Zebrafish as a Model for Studying Cardiac Regeneration

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Abstract Heart failure caused by cardiomyocyte loss after ischemic tissue damage is a leading cause of death worldwide. Although adult mammalian cardiomyocytes are able to proliferate during normal aging and after infarction, they do so at insufficient rates for effective cardiac regeneration. Likewise, none of several described cardiac stem cell populations appear to form significant numbers of cardiomyocytes in ischemic hearts. Thus, adult mammals cannot regenerate heart injuries. Zebrafish, on the contrary, are able to regenerate multiple organs including amputated fins, lesioned brain, retina, spinal cord as well as the heart. In response to injury, spared zebrafish cardiomyocytes re-enter the cell cycle, proliferate and form new cardiomyocytes robustly, resulting in full morphological and functional recovery. Since zebrafish heart regeneration was first described about a decade ago, this model has significantly improved our knowledge about the underlying mechanisms of natural heart regeneration. Here, we describe different heart injury techniques and review our current knowledge about cellular and molecular mechanisms of regeneration, with a focus on factors regulating cardiomyocyte proliferation.

Keywords Zebrafish · Heart regeneration · Cardiomyocytes · Cell proliferation · Model organism

Introduction

Myocardial infarction (MI), typically caused by the blockage of coronary arteries and ensuing ischemic tissue damage, is a leading cause of death worldwide. Although other mammalian organs possess some regenerative capacity, lost mammalian cardiomyocytes (CMs) can largely not be replaced after MI. Infarction thus results in muscle death, irreversible scarring and eventually heart failure. In contrast, natural cardiac regeneration after injury appears to be a conserved feature among lower vertebrates such as zebrafish and salamanders, but also occurs in neonatal mice (until postnatal day 7). In these organisms, lost CMs are replenished by proliferation of spared CMs as described in more detail below [1•, 2••, 3–6]. Adult mammalian heart regeneration by CM proliferation does not naturally occur, and it has indeed been a long-held belief that CMs are terminally differentiated and thus unable to re-enter the cell cycle in adult mammals (reviewed in [7]). However, recent studies taking advantage of inadvertent radioactive isotope pulse-chase labeling of cells as a consequence of atmospheric nuclear bomb testing in the 1950s and 1960s have shown that new CMs form at a low but measurable rate in humans throughout their lifespan (about 1 % annual turnover at the age of 25–0.45 % at the age of 75) [8•]. Likewise, lineage-tracing approaches in the adult mouse heart have revealed that pre-existing CMs are the dominant source of CM replacement during normal aging as well as CM replenishment after MI via cell division [9••]. The rate of natural adult CM proliferation, however, is too low for effective cardiac regeneration after MI [9••].

A number of studies have demonstrated that addition of exogenous factors such as neuregulin 1 or several microRNAs significantly induces CM proliferation in culture

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[10–12]. Although in vivo administration of these factors reduces infarct size and improves functional recovery after infarction, it is difficult to imagine that these effects could be attributed to the minimal elevation of CM proliferation rates seen in vivo (typically less than 1 % of CMs were proliferative in these studies). Furthermore, with many markers commonly used in cell proliferation studies, it is difficult to ascertain whether those CMs actually productively divide and do not only undergo hypertrophy (reviewed in [7]). Thus, genetic lineage-tracing experiments would be required to show that a significant number of new CMs are actually produced from existing ones in vivo in response to the treatments described above.

Nevertheless, such results have fostered the hope that it could be possible to therapeutically induce adult mammalian CM proliferation. This idea has recently been strengthened by Martin and colleagues who identified Hippo signaling as an endogenous inhibitor of adult mouse CM proliferation [13]. Efficient deletion of Hippo components (*Salv* and *Lats1/2*) in CMs at 3 months of age promotes adult CM renewal via cell cycle re-entry and progression through mitosis and cytokinesis. In addition, removal of Hippo components at P7 (a time at which natural cardiac regeneration via CM proliferation is severely compromised in mice [3]) followed by apical resection at P8 results in significant muscle regeneration from spared CMs and improved functional recovery [13]. It is therefore reasonable to speculate that a subset of adult mammalian CMs retains the ability to proliferate and can potentially be stimulated by removal of natural inhibitors and/or addition of factors promoting proliferation. Identification of such factors will be aided by a thorough understanding of the mechanisms underlying natural heart regeneration. Owing to its robust regenerative capability and its propensity for genetic manipulation (reviewed in [14]), zebrafish has developed rapidly over the past years as an important heart regeneration model. In this review, we summarize recent discoveries concerning the mechanisms of zebrafish heart regeneration with a focus on CM proliferation.

Injury Models in Zebrafish Heart Regeneration

The ability of zebrafish to fully regenerate the adult heart was first reported by Poss and colleagues in 2002 [5]. After surgical removal of around 20 % of the apex of the ventricle, a fibrin-rich clot seals the injury within days. In contrast to mammalian hearts after MI, little or no collagen deposition is found 1 or 2 months after injury, indicating that permanent scarring does not occur. Rather, the damaged area appears to be completely replaced by new muscles because of elevated CM proliferation, which will be discussed in more detail later [5]. Apical resection has

since been the most frequently used injury model, but recent studies have suggested new ways to damage the heart, which better mimic aspects of mammalian cardiac injury.

Cryoinjury

In contrast to most mammalian heart injury models, apical resection does not cause widespread tissue death, and thus its value as a clinically relevant model has been questioned. Hence, cryoinjury has been employed in zebrafish as an alternative to better mimic certain aspects of MI [15•, 16•, 17•]. In these models, either a small piece of dry ice [17•] or a thin metal filament cooled by liquid nitrogen [15•, 16•] is applied to the surface of the ventricle to cause a sudden change of temperature, which results in extensive cell necrosis. Fish can tolerate cryoinjury-induced death of approximately 25 % of the ventricle. Complete regeneration is observed between 60 and 130 days post-injury (dpi). Immune cells that are possibly responsible for clearing cellular debris and necrotic cells from the affected area infiltrate the involved myocardium after cryoinjury [17•]. This might explain, at least in part, the longer time required for cryolesioned hearts to fully regenerate compared to resected hearts, which need only about 30 days. Nevertheless, other cellular responses are basically identical between the two models including elevated CM proliferation and reactivation of developmental gene programs in the epicardium and endocardium [18•, 19]. One intriguing feature of the cryolesioned heart is that the affected area does show signs of collagen deposition consistent with scar formation starting as early as 3 dpi; however, this is progressively resolved during the course of regeneration, resulting in scar-free recovery [15•, 16•]. This implies that scarring could be reversible and does not necessarily inhibit CM proliferation and regeneration as has been suggested in the mammalian heart. Interesting questions for future research include whether the transient scars observed in zebrafish differ in their cellular composition from those observed in the mammalian heart and what molecular mechanisms assure scar resolution.

Genetic Ablation

Mechanical zebrafish heart injury models (apical resection and cryoinjury) not only cause the loss of CMs, but also other cardiac cell types as well. It is therefore unclear whether CM cell death alone is sufficient to trigger regenerative responses. Poss and colleagues addressed this question by generating an inducible CM-specific genetic ablation system, Z-CAT, which targets expression of diphtheria toxin a chain (DTA) to Cre-expressing CMs upon 4-hydroxytamoxifen (4-HT) induction [20]. This

system rapidly depletes around 60 % of total CMs and results in lethargy, severe stress hypersensitivity, gasping phenotypes and reduced swimming performance, which are classical signs of heart failure and are not seen in mechanical injury models. Despite significant muscle damage, cellular responses similar to other injury models and CM proliferation are rapidly initiated. Full muscle regeneration and functional recovery are observed around 30 and 45 days post-depletion, respectively [20].

While this genetic ablation system has the disadvantage of lacking a defined, localized injury area due to the stochastic nature of the induced cell death, it will be a valuable tool for high-throughput experimental approaches, and it will also enable studies on the limitations, if any, of zebrafish CM's proliferative and regenerative potential through repeated injuries.

Cellular Responses

Zebrafish heart injury, induced by any of the models described above, triggers organ-wide responses from all three primary layers of the heart, namely endocardium, epicardium and myocardium. In this part, we summarize the recent discoveries of various cellular responses.

Endocardium

Endocardial cells are the first to respond to heart injury after apical resection [18•]. As early as 1 hour post-injury (hpi), endocardial cells near the amputation plane, which typically possess elongated nuclei and thin cell bodies tightly adhering to CMs, appear to round up and detach from the CMs as seen by transmission electron microscopy (TEM). In addition, the retinoic acid (RA) synthesizing enzyme *aldh1a2* (*raldh2*) is upregulated in the whole endocardium at 3 hpi, which represents an intriguing organ-wide response to injury. From 1 dpi onwards, *raldh2* expression becomes confined to both endocardial cells near the amputation plane and to the entire epicardium, which is retained throughout the early stage of regeneration (until 14 dpi). Inhibition of RA signaling by overexpression of dominant-negative retinoid acid receptors or the RA-degrading Cyp26 enzyme significantly inhibits CM proliferation at 7 dpi, while agonist treatment (RA injection) does not induce CM proliferation. This suggests that RA is a permissive but not an instructive signal for CM proliferation and regeneration [18•]. However, how endocardial or epicardial RA signaling regulates CM proliferation has not been tested. Many open questions about the role of the endocardium in heart regeneration remain, including which signals trigger its activation, what is the significance of its organ-wide response and what roles it plays in the regulation of CM proliferation.

Epicardium

Epicardial cells respond to heart injuries by reactivating expression of genes associated with epicardial development, including *tbx18*, *wt1b* and *raldh2*, and they become proliferative from 3 dpi [16•, 17•, 18•, 19]. Genetic lineage-tracing experiments using inducible Cre recombinase driven by regulatory sequences of *tcf21*, an epicardial marker, revealed that *tcf21*-expressing epicardial cells give rise to perivascular cells but not CMs after injury [21•]. Mercader and colleagues supported this finding with transplantation experiments and in addition identified myofibroblasts as progeny of epicardial cells [22]. Thus, zebrafish and mouse epicardial cells appear to have similar potential, since reports of CM formation from epicardial cells during mouse development have recently been attributed to artifacts caused by ectopic expression of Cre drivers in CMs [23, 24].

Very little is known about the molecular roles of the epicardium during heart regeneration, in particular whether it acts as a source of factors regulating myocardial regeneration. A recent report by Poss and colleagues has shown that fibronectin, a main component of the extracellular matrix, is deposited by injury-activated epicardial cells [25]. Overexpression of a dominant-negative human fibronectin fragment significantly inhibits muscle regeneration and results in fibrosis at 30 dpi, while, surprisingly, CM proliferation is not affected. The same is the case for fibronectin (*fn1*) mutant zebrafish. Quantification of CM density at 30 dpi revealed that a higher number of CMs are present in the area flanking the injury in *fn1* mutants compared to heterozygous clutchmates, suggesting that fibronectin might be required for CM mobilization and integration into the injury site. However, the molecular mechanisms underlying the pro-regenerative effect of fibronectin have not been identified. Since fibronectin is deposited by injury-activated epicardial cells, it would be interesting to investigate its role in regeneration of epicardial cells and vascularization of the regenerate, which might in turn affect myocardial regeneration. Altogether, these data suggest that the epicardium promotes myocardial regeneration via a number of mechanisms.

Myocardium

For a period of time, it remained unclear whether the newly forming CMs are derived from existing differentiated CMs or other sources such as stem cells. After the Poss group had suggested a non-CM source for regenerating muscle in an earlier study [19], Belmonte and colleagues found that the resected myocardium is replaced mainly by cells derived from *cmhc2*-expressing CMs, using genetic lineage-tracing experiments with an inducible CM-specific Cre

driver (*cmhc2:CreERT2*) [1•]. However, in this study, Cre activity was induced during embryonic development. Although *cmhc2:CreERT2* expression is restricted to the heart during development, it is not entirely clear whether those labeled cells remain lineage-restricted until adulthood, and therefore this result needs to be interpreted with care. The Poss group, on the other hand, demonstrated that CMs labeled 5 days before resection in adult fish by *cmhc2:CreERT2* contribute to the vast majority of the regenerated myocardium [2••], in contrast to their earlier hypothesis. They also found that *gata4*, a cardiac transcription factor expressed in the developing heart, is re-expressed in a subpopulation of CMs in the compact myocardium near the wound from 3 to 7 dpi. Some of these *gata4*-expressing CMs are proliferative, and their descendants, genetically marked by inducible *gata4:CreERT2* at 5–7 dpi, contribute significantly to the regenerated myocardium [2••].

Overexpression of a dominant-negative form of Gata4, which is able to block wild-type Gata4 from interacting with its target sequences, in CMs prior to apical resection significantly impairs proliferation of CMs in the compact myocardium, while trabecular CM proliferation is not affected [26]. This results in inhibition of myocardial regeneration and scarring at 30 dpi, suggesting that *gata4*-regulated proliferation of the compact myocardium is crucial for successful regeneration. Thus, the zebrafish myocardium appears to regenerate from spared cardiomyocytes, which reactivate *gata4* expression after injury. However, whether all CMs can contribute to the regenerated tissue or whether only a subpopulation of CMs retains the ability to proliferate in adults requires further investigation.

It was reported that zebrafish CMs undergo dedifferentiation after apical resection as evidenced by disorganized sarcomeric structures [1•, 2••]. Belmonte and colleagues further suggested that dedifferentiation is the prerequisite of CM proliferation since no mitotic CMs display highly organized sarcomeric structures [1•]. Mammalian CMs are thought to dedifferentiate after infarction as well [27], which is associated with transcriptional changes, in addition to disorganization of sarcomeric structures, involving isoform switching of genes required for metabolism, sarcomeric components (e.g., loss of α -MHC and expression of β -MHC) and reactivation of developmental gene programs [e.g., *atrial natriuretic peptide (ANP)*, *destrin*, *runx1* etc.] (reviewed in [7]). Dedifferentiation thus might represent an injury response shared by mammalian non-regenerative and regenerative zebrafish CMs. More detailed studies into the molecular changes induced by injury in mammalian and zebrafish CMs will be essential to identify the determinants of regenerative CM proliferation and to distinguish these from non-regenerative injury responses.

In addition to morphological restoration, functional incorporation of newly formed CMs is crucial for successful myocardial regeneration. Although methods for evaluating functional recovery by echocardiography have not yet been developed in zebrafish, full electrical coupling of regenerated CMs has been demonstrated by voltage mapping at 30 dpi, implying functional incorporation of newly formed CMs [2••].

Migration of CMs into the injury site has been proposed to represent another important cellular event for myocardial regeneration and is supposedly regulated by the Cxcl12-Cxcr4 system [28]. Pharmacological blockage of Cxcr4 significantly reduces the number of CMs present in the injury site at 14 dpi and inhibits complete myocardium regeneration at 60 dpi, but has no effect on CM proliferation. While these data are indicative of the importance of CM migration for regeneration, the actual occurrence of active CM migration will have to be further supported using live cell tracking of marked CMs.

Do all Zebrafish Cardiomyocytes Proliferate and Contribute to Regeneration?

Although our knowledge about zebrafish CMs during heart regeneration has improved greatly, several findings regarding their proliferation require careful interpretation to avoid premature conclusions. Most publications have used BrdU incorporation or PCNA expression to detect proliferating CMs, reporting a frequency between 20 and 40 % of actively proliferating CMs after mechanical injury and genetic ablation, respectively [1•, 2••, 15•, 16•, 17•, 20, 29]. However, both BrdU and PCNA are S-phase makers, which do not indicate whether a given cell will undergo karyokinesis and cytokinesis and thus truly proliferates. Indeed, despite a significant elevation of the percentage of cycling zebrafish CMs, only a handful of them (a few cells per heart section analyzed) express the mitotic marker phospho-histone 3 [1•, 5]. One possible explanation is that the M-phase could be very short compared to the rest of the cell cycle; mitotic markers would therefore only be transiently expressed in a short time window during the cell cycle compared to S-phase markers. However, since we only know very little about the cell cycle of zebrafish CMs (e.g., its length and duration of different phases), it is too early to judge whether this is indeed the case. Another possible explanation is that only a small population of CMs actually undergoes mitosis after injury. It is believed that in injured mammalian hearts, many CMs undergo hypertrophy, which includes DNA replication with or without karyokinesis, but no cytokinesis, and thus results in polyploidization or binucleation (reviewed in [30]). Although non-injured adult zebrafish CMs are predominantly mononucleated [31], ploidy analyses have not been

performed to check whether polyploidization or binucleation could happen during regeneration. Until these issues are resolved, any proliferation analysis with S-phase markers alone should be interpreted as cell cycle re-entry but not true proliferation.

Factors Regulating Cardiomyocyte Cell Cycle Re-Entry

As mentioned, CM proliferation is central to the regenerative capacity of the zebrafish heart, and recent studies have identified new factors that regulate CM cell cycle re-entry. Poss and colleagues have adapted the fluorescent ubiquitylation-based cell cycle indicator (FUCCI) system to perform a chemical screen in embryonic zebrafish hearts and have identified hedgehog (Hh), insulin-like growth factor (Igf) and transforming growth factor β (Tgfb) signaling pathways as modifiers of CM proliferation during development [29]. These signaling pathways are also reactivated in the adult zebrafish heart after injury and are required for CM cell cycle re-entry. Lien and colleagues further showed that continuous pharmacological inhibition of Igf signaling by treatment with an Igf1 receptor inhibitor significantly impairs heart regeneration, possibly via inhibiting CM cell cycle re-entry and/or migration of *gata4*-positive CMs after injury [32]. In addition to its mitogenic role, Tgfb signaling has been shown to be important for transient scar formation after cryoinjury [33]. While control cryolesioned hearts display scar tissue with collagen deposition at 14 dpi (which is cleared at later stages), treatment with a Tgfb/activin pathway receptor (Alk5/4-i) inhibitor abrogates such transient scar formation, resulting in an infarct bulging phenotype and a lack of regeneration by 30 dpi. This is accompanied by reduced production of cardiac extracellular matrix (ECM) including fibronectin and tenascins. How these processes are related to myocardial regeneration however requires further investigation.

Although these pathways have a positive influence on heart regeneration in fish, their functions are not necessarily conserved in the mammalian heart after MI. Tgfb signaling mediates rapid cardiac remodeling including fibrosis [34] and hypertrophy (reviewed in [35]) in mammals after MI. As a result, pharmacological inhibition of Alk5 in a rat MI model significantly inhibits ECM and collagen deposition and CM hypertrophy, which leads to reduction of infarct size and improvement of heart function [36], implying a pro-remodeling and anti-regeneration role of Tgfb signaling in the mammalian heart. Thus, while inhibition of Tgfb signaling appears to reduce ECM deposition and scarring in both fish and mammals, the consequences for heart recovery seem to be radically different. It will be important to learn more about the importance of transient ECM deposition for successful

regeneration in fish and whether and how the molecular composition, morphology, formation and remodeling of such scars differ from those in mammals.

On the other hand, activation of Hh and Igf signaling is beneficial to heart recovery in both the zebrafish and mammalian models, but their mode of action seems to be different. In mammals, Hh signaling is activated in the myocardium after MI, and injection of Sonic hedgehog (Shh) plasmid is sufficient to promote neovascularization and protects CMs from apoptosis [37]. Similar cytoprotective effects are also observed after exogenous administration or transgenic overexpression of Igf1 in the mammalian heart after MI [38, 39]. Activation of both signaling pathways reduces post-MI remodeling and improves functional recovery [37–39]. In zebrafish, on the other hand, both Smoothened and Igf agonist treatments are sufficient to induce CM cell cycle re-entry during heart regeneration [29]. These data together imply differential mechanisms of Hh and Igf signaling to promote heart recovery. It would be interesting to identify the interacting partners and downstream targets of Hh and Igf signaling in both models that could result in either CM hyperplasia (zebrafish) or nonhyperplastic remodeling (mouse).

In contrast to the signaling pathways described so far that are required for CM cell cycle re-entry in both the developing and regenerating heart, Poss and colleagues recently suggested that the Janus kinase 1/Signal transducer and activator of transcription 3 (Jak1/Stat3) pathway is only essential for zebrafish CM cell cycle re-entry during regeneration but not during heart growth [31, 40]. Overexpression of a dominant-negative Stat3 3 days before apical resection significantly inhibits muscle regeneration and results in extensive scarring at 30 dpi, suggesting an anti-remodeling role of Jak/Stat signaling similar to that observed in the mammalian heart after MI [41, 42]. Concomitantly, cell cycle re-entry of CMs is also inhibited at 7 dpi in the same experiment [40]. It is however unclear whether Jak1/Stat3 signaling is mitogenic to CMs since cardiomyocyte proliferation assays upon short-term Jak1/Stat3 LOF (e.g., 24 h before analysis) have not been performed. Recent studies have also uncovered other factors that influence zebrafish heart regeneration. In brief, hypoxia and inflammatory responses appear to positively regulate CM cell cycle re-entry, whereas hyperoxia, p38 α MAPK and miR-133 have negative roles [43–46].

Conclusions

The zebrafish has developed rapidly over the last 10 years as a model to study cardiac regeneration. Recent discoveries have advanced our understanding of how natural heart regeneration is regulated (Fig. 1). Several signaling

Fig. 1 In response to injury (e.g., cryoinjury), the wound is first sealed by a fibrin-rich clot (from 3 days post-injury, dpi). According to the current model of the cellular events of heart regeneration, spared cardiomyocytes (CMs) are activated to proliferate, and their progeny regenerate the myocardium, resulting in scar-free recovery at 30–60 dpi. Tgf β , Hh, Igf, RA, Jak1/Stat3 signaling pathways, hypoxia and inflammatory responses have been described to positively influence CM cell cycle re-entry, while p38 α MAPK and miR133 negatively regulate myocardial regeneration. Currently, it is unclear which fraction of cycling CMs actually undergoes cytokinesis, but not polyploidization and binucleation

pathways have been shown to have a positive influence on CM cell cycle re-entry, including Tgf β , Hh, Igf and RA. It will be interesting to see whether activation of these signaling pathways in injured mouse hearts, where natural inhibitors such as the Hippo pathway are removed, could induce sufficient CM proliferation for effective regeneration [13]. Studies in zebrafish might also provide clues to important questions for guiding mammalian heart regeneration such as: (1) Are there natural mechanisms ensuring cell cycle completion to prevent binucleation and polyploidization in zebrafish CMs? (2) What are the underlying mechanisms of scar removal? (3) Does the zebrafish heart also undergo remodeling after injury (e.g., hypertrophy)? If so, what would the checks and balances system between

excessive remodeling and hyperplasia? We therefore believe the zebrafish will continue to thrive as an important cardiac regeneration model for many more years to come.

Compliance with Ethics Guidelines

Conflict of Interest Gilbert Weindinger and Chi-Chung Wu declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors. Research in the Weindinger Laboratory is performed in accordance with the animal rights protection regulations of Ulm University and the state of Baden-Württemberg.

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