Published in final edited form as:

Drug Discov Today Dis Models. 2007 ; 4(4): 219–225. doi:10.1016/j.ddmod.2007.09.002.

Zebrafish Heart Regeneration as a Model for Cardiac Tissue Repair

Robert J. Major and Kenneth D. Poss

Department of Cell Biology, Duke University Medical Center, Durham, NC 27710 USA

Abstract

Heart disease remains the leading cause of mortality throughout the world. Mammals have an extremely limited capacity to repair lost or damaged heart tissue, thus encouraging biologists to seek out models for heart regeneration. Zebrafish exhibit a robust regenerative capacity in a variety of tissues including the fin, spinal cord, retina, and heart, making it the sole regenerative vertebrate organism currently amenable to genetic manipulation. Future studies will utilize functional approaches to tease apart zebrafish heart regeneration in hopes of unlocking our own regenerative potential.

Introduction

Tissue regeneration has fascinated biologists for centuries. Despite this interest, the cellular and molecular events driving the recovery of lost tissues are poorly understood. At the root of many of these events lie potent progenitor or stem cells capable of reconstituting cell populations of the newly regenerated tissue. This potential, along with the isolation of these progenitor cells from a wide variety of tissues, has impelled the field of regeneration with hopes of future uses in medicine.

Although robust replacement of structural cells has been discovered in mammalian tissues, such as the liver, blood, and skin, most tissues do not share this remarkable ability[1–4]. Perhaps most prominently, the mammalian heart is incapable of significant regeneration following an injury such as an acute myocardial infarction[5,6]. Loss of oxygenation to ventricular muscle, usually due to occlusion of a coronary artery, will result in necrosis of that tissue. Too often, these injuries result in immediate death. Those fortunate to survive a myocardial infarction replace lost muscle with a scar, and typically are susceptible to compensatory pathology and/or future infarctions. Unlike the mammalian heart, the injured zebrafish heart normally undergoes minimal scarring[7]. Instead, a transient fibrin clot is replaced with new contractile muscle (Fig. 1). This review will focus on recent progress in the field of cardiac regeneration with an emphasis on the zebrafish model system.

Mammalian and Amphibian Heart Regeneration

As discussed above, mammalian species have little or no ability to replace lost cardiac muscle. This poor regenerative capacity is due in part to the failure for adult cardiomyocytes to undergo proliferation[8]. It is possible that the proliferation of adult cardiomyocytes may be

Corresponding Author: Kenneth D. Poss (k.poss@cellbio.duke.edu), 919-681-8457 (phone) 919-684-5481 (fax).

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therapeutically stimulated. In support of this, recent reports have shown that although adult mammalian cardiomyocytes show very little or no proliferation when cultured, FGF1 treatment concomitant with p38 MAP kinase inhibition can stimulate their proliferation in culture[9, 10]. In other studies, the discovery of putative progenitor cells within the hearts of adult mammals has led to the suggestion that the heart has the potential for homeostatic or regenerative renewal[11–15]. Yet, it is unclear why such progenitor cell populations are incapable of responding to injury with replacement by new cardiac muscle. Future therapeutic advancements may aim to “switch” on the appropriate programs within these progenitor cells or their environment and stimulate such replacement.

Urodele amphibians, due to their robust capacity to regenerate a wide variety of tissues, including spinal cord, brain, limb, retina, and lens, have historically been a key model system driving regeneration research[16–18]. Unlike adult mammalian cardiomyocytes, newt cardiomyocytes will readily undergo proliferation in tissue culture, thus representing one possible key difference that reflects regenerative capacity[19,20]. However, although newt heart regeneration does occur to some extent after partial resection of ventricular myocardium, there is only a modest level of tissue replacement that accompanies scarring[21–23]. One suspected conduit for amphibian regenerative success is dedifferentiation, the process by which cells within injured tissue lose the expression of key genes necessary for function of that particular cell type (e.g. contractile genes for cardiac muscle). This functional reduction is thought to reprogram the cells, facilitating proliferation or differentiation to other cell types (transdifferentiation) in support of tissue regeneration. Newt lens, tail, and limb have been shown to undergo dedifferentiation during regeneration[24–26]. Recent data has shown that newt cardiomyocytes have an ability to lose contractile gene expression rapidly following a mechanical injury to the heart[27]. Therefore, regeneration of the urodele heart may be accomplished, at least partially, through dedifferentiation events.

Teleost Heart Regeneration

A combination of forward genetic screens, large clutch size, and external development has made the zebrafish a popular model system for ontogenetic development[28]. In particular, our understanding of heart development has benefited greatly from zebrafish mutants that specifically disrupt cardiovascular form and function[29–31]. Genetic approaches also make zebrafish a favored model system for studying tissue regeneration[32]. Indeed, it remains the only laboratory model system that is both amenable to genetic manipulation and capable of carrying out a robust regenerative response after the loss of complex tissue[7,33–37].

After acute myocardial infarction in mammalian hearts, fibrin deposition occurs at the injury site. Then, fibrin is replaced by scar tissue, thought to be permanent[5,6]. In zebrafish, after surgical removal of up to 20% of the ventricle by iridectomy scissors, an initial fibrin clot forms at the wound site. Unlike in mammal infarcts, the fibrin clot is subsequently replaced with new cardiac muscle in a process that takes 1–2 months (Fig. 1)[7].

Zebrafish heart regeneration proceeds through injury-induced proliferation of cardiomyocytes. For instance, BrdU labeling studies revealed labeled cardiomyocytes along the leading edge of the regenerating heart[7]. However, it had not been known until recently whether these BrdU-positive cardiomyocytes result from proliferation of differentiated cardiomyocytes, proliferation of progenitor cells forming new heart tissue, or dedifferentiation, proliferation, and redifferentiation of spared cardiomyocytes. Recent work has tried to address these questions through *in vivo* developmental timing assays that employed double transgenic zebrafish strains carrying reporter constructs for the *cmlc2* (*cardiac myosin light chain 2*) promoter[38]. In these experiments, transgenes for *EGFP* and *nuclear-DsRed2*, both simultaneously driven by separate *cmlc2* promoters, reported the contractile state of cardiac

cells. Because GFP folds and fluoresces more rapidly than DsRed2, any cardiac progenitors that begin to turn on the *cmhc2* promoter, and thus change their contractile status, will be transiently marked as GFP-positive/DsRed2-negative in nascent myocardium[39–42]. Indeed, this was the case in the double-transgenic embryo, as GFP fluorescence is observed up to a day prior to DsRed2 fluorescence within developing cardiomyocytes. Within five days of ventricular resection, a front of GFP-positive/DsRed2-negative cardiomyocytes was found at the apical edge[38]. This suggests that regenerating myocardium matures from undifferentiated, *Cmhc2*-negative, progenitor cells at the leading edge. In support of this mechanism, this leading edge was found to express a variety of molecular markers for the embryonic heart field, such as *hand2*, *tbx20*, and *nkx2.5*, suggesting that these cells acquire cardiac fate reminiscent of cardiac progenitors that provide the first cardiomyocytes of the developing embryo (Fig. 1).

It is not known from what source(s) these apparent progenitor cells are derived. One possibility is that dedifferentiation of mature cardiac cells supply the regenerate with new progenitors. Although this possibility was examined by developmental timing assays similarly as above and was not witnessed, it cannot be ruled out entirely[38]. Lineage analyses will be necessary to determine possible contributions of dedifferentiation. For example, experimentally pulsing the expression of a permanent genetic marker within mature cardiomyocytes just before injury will help determine whether new muscle cells are derived from existing cardiomyocytes during regeneration.

The Role of the Epicardium During Zebrafish Heart Regeneration

Recent results have shed light on the role of the outer non-muscle layer of the heart, the epicardium, in cardiac regeneration. During embryogenesis, the epicardium migrates out as a sheet from the proepicardium, a cluster of mesoderm-derived cells near the liver primordium and the septum transversum, to envelop the developing myocardial tube[43]. Following this encasement, some epicardial-derived cells undergo an epithelial-to-mesenchymal transition (EMT) into the subepicardial space and invade the myocardium to contribute endothelial and smooth muscle tissue of the coronary vasculature[44,45].

Interestingly, adult zebrafish epicardial cells retain this plastic behavior. Perhaps the most striking aspect of the zebrafish epicardium during regeneration is that it undergoes an extremely rapid and dynamic response to injury[38]. Its developmental activation can be assayed by *raldh2* and *tbx18* expression, two genes known to be expressed within the embryonic epicardium (Fig. 2)[46,47]. As early as six hours post-ventricular resection, *raldh2* expression initiates within the epicardium of the outflow tract and atrium and then follows within the ventricle by 24 hours. In addition, *tbx18* expression is observed within the atrial and ventricular epicardium by 1–2 days. Later on, cells positive for *tbx18* and/or *raldh2* appear at the wound site. This organ-wide response is intriguing for multiple reasons. First, the response is extremely rapid. The molecular details that connect the injury to this early response are unknown but are of interest, as their understanding will shed light on how heart regeneration is initiated. Second, the response is seen first distant from the wound site, within the atrium. The significance of this distal activation and how this signal is transferred over these distances are unknown, but are of interest to the field.

After ventricular resection induces developmental gene expression and proliferation within the epicardial layer, activated epicardial cells soon surround the wound with a portion of them penetrating several cell layers deep into the wound and regenerating muscle. Concomitantly, the new myocardium is substantially vascularized[38]. Thus it is assumed that the epicardial cells have similar roles as in the embryonic heart; that is, as a progenitor tissue that contributes smooth muscle and/or endothelial cells during neovascularization.

In the regenerating zebrafish heart, Fgf receptors 2 and 4 are expressed in epicardial or epicardial-derived cells at or near the injury site, which was shown to express at least one Fgf ligand. Furthermore, signaling by Fibroblast growth factors (Fgfs) is necessary for epicardial cell activity during regeneration, as ectopic expression of a dominant negative transgene that inhibits signaling through Fgf receptors disrupts this invasion of epicardial-derived cells, arresting regeneration (Fig. 3). This stimulatory role for Fgf signaling in adult zebrafish epicardial cells *in vivo* appears to mirror its effects on cultured epicardial cells in *in vitro* EMT assays[48]. Together, these data indicate a specific role for Fgf signaling in directing the EMT of epicardial-derived cells that ultimately vascularize the regenerate. Interestingly, treatment of injured rodent hearts by Fgf supplementation along with p38 MAP kinase inhibition stimulated neovascularization, decreasing infarct size and the level of scarring (Fig. 3)[9,10]. These results suggest that both mammalian and non-mammalian vertebrates are responsive to Fgfs after injury as a means to increase neovascularization, but only selected species like zebrafish naturally utilize Fgfs to support myocardial regeneration. It is likely that additional factors will emerge that affect the production of coronary vessels in both zebrafish and mammalian models.

Other recent studies support the idea that zebrafish have naturally optimized regenerative machinery that can function in both non-mammalian and mammalian injured hearts. The G-actin sequestering protein, Thymosin- 4, induces outgrowths from mammalian epicardial explants *in vitro* (Fig. 2)[49,50]. Treatment of epicardial explants with Thymosin- 4 induces the differentiation of fibroblasts, endothelial, and smooth muscle cells as assessed by gene expression and cellular morphology (Fig. 2). In addition, *in vivo* Thymosin- 4 treatment can partially restore cardiac survival and function following coronary ligation in the mouse heart [51]. Notably, during zebrafish heart regeneration, Thymosin- 4 expression is induced in the wound and compact myocardium, indicating that fish naturally release this epicardial stimulant upon injury (Fig. 2)[52].

Genetic Approaches to Heart Regeneration in Zebrafish

Genetic approaches in zebrafish have revealed a large number of mutants affecting embryonic development. Most of these mutants exhibit embryonic or larval lethality, thus making it impossible to assess roles for specific genes in adult tissue regeneration, unless such mutations have effects in heterozygous animals. Investigators have circumvented this issue in a small number of studies by searching for conditional (temperature-sensitive) or hypomorphic alleles of genes that may be important for regeneration. Unfortunately, the idea of a direct screen for heart regeneration mutants comes with many challenges. Transgenic fish allowing inducible ectopic expression of a wild-type gene or a dominant-negative construct have been used recently and give similar benefits as conditional mutants[7,38,52,53]. Future studies are also likely to take advantage of lineage tracing tools that have been utilized in mice for progenitor cell studies, to define progenitor/progeny relationships during heart regeneration[54–57].

It will be important to examine new cardiac injury models in zebrafish. The current models produce a mechanical injury[7]. While this injury might indeed stimulate the strongest possible regenerative response, it will also be interesting to better mimic the infarct models utilized in mammals. A recent study used the reducing power of Nitro-reductase (NTR), expressed by a tissue-specific promoter, to convert non-toxic metronidazole (Mtz) into a cytotoxic substance in transgenic zebrafish[58]. This approach facilitated cardiac lesions in embryonic zebrafish under temporal control determined by Mtz addition, and may have additional uses in studies of adult heart regeneration[58]. For instance, one could create patches of lesioned myocardium, or specifically ablate different cardiac cell types to address their roles in regeneration. Potentially, the NTR/Mtz system or other genetic toxins might be adapted to expedite a genetic screen for heart regeneration mutants.

Conclusions

Within the past few years, we have witnessed a growing interest in regeneration biology. Amphibians, mammals, and teleosts have different capacities to repair lost cardiac tissue, and it is from these differences we can learn best how to minimize scarring and maximize regeneration after injury. Zebrafish represent a highly useful system because of the combination of available genetic tools and a robust regenerative capacity. Through continued studies of zebrafish heart regeneration, we stand to learn how cardiac progenitor cells are successfully utilized for regeneration, and how the epicardium is optimally activated and directed to support this regeneration. Additional molecular studies in the future will increase the resolution of regenerative pathways and bring the idea of cardiac regeneration in humans closer to reality.

Acknowledgements

We thank Felix Engel, Ellen Lien, and Paul Riley for original figure panels, and the National Heart, Lung, and Blood Institute, the American Heart Association, the Whitehead Foundation, and the Pew Charitable Trusts for funding our research on heart regeneration.

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Figure 1. Regeneration of the zebrafish heart

(a) Images of a regenerating zebrafish ventricle following 20% ventricular resection. Tissues were stained for myosin heavy chain to identify cardiac muscle (brown) and aniline blue to identify the fibrin clot (blue). By 7 dpa, the wound is sealed by fibrin and is replaced by cardiac muscle by 30 dpa. (b) Proliferation, based on BrdU incorporation, is activated in cardiomyocytes by 7 dpa. The ventricular wall is restored by proliferation at the leading edge of the regenerating tissue. (c) Expression of embryonic heart field markers, *hand2*, *nkx2.5*, and *tbx20* at the apical edge of the regenerate (brackets). Reproduced with permission from Refs. [7,38].

Figure 2. Participation of the epicardium during regeneration

(a) Expression of the embryonic epicardial markers, *raldh2* (top) and *tbx18* (bottom) are induced in the adult epicardium after ventricular resection. Expression of both genes becomes localized to the wound by 14 dpa. The outflow tract (o), atrium (a), and ventricle (v) are labeled accordingly. (b) Radioactive *in situ* hybridization for Thymosin- 4 in sham operated control and amputated hearts 3 and 7 dpa. Expression of Thymosin- 4 is upregulated in regions surrounding the wound and the compact myocardium following injury. (c) Adult mouse heart explants cultured *in vitro* show increased migration of epicardial cells (confirmed by staining for epicardin – data not shown here) following treatment with Thymosin- 4. These cells then begin to express markers for smooth muscle cells (SM A – smooth muscle alpha-actin), fibroblasts (procollagen type I), and endothelial cells (Flk-1). Reproduced with permission from Refs. [38,50,52].

Figure 3. Fgf signaling is necessary for zebrafish heart regeneration and cardioprotection in rat hearts. Myocardial infarction was induced and treated *in vivo*

(a) Trichrome stains on heart sections to indicate scar tissue (blue). Sections shown were taken from base to apex. FGF1, p38 MAP kinase inhibition (p38i), and combined treatment significantly reduced the level of scar tissue formed after two weeks. This result is depicted graphically in panel (b). (c) *cmlc2* expression at 7, 14, and 30 dpa in control and *hsp70:dn-fgfr1* zebrafish. Treated animals failed to complete regeneration leaving a large wound by 30 dpa. Reproduced with permission from Refs. [10,38].