Stem Cell Research (2014) 13, 542–555

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Advances in understanding the mechanism of zebrafish heart regeneration

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Received 13 June 2014; received in revised form 11 July 2014; accepted 13 July 2014
Available online 19 July 2014

Abstract The adult mammalian heart was once believed to be a post-mitotic organ without any capacity for regeneration, but recent findings have challenged this dogma. A modified view assigns the mammalian heart a measurable capacity for regeneration throughout its lifetime, with the implication that endogenous regenerative capacity can be therapeutically stimulated in the injury setting. Although extremely limited in adult mammals, the natural capacity for organ regeneration is a conserved trait in certain vertebrates. Urodele amphibians and teleosts are well-known examples of such animals that can efficiently regenerate various organs including the heart as adults. By understanding how these animals regenerate a damaged heart, one might obtain valuable insights into how regeneration can be augmented in injured human hearts. Among the regenerative vertebrate models, the teleost zebrafish, *Danio rerio*, is arguably the best characterized with respect to cardiac regenerative responses. Knowledge is still limited, but a decade of research in this model has led to results that may help to understand how cardiac regeneration is naturally stimulated and maintained. This review surveys recent advances in the field and discusses current understanding of the endogenous mechanisms of cardiac regeneration in zebrafish.

Contents

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http://dx.doi.org/10.1016/j.scr.2014.07.003

Introduction

Acute myocardial infarction (MI), typically caused by coronary artery occlusion and ischemia, is a leading cause of death worldwide. For those fortunate enough to survive MI, necrotic muscle induces a massive inflammatory response that activates reparative mechanisms including recruitment and activation of local fibroblasts, leading to the replacement of lost myocardium with collagen-rich scar tissue. The scar provides a rapid solution to cardiac injury by stabilizing the wound area; however it is not contractile and weakens cardiac output, increases susceptibility to aneurysm, induces compensatory pathology and eventually leads to heart failure. Therapies that facilitate survival or replacement of myocardium after ischemic injury in the human heart are urgently needed and would have enormous social and economic impact.

Patients with acute MI represent a significant test-bed for regenerative medicine. In principle, cardiomyocytes could be generated from a variety of cellular sources and transplanted into damaged tissues to restore functional myocardium. To date, multiple endogenous stem and progenitor cell populations have been isolated from the postnatal mammalian heart and these cells to varying degrees can differentiate into cardiomyocytes when transplanted into infarcted hearts (Bearzi et al., 2007; Beltrami et al., 2003; Bu et al., 2009; Chong et al., 2011; Ellison et al., 2013; Goumans et al., 2007; Hierlihy et al., 2002; Laugwitz et al., 2005; Matsuura et al., 2004; Messina et al., 2004; Oh et al., 2003; Pfister et al., 2010; Smith et al., 2007; Uchida et al., 2013; van Berlo et al., 2014; Ye et al., 2012). Other promising sources for generating cardiomyocytes in vitro are embryonic stem cells (He et al., 2003; Kattman et al., 2006; Kehat et al., 2001; Mummery et al., 2003; Yang et al., 2008) and induced pluripotent stem cells (Mauritz et al., 2008; Narazaki et al., 2008; Zhang et al., 2009), which have been used for treating damaged hearts in animal models (Chong et al., 2014; Laflamme et al., 2007). Cardiac stem and progenitor cells are extremely rare populations which diminish in quality with age, which makes it reasonable to explore other cellular targets for regenerative therapies. The epicardium, a mesothelial layer covering the heart, has been shown to be capable of contributing to the myocardial lineage at low frequency in infarcted mouse hearts pretreated with the natural secreted signaling peptide Thymosin β4 (Smart et al., 2011). More recently, cardiac fibroblasts have been induced to transdifferentiate into cardiomyocytes in vitro and in vivo when defined cardiac transcription factors (Ieda et al., 2010; Nam et al., 2013; Qian et al., 2012; Song et al., 2012) or miRNAs (Jayawardena et al., 2012) are overexpressed.

An alternative approach would be to identify successful examples of organ regeneration in nature, dissect their mechanisms, and then attempt to apply gained insights to humans via the provision of the appropriate regenerative stimuli. Urodele amphibians and teleosts are well-known examples of animals that possess remarkable regenerative capacity in a variety of structures and organs as adults (Brockes and Kumar, 2008; Poss, 2010). Among these, the zebrafish (*Danio rerio*) is a relatively new experimental model in regeneration biology, and has been quickly established as the standard for investigating mechanisms of natural organ regeneration, primarily due to its amenability to genetic approaches. Zebrafish are highly regenerative as adults and regrow injured or amputated tissues such as fins (Johnson and Weston, 1995), maxillary barbel (LeClair and Topczewski, 2010), retinae (Vihtelic and Hyde, 2000), optic nerves (Bernhardt et al., 1996), spinal cord (Becker et al., 1997), heart muscle (Poss et al., 2002), brain (Kroehne et al., 2011), hair cells (Ma et al., 2008), pancreas (Moss et al., 2009), liver (Sadler et al., 2007), and kidney (Diep et al., 2011).

Although cardiac regeneration and repair have been investigated in other teleost models (Grivas et al., 2014; Ito et al., 2014; Lafontant et al., 2012), zebrafish arguably display the most robust and best characterized cardiac regenerative responses known to date among non-mammalian vertebrate models (Chablais et al., 2011; González-Rosa et al., 2011; Parente et al., 2013; Poss et al., 2002; Schnabel et al., 2011; Wang et al., 2011). This review will summarize recent advances in the field of regenerative medicine and discuss cellular and molecular mechanisms underlying the cardiac regenerative response in zebrafish.

Origins of regenerated myocardium

Fate-mapping studies

Identifying cellular origins of regenerating tissues is a fundamental question in regeneration biology. Several studies have performed fate-mapping analyses to investigate the cellular origin(s) of cardiac muscle during heart regeneration in zebrafish. By using inducible genetic fate-mapping techniques (Buckingham and Meilhac, 2011), two studies directly examined the contribution of cardiomyocytes to the regenerating zebrafish heart (Jopling et al., 2010; Kikuchi et al., 2010). In both cases, two transgenic lines were used: one line carries a 4-hydroxytamoxifen (4-HT) – inducible Cre recombinase (CreER) gene from which expression is driven in cardiomyocytes by the promoter of the *cardiac myosin light chain 2* (*cmlc2*) gene, also known as *myosin light chain 7* (*myl7*) gene; and the other is an indicator line in which enhanced green fluorescent protein (EGFP) reporter expression can be induced in CreER-

expressing cells after excision of the *loxP*-flanked transcriptional stop cassette by 4-HT treatments (Jopling et al., 2010; Kikuchi et al., 2010). Using this system, almost all cardiomyocytes expressing *cmlc2* were pre-labeled with EGFP by 4-HT treatments before injury, and regeneration experiments were performed 30 days after ventricular resection. The results from both studies clearly showed that the vast majority of regenerated myocardium is labeled with EGFP, with no significant difference detected in the proportion of EGFP$^+$ cardiomyocytes in the regenerated tissue compared

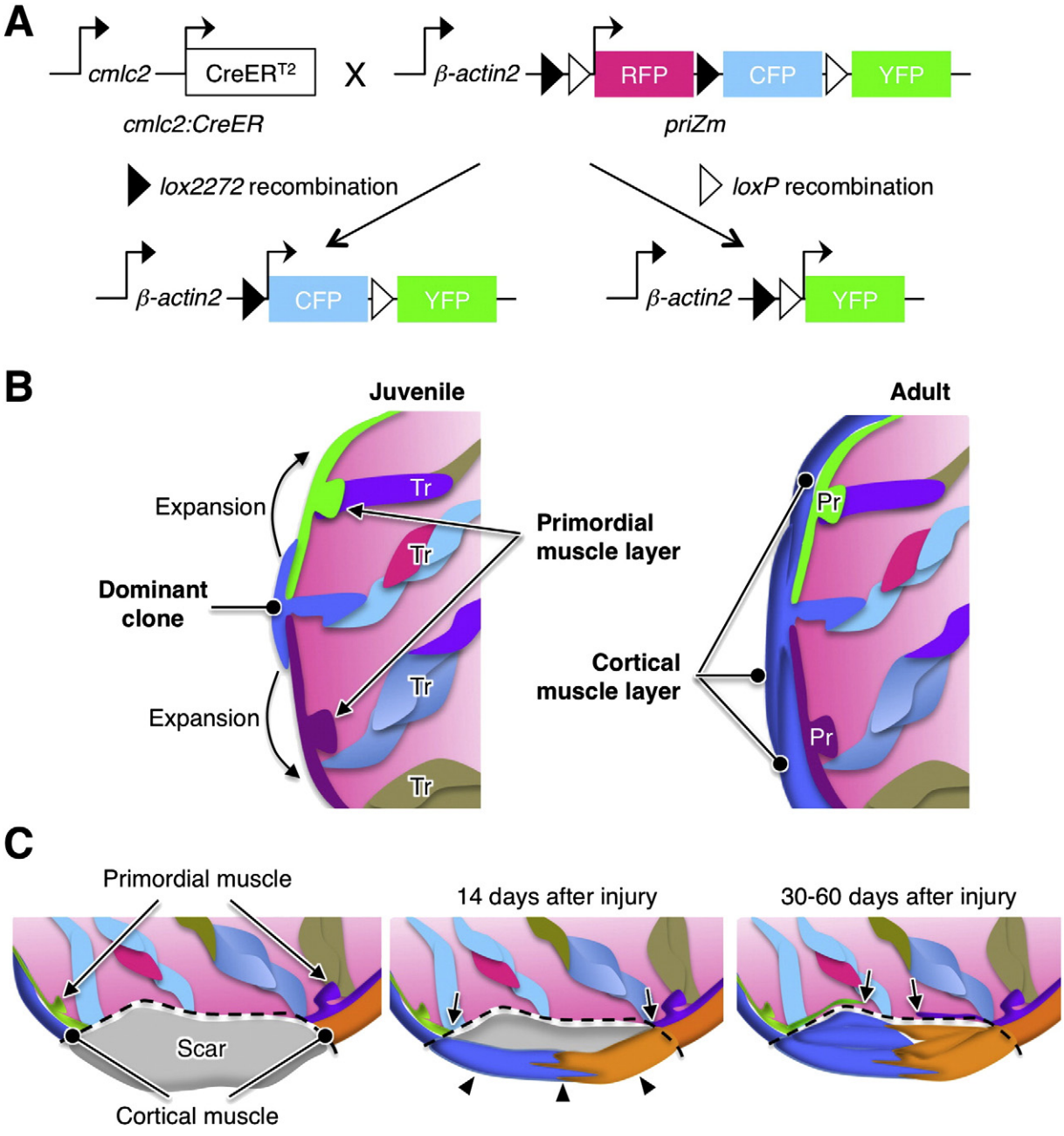

Figure 1 New cellular sources for cardiac muscle. (A) A transgenic system for multicolor clonal labeling of cardiomyocytes. The *priZm* cassette expresses a red fluorescent reporter protein (RFP) as a default color under the control of the *β-actin2* promoter. Differential recombination between paired *lox2272* (black triangle) or *loxP* (white triangle) sites induces cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) expression, respectively. The *priZm* transgenic strain carries multicopy tandem insertions of the indicated cassette at a single genetic locus, and limited Cre-mediated excision events at the *loxP* or *lox2272* sites produce many possible permanent colors with various combinations of RFP, CFP, and YFP expression. This strategy allows multicolor clonal labeling of cardiomyocytes in the double transgenic context with the *cmlc2:CreER* strain (Kikuchi et al., 2010) after tamoxifen treatments (Gupta and Poss, 2012). (B) Muscle lineages in the zebrafish ventricle. Tr, trabecular muscle; Pr, primordial muscle. (C) Regeneration of primordial and cortical muscle cells. Arrows and arrowheads in the middle and right panels indicate the edge of migrating clones of the primordial muscle layer and the ventricular wall repaired by cortical muscular layer, respectively. Dotted line: amputation plane. The figure is a summary of the work by Poss and colleagues (Gupta and Poss, 2012).

with uninjured ventricles, indicating that existing *cmlc2*$^{+}$ cardiomyocytes, but not *cmlc2*$^{-}$ non-myocytes, are the major source for new muscle during zebrafish heart regeneration.

Epicardial derived cells (EPDCs) have previously been shown to contribute to and regulate vascularization during cardiac regeneration in zebrafish (Kim et al., 2010; Lepilina et al., 2006); however, their contribution to regenerating myocardium has not been directly tested. Recent studies have addressed this issue using two different approaches: genetic fate-mapping (Kikuchi et al., 2011a) and transplantation (González-Rosa et al., 2012). In the inducible genetic fate-mapping study, the *cis*-regulatory sequences of *transcription factor 21* (*tcf21*), also known as *Epicardin* (Robb et al., 1998), *Pod1* (Quaggin et al., 1999), *Capsulin* (Hidai et al., 1998; Lu et al., 1998), were used to target CreER to the zebrafish epicardium (Serluca, 2008), and its progeny were traced during regeneration following CreER-dependent labeling with EGFP, which was induced from an indicator line with a transient 4-HT treatment. In the transplantation assay, a piece of cardiac tissue was collected from the injured ventricle of the *β-actin: EGFP* strain, in which most cardiac cells, including epicardial cells, were labeled with EGFP, and transplanted into a freshly injured wild-type heart that had been irradiated prior to the injury to suppress proliferation of cells in the recipient tissue. In both studies, many fate-mapped cells were detected in the regenerate and histologically characterized as myofibroblasts (González-Rosa et al., 2012) and perivascular cells (González-Rosa et al., 2012; Kikuchi et al., 2011a), but not as cardiomyocytes (González-Rosa et al., 2012; Kikuchi et al., 2011a). These results indicate that epicardium or EPDCs do not give rise to cardiac muscle during zebrafish heart regeneration, which is consistent with the finding that cardiomyocytes are the major source for regenerating myocardium.

The lineage tracing experiments described above did not address whether the *cmlc2*$^{+}$ population includes multiple muscle cell types that may contribute differently to regeneration. Recently, Poss and colleagues have generated a transgenic system that allows multicolor clonal labeling of cardiomyocytes, and defined previously uncharacterized myocardial lineages, while revealing their behaviors during morphogenesis of the zebrafish heart (Gupta and Poss, 2012). This system consisted of two transgenic alleles, *cmlc2:CreER* (Kikuchi et al., 2010) and a multicolor reporter cassette termed *priZm*, which was constructed by utilizing the Brainbow technology originally developed to visualize individual neurons and connections in the mouse brain (Livet et al., 2007) (Fig. 1A). Using this system, the authors induced multicolor clonal labeling at 2 days post fertilization, and performed image analyses at various developmental stages to characterize in detail how cardiomyocyte clones behave during morphogenesis of the heart.

The zebrafish ventricle has been recognized to contain two types of cardiac muscle, comprising a peripheral wall termed the compact layer and an inner trabecular layer (Hu et al., 2001). Formation of trabeculae has been shown to occur through delamination of cardiomyocytes from the ventricular wall muscle (Liu et al., 2010). The clonal labeling approach described above refined this anatomical and developmental understanding of the zebrafish heart. First, the authors found that the outer muscular wall of the embryonic heart tube persists even in the adult ventricle. To accommodate cardiac growth, this muscle layer expands only circumferentially throughout the life of the fish, maintaining a single-cardiomyocyte thickness as seen in the embryonic heart. This muscle lineage, termed primordial muscle, persists to form the innermost layer of the peripheral muscular wall of the adult ventricle (Fig. 1B, right panel). Second, the authors observed that at the juvenile stage, the primordial muscle layer is penetrated by a few trabecular cardiomyocyte clones – ~8 clones per ventricle – as rare and spatially segregated events, and these dominant cardiomyocyte clones grow over the entire primordial layer, creating another muscular wall that constitutes the outermost layer of the peripheral muscular wall of the adult ventricle (Fig. 1B). This muscle lineage is clearly distinct from the trabecular and primordial muscle lineages, and has been termed the cortical muscle layer. Thus, in the refined view, the adult zebrafish ventricle consists of three different muscle lineages, primordial, trabecular, and cortical muscle, and these lineages develop in this temporal sequence during development (Gupta and Poss, 2012).

After definition of the distinct muscle lineages, an interesting question to ask is whether these lineages differentially contribute to heart regeneration. The authors performed regeneration experiments using ventricles of adult *cmlc2: CreER*; *priZm* fish in which multicolor clonal labeling had been induced at an embryonic stage as described above. At 14 days post injury, cortical muscle clones adjacent to the wound expanded in lateral and radial directions into the injury site, but integration of primordial muscle cells did not occur at this time point (Fig. 1C, middle panel). By 30 days after injury, as cortical muscle reconstructed the muscular wall, clones of the primordial muscle layer became detectable in the regenerate, and a complete primordial layer was restored, forming a single cell layer boundary between trabecular and regenerated cortical muscle by 60 days after trauma (Fig. 1C, right panel). Thus, both primordial and cortical muscle layers contribute to the regenerate but they are formed in reverse order compared to the sequence of normal development. Primordial muscle expands only circumferentially, as observed during cardiac morphogenesis, whereas cortical muscle expands in a less restricted manner and contributes the greater mass of the new ventricular wall. This result is consistent with the previous observation that regenerated muscle in amputated hearts is primarily derived from a myocyte sub-population in the lateral wall of the ventricle in which the cardiogenic transcription factor *gata4* is activated (Kikuchi et al., 2010).

More recently, the same group has performed similar multicolor clonal analyses on *gata4*$^{+}$ and *cmlc2*$^{+}$ cortical muscle cells with labeling induced at the adult stage, and these experiments revealed that unlike the initial ventricular morphogenesis, there is no clonal-dominance in cortical muscle cells during regeneration (Gupta et al., 2013), suggesting that the proliferation mechanism utilized by cardiomyocytes during regeneration is distinct from the one used during cardiac development.

Dedifferentiation

Dedifferentiation is a cellular process in which a specialized cell reverts to an earlier developmental stage in response to a

stimulus. Cardiomyocyte dedifferentiation, which is typically characterized by a reduction of sarcomere structures and expression of developmental marker genes, appears to be the dominant mechanism for heart regeneration in zebrafish. In the injured zebrafish heart, transmission electron microscopy and immunofluorescence revealed that cardiomyocytes acquire a less-organized sarcomeric structure in the regenerating area (Jopling et al., 2010; Kikuchi et al., 2010). In addition, as mentioned above, analysis using a reporter strain has shown that most regenerating myocytes induce the regulatory sequences of *gata4*, a gene essential for cardiac development, and maintain this expression throughout the regeneration process (Kikuchi et al., 2010). Together with the previous observation that other embryonic cardiogenic genes are also induced upon injury (Lepilina et al., 2006), the prevailing model is that in response to injury, existing cardiomyocytes reduce their contractile state to acquire an immature form in which cell division is facilitated.

The dedifferentiation phenotypes described above are reminiscent of those seen in embryonic mammalian cardiomyocytes. Disassembly of sarcomere structures has been shown as a feature of proliferating cardiomyocytes in the embryonic mouse heart (Ahuja et al., 2004). *Gata4* gene expression is also induced in myocardium during the embryonic development of the mouse heart, and its function is required for cardiomyocyte proliferation (Zeisberg et al., 2005). These results might suggest that mechanisms used for regenerating the zebrafish heart are also conserved in the mammalian hearts. It would be interesting to address how injury stimulus can activate the regenerative mechanism in the zebrafish heart and why such mechanism cannot be re-activated in the postnatal mammalian heart to induce cardiomyocyte proliferation.

The functional roles of the upregulated embryonic genes in the regenerating zebrafish heart have not been fully characterized, but a recent study has demonstrated that Gata4 is essential for regeneration. Myocardial overexpression of a dominant-negative form of Gata4 induced an arrest of regeneration after ventricular resection with collagenous scar forming at the wound area. Interestingly, histological analyses revealed that Gata4 inhibition specifically blocks the proliferation of cortical cardiomyocytes, but not the trabecular cardiomyocytes (Gupta et al., 2013), consistent with the specific activation of the *gata4* promoter in the cortical area (Kikuchi et al., 2010).

Dedifferentiation characteristics are also detected in the adult mammalian heart under some pathological situations. Braun and colleagues investigated heart tissue samples from chronic dilated cardiomyopathy patients to address what factor(s) can cause dedifferentiated phenotypes in human cardiomyocytes (Kubin et al., 2011). By using proteomics and biochemical approaches, Oncostatin M (OSM), an interleukin-6 family cytokine with pleiotropic functions (Heinrich et al., 2003; Tanaka and Miyajima, 2003), was found to be highly expressed in hearts with dilated cardiomyopathy but not in healthy hearts. The authors revealed that OSM induces loss of sarcomeric structures and re-expression of fetal marker genes in adult cardiomyocytes in vitro and in vivo through signals mediated by the OSM receptor (Oβ). OSM could also enhance cell cycle re-entry at least in neonatal cardiomyocytes in vitro, and Oβ was required for the acquisition of dedifferentiation phenotypes in cardiomyocytes at the border zone in the infarcted mouse heart. Consistently, the activation of OSM signaling led to increased animal survival after acute MI in the mouse model. However, in a model of dilated cardiomyopathy, the continuous activation of the OSM pathway reduced both cardiac function and animal survival, which is possibly due to the reduction of contractile force and the acceleration of ventricular dilation by the persistence of dedifferentiated cardiomyocytes (Kubin et al., 2011; Pöling et al., 2012, 2014). Thus, the activation of dedifferentiation pathways alone may not simply lead to regeneration in mammals, a notion that may need to be considered as results obtained in zebrafish are translated to clinically relevant situations.

Transdifferentiation

Transdifferentiation is a regenerative phenomenon in which one cell type converts to another, sometimes via an undifferentiated intermediate. A classical example is during lens regeneration in adult newts, where a new lens is created by the dorsal pigmented iris tissue (Eguchi et al., 1974; Grogg et al., 2005). Given our understanding of the epigenetic stability of differentiated states, it would seem that such a change would only occur in developmentally related lineages, and so this phenomenon may not be commonly observed in other regeneration models under natural conditions. However, it is increasingly recognized that experimental manipulations can reprogram epigenetic states, which was most notably demonstrated by the induction of pluripotent stem cells (induced pluripotent stem cells; iPSC) from adult somatic cells by expression of defined transcription factors (Takahashi and Yamanaka, 2006). To date, various cellular sources have been shown to transdifferentiate into cardiomyocytes using similar strategies. By expressing combinations of cardiac transcription factors, cardiac or dermal fibroblasts were shown to transdifferentiate into cardiomyocytes in vitro (Ieda et al., 2010) and non-cardiogenic mesoderm into beating myocardium in vivo (Takeuchi and Bruneau, 2009). More recent studies have demonstrated that the overexpression of transcription factors (Nam et al., 2013; Qian et al., 2012; Song et al., 2012) or miRNAs (Jayawardena et al., 2012) directly induces lineage reprogramming of cardiac fibroblasts into cardiomyocytes in vivo.

Interestingly, transdifferentiation can also occur naturally to accommodate extreme loss of a particular cell lineage. Upon genetic ablation of nearly all of the pancreatic β cells in the adult mouse, glucagon-producing α cells, a distinct endocrine cell type in the pancreas, gain the expression of β cell specific transcription factors and differentiate into insulin producing cells (Thorel et al., 2010). In a recent study, Chi and colleagues have examined this phenomenon in the context of cardiac regeneration, and discovered a novel regeneration mechanism in the zebrafish heart (Zhang et al., 2013) (Fig. 2). The authors first generated a genetic ablation model in which the bacterial enzyme Nitroreductase is expressed only in ventricular muscle under the control of regulatory sequences of the *ventricular myosin heavy chain* (*vmhc*) gene. Nitroreductase can convert a prodrug into a cytotoxic agent and its transgenic expression enables one to perform cell type specific ablation in an inducible manner (Curado et al., 2007). Using this model, the authors analyzed regenerative responses with a particular focus on the atrium, as

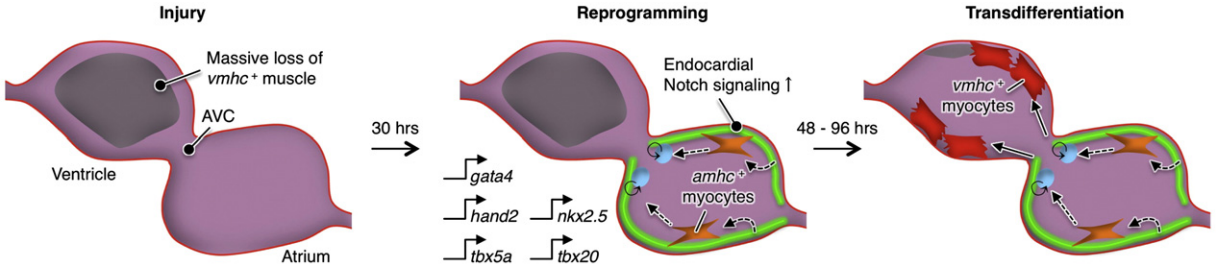

Figure 2 Transdifferentiation of atrial myocytes to ventricular muscle. After massive genetic ablation of ventricular muscle (left), Notch signaling is activated in atrial endocardial cells with upregulation of multiple heart field markers in the entire heart (middle). Notch signaling indirectly regulates the transdifferentiation of *amhc*$^{+}$ myocytes to *vmhc*$^{+}$ myocardium possibly through intermediate proliferative progenitor-like cells expressing cardiogenic transcription factors (right). The figure is a summary of the work by Chi and colleagues (Zhang et al., 2013).

atrial cardiomyocytes have been shown previously to proliferate in response to ventricular injury in vertebrate hearts (McDonnell and Oberpriller, 1983, 1984; Oberpriller et al., 1987). Ablation was induced in zebrafish embryos at 3–4 days post-fertilization, an early developmental stage in which cardiac chamber specification is completed. The results suggest that new ventricular cardiomyocytes arise from the area adjacent to the atrioventricular canal (AVC) and expand across the chamber to restore lost ventricular myocardium. Inducible genetic lineage tracing using the regulatory sequences of *atrial myosin heavy chain* (*amhc*) gene, also known as *myosin heavy chain 6* (*myh6*) gene, and live-imaging analyses provided evidence that *amhc*$^{+}$ atrial cardiomyocytes acquire ventricular muscle fate and migrate into the injured area to restore lost ventricular muscle (Zhang et al., 2013) (Fig. 2, right panel). When ablation was induced in the adult heart, *amhc*$^{+}$ cardiomyocytes did not give rise to ventricular muscle (Zhang et al., 2013), suggesting that the atrial-to-ventricular transdifferentiation is an age-dependent mechanism.

To understand the molecular mechanism of this event, the authors performed gene expression analyses and found that ventricular injury induces the expression of the genes for Notch1b and its ligand DeltaD in the atrium, concomitant with re-induction of various heart field markers in the entire heart. Pharmacological inhibition of Notch signaling impaired the recovery of ventricular morphology and function of the ablated heart, indicating that the Notch pathway is essential for the regeneration mechanism. Interestingly, the analysis of a transgenic strain in which Notch signaling activity can be monitored by reporter gene expression revealed that this signaling pathway is activated in the atrial endocardium, suggesting that atrial-to-ventricular transdifferentiation is indirectly regulated by the endocardium through the Notch pathway (Zhang et al., 2013) (Fig. 2, middle and right panels).

Regulations by epicardial and endocardial cells

Organ-wide injury responses

Injury responses in the zebrafish heart are initiated in an organ-wide manner before restricting to the wound area. All major cardiac tissues – epicardium, endocardium, and myocardium – employ this strategy in response to injury (Kikuchi et al., 2010, 2011b; Lepilina et al., 2006). Within an hour of injury, endocardial cells throughout the heart undergo morphological changes, such as rounding up and detachment from myocardial cells, and induce the expression of developmental marker genes such as *raldh2* (*retinal aldehyde dehydrogenase 2*), also known as *alh1a2* (*aldehyde dehydrogenase 1 family, member A2*), and *heg* (*heart of glass*) in the entire heart by 3 h post-injury (Kikuchi et al., 2011b). Similarly, the whole epicardium upregulates *tbx18* (*T-box transcription factor 18*) and *raldh2* expression by 3 days post-trauma (Lepilina et al., 2006). By 7 days after injury, the myocardium activates *gata4* regulatory sequences in the lateral wall of the ventricle, which is now defined as the cortical muscle layer (Gupta et al., 2013; Kikuchi et al., 2010). At different time points, depending on the cell type, these global expression signatures regress although they are maintained at the injury site, where they aid or indicate cardiac muscle regeneration (Kikuchi and Poss, 2012).

The organ-wide activation pattern is not limited to the induction of developmental marker genes. As mentioned in later sections, Fibronectin, a major component of extracellular matrix, as well as inflammatory cytokines and their downstream signaling molecules have also been shown to follow organ-wide activation patterns during zebrafish heart regeneration (Fang et al., 2013; Wang et al., 2013). The mechanisms underpinning these responses remain unclear; however, when zebrafish were intraperitoneally injected with the inflammatory agent Lipopolysaccharide, *raldh2* expression was induced in the entire epicardium and endocardium of the uninjured heart, indicating that inflammatory signals might be involved (Kikuchi et al., 2011b). This speculation seems consistent with the aforementioned organ-wide activation of inflammatory genes (Fang et al., 2013).

At first sight, it seems natural to imagine that in tissues that are competent for regeneration, local signals provoked by injury, control the key regenerative events. However, as noted, during heart regeneration in zebrafish the response is first initiated in an area including the injury site but also distant from the injury. Accumulating evidence suggests that an organ-wide injury response is neither unique to zebrafish nor to the heart. Ancient fish *Polypterus senegalus* also induce expression of *raldh2* in the entire epicardium and endocardium in response to local injury (Kikuchi et al., 2011b). The neonatal mouse induces cardiomyocyte dedifferentiation phenotypes (Porrello et al., 2011b) and accumulation of angiogenic macrophages (Aurora et al., 2014), not only near the injury area, but also in the entire ventricle after injury. During

liver regeneration, partial hepatectomy is known to affect tissue distant from the trauma and to activate compensatory hepatocyte proliferation in spared lobes (Taub, 2004). More recently, injury to the *tibialis anterior* muscle of the mouse hind limb has been shown to potentiate the regenerative capacity of satellite cells that reside in the intact contralateral limb (Rodgers et al., 2014). Thus, the capacity to respond to distant injury signals might be a common feature conserved in regenerative animals and tissues, and might be a key to mounting effective regenerative responses against injury.

Neovascularization

After responding to injury in an organ-wide manner, epicardial and endocardial cells retain the activation signatures at the wound area to facilitate heart regeneration. A notable regenerative event to which these cells contribute is cardiomyocyte proliferation. A number of molecular mechanisms have been identified whereby epicardial and endocardial cells stimulate cardiomyocyte proliferation, which will be discussed separately in a later section. Another important event during heart regeneration is the establishment of new vasculature. To date, multiple developmental signaling pathways have been implicated in this process during zebrafish heart regeneration. Epicardial cells appear to facilitate the creation of new vascular components, as in heart development in higher vertebrates (Gittenberger-de Groot et al., 2000; Mikawa and Fischman, 1992; Mikawa and Gourdie, 1996; Pérez-Pomares et al., 2002; Tevosian et al., 2000). As mentioned earlier, the results of genetic fate mapping analysis and transplantation have revealed that epicardial cells do not readily acquire endothelial fate, but rather contribute to perivascular components (González-Rosa et al., 2012; Kikuchi et al., 2011a). Moreover, they likely also promote the formation of new vasculature during regeneration through paracrine mechanisms, as observed during the repair of infarcted mouse hearts (Zhou et al., 2011). Members of the fibroblast growth factor (Fgf) signaling pathway are upregulated after resection injury and this pathway seems to be involved in the repair process. The expression of Fgf ligand gene *fgf17b* is induced in injured myocardium, and Fgf receptor genes *fgfr2* and *fgfr4* are correspondingly upregulated in epicardial cells within the regenerate. Inhibition of Fgf signaling by transgenic overexpression of a dominant-negative Fgfr inhibits epicardial cell integration into the wound area and also blocks neovascularization, which leads to regeneration arrest and scar formation (Lepilina et al., 2006). Platelet-derived growth factor (Pdgf) signaling also seems to be important during regeneration. The expression of a Pdgf receptor gene *pdgfrβ* is induced in the regenerating heart and pharmacological blockade of receptor function inhibits proliferation in epicardial cells and coronary vasculature formation (Kim et al., 2010). Thus, both Fgf and Pdgf signaling appear to reactivate vascular development during heart regeneration in zebrafish.

Cardiomyocyte migration

As described above, the lineage tracing studies have demonstrated that cardiomyocytes generate new myocardium through proliferation; however, the mechanism by which proliferating cardiomyocytes integrate into the wound area remains poorly understood. Kawakami and colleagues have recently shown that epicardial cells provide a critical support for cardiomyocyte migration during regeneration (Itou et al., 2012). The authors found that after apical resection of the ventricle, a chemokine ligand gene *cxcl12a* is transiently induced in epicardial cells between 3 and 5 days after trauma, concomitantly with the expression of its receptor *cxcr4b* in cardiomyocytes. The authors performed loss-of-function analyses of the Cxcr4 pathway using either pharmacological inhibition of Cxcr4 or in *cxcr4b* null mutant zebrafish, and observed less integration of tissue stained for muscle myosin at the wound area. Interestingly, with Cxcr4 inhibition, the total extent of cardiomyocyte proliferation, neovascularization, and fibrin clearance – phenotypes relevant to regeneration – was normal. Instead, the ratio of proliferating cardiomyocytes in the wound area versus other regions appeared significantly reduced, suggesting that migration of proliferating cardiomyocytes is impaired. This hypothesis was directly addressed using a new transgenic strain in which a photoconvertible fluorescent reporter, Kaede (Ando et al., 2002), is specifically expressed in cardiomyocytes. In this strain, irradiation can permanently convert native green fluorescence of the reporter to red fluorescence, which enables tracing of the behavior of irradiated cardiomyocytes during regeneration. After injury, photolabeled cardiomyocytes bordering the edge of the lesion did not relocate into the wound area in the presence of Cxcr4 inhibition at all time points analyzed (Itou et al., 2012). This result indicates that epicardial cells contribute to heart regeneration in part by regulating cardiomyocyte migration through chemokine signaling.

The extracellular matrix is the non-cellular tissue structure that provides essential scaffolds for cell growth and differentiation. The importance of extracellular matrix for the formation of cardiac muscle is highlighted by the demonstration that a de-cellularized rat cardiac matrix can reconstitute a beating heart-like organ when re-seeded with neonatal cardiac cells (Ott et al., 2008). A recent study published by Wang et al. has found that during zebrafish heart regeneration, extracellular matrix deposition is dynamically regulated by epicardial cells and this is essential for new myocardium to be properly restored in the ventricular wall (Wang et al., 2013). Using a proteomics approach, the authors found that the production of Fibronectin, a major component of the extracellular matrix, is prominently upregulated in the regenerating zebrafish heart. Histological analyses revealed that expression of fibronectin paralogous genes, *fn1* and *fn1b*, is initially induced in the epicardium in the entire heart and then localized to the wound area, whereas the integrin gene, *itgb3*, encoding a Fibronectin receptor, is expressed in cardiomyocytes. With Fibronectin loss-of-function, which can be induced either with a temperature sensitive mutant allele of *fn1* (Trinh and Stainier, 2004) or by expressing dominant-negative Fibronectin, new myocardium was not properly formed in the wound area after apical resection. Interestingly, cardiomyocyte proliferation was not impaired by Fibronectin knockdown, a phenotype reminiscent of the migration defect described above, indicating that Fibronectin is not required for promoting cardiomyocyte proliferation. Thus, epicardial cells play a critical role in establishing and providing extracellular matrix cues that support heart regeneration in zebrafish.

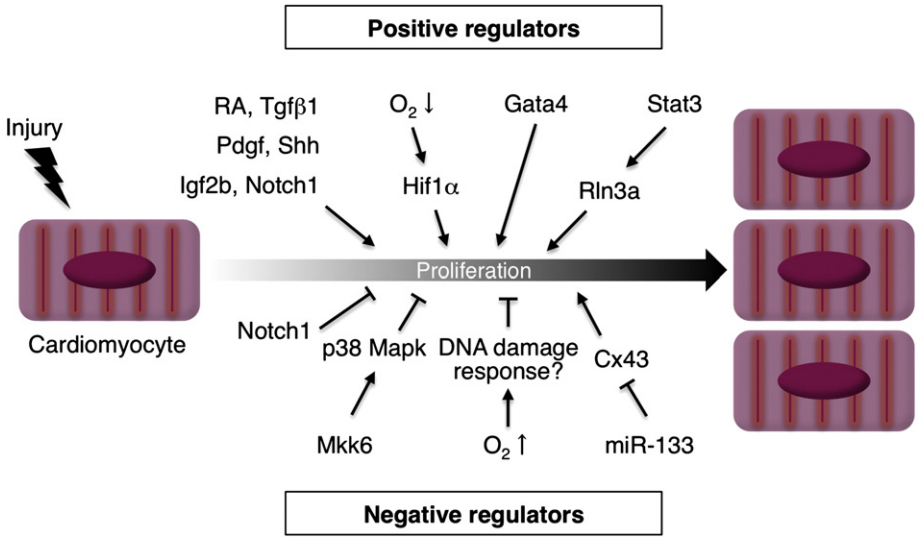

Figure 3 Signaling pathways regulating cardiomyocyte proliferation during zebrafish heart regeneration. See text for details.

Molecular mechanisms of cardiomyocyte proliferation

Positive regulators

Given the identification of existing cardiomyocytes as the dominant source for regenerating myocardium, the mechanism of cardiomyocyte proliferation is increasingly recognized as a major area of interest within the field. Thus far, a number of pathways that positively regulate cardiomyocyte proliferation have been identified (Fig. 3). Upon injury, retinoic acid (RA) synthesis (Kikuchi et al., 2011b) and expression of various developmental growth factor genes such as *transforming growth factor β1* (Chablais and Jaźwińska, 2012), *pdgf* (Lien et al., 2006), *sonic hedgehog* (Choi et al., 2013), and *insulin-like growth factor 2b* (Choi et al., 2013; Huang et al., 2013) are upregulated at the injury site, where these factors promote proliferation of cardiomyocytes. The expression of Notch family genes, *notch1a*, *notch1b*, and *notch3*, has also been shown to be induced in epicardial and endocardial cells at the injury site (Raya et al., 2003; Zhao et al., 2014). Interestingly, cardiomyocyte proliferation and myocardial regeneration are similarly impaired by either inhibiting or activating the Notch pathway, indicating that the balance between activation and inhibition of Notch signaling is critical for a successful regeneration of the zebrafish heart (Zhao et al., 2014).

Molecular mechanisms of cardiomyocyte proliferation have been addressed by unbiased gene expression analyses (Lien et al., 2006; Sleep et al., 2010). However, since the heart is highly heterogeneous in cellular components, gene expression changes in cardiomyocytes would be better pursued by a myocardial-specific approach. One such approach is translating ribosome affinity purification (TRAP), which can enrich cell type specific mRNAs by transgenically expressing EGFP-tagged ribosomal proteins under a tissue-specific promoter and purifying polysomal mRNAs using anti-EGFP antibodies (Heiman et al., 2008). Fang et al. have recently applied this technology to obtain gene expression profiles of regenerating cardiomyocytes (Fang et al., 2013). In the resulting profiles, the most prominently upregulated genes upon injury were involved in the Jak1 (Janus kinase1)/Stat3 (signal transducer and activator of transcription 3) pathway, including *il6st* (*interleukin 6 signal transducer*), a cytokine receptor gene, *jak1*, *stat3*, and a Stat3 target gene *socs3b* (*suppressor of cytokine signaling 3b*). The expression of multiple Il6st co-receptor genes was also confirmed, but interestingly, the zebrafish paralog of the OSM receptor, a molecule regulating dedifferentiation phenotypes in mammalian cardiomyocytes (Kubin et al., 2011), was not detected. As mentioned above, the expression of these genes followed an organ-wide activation pattern: expression was initially induced in the entire heart, then maintained in the vicinity of the regenerating myocardium.

To perform cardiomyocyte-specific loss-of-function analysis of the Stat3 pathway, the authors generated a transgenic line that enables inducible expression of dominant-negative Stat3 (dnStat3) after excision of *loxP*-flanked stop sequences, and crossed this line with the *cmlc2:CreER* line (Kikuchi et al., 2010). Regeneration experiments revealed that with Stat3 inhibition, cardiomyocyte proliferation was reduced to nearly 20% of the control level and myocardial regeneration was blocked with the formation of massive collagenous scar tissue at the injury site, indicating that myocardial Stat3 function is essential for cardiomyocyte proliferation and regeneration. Further gene expression analyses of the hearts expressing dnStat3 revealed that Stat3 function is required for the production of Relaxin 3a (Rln3a), a Relaxin family hormone that activates cAMP dependent pathways through G protein-coupled receptors (Hsu et al., 2002). Chromatin immunoprecipitation showed that Stat3 is recruited to the *rln3a* promoter upon cardiac injury, and injection of recombinant human RLN3 peptides partially rescued the impaired myocardial proliferation in the presence of dnStat3. Thus, Stat3 regulates cardiac muscle regeneration by promoting myocardial release of a paracrine factor, Rln3a, which in turn induces regenerative proliferation of cardiomyocytes (Fang et al., 2013).

The systemic environment of the body and its overall physiology can be altered after injury, and this may play a

significant role in cardiac regeneration. Given the high oxygen demand in contracting myocardium, one of the environmental changes that is likely to occur in the heart after injury is an insufficient oxygen supply due to reduced cardiac function. Belmonte and colleagues have recently addressed how the low oxygen condition, hypoxia, affects heart regeneration in zebrafish (Jopling et al., 2012a). Using hypoxyprobe, a chemical reagent that can be used to visualize hypoxic cells, the authors showed that hypoxia, while ubiquitous in injured ventricular tissue, was more elevated within the clot and injury border zone areas, where regeneration events are taking place. Since one of the cell types at the injury site with enhanced hypoxyprobe signal was confirmed as cardiomyocytes, the authors next examined the effect of hypoxia on cardiac muscle regeneration. To mimic systemic hypoxia, the authors induced severe anemia by treating zebrafish with Phenylhydrazine, a hemolytic chemical reagent, and found that cardiomyocyte proliferation was elevated during regeneration in the anemic zebrafish. Consistently, hypoxic culture conditions increased the number of cardiomyocytes in mitosis and showing dedifferentiation phenotypes in vitro, whereas hyperoxic conditions strongly inhibited cardiomyocyte proliferation both in vitro and in vivo. Collectively, these results suggest that environmental oxygen concentration is a critical factor for heart regeneration in zebrafish.

The cellular response to hypoxia is regulated by a family of transcription factors, hypoxia-inducible transcription factors (Hifs), which consist of α and β subunits that form stable heterodimers under hypoxic conditions and activate genes for cellular adaptations to low oxygen (Semenza, 2014). A transgenic strain was generated that enables conditional Cre-dependent myocardial expression of a dominant-negative form of Hif1α (dnHif1α), and crossed with a muscle-specific inducible Cre driver strain (Jopling et al., 2010). With dnHif1α expression, DNA synthesis of cardiomyocytes was reduced and myocardial regeneration appeared to be incomplete at 30 days after injury, indicating that Hif1α-mediated signaling is positively regulating cardiac muscle regeneration in zebrafish.

Sadek and colleagues have recently reported that the oxygen-rich environment of the postnatal mammalian heart contributes to the generation of reactive oxygen species (ROS), and have shown that ROS induces oxidative DNA damage and subsequently a DNA damage response, contributing to cell cycle arrest in mammalian cardiomyocytes (Puente et al., 2014). By contrast, such responses seem to be prevented in the regenerative zebrafish heart, likely due to low-oxygen saturation of the aquatic environment that they live in and the inherently low oxidative metabolism of zebrafish cardiomyocytes (Puente et al., 2014). As described above, the level of hypoxia is elevated in the vicinity of regenerating area in the zebrafish heart, which might help to further prevent the activation of the DNA damage response in proliferating cardiomyocytes.

Negative regulators

Compared to positive regulations, negative regulators of the regenerative proliferation of cardiomyocytes in zebrafish have not been well characterized (Fig. 3). One of the molecular mechanisms that contributes to cell cycle arrest in postnatal mammalian cardiomyocytes is the activity of p38 MAP kinase (p38 MAPK) (Engel et al., 2006, 2005). p38 MAPK activation inversely correlates with growth of mammalian hearts and its inhibition increases mitosis in adult mammalian cardiomyocytes both in vitro and in vivo (Engel et al., 2005). A recent zebrafish study has also reported that phosphorylated-p38 Mapk, an activated form of this kinase, is present in the nuclei of non-proliferating cardiomyocytes, but disappears when cardiomyocytes enter the mitotic cycle in vitro and in vivo (Jopling et al., 2012b). When a constitutively active form of Mkk6, an upstream kinase in the p38 Mapk pathway, was overexpressed in the myocardium, cardiomyocyte proliferation was severely impaired and cardiogenesis did not occur at the wound area. These results suggest that p38 Mapk also plays a crucial role in the negative regulation of cardiomyocyte proliferation during heart regeneration in zebrafish.

MicroRNAs (miRNAs) are increasingly recognized as critical regulators in diverse biological processes, including the proliferative arrest of cardiomyocytes in the postnatal mammalian heart (Liu et al., 2008; Porrello et al., 2011a). Yin et al. have recently reported that miRNAs show dynamic regulation during cardiac regeneration and contribute to modulating cardiomyocyte proliferation (Yin et al., 2012). The authors performed unbiased miRNA expression analyses comparing uninjured and injured ventricles, and identified expression changes of many miRNAs in response to injury. One showing differential expression was miR-133, which has been shown to suppress caudal fin regeneration in zebrafish (Yin et al., 2008) and mammalian orthologs of which contribute to cardiac development and diseases (Liu et al., 2008; Wystub et al., 2013). Validation experiments revealed that miR-133 is expressed in cardiomyoctyes and its expression inversely correlates with the progress of heart regeneration in zebrafish, suggesting an inhibitory role on heart regeneration. When miR-133 was overexpressed under the control of heat-inducible promoter sequences, cardiomyocyte proliferation was reduced at the wound area by nearly 50%, and myocardial regeneration was blocked with the formation of collagenous scar. By contrast, when miR-133 was depleted by expressing target-specific miRNA "sponge" sequences, which contain triplets of perfectly matched binding sites for miR-133, the proliferation of cardiomyocytes was elevated throughout the regeneration process, even at 30 days after injury, a time point at which regenerative responses are largely complete.

Using injured ventricular samples with or without miR-133 function, gene expression analyses were performed to identify miR-133 target genes during regeneration. In addition to various known regulators of the cell cycle machinery, the authors found that the expression of cell junction molecule genes was significantly changed depending on miR-133 expression. One of these, *connexin 43* (*cx43*) was particularly interesting, as it had not been reported as a target gene for miR-133 or its close relatives. A sensor assay using a reporter construct containing *cx43*-3′UTR sequences confirmed that *cx43* is an in vivo target of miR-133, and pharmacological inhibition of Cx43 suppressed cardiomyocyte proliferation in the injured ventricle. Although the mechanism by which Cx43 regulates regeneration needs further investigation, these results indicate that miR-133 negatively regulates cardiomyocyte proliferation, partly through modulating Cx43 level (Yin et al., 2012).

Conclusions and perspectives

The view of the mammalian heart as a post-mitotic organ with no regenerative capacity has been revised as a result of recent studies. The neonatal mouse heart has been shown to possess robust regenerative capacity during a short window of time after birth (Porrello et al., 2011b). This capacity diminishes within a week, which seems to coincide with binucleation and loss of proliferative capacity of cardiomyocytes (Laflamme and Murry, 2011). In contrast, the majority of cardiomyocytes are mononucleated in the zebrafish heart (Wills et al., 2007). In the adult mouse heart, a small population of mononucleated cardiomyocytes has been shown to proliferate in the presence of growth factor stimulation (Bersell et al., 2009), supporting the notion that mononucleation is a prerequisite for cardiomyocyte proliferation. However, a recent study has reported the surprising finding that both mononucleated and binucleated mouse cardiomyocytes retain robust proliferative capacity beyond the neonatal period, being temporarily reactivated at postnatal day 15, and contributing to a substantial increase in new cardiomyocytes in the preadolescent heart (Naqvi et al., 2014). Significant proliferation of cardiomyocytes does not seem to occur after this period, but it has been recognized that the heart maintains self-renewal capacity at a measurable level throughout life (Bergmann et al., 2009; Senyo et al., 2013). Collectively, these findings illuminate the possibility of reactivating endogenous regenerative capacity in the human heart as a novel therapeutic strategy to treat cardiac diseases.

Successful stimulation of endogenous regenerative capacity in injured human hearts will benefit from studies discussed above on the robust regenerative responses observed in the adult zebrafish heart. Previous genetic fate-mapping studies have shown that the zebrafish heart utilizes cardiomyocyte proliferation as the dominant mechanism to regenerate myocardium. Understanding intrinsic and extrinsic molecular signals that control cardiomyocyte proliferation and differentiative quiescence in this model will have direct implications for how regeneration can be stimulated in the injured human heart through cardiomyocyte proliferation. Chemical genetics approaches are an interesting strategy to dissect such mechanisms, which may be facilitated by recently developed transgenic reporter strains in which cardiomyocyte proliferation and regeneration can be monitored in live animals (Chen et al., 2013; Choi et al., 2013). Novel lineage tracing studies have identified several previously unknown sources for regenerating muscle, which will promote further inquiry into the cellular mechanism of heart regeneration. Identifying pathways affecting non-myocytes such as epicardial and endocardial cells, will provide further interesting molecular candidates that modulate cardiomyocyte proliferation, migration or neovascularization. Results obtained from the zebrafish model will complement those from other models and contribute valuable insights for better understanding heart regeneration toward the ultimate goal of treating heart failure.

Acknowledgments

I apologize to my colleagues whose work was not discussed due to space limitations. I thank R. Harvey, G. Chapman, S. Hui, and S. Phan for reading and commenting on the manuscript. K. K. is an Australian Research Council Future Fellow (FT110100836) and acknowledges the grant support from the National Health and Medical Research Council (1032522 and 1046469).

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