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Mechanisms of Cardiac Regeneration

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Abstract

Adult humans fail to regenerate their hearts following injury, and this failure to regenerate myocardium is a leading cause of heart failure and death worldwide. Although all adult mammals appear to lack significant cardiac regeneration potential, some vertebrates can regenerate myocardium throughout life. In addition, new studies indicate that mammals have cardiac regeneration potential during development and very soon after birth. The mechanisms of heart regeneration among model organisms, including neonatal mice, appear remarkably similar. Orchestrated waves of inflammation, matrix deposition and remodeling, and cardiomyocyte proliferation are commonly seen in heart regeneration models. Understanding why adult mammals develop extensive scarring instead of regeneration is a crucial goal for regenerative biology.

Introduction

The intricate process of regeneration restores tissue architecture through a sequential orchestration of events including cellular proliferation, differentiation and dedifferentiation and coordinated morphogenic rearrangements. In a vital organ like the heart, regeneration is not only fascinating but also clinically relevant. Lower vertebrates such as the newt and zebrafish have an astonishing ability to replace lost cardiac tissue (Gamba et al., 2014; Poss et al., 2002; Witman et al., 2011), but there has been a longstanding dogma that mammalian heart tissue could never regenerate, reinforced by the belief that adult mammalian cardiac cells are incapable of cell division. In response to cardiac injury, adult mammals—including humans—fail to regenerate the majority of the lost cardiomyocytes and instead replace necrotic muscle with scar tissue. The loss of cardiomyocytes eventually compromises contractility of the remaining myocardium, leading to heart failure and death when the extent of injury is severe (Porrello and Olson, 2014). However, recent data indicate that mammalian cardiogenesis occurs during adult life, including in humans (Bergmann et al., 2009, 2015). In addition, the neonatal mouse heart has a regenerative response immediately after birth (Porrello et al., 2011a). Thus, regeneration of myocardial tissue is an exciting

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therapeutic goal. We are far from a complete understanding of how heart tissue can regenerate, but we are now defining molecular mechanisms that could open the door to stimulating adult mammalian heart regeneration.

Heart Regeneration in Lower Vertebrates

Teleosts

Teleost fish can effectively regenerate many body parts including brain (Kroehne et al., 2011), retina (Vihtelic and Hyde, 2000), fins (Johnson and Weston, 1995), spinal cord (Becker et al., 1997) and heart (Poss et al., 2002). Availability of genetic and molecular tools as well as the extensive regenerative capability even into adulthood have made the zebrafish the best characterized heart regeneration model system to date. Teleosts have 2 chambered hearts that pump blood to the body and the gills. As shown in seminal studies by Poss and colleagues (Lepilina et al., 2006; Poss et al., 2002), within seconds after resection of the zebrafish ventricular apex, profuse bleeding from the ventricle is stopped by clotting in wound. Following fibrin deposition, the zebrafish heart does not go through the intense collagen deposition and scarring seen in mammalian hearts after injury. Instead, cells proliferate to replace lost cardiomyocyte tissue. The proliferation in cardiomyocytes peaks at 14 days post resection. By 60 days post resection, almost all the lost muscle tissue is replaced, with contractile function of hearts appearing grossly normal (Kikuchi and Poss, 2012; Poss et al., 2002). Studies in zebrafish have not supported stem cells as the source of regenerating myocardium. Cre-based genetic fate mapping has shown that pre-existing cardiomyocytes reduce organization of their sarcomeric structures and dedifferentiate to a more embryonic form, followed by cell division and maturation that recapitulates the developmental program (Jopling et al., 2010; Kikuchi et al., 2010).

In addition to the apical resection approach, zebrafish myocardial injury can also be achieved through genetic ablation(Wang et al., 2011) and cryoinjury (Chablais et al., 2011; González-Rosa et al., 2011; Schnabel et al., 2011) (Figure 1). In both cases robust myocardial regeneration is observed, although the dynamics of the regenerative process may differ. In the genetic ablation experiments by Wang et al, cardiomyocyte specific Cre recombinase activity from cmlc2 promoter drove the expression of a cytotoxic DTA (diphteria toxin A chain) gene that led to cardiomyocyte death. When more than 60% of cardiomyocytes were eliminated with this technique, tissue was replaced through regeneration with minimal scarring and restored function (Wang et al., 2011). In cryocauterization (or cryoinjury), the heart was probed with a flash frozen metal filament, causing local but massive death of cardiomyocytes (approximately 25% of ventricular muscle) as well as other cell types (Chablais et al., 2011). In the cryoinjury model, the course of healing includes an initial deposit of collagen that is later cleared, and the heart muscle renews itself 130 days after cryoinjury (González-Rosa et al., 2011). It is possibly due to the clearing of the necrotic tissue that the cryoinjury injury model follows a different timeline and process of regeneration compared to the resection model. In all three injury models, the zebrafish heart is able to regain functional as well as physical integrity.

It should be noted that while organ regeneration studies in teleosts have not been limited to zebrafish (Nabrit, 1929), heart regeneration in medaka, another teleost model species, have

revealed differential reparative phenotypes: medaka fish failed to regenerate its heart upon injury and responded with excessive fibrosis (Ito et al., 2014). Future studies need to investigate whether regenerative capacity differs with age or injury type in different teleost species.

Urodeles

Urodeles including newts and salamanders have been regarded as the champions of regeneration due to their extensive ability to replace body parts after amputation or injury of different tissues, including the lens, jaw, limbs, tail and heart (Faber, 1960; Suetsugu-Maki et al., 2012). The phenomenon of limb regeneration in salamanders was described in 1769 (Spallanzani, 1769), and classical experiments by Paul Weiss and colleagues in the 1930's in the regenerating urodele limb described the kinetics of regeneration (Weiss and Walker, 1934). Many processes that were initially characterized in urodele limb and tail regeneration, such as wound healing and innervation, have now been shown to play a role in heart regeneration across multiple species.

The newt and aquatic axolotl hearts have two atria and a single avascular ventricle. Following axolotl or newt heart injury, the heart can replace lost tissue and function within 90 days without evidence of scarring (Becker et al., 1974; Flink, 2002; Oberpriller and Oberpriller, 1974). Regeneration in the urodele heart starts with the formation of a clot to prevent blood loss, followed by fibrin and collagen deposits at 7 days post injury (Witman et al., 2011). Gene expression changes associated with increased extracellular matrix (ECM) are prominent responses to cardiac injury; matrix metalloproteinases, collagen, keratin, tenascin-C and other ECM components are expressed immediately and serve to provide a structural framework for the regeneration (Gamba et al., 2014; Piatkowski et al., 2013). Cellular proliferation increases in the epicardium and cardiomyocytes (both atrial and ventricular) (Flink, 2002) and within the next 50 days the matrix deposition is gradually replaced with cardiomyocytes (Witman et al., 2011). Interestingly, distinct populations of cells in the regenerate have been identified to express Islet1 and Gata4 , which are markers for cardiac progenitors (Witman et al., 2011), suggesting recapitulation of developmental cardiogenesis during regeneration. Notably, Laube et al showed that the newt myocardium downregulates expression of sarcomeric genes during regeneration, supporting the hypothesis that cardiomyocytes go through at least partial de-differentiation during regeneration (Laube et al., 2006). After 60-90 days, the tissue is regenerated completely without scarring (Witman et al., 2011); however, the robustness of the regenerative response and degree of scarring can vary depending on the location and size of the resected tissue (Kikuchi and Poss, 2012; Witman et al., 2011).

Anurans

Anurans are an order of Amphibians distinct from urodeles and includes toads and frogs. In the late 1960s and the early 1970s, the Soviet scientist Rumyantsev characterized cellular proliferation and ultrastructure of muscle fibers following heart injury in adult frogs (Rumjancev and Carlson, 1991; Rumyantsev, 1973). Adult anuran cardiomyocytes are capable of DNA synthesis, cellular proliferation and dedifferentiation to some extent, but

fail to carry out complete heart regeneration, leading to formation of scar tissue in the injured frog heart (Rumjancev and Carlson, 1991; Rumyantsev, 1973).

There is a remarkable difference in the regenerative capacity of the heart in anuran and urodele species of Amphibia. Anurans lose their overall regenerative capacity progressively as they go through metamorphisis from the larvae stage to the adult, concominant with maturation of the immune system (Izutsu and Yoshizato, 1993; Rollins-Smith, 1998). Urodeles, which have strikingly different immune systems compared to anurans, also go through metamorphosis, but their immune system is more stable (Godwin and Rosenthal, 2014). The differences in regenerative capacity and adaptive immunity in adult anurans and urodeles may be mechanistically related. In specificity, speed of onset and memory, the adult frog immune system is more comparable to mammals (Godwin and Rosenthal, 2014). Salamanders, on the other hand, are considered to have subdued immune systems; the humoral response is relatively slow, and despite a large T-cell and B-cell repertoire, immune memory of salamanders is low (Tournefier et al., 1998). These observations are consistent with the concept that the immune system is critical for the regenerative response in myocardium, as discussed below.

Heart Regeneration in Rodents

Cardiac regeneration in the fetal mouse heart

While cardiac development and post-natal heart regeneration have been extensively studied, a remarkable study by Drenckhahn et al. explored the regenerative capacity of the heart during development (Drenckhahn et al., 2008). A cardiomyocyte-lethal mutant gene in the X chromosome was conditionally expressed at embryonic day 12.5 (E12.5) in only half of the cardiomyocytes in female embryos due to random X inactivation. Fetal hearts that had undergone this genetic ablation were able to restore approximately 50% of lost cardiomyocyte mass, indicating that the embryonic environment and the transcriptional state of embryonic cardiomyocytes faciliate cardiomyocyte cell cycle re-entry and repopulation of the heart (Figure 2). This highlights the importance of understanding the dramatic change in cardiac regenerative potential from the embryo through the first weeks of life.

Cardiac regeneration in neonatal mammals

Adult mammalian hearts fail to show significant regenerative capacity in different injury models (Kikuchi and Poss, 2012; Rumyantsev, 1977), but the possibility of a regenerative window in children has been proposed for almost a century in analyses of post-mortem histological specimens (Macmahon, 1937; Warthin, 1924). These studies inspired Robledo to explore cardiac regeneration in young rats in the 1950s, but he observed only incomplete regeneration following a myocardial burn injury in rats day 4-7 after birth (Robledo, 1956). More recently, case reports from corrective heart surgeries in infants (Fratz et al., 2011) and myocardial infarction of a newborn child (Haubner et al., 2015) have suggested that human neonatal heart can also functionally recover and may have a higher regenerative potential. Recently, direct experimental evidence for a robust regenerative capacity in neonates has been reported in mice in a range of injury models including ventricular resection (Porrello et

al., 2011a), myocardial infarction (Haubner et al., 2012), cryoinfarction (Jesty et al., 2012; Strungs et al., 2013) and clamping (Bryant et al., 2014).

Neonatal heart regeneration studies in mice were reported by Porrello et al in resection and myocardial infarction (MI) models (Porrello et al., 2011a, 2013). These studies identified a time window immediately after birth when the mammalian heart mounts a robust regenerative response. Neonatal animals that have undergone resection or MI surgery exhibit increased cardiomyocyte proliferation and robust angiogenic growth. Although some scarring may occur, substantial lost tissue is restored within 3 weeks, in both MI and the ventricular resection model when 15% of the ventricular apex is resected (Porrello et al., 2011a). Similar to the regenerative response in teleosts and axolotls, the neonatal mouse injury response is initiated with rapid clotting, inflammatory cell infiltration to the injury site, epicardial activation and initiation of cardiomyocyte proliferation. In the MI model, ischemia is routinely induced by ligation of the left anterior descending coronary artery soon after birth. Although the infarction model initially induces myocyte necrosis and collagen deposition, 95% of lost tissue is replaced within 3 weeks with minimal fibrosis, and cardiac function is normal 9 months after surgery (Porrello et al., 2013). Genetic fate mapping by Porrello et al and others has shown that the major source of cardiomyocyte repopulation is pre-existing cardiomyocytes that re-enter cell cycle (Haubner et al., 2012; Porrello et al., 2013), as in zebrafish (Jopling et al., 2010; Lepilina et al., 2006). Interestingly, this robust regenerative response is not elicited in mice injured at post-natal day 7 or day 14, revealing that soon after birth there is a sharp decline in heart regenerative potential.

Controversy arose in the field when Andersen et al reported extensive scarring and limited regeneration following neonatal ventricular resection (Andersen et al., 2014). Bryant et al conducted a systemic analysis of technical considerations in the surgery and demonstrated that experimental issues such as the size of apical resection can lead to variations in the regenerative response, including some degree of scarring at 21 days post operation (Bryant et al., 2014). There is clear evidence for myocardial regeneration after ventricular resection in neonatal mice as demonstrated by several independent groups (Bryant et al., 2014; Han et al., 2015; Porrello et al., 2011a), although technical issues can affect the regenerative response. In contrast, cardiomyocyte proliferation in neonatal mice does not increase significantly following cryoinjuries and transmural cryoinjury fails to elicit regenerative response, while non-transmural cryoinjury models can fully recover (Darehzereshki et al., 2015). This underscores the importance of carefully controlled experiments with consistent choice of injury type in order to limit the impact of technical factors in any mouse heart regeneration experiment.

Evolutionarily Conserved Mechanisms in Heart Regeneration

Sources of regenerated myocardium

An explosion of interest in adult stem cell biology over the past 15 years has driven interest in the concept that adult stem cells activated following injury can be a source of new cardiomyocytes. However, as described above, fate mapping studies in zebrafish heart regeneration reveal that fish myocardium replaces lost cardiomyocytes through proliferation of existing cardiomyocytes. In two independent studies (Jopling et al., 2010; Kikuchi et al.,

2010), inducible Cre recombinase (CreER) expression was driven by the cardiac myosin light chain 2 (*cmlc2*) promoter sequence, resulting in the expression of a loxP flanked EGFP reporter gene. Before cardiac injury, all cardiomyocytes expressing Cmlc2 were labeled by EGFP expression, and 30 days post ventricular resection, the majority of the regenerated tissue was labeled with EGFP expression, showing that cmlc2+ cardiomyocytes were the major source for regenerated myocardium.

A study from Poss and colleagues further dissected the contribution from subpopulations of cardiac muscle into the regenerate (Gupta and Poss, 2012). The authors employed a multicolor clonal analysis sytem in order to identify 3 distinct muscle lineages in the zebrafish heart: primordial, trabecular, and cortical muscle, distinct in their order of development during cardiac morphogenesis. The multicolor clonal analysis technique employed in this study was adapted from the Brainbow technology that was initially developed in the mouse (Livet et al., 2007). Cre/lox recombination was used to create a stochastic gene expression pattern from 3 tandem fluorophores, allowing one to distinguish clones from a specific recombination event (Gupta and Poss, 2012). Clonal labeling in zebrafish cardiomyocytes revealed that during regeneration, proliferation of cortical muscle in the wound area is detectable at 14 days post amputation and constitutes the primary component of the regenerate wall, whereas the primordial layer of muscle is first detected at 30 days post amputation in a restricted lateral expansion, as observed during embryogenesis. However, the appearance of muscle layers during regeneration is in reverse order to the order in cardiac development (Gupta and Poss, 2012).

Comparable fate mapping analysis has also been performed in the neonatal mouse (Senyo et al., 2013). Using genetic fate mapping and stable isotope imaging technology, Senyo et al showed during normal myocardial homeostasis in the adult mouse, new cardiomyocytes arise from pre-existing cardiomyocytes. In addition, after injury, modest new cardiomyocyte regeneration near the injury increases, and these myocytes appeared to arise from pre-existing cardiomyocytes. A recent study by Kimura et al. reported fate mapping of cycling cardiomyocytes following injury to a subset of hypoxic cardiomyocytes that express HIF1-α (Kimura et al., 2015). The extent to which a progenitor cell pool contributes to cardiomyocyte renewal remains controversial: c-kit+ cells were previously reported to mark cardiomyocyte progenitors (Angert et al., 2011; Beltrami et al., 2003; Hatzistergos et al., 2010; Orlic et al., 2001) but a quantitatively rigorous genetic fate mapping study showed that this contribution is functionally negligible (van Berlo et al., 2014). Hatzistergos et al reported that c-kit+ cells are of cardiac neural crest origin in development and their limited contribution to cardiac progenitors is due to a nonpermissive environment in the developing heart (Hatzistergos et al., 2015). In contrast, a new study of multiple lines of genetically engineered mice showed no significant cardiogenesis from c-kit+ cells, consistent with van Berlo et al study (Sultana et al., 2015). The role of ckit+ cells in post-infarction myogenesis was explored in 2012 by Jesty et al, who reported that ckit+ cells partially support repopulation of the myocardium following injury in the neonatal heart, but that ckit+ cells do not adopt cardiomyocyte cell fate during myogenic repair in adult mice that harbor a transcription marker for ckit (ckit^{BAC}-EGFP) (Jesty et al., 2012).

A major difference between lower vertebrates such as the zebrafish and newt and mammals may be their ability to complete the cell cycle. In contrast to the mononucleated zebrafish cardiomyocytes that can reenter the cell cycle in adulthood, many of the cardiomyocytes in the mammalian heart become binucleated either before (Jonker et al., 2007) or shortly after birth (Soonpaa et al., 1996). In rodents, up to 95% of cardiomyocytes are binucleated (Soonpaa et al., 1996), while binucleation is much lower in human cardiomyocytes at 30-40% (Mollova et al., 2013). A study by Bersell et al. showed that Neuregulin1 can induce proliferation of differentiated adult cardiomyocytes in cell culture and in vivo (Bersell et al., 2009). Interestingly, Neuregulin1 (NRG1) appears to affect the mononucleated subpopulation of differentiated cardiomyocytes, supporting the premise that mononucleated cardiomyocytes may be more receptive to cell cycle reentry. However, D'Uva et al recently showed that cytokinesis in bi-nucleated cardiomyocytes are also possible with constitutive activation of the NRG1 co-receptor ERBB2 (D'Uva et al., 2015).

The curious role of nerves

Over the last century, studies across multiple species has shown that nerves are indispensible for regeneration in many organs. Classical experiments in newt limbs show that denervated limbs cannot regenerate (Todd, 1823). Newt and salamander lens, retina and tail are other systems where adequate number of nerve fibres are required to guide regeneration To date, several nerve-derived factors have been shown to function in regeneration, including fibroblast growth factors (FGFs) in vertebrate limb regeneration(Gospodarowicz and Mescher, 1980), glial growth factor (GGF) in zebrafish tail regeneration and vertebrate limb regeneration (Rojas-Muñoz et al., 2009; Wang et al., 2000), nAG in newt limb regeneration (Kumar et al., 2007) and glial-derived neurotrophic factor (GDNF) in hematopoietic regeneration in mammals (Lucas et al., 2013).

Recently, the role of nerves in heart regeneration has also been explored. Mahmoud and O'Meara et al. mechanically interrupted the left vagus nerve and found that this suppresses the heart regenerative response upon injury in neonatal mice (Mahmoud et al., 2015). This suggests a role for parasympathetic nerve function in heart regeneration. In another recent study, White et al. explored the influence of sympathetic nerves in cardiac regeneration (White et al., 2015). Mice that undergo chemical sympathectomy have inhibited sympathetic regrowth and failed cardiac regeneration following apical resection surgery (White et al., 2015). These new findings suggest that nerves may function in the regenerative process, although this does not appear restricted to sympathetic vs. Parasympathetic nerves. It is possible that a critical density of nerve factors is necessary to support regeneration. The concept of a critical nerve density rather than specific nerve synaptic activity in driving regeneration is consistent with findings in axolotl limb regeneration (Kumar and Brockes, 2012; Kumar et al., 2007; Litwiller, 1938). Thus, regulation of the regenerative response in many tissues by nerves appears to be an evolutionarily conserved pathway among different species including lower vertebrates, and the study of nerves in cardiac regeneration could shed light into conserved regenerative molecular pathways.

Inflammatory and immune response in heart regeneration

Unlike embryonic development, tissue growth in regeneration is initiated by an injury, and the inflammatory response to that injury is a critical regulator of the regenerative process. Inflammation can drive regeneration but can also inhibit it under some circumstances. Godwin et al. reported that following macrophage depletion, newt limb regeneration fails and leads to extensive fibrosis (Godwin et al., 2013). In contrast, evidence for the constraining effects of a developed immune system on regeneration come from studies of *Xenopus* limbs. As young *Xenopus* larvae transition into adulthood through metamorphosis, their immune system also passes through gradual maturation; concominantly, they lose their regenerative capacity (Godwin and Rosenthal, 2014). Grow et al. performed gene expression analysis comparing earlier stage regeneration-competent xenopus limbs and later stage regeneration-incompetent xenopus limbs and reported a higher level of pro-inflammatory genes expression 1 day post amputation in the regeneration incompetent limbs (Grow et al., 2006). Limb amputation experiments in the developing xenopus limb suggest that it is the local inflammatory response subsequent to the wound formation that exerts a constraining effect on the regenerative capacity (Godwin and Rosenthal, 2014; King et al., 2012). Together, the studies in frogs and salamanders show that the early inflammatory response plays a crucial role in the intiation of regenerative processes.

In both lower vertebrates and mammals, cardiac injury is also associated with an initial wave of inflammation. The consequences of the inflammatory/immune response is *remodeling through scarring* in non-regenerating animals, versus *remodeling through cellular repopulation* in regenerating animals. Infiltration by inflammatory cells in injured heart peaks around 3 days post-amputation in zebrafish hearts (Lien et al., 2006). The inflammatory system responds immediately to cardiac injury in all vertebrate species studied (Kyritsis et al., 2012; Xin et al., 2013a). In mice, monocyte and macrophages are required for cardiac regeneration (Aurora et al., 2014), and injury-induced cardiac proliferation is inhibited by immunosuppression (Han et al., 2015). Acute inflammation is required for neonatal heart regeneration, and in the absence of interleukin 6 (IL-6), cardiomyocytes fail to proliferate upon injury (Han et al., 2015).

The relationship observed in anurans between maturation of the immune system and the decrease in regenerative capacity has been explored in mammals in the context of postnatal changes in the immune system and the loss of capacity for cardiac regeneration (Aurora et al., 2014). Aurora et al. identified differences in the cellular immune response to MI in 1 day old and 14 day old mice; they used a macrophage depletion model in neonatal mice to show that macrophages are required for regeneration and neoangiogenesis in the injured heart. Interestingly, macrophage depletion did not influence cardiomyocyte proliferation following infarction. The molecular profiling of macrophages suggested that secretion of pro-angiogenic cytokines may be responsible for their important role in cardiac regeneration (Aurora et al., 2014) (Figure 3).

Angiogenesis and heart regeneration

Throughout phylogeny, formation of new vasculature following injury is vital for regeneration (Kleinheinz, 2013). As new tissue with complex architecture is restored, the

regenerating tissue needs a continuous supply of energy and substrates as well as routes for eliminating metabolic products. Therefore, a functioning dynamic vasculature is critical for a successful regenerative response. In the absence of neovascularization following injury, the zebrafish heart fails to regenerate and instead forms extensive fibrotic scarring (Lepilina et al., 2006). Gene expression and genetic analyses in zebrafish have established regulators of angiogenesis that are essential for the response to cardiac injury. FGF receptor expression in the epicardium and FGF ligand expression in the myocardium appear to be required for formation of new vasculature, and this process is thought to regulate epithelial to mesenchymal transition (EMT) of the epicardium in order to form coronary vasculature (Lepilina et al., 2006). Additionally, Pdgf signaling is required for epicardial proliferation and new blood vessel formation in cardiac regeneration (Kim et al., 2010), suggesting that new vascular formation during regeneration recapitulates molecular mechanisms that govern developmental processes of blood vessel formation. Interestingly, cxcr4a mutant zebrafish that fail to develop coronary vasculature in the myocardium can be viable as adults, but the mutants that survive into adulthood fail to regenerate their hearts, suggesting loss of this oxygen supply is especially critical for regeneration (Harrison et al., 2015).

In neonatal mice, robust neovascularization is evident during the regenerative response to either apical resection or MI injuries (Porrello et al., 2011a, 2013). In adult mice where the regenerative response is not evident, the neovascular response is also not observed, suggesting that lack of blood vessel formation after injury could contribute to loss of cardiac regenerative capacity (Epelman et al., 2015; Lavine et al., 2014). Factors inducing vascular regeneration have been shown to improve tissue renewal and cardiac functional restoration in adult mice following MI (Zangi et al., 2013). Zangi et al. have employed modified RNA (modRNA) technology to induce VEGF expression in a spatiotemporally controlled manner and thereby improve the regenerative response following injury, through neoangiogenesis (Zangi et al., 2013).

Extracellular matrix and heart regeneration

During normal cardiac development, signaling from extracellular matrix (ECM) provides structure and guidance for cellular migration, proliferation and differentiation. For example, fibronectin is a key cue for migration of cardiomyocytes towards the midline (Trinh and Stainier, 2004) and is a regulator of cardiomyocyte proliferation (Ieda et al., 2009). Similarly, changes in the tissue microenvironment comprise a crucial component of the regenerative response. In neonatal mice and lower vertebrates, the myocardium goes through an extensive remodeling process and scar formation is minimized compared with adult mammalian myocardium, but new extracellular matrix deposition is nevertheless considerable (Piatkowski et al., 2013; Porrello et al., 2011a; Wang et al., 2013). Ablation of transient scar formation in zebrafish through pharmacological inhibition of TGFβ signaling abolishes cardiac regeneration (Chablais and Jazwinska, 2012). Studies in the newt have revealed that an increase in ECM components precedes cardiomyocyte proliferation after injury, and that tenascin-C is sufficient to induce proliferation *in vitro* (Mercer et al., 2013). In the newt and zebrafish, ECM components and ECM-modifying proteases are among the most robustly enriched genes expressed in response to local injury; in contrast, in adult mammalian myocardium, inflammation and metabolic genes comprise the most significantly

enriched transcripts (Mercer et al., 2013). Over several weeks following injury, regeneration-competent hearts marginalize fibrotic ECM deposition to the periphery and replace it with regenerated myocardium (Porrello et al., 2013), as observed in newts and zebrafish (González-Rosa et al., 2011; Mercer et al., 2013).

ECM components secreted from embryonic fibroblasts include fibronectin, collagen and heparin-binding EGF-like growth factor, and these factors can promote cardiomyocyte proliferation in a paracrine fashion(Ieda et al., 2009). Therefore, it is possible that post-natal changes to ECM composition alter the proliferative capacity of cardiomyocytes. Studies in zebrafish have revealed that epicardium is especially important for initiating extracellular matrix deposition (Wang et al., 2013): injury in the myocardium induces epicardial cells to express fibronectin paralogues, Fn1 and Fn1b, within a day of injury. Through genetic ablation of *fn1*, Wang et al showed that fibronectin is required for heart regeneration, although not through the regulation of cardiomyocyte proliferation (Wang et al., 2013).

Periostin is another secreted extracellular matrix component that has been shown to increase cardiomyocyte cell cycle activity (Kühn et al., 2007). While in development periostin plays a role in epithelial mesenchymal transition (EMT) (Litvin et al., 2005), periostin is expressed in adult myocardium upon injury (Butcher et al., 2007; Stanton et al., 2000). Promoting collagen cross-linking in the ECM, periostin accelerates fibrillogenesis and contributes to scar formation (Oka et al., 2007). In a study by Kühn et al using the adult mouse MI model, exogenous expression of periostin enhanced post-injury myocardial proliferation and promoted cardiac repair, which resulted in improvement in ventricular remodeling and function (Kühn et al., 2007). Periostin function was mediated through activation of β integrins on cardiomyocytes; in its absence, activation of phosphatidylionsitol-3-OH kinase was sufficient for cell cycle reentry (Kühn et al., 2007). Controversy arose in the field when genetic manipulation of periostin did not alter cell cycle activity, cardiomyocyte content or cardiac repair in mice, in either ablation or overexpression (Lorts et al., 2009).

Cell type composition also appears to have importance in the heart regenerative response due to their influence on the extracellular matrix. Fibroblasts greatly influence the composition of ECM deposition, and their presence has been shown to impact the proliferative capacity of cardiomyocytes *in vitro* (Ieda et al., 2009). Fibroblasts are more abundant in the adult mammalian heart compared to fetal mammalian or adult non-mammalian hearts. Thus, changes in fibroblast abundance or composition may be responsible for the permissive versus non-permissive cardiac environment for regenerative growth.

Development vs. Regeneration in the heart

Transcriptional profile of cardiomyocytes in development and regeneration

A common principle observed in tissue regeneration is the reactivation of the previously employed developmental transcriptional programs. Studies in different model organisms have shown that mechanisms in developmental cardiogenesis also govern morphological regeneration of the injured heart. As discussed previously, in lower vertebrates and

mammals, the source of new cardiomyocytes appears to be predominantly pre-existing cardiomyocytes that re-enter the cell cycle, although a role for progenitors and stem cells remains hotly debated in the cardiovascular community.

The cardiomyocyte lineage originates from mesodermal cells that express T-box transcription factor Eomes and Mesp1 (Bondue et al., 2008), which is thought to be a regulator of cardiac progenitor cell fate. These precursors are later allocated to two major populations designated as the first heart field and the second heart field, defined by their crescent shapes distinct on day E7.5 in mouse embryogenesis. As the heart tube forms and then loops to form the chambers, molecular cues further induce and define mesodermal progenitors to different cardiac cell types. An intricate gene regulatory network refines progenitor boundaries, terminal differentiation and transcriptional identities specific to each cell type during heart organogenesis. At early stages of development, NKX2-5 and ISL1 expression define cardiac progenitor cells (Cai et al., 2003; Ehrman and Yutzey, 1999). This is followed by cardiomyocyte specific expression of Hopx in a subset of progenitors (Jain et al., 2015). Hopx defines cardiomyocyte cell fate and coordinates an antagonistic crosstalk between BMP and WNT pathways by physically interacting with BMP effector SMADs to repress WNT genes (Jain et al., 2015) and promote cardiomyogenesis. During cardiomyocyte differentiation, zinc finger transcription factor GATA4 has an essential role in regulation of structural genes including α-myosin heavy chain (*α-mhc*) and cardiac troponin C (*Ctnc*)(Molkentin et al., 1997; Temsah and Nemer, 2005). Elucidating transcriptional profile of cardiac progenitors has enabled derivation of cardiomyocytes from embryonic stem cells (Qian et al., 2012), and their therapeutic potential is being tested in grafts into non-human primate models (Chong et al., 2014).

Reactivation of a GATA4-driven gene expression program plays a role in the context of regeneration as well (Gupta et al., 2013; Kikuchi et al., 2010), consistent with the observation that the tissue activates an embryonic differentiation program for regenerating cardiomyocytes that acquire less organized sarcomeric structures (Jopling et al., 2010). Another developmental gene, *Hand2*, induces cardiomyocyte proliferation during regeneration (Schindler et al., 2014). Homeodomain transcription factor MEIS1, which is required for normal cardiac development (Azcoitia et al., 2005; Stankunas et al., 2008), has been shown to orchestrate postnatal cell cycle arrest and maturation (Mahmoud et al., 2013). Strikingly, deletion of *Meis1* in the adult heart is sufficient to induce cardiomyocyte cell cycle re-entry in adult cardiomyocytes. In addition to activation of developmental programs, expression of cardiac muscle genes and dedifferentiation facilitate mitotic and morphogenetic activity during post-injury response in newt regeneration (Pesce et al., 2011). Dedifferentiation has also been explored in mammalian cardiomyocytes (Szibor et al., 2014), and many laboratories are now trying to dissect the transcriptional program that renders adult mammalian cardiomyocytes dedifferentiated sufficiently to be more permissive for cell cycle reentry. Recently, an analysis of global transcriptional programs in mammalian cardiomyocyte differentiation and regeneration has revealed that the regenerating mouse heart reverses the transcriptional processes of cardiomyocyte differentiation, with reactivation of latent developmental programs (O'Meara et al., 2015).

Disruption of miRNA machinary in development and homeostasis results in cardiac abnormalities (da Costa Martins et al., 2008). A role for microRNAs (miRNA) in cardiomyocyte proliferation and regeneration was revealed by numerous studies including a study by Porrello et al in 2011 (Porrello et al., 2011b) and a fluorescent microscopy based screen (Eulalio et al., 2012). MiRNAs are short RNA sequences that base-pair partially with messenger RNAs of target genes and thereby regulate gene expression (Bartel, 2009). To date, dozens of miRNAs have been identified to play critical roles in not just cardiomyocyte DNA synthesis and cytokinesis but also post-natal mitotic arrest (Chen et al., 2013; Eulalio et al., 2012; Porrello et al., 2011b; Wang et al., 2010; Yin et al., 2012).

Epigenetic control of cardiogenesis has also gained considerable attention, particularly because epigenetic events regulate maintanence of the proliferative state of cardiomyocytes through several mechanisms such as DNA methylation, chromatin remodeling, or covalent histone modifications including acetylation and methylation. The chromatin remodeling BAF complex and its subunit Baf60c have specific roles for transcriptional regulation at cardiac specific enhancer sites, and thus are essential for cardiac morphogenesis (Lickert et al., 2004). Deletion of histone deacetylase *Hdac2* with *Hopx* during cardiogenesis results in increased cardiomyocyte proliferation, and this effect is mediated through deacetylation of *Gata4* (Trivedi et al., 2010). Histone acetyltransferase P300 directly regulates cardiac specification through *Gata4*, *Mef2c* and *Srf* (Takaya et al., 2008). Mice null for histone methyltransferase Smyd1 are embryonic lethal, and mice deficient in histone demethylase *Jarid2* die immediately after birth (Gottlieb et al., 2002; Mysliwiec et al., 2012). Interestingly, JARID2 directly represses Notch target genes, thereby changing responsiveness of cells to the signaling events in the heart. Genetic deletion of Brahma-related gene 1, an ATP-dependent chromatin-remodeling factor, results in proliferation defects in myocardium (Hang et al., 2010). Thus, epigenetic events in the heart are dynamic and directly control cardiomyocytes and their proliferative state. Manipulating the epigenetic state of cardiomyocytes during the critical post-injury period is an attractive regenerative strategy, and histone deacetylase inhibitors are potential therapeutic agents (Xie and Hill, 2013).

Epicardium in regeneration

The epicardium is the external epithelial layer that contributes to myocardial growth through secretion of soluble growth factors. For example, retinoic acid-mediated production of FGF ligands and insulin-like growth factors (IGF) by epicardium drives growth in the underlying myocardium during development (Lavine et al., 2005; Li et al., 2011), thereby contributing to the morphogenesis of the ventricles during development. Recent studies reveal that epicardium participates in mitogen secretion during heart regeneration. Huang et al. reported that IGF signaling from the epicardium is required for adequate cardiomyocyte proliferation in zebrafish heart regeneration (Huang et al., 2013); the contribution of cells that are *gata4* lineage is impaired in regeneration when IGF signaling is inhibited. This finding suggests that epicardium may participate in initiation of the developmental gene program in adult cardiomyocytes during post-injury remodeling of the heart. When the epicardial cell population is genetically ablated in adult zebrafish, cardiac proliferation is impaired and regeneration is delayed (Wang et al., 2015).

Wei et al. reported that follistatin-like 1 (FSTL1) is an epicardial cardiomyogenic factor that dramatically improves regenerative repair and function upon dynamic expression following myocardial injury (Wei et al., 2015). Prior studies had identified anti-apoptotic effects of FSTL1 following ischemia-reperfusion injury in the myocardium (Ogura et al., 2012; Oshima et al., 2008). In the context of myocardial infarction, application of an epicardial patch of FSTL1 following injury significantly improved myocardial proliferation, survival and function in mice and swine models (Wei et al., 2015).

Distinct role of Neuregulin1 in development and regeneration

NRG1 is a member of the epidermal growth factor family with crucial roles in cardiac development and homeostatic cardiac function. Evidence from studies in zebrafish and mice reveals that signaling through NRG1 and its ERBB receptor tyrosine kinases is crucial for proper heart formation, cardiomyocyte proliferation and morphology (Hertig et al., 1999; Reischauer et al., 2014). In zebrafish with *erbb2*−/− cardiomyocytes, myofibril organization is disrupted due to abnormalities in spatiotemporal organization along the apico-basal axis of the heart (Reischauer et al., 2014). In mouse studies where the effects of NRG1 in later development were explored, NRG1 signaling is necessary for trabeculation of the ventricular wall and compact zone expansion (Hertig et al., 1999). Thus NRG1 plays a major role in heart development and signaling between endocardium and myocardium as well as regulating differentiation and maturation during morphogenesis (Grego-Bessa et al., 2007; Xin et al., 2013a). *In vitro*, treatment of rat neonatal cardiomyocytes with NRG-1 results in increased F-actin organization, proliferation, protein production and hypertrophy (Baliga et al., 1999; Reischauer et al., 2014).

The cardiomyocyte mitogenic property of NRG1 *in vitro* has motivated studies on a potential role of NRG-1 in cardiac regeneration. In injured zebrafish hearts, NRG1 protein expression is induced in the cells of the outer layer of the heart wall (Gemberling et al., 2015). In uninjured zebrafish hearts, exogenous expression of Nrg1 promotes cardiomyocyte proliferation and dedifferentiation. In adult mice, NRG1 mediated activation of the ERBB2-ERBB4 receptor heterodimer stimulates cardiomyocyte proliferation (Bersell et al., 2009) and transient induction of a constitutively active ERBB2 receptor was shown to be sufficient to reactivate cardiomyocyte proliferation in adults (D'Uva et al., 2015) . However, another study found no induction of cardiomyocyte DNA synthesis with neuregulin-1 treatment in injured mouse hearts (Reuter et al., 2014).

The Hippo pathway controls heart development and regeneration

Cell-intrinsic signaling pathways that regulate cardiomyocyte proliferation during development have an active role in regeneration. The Hippo pathway is an evolutionarily conserved pathway that regulates organ size and growth during development in a range of animals from *Drosophila* to humans by restraining cellular proliferation, inducing apoptosis and regulating cell fate decisions (Zhao et al., 2011). Mechanical stress, cell polarity, cell adhesion and cell junction proteins activate the Hippo pathway, which acts through phosphorylation of transcription co-activators YAP and TAZ in mammals. When the Hippo pathway is off and YAP/TAZ proteins are dephosphorylated, they are translocated to the nucleus and induce pro-proliferation gene expression. The Hippo pathway participates in

cardiac organogenesis in a range of events from progenitor migration to the midline in early cardiac development (Miesfeld and Link, 2014), to cardiac proliferation (von Gise et al., 2012). Phenotypical analyses of Hippo pathway mutant mouse models suggest that disrupting Hippo effectors in cardiomyocyte precursors regulates proliferation and cell size (Zhou et al., 2015). Moreover, Hippo signaling interacts with other signaling pathways, such as Wnt, to orchestrate proliferation and size in the developing heart (Heallen et al., 2011). Mice that are null for the Hippo pathway component protein salvador homolog1 (*Sav1)* (systemic deletion and cardiomyocyte specific deletion) have improved heart regenerative capacity and reduced scar size following adult myocardial infarction and post natal day 8 post apical resection, when control mice have very limited ability to regenerate resected tissue (Heallen et al., 2013). Similar results are obtained with MI injury in *Sav1* KO as well as *Yap*-overexpressing transgenic animals, at ages as old as 1 or 2 months (Xin et al., 2013b), and enhanced regeneration appears to be due to enhanced cardiomytocyte proliferation following injury(Heallen et al., 2013; Xin et al., 2013b). More recently, Lin et al showed that cardiac-specific YAP activation following MI improves cardiac function and leads to better survival, indicating that repressing Hippo pathway can enhance regenerative outcome in adults following injury (Lin et al., 2014).

Conclusion

New research in heart regeneration in different experimental models is revealing that many molecular mechanisms may be shared by organisms that are able to regenerate their hearts. These mechanisms include stimulation of an essential immune response, regulation by extracellular cues, a role for nerves and a critical contribution for cardiomyocyte division. We still do not understand the barriers to heart regeneration that lead to extensive scarring and eventual heart failure in humans who have major cardiac injuries. The imperative for the field is to learn from the developmental biology of the heart and define the regenerative pathways. Currently, after blood flow is restored in heart attack patients, we largely watch and see how extensive the injury become. In this critical time window, there is an opportunity to identify which patients may develop heart failure in the future and treat them with a regenerative therapy. Should we deliver exogenous cells to replace the lost cardiomyocytes, or can we activate endogenous regeneration pathways at just the right moment?

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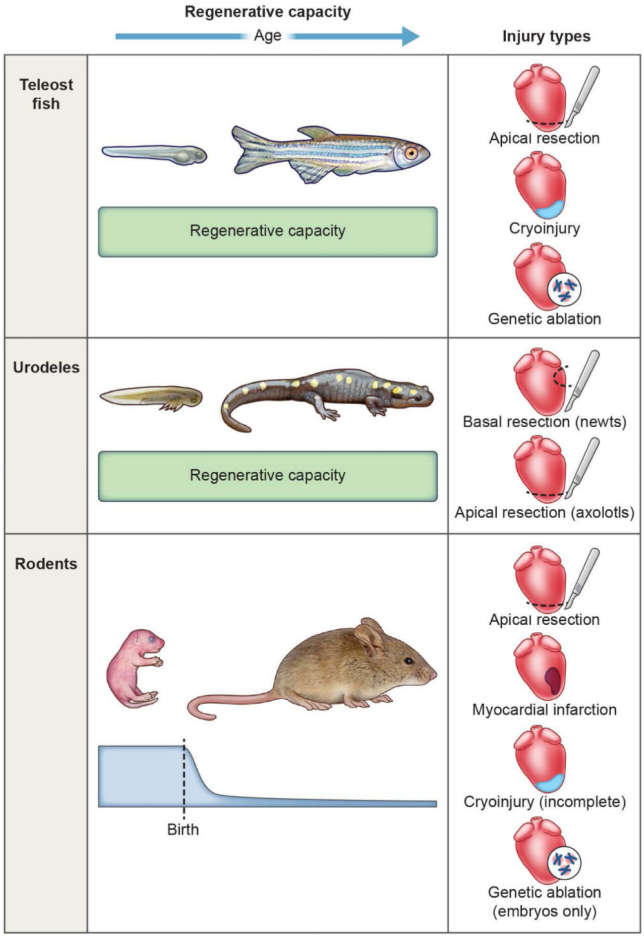

Figure 1. Cardiac Regeneration Across Model Organisms

Cardiac regeneration has been studied in a number of model systems. While lower vertebrate model species like teleost fish and urodeles retain regenerative capacity throughout adult life, anurans and mammals lose this ability in adulthood. It should be noted that not all teleost fish have been reported to have complete regenerative response upon heart injury. Future studies on different species and different injury types will broaden our understanding of evolutionary conservation of capacity for cardiac regeneration.

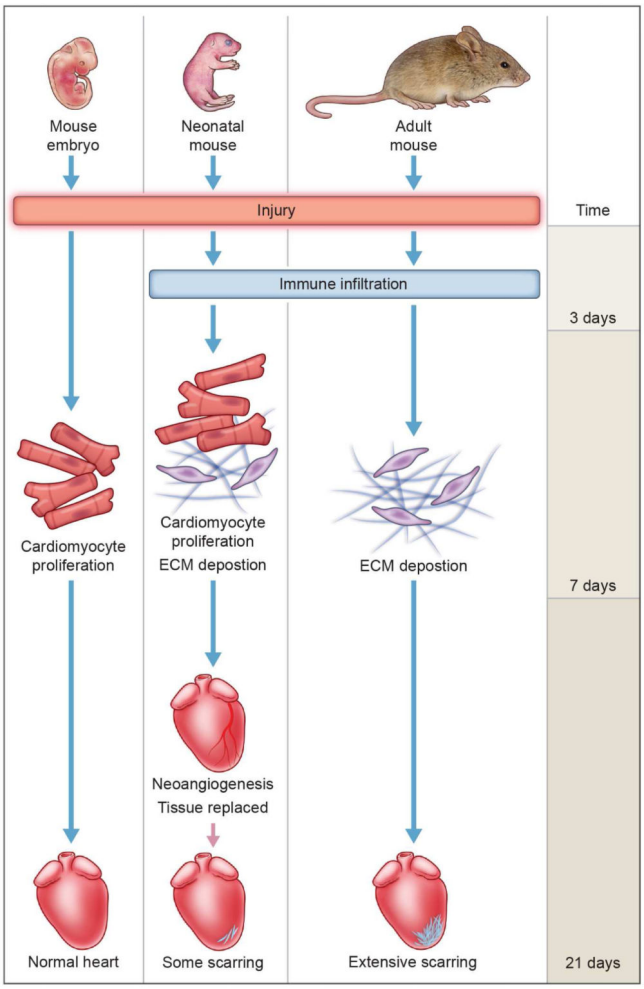

Figure 2. Mammalian response to injury

Cardiac regeneration has been explored in fetal, neonatal and adult mammals and occurs to a different extent in each model. At embryonic stages, compensatory growth in cardiomyocytes restores up to 50% of lost tissue. In the neonatal mouse, tissue can replace a majority of lost cardiomyocytes with minimal scarring in myocardial infarction and ventricular resection models. In the adult mouse, cardiomyocyte proliferation is insufficient to replace lost tissue, and extracellular matrix deposition following injury leads to extensive scarring.

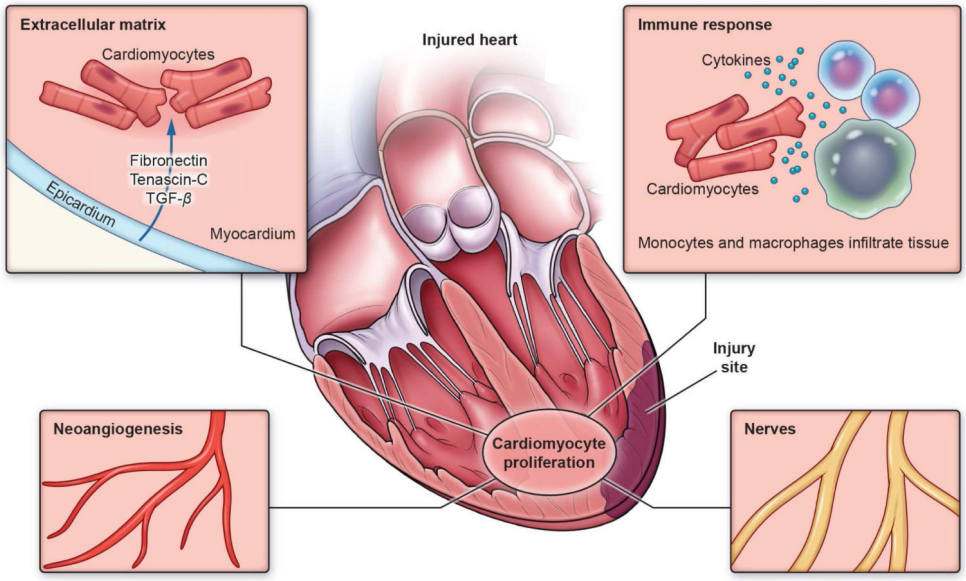

Figure 3. Evolutionarily conserved mechanisms of cardiac regeneration

Cardiomyocyte proliferation is central to the process of cardiac regeneration, and several processes have been shown to modulate proliferation upon injury in a range of organisms that are capable of a complete regenerative response. An inflammatory response tightly regulates a fine balance between proliferation and repair. Extracellular matrix deposition following injury creates a permissive environment for cellular proliferation and is influenced by signals from the epicardium following injury. Neoangiogenesis is triggered by FGF and PDGF signaling and provides a supply of oxygen and nutrients to the regrowing tissue, and new studies emphasize the requirement of nerves in the regenerate.