markers *hand2*, *nkx2.5*, and *tbx20* are each present in a domain of enhanced expression at the apical edge of the regenerate (brackets), a region of the most recent myocardial differentiation events. Figure adapted with permission from Ref. [34].

provocative finding that lends even more optimism to the idea that mammalian cardiac stem cells may someday be coaxed toward therapeutic regeneration.

6. Non-myocardial influences on heart regeneration

How are zebrafish cardiac progenitor cells successfully propelled to regenerate? Determining non-myocardial influences,

or describing the niche, is of equal importance to characterizing the progenitors themselves. Prominent non-myocardial tissues in the adult zebrafish heart are the epicardium, a thin epithelial layer enveloping the chambers, and the endocardium, a layer of endothelial cells that lines the inner trabecular myofibers.

The epicardium is not simply a bystander to myocardial regeneration. By contrast, the epicardium exhibits a rapid and robust response to injury, proliferating and expressing markers

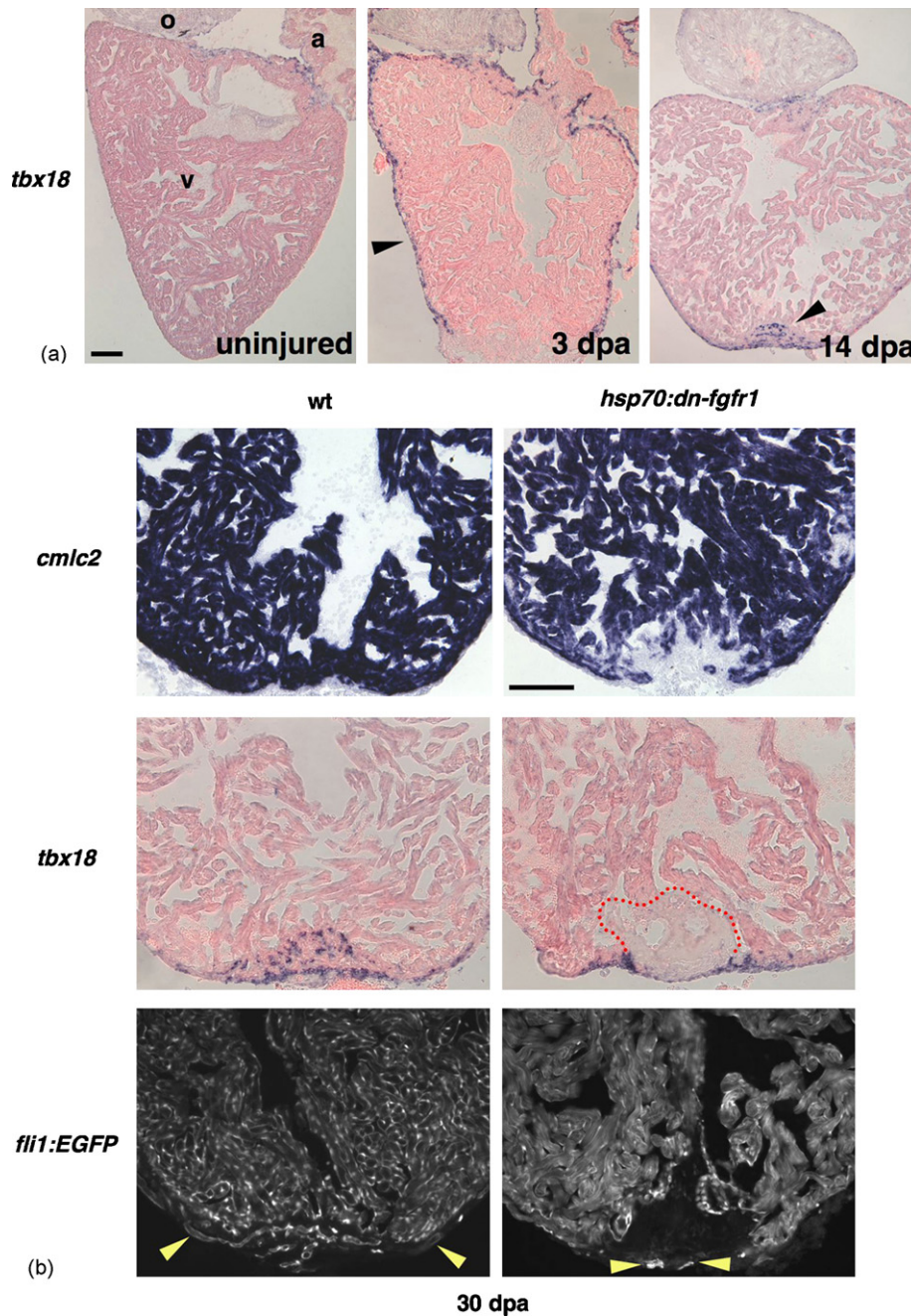


Fig. 4. Epicardial support of regeneration. (a) Expression of the embryonic epicardial marker *tbx18* (arrowheads) is induced in the adult ventricular and atrial epicardium after apical injury. By 14 dpa, developmentally activated epicardium is localized to the injury. (b) Fgf signaling through Fgf receptors, localized to epicardial-derived cells (not shown), is required for normal epicardial cell invasion and myocardial regeneration. (Top) Blockade of Fgf signaling by use of *hsp70:dn-fgfr1* transgenic zebrafish arrests myocardial regeneration, assessed by *cmc2* expression. (Middle) Instead of integrating into the regenerate as in wildtype fish, *tbx18*-positive epicardial cells accumulate at the apical edge of the transgenic wound. Clot material is outlined in red. (Bottom) Wildtype regenerates are well-vascularized (region within arrowheads), as visualized by the endothelial cell reporter strain *flt1:EGFP*. By contrast, *hsp70:dn-fgfr1* transgenic wounds developed little or no organized endothelial structures in the vicinity of muscle. Scale bar = 100 μ m. Figure adapted with permission from Ref. [34].

of the embryonic epicardium like *tbx18* and *raldh2* within 1–2 days of resection [34]. Both cardiac chambers are enveloped by this activated epicardium, which covers the injured apex within 7–14 dpa (Fig. 4a). A subpopulation of cells from the overlying epicardium then invades the regenerating tissue (Fig. 4a and b), a process highly reminiscent of epicardial behavior during embryonic heart development, during which epicardial cells undergo epithelial-to-mesenchymal transition (EMT), invade the underlying subepicardial space and myocardium, and contribute endothelial and smooth muscle cells to new coronary vessels [43,44]. The epicardial-derived cells in the regenerate appear to possess a similar role, as the regenerate becomes heavily vascularized by 14 dpa (Fig. 4b) [34].

This response of the zebrafish epicardium provokes several questions. For instance, how is the entire epicardium, including that surrounding the atrium, activated by resection of the ventricular apex? Also, how does the epicardium target the injury, and to what extent is this response required for myocardial regeneration? Lepilina et al. addressed these latter questions by examining participation of the Fibroblast growth factor (Fgf) signaling pathway during regeneration [34]. Fgfs have been shown to promote EMT in cultured epicardial cells, and thus presented a candidate for recruiting epicardial cells to the site of regeneration [45]. In support of this idea, the investigators found that an Fgf ligand, *fgf17b* was induced in the myocardium during regeneration, while Fgf receptors *fgfr2* and *fgfr4* were expressed in what appeared to be epicardial cells invading the regenerate. To test the idea that Fgfs target the epicardial cells to the regenerate, they used an inducible transgenic line that allowed heat-activated expression of a dominant-negative Fgf receptor [46]. In animals undergoing Fgfr inhibition after ventricular resection, myocardial regeneration arrested by ~14 dpa, and associated with the defect were both a failure of epicardial EMT and neovascularization of the regenerate (Fig. 4b). These results indicate that the epicardial response and formation of coronary vessels are required to maintain regeneration by progenitor cells. The authors proposed that Fgf signaling acts to support regenerating progenitor cells by directing the neovascularizing activity of developmentally activated epicardial cells. Certainly, epicardial cells may have additional functions during regeneration, and it will be fascinating to understand those functions as well as those of other cardiac cell types in future studies that functionally dissect zebrafish heart regeneration.

7. Molecular genetic approaches to regeneration in zebrafish

The ability to observe CM division in vitro, as well as the potential for grafting and lineage studies shown beautifully in studies of appendage regeneration [24,33,47], represent strengths of the amphibian model systems for studying heart regeneration. In fact, these approaches would be more difficult to apply in much smaller, fully aquatic adult zebrafish.

The strengths of the zebrafish system for approaching regeneration lie in its amenability to molecular genetic approaches, a feature not readily available in amphibian model systems. Thus, the molecular mechanisms of zebrafish heart regeneration are

beginning to receive attention. As mentioned above, the use of an inducible transgenic approach was effective in identifying a requirement for Fgf signaling during heart regeneration [34]. An earlier study found an induction of *notch1b* receptor mRNA and *deltaC* ligand mRNA in the endocardial cell layer of the injured heart, suggesting roles for Notch signaling that require functional confirmation [30]. Lien et al. recently examined the gene expression profiles of heart regeneration at 3, 7, and 14 dpa using Affymetrix microarrays [48]. Several genes differentially expressed at these times were identified, predicted to be involved in wound healing, tissue remodeling, or CM hyperplasia. Interestingly, by comparing heart regeneration gene expression profiles with those obtained from regenerating fin tissue, the authors suggested the existence of a set of “regeneration core molecules”. The authors focused their attention on two members of the Platelet derived growth factor (Pdgf) family, Pdgf-a and Pdgf-b, which showed increased expression after injury by microarray and/or RT-PCR. Pdgf treatment was shown by others to increase DNA synthesis in cultured newt CMs [49], and the authors found a similar effect on isolated zebrafish CMs, supporting the idea that Pdgf is an injury-activated CM mitogen. Future studies are thus likely to focus on in vivo functions of Pdgf. This study illustrates how microarray and in situ hybridization screens can yield many candidate genes during heart regeneration.

Multiple recent studies have employed forward genetics to identify genes essential for regeneration of the caudal fin, using ethylnitrosourea, an alkylating point mutagen, to mutagenize the adult male germline. In 1995, Johnson and Weston pioneered this approach, showing that (1) screens for temperature-sensitive (ts) mutations can be fruitful in zebrafish and (2) these screens can identify mutants with conditional defects in appendage regeneration [50]. One assumption of this type of screen is that many genes important for regeneration also have earlier roles in ontogenetic development; hence, the employment of a temperature change to identify conditional alleles. Importantly, this screen is designed to be inclusive, able to also identify strong, non-ts mutations in those hypothetical regeneration-specific genes, or non-ts alleles that affect only regeneration in genes that function in both embryogenesis and regeneration. A later mutagenesis screen found additional mutants, and, with the establishment of genetic markers and progress of genome sequencing efforts, the disrupted genes were readily mapped to chromosomal loci [32]. Positional cloning revealed the affected genes for four of these mutants: (1) *mps1*, a cell cycle regulator essential for the mitotic checkpoint [32], (2) *sly1*, involved in intracellular vesicular trafficking [51], (3) *hsp60*, a chaperone essential for the response to stress [52], and (4) *fgf20a*, one of many zebrafish Fgfs [53].

Can forward genetics be applied to discover new genes essential for heart regeneration? The best genetic screens identify mutations affecting a consistent and easily observed trait. By contrast, there are several hurdles to overcome in creating an effective screen for new heart regeneration mutants. First, the current resection injury model lacks precision, given that the target is tiny, it is contracting, and that ventricular size and shape varies from animal to animal. Also, when compared to fin regeneration, heart regeneration requires a great deal of

time, 1–2 months, to follow to approximate completion. Third, assessment of regeneration is tedious, requiring histological processing of the entire ventricle. Finally, while fin regeneration mutants can be visually assessed without invasive procedures, heart regeneration mutants would definitively be identified only after sacrificing the animal. Thus, founder lines would need to be established by siblings or by animals saved from a previous generation. In fact, one might expect that many heart regeneration mutants would fail to survive, given that the heart, unlike the fins, is an essential organ. These technical disadvantages are by no means impenetrable, but deserve consideration when designing forward genetic screens. It is interesting that two of the four genes shown to be essential for fin regeneration were also found to be critical for heart regeneration [25,52]. Therefore, a subset of heart regeneration mutants can be obtained through secondary screening of fin regeneration mutants.

The use of transgenic reporter strains should facilitate directed forward genetic screens for heart regeneration mutants. For instance, the *fil:EGFP* marks endothelial cells and has been used to identify mutants in vascular development [54,55]. Similarly, a “gut” GFP strain that marks the liver, pancreas, and intestine has been employed to reveal new mutations that disrupt development of these normally concealed structures [56]. The appropriate fluorescent reporter strains could potentially enhance identification and/or characterization of heart regeneration mutants.

To supplement inducible transgenesis targeting candidate genes and forward genetics, heart regeneration can potentially be dissected through antisense technology and chemical genetics. For antisense approaches, most zebrafish embryologists employ morpholinos, which are stable, modified oligonucleotides complementary to start or splice sites that block translation or splicing [57]. Morpholinos have also been delivered to the regenerating spinal cord or fin with some effects [58,59]. Whether morpholinos can be used to knock down adult cardiac gene expression is unknown. A more commonly used antisense technology for other model systems is RNA interference, which takes advantage of the cellular machinery that generates microRNAs [60]. However, RNA interference was not immediately effective in zebrafish [61] and has yet to be adopted by zebrafish researchers. Interfering RNAs have an advantage over morpholinos in that they could potentially be expressed behind a tissue-specific or inducible promoter as part of a transgenic construct to knockdown expression in adult fish. With respect to chemical genetics, approaches relevant to regeneration have already been performed. Myoseverin was identified from a library of purine compounds for its ability to initiate cleavage of cultured, multinucleate myotubes into proliferative, mononuclear cells [62]. Relevant to cardiovascular biology in zebrafish, chemical modulators of heart rate [35] and vascular development [63] have been identified. While chemical genetics may seem a tedious way to dissect cardiac regeneration, it is possible that its combination with transgenic reporter strains would be fruitful. For instance, an injected compound, analogous to a mutation, may block expression of a regeneration marker in an injured fish, or even induce expression of a regeneration marker in an uninjured animal. An entire library could be screened in

such a way to find those compounds that enhance or inhibit some aspect of regeneration.

8. Application to heart regeneration in mammals

There are obvious applications for information gathered from zebrafish heart regeneration studies. After just a small number of studies, we now know that both zebrafish and mammalian adult hearts contain epicardial tissue and cardiac progenitors; yet, only zebrafish have found ways to activate and utilize these tissues toward successful regeneration [34]. These results point for the first time to the adult epicardium as a potential source of new vascular tissue that may aid survival and regeneration in the injured mammalian heart. Furthermore, we can definitively implicate Pdgf and Fgf signaling pathways as positive influences on regeneration, findings that will stimulate their manipulation in mammalian MI models [34,48]. In fact, a very recent study found that slow release of Fgf in the injured rodent heart can increase vascularization and reduce infarct size [64]—a result that is predicted from zebrafish studies. As the field expands and matures, we will learn much more from zebrafish that can be applied to mammalian models and even humans.

It is reasonable to point out that there are numerous differences in the biology of teleosts and mammals, as well as specific differences in CM cellular structure and anatomy, all of which might contribute to regenerative capacity. Unlike mammals, zebrafish can grow throughout most of adulthood, a phenomenon called “indeterminate growth”. In fact, their growth can be affected markedly by changes in nutrition and population density [65]. It is thus possible that the capacity to rapidly replace cardiac tissue in teleosts has been retained in evolution as a function of the need for rapid cardiac growth during adult growth phases.

Also, there are differences in cardiac biology between mammals and teleosts. For instance, the zebrafish ventricle has a thin wall of compact muscle surrounding a much larger compartment of myofibers organized into elaborate trabeculae. It is intriguing that this structure is very similar to that of the embryonic mammalian ventricle prior to its septation and fusion of trabecular myofibers into a thick, vascularized wall muscular wall [66]. That the mammalian heart has a more differentiated, contractile anatomy is apparent not only in gross cardiac structure, but also in cellular features. Teleost CMs are 2–10 times smaller, mononucleated, have a greatly reduced sarcoplasmic reticulum, and lack the T-tubule system found in skeletal muscle and mammalian cardiac muscle [67]. At a glance, it would appear that the teleost heart is better designed for growth and regeneration, while the mammalian heart better designed for sheer contractile force. Nevertheless, none of the mentioned differences between lower and higher vertebrate hearts preclude the idea that the mammalian heart is capable of being stimulated to regenerate, especially if that regeneration results from undifferentiated progenitor cells.

One interesting concept that has emerged from initial findings is that regeneration and fibrosis are competing events in the vertebrate heart. That is, if there is a capacity for injury-stimulated CM hyperplasia beyond a certain threshold, regenerative mechanisms can overcome scarring. Results consistent with this idea

came from experiments using a hypomorphic mutant in the cell cycle checkpoint kinase *Mps1* [25]. As mentioned earlier, *mps1* mutants were initially identified as having a ts defect in caudal fin regeneration. Serendipitously, *mps1* mutants also showed defects in cardiac regeneration at the restrictive temperature. Instead of regenerating muscle in response to ventricular resection injury, *mps1* mutants repaired wounds by forming large injury scars. These results indicate that even vertebrates with high cardiac regenerative capacity have a default scarring mechanism; normally, regeneration somehow restricts this pathway. The implication is exciting: perhaps by stimulating regeneration in a poorly regenerative system like the mammalian heart, scarring events characteristic of MI would be restricted by new muscle formation.

9. Concluding remarks

Our understanding of cardiac regeneration in zebrafish is still limited, but the motivation for moving forward is clear. Indeed, there is no question that what is learned about natural cardiac regeneration in zebrafish will continue to provide insights into regenerative failures in humans, and possible therapies. I close by summarizing a few different ways by which we must broaden our understanding of zebrafish heart regeneration.

First, how zebrafish cardiac tissue responds to an infarction injury is a critical, unanswered question. Multiple injury models should be examined in attempts to parallel human MI; i.e. models that focally destroy myocardium without its removal. Second, optimal culture conditions for adult zebrafish cardiac cells should be established, so that in vitro regenerative paradigms or chemical genetic strategies may be explored. Third, genetic lineage tracing tools (e.g. the Cre-loxP system) that have been so successful for elucidating progenitor cell biology in mammalian systems must be introduced to the zebrafish system. Combined with the identification of additional molecular markers, these experiments would define the cellular origin of regenerated myocardium. Fourth, although the tools for the appropriate genetic screen may not yet be available, innovation and creativity should yield a screening strategy that reveals mutations detrimental to cardiac regeneration. As mechanisms of heart regeneration in zebrafish achieve higher cellular and molecular definition, increasingly detailed comparisons with the non-regenerative mammalian heart will be made, and attempts to equalize our own cardiac regenerative capacity may be within grasp.

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