

## Cellular and molecular basis of cardiac regeneration

Justin JUDD, Wanling XUAN, Guo N. HUANG\*

Cardiovascular Research Institute, School of Medicine, University of California, San Francisco, CA, USA

Received: 13.04.2015 • Accepted/Published Online: 26.06.2015 • Final Version: 23.02.2016

**Abstract:** Heart disease is one of the largest causes of death in humans throughout the developed world. Despite its critical role in maintaining physiologic homeostasis, the adult mammalian heart does not exhibit a significant capacity for regeneration. By contrast, several lower vertebrates exhibit a remarkable ability to regenerate the aging myocardium in response to severe injury. Additionally, recent evidence has shed light on a transient regenerative window in postnatal mammals. In this review, we discuss key findings that help to unravel the differences in cardiac regeneration across phylogeny and ontogeny. Furthermore, we highlight recent significant progress in the pursuit of adult mammalian cardiac regeneration. Collectively, this important body of work has great potential impact on human therapy for heart failure.

**Key words:** Heart, cardiac, mammal, vertebrate, regeneration

### 1. Introduction

Historically, adult cardiac muscle was believed to be terminally differentiated, with little potential for renewal. In contrast, an early report in 1956 described myocardial cell division and some degree of regeneration in young rats (between 4 and 7 days of age) in response to burn injury (Robledo, 1956). Subsequently, several lower vertebrates, such as the frog (Rumyantsev, 1973), newt (Oberpriller and Oberpriller, 1974), zebrafish (Poss et al., 2002), and axolotl (Vargas-González et al., 2005; Cano-Martínez et al., 2010) were found to exhibit some capacity to regenerate severely injured cardiac tissue. More recently, it was demonstrated that neonatal mice also exhibit a robust regenerative response to cardiac injury (Porrello et al., 2011b). Many organ systems exhibit ongoing renewal throughout adult life in mammals: the skin, gut, liver, and blood. However, despite its critical role in organismal homeostasis, the adult mammalian heart does not efficiently regenerate naturally.

Since heart failure is one of the largest causes of death in the world, considerable effort has been devoted to identifying potential ways to stimulate cardiac regeneration in adult humans. In this review, we discuss the current state of cardiac regeneration with respect to natural differences across phylogeny and ontogeny, as well as efforts to synthetically induce cardiac regeneration in mammals.

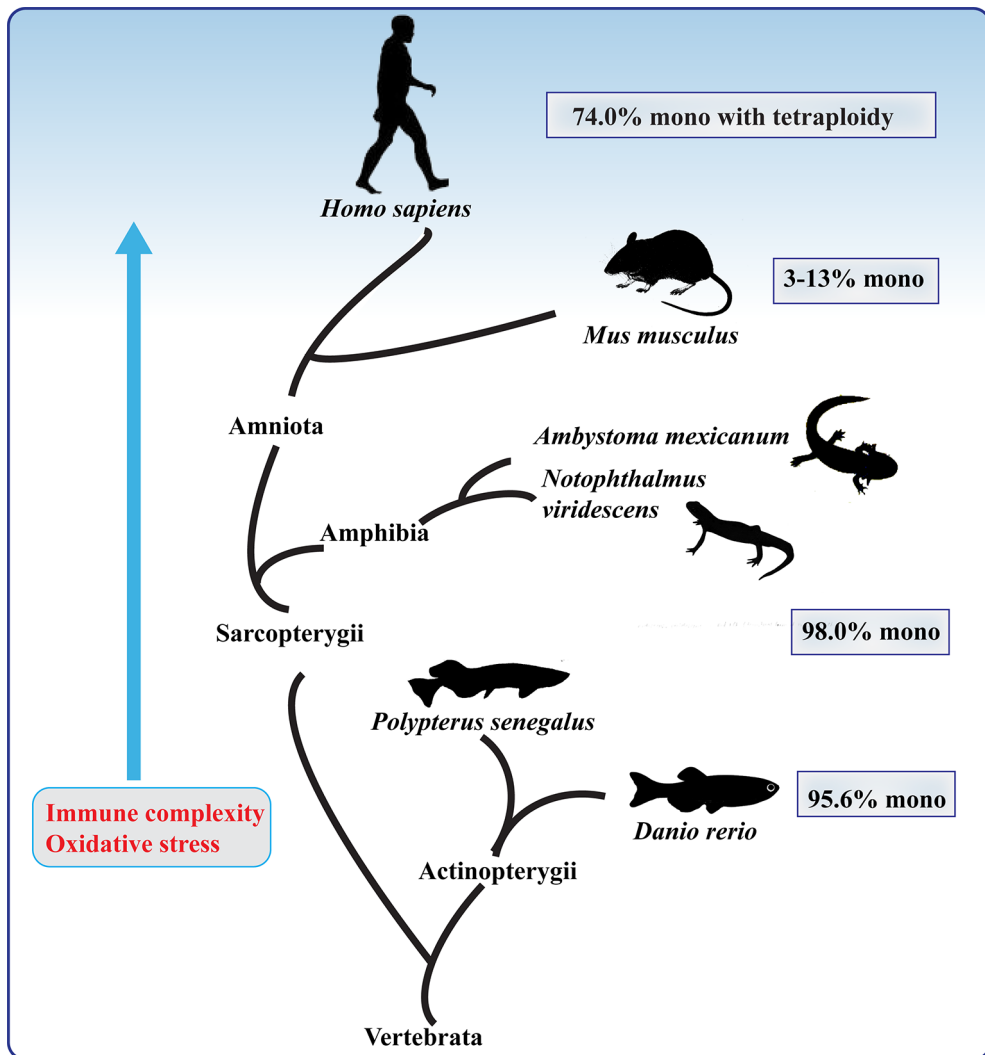
### 2. Natural regenerative mechanisms

#### 2.1. Lower vertebrates

Several lower vertebrates are known to retain the ability to efficiently regenerate injured myocardial tissue, in addition to central nervous system and appendages, throughout adulthood. These include some urodele amphibians, such as the newt (Witman et al., 2011) and axolotl (Cano-Martínez et al., 2010), as well as zebrafish (Poss et al., 2002; Jopling et al., 2010; Kikuchi et al., 2010) and *Polypterus senegalus* (Kikuchi et al., 2011) (Figure). A phylogenetic tree would suggest that the ability to regenerate cardiac tissue may have been present in a common ancestor between these species and mammals.

Several theories have been proposed to explain the source of cardiomyocyte replacement in these regenerative organisms, including circulating stem cells, resident stem/progenitor cells, and the dedifferentiation, proliferation and redifferentiation of mature cells. There seems to be a growing consensus that preexisting cardiomyocytes are the predominant source for new myocardium in zebrafish heart regeneration (Jopling et al., 2010; Kikuchi et al., 2010) (in addition to neonatal mice, discussed below). Jopling et al. (2010) and Kikuchi et al. (2010) both used genetic lineage tracing experiments to track the fate of cardiomyocytes during regeneration in an apical resection model. Jopling et al. (2010) labeled cardiomyocytes 48 h after fertilization by tamoxifen pulsing transgenic *cmlc2a-Cre-Ert2;cmlc2a-LnL-GFP* zebrafish and performed 20%

\* Correspondence: guo.huang@ucsf.edu



**Figure.** Phylogenetic relationship between mammals and organisms that exhibit significant cardiac regeneration. Note that parsimony dictates ancient regenerative ability within early Vertebrata and loss within Amniota where oxidative stress (Puente et al., 2014) and immune complexity (Aurora et al., 2014) become potentially relevant to regeneration. The percent of mononucleate cardiomyocytes in adults is shown at right for *Homo sapiens* (Olivetti et al., 1996), *Mus musculus* (Soonpaa, Field, 1994), *Notophtha viridescens* (Bettencourt-Dias et al., 2003), and *Danio rerio* (Wills et al., 2008).

apical resection in adulthood (3 months old). A fibrin clot was observed at 7 days post amputation (dpa), followed by expansion of predominantly GFP<sup>+</sup> positive cardiomyocytes into the resected area at 14 and 21 dpa, leaving only a small scar. BrdU incorporation in cardiomyocytes increased ~4-fold in resected hearts compared to the control, with highest activity near the resected tissue. Scanning electron micrographs showed significant cardiomyocyte dedifferentiation and loss of sarcomeric organization in regenerating myocardium. Furthermore, cell cycle regulator *plk1* was upregulated during and was required for regeneration.

Kikuchi et al. (2010) found that *gata4* promoter activity (using a transgenic reporter, *gata4-GFP*) increased significantly in cardiomyocytes in the compacted myocardium at 3–7 dpa, and these cells migrated into the wound site by 14 dpa. They used a transgenic *gata4-ERC<sub>Cre</sub>ER;β-actin-LoxP-DsRed-STOP-loxP-EGFP* zebrafish line to track the fate of Gata4<sup>+</sup> cardiomyocytes during regeneration. Pulsing with tamoxifen at 4–7 dpa (before Gata4<sup>+</sup> cardiomyocytes were observed in the wound site) revealed that Gata4<sup>+</sup> cardiomyocytes migrated from outside the wound site and expanded to generate new myocardium. Since noncardiomyocytes could potentially

express *Gata4*<sup>+</sup> and subsequently differentiate into cardiomyocytes, they then used a specific *cmlc2* driven Cre reporter line to show that new myocardium was indeed derived from preexisting cardiomyocytes.

These above studies provide convincing evidence that regeneration in zebrafish occurs through dedifferentiation and proliferation of preexisting cardiomyocytes, similar to mechanisms seen in axolotl limb regeneration (Kragl et al., 2009; Wu et al., 2015). However, the humoral and molecular signaling mechanisms that allow cardiomyocytes to proliferate in lower vertebrates are still being unraveled.

Recently, the role of mitochondria and oxidative damage in controlling cardiomyocyte proliferation and cardiac regeneration is gaining appreciation (Puente et al., 2014). It seems that high levels of radical oxygen species (ROS, such as from elevated mitochondrial respiration) lead to a DNA damage response that prevents cell division and signals cell cycle exit. The low metabolic demand and hypoxic environment of zebrafish seems to curtail the DNA damage response and enable cardiomyocyte proliferation. Retinoic acid signaling in the endocardium and epicardium is also thought to play a critical role in directing cell migration during zebrafish heart regeneration and has been reviewed elsewhere (Masters and Riley, 2014).

Although the exact molecular cues that drive cardiac regeneration are still being delineated, it is tempting to hope that the efficient regeneration seen in these organisms indicates a quiescent primitive regenerative mechanism may exist in a greater portion of Animalia, including humans, which merely needs reawakening. Parsimony amongst phylogenetic relationships between urodele amphibians, zebrafish, and mammals suggests that regeneration was present in a common ancestor, but was lost somewhere in Amniota in adults (Figure). Furthermore, it seems that neonatal mice regenerate via a mechanism similar to that of zebrafish (Porrello et al., 2011b). If future work shows via lineage tracing experiments that axolotl and newt hearts also regenerate via dedifferentiation and proliferation of preexisting cardiomyocytes, it would support the hypothesis that these regenerative species use a conserved regenerative program, which may be accessible in adult humans.

## 2.2. Mammals

Adult mammals are not known to efficiently regenerate injured myocardium. However, several studies have shown a very modest contribution of preexisting cardiomyocytes to new mononucleate myocardial cells, both in the normal and injured adult mammalian heart (Senyo et al., 2013).

Like zebrafish and urodele amphibians, neonatal (up to postnatal day 7) (Porrello et al., 2011b) and fetal (Drenckhahn et al., 2008) mice exhibit a remarkable ability to regenerate myocardium after severe injury. As

in zebrafish, preexisting cardiomyocytes make a major contribution to regenerated myocardium in neonatal mice (Jopling et al., 2010; Kikuchi et al., 2010). However, shortly after birth, mice experience a drastic decline in regenerative capacity, as the majority of cardiomyocytes exit the cell cycle. It should be noted that some controversy remains over the extent of regeneration in mouse neonates that concerns not only surgical techniques, but also the definition of regeneration versus enhanced healing (Andersen et al., 2015). Regeneration has been previously distinguished from wound repair as a process that results in complete restoration of gross normal tissue architecture and function (Clark et al., 1998). Regeneration typically involves progenitors originated from stem cells or dedifferentiated from mature cells that lead to the formation of new tissue. Nonregenerative healing has been proposed to proceed through a distinct mechanism, characterized by a fibrin clot that leads to scar formation with altered extracellular matrix composition and a reduction in functional parenchyma. It is possible that a continuum between the two extremes is responsible for different degrees of regeneration that can occur across phylogeny and ontogeny.

### 2.2.1. Humans

The high incidence of heart failure in aging humans underscores the lack of significant regenerative capacity. However, despite historical skepticism, recent evidence reveals that some degree of cardiomyocyte renewal does exist in the aging human. As in mice, estimates of human cardiomyocyte turnover vary (Yacoub, 2015), though most reports point toward a level consistent with the inability of efficient human heart regeneration.

Perhaps the most compelling evidence to support myocardial turnover in humans was revealed in a recent radiometric dating study (Bergmann et al., 2009). A period of heavy nuclear testing between 1955 and 1963 resulted in massive <sup>14</sup>C radioisotope pulse labeling on earth. This allowed Bergmann et al. (2009) to calculate the rate of cardiomyocyte turnover in humans based on the level of isotope incorporation in myocardial samples. To reduce the contamination of sorted cardiomyocytes with noncardiomyocytes, for use in biochemical and radioisotope assays, the authors developed a cardiomyocyte nuclei sorting method using nuclear localized cardiac troponin I and T as markers, resulting in ~96% purity. From sorted nuclei, they determined that the fraction of multiploid (>2n) cardiomyocytes increased drastically from 0 to 10 years and then remained unchanged throughout life, consistent with other reports (Olivetti et al., 1996; Mollova et al., 2013). Using a mathematical model, they also estimated the rate of cardiomyocyte turnover to be 1% per year at 20 years of age, declining to 0.45% by 70 years of age. At this estimation, the human heart (with ~1 billion

cardiomyocytes (Porrello and Olson, 2014)) creates up to 10 million new cardiomyocytes per year. Furthermore, Bergmann et al. estimated that approximately 45% of cardiomyocytes in the heart are renewed throughout human life. Interestingly, they reported that the human heart is homogeneous in proliferative potential, with no identifiable subset of cells containing a disproportionate ability to renew; this is in contrast to the preadolescent burst of cardiomyocyte proliferation in mice occurring predominantly in the subendocardium (Naqvi et al., 2014). This study was significant in that it provided quantitative insight into the ability of the healthy human heart to renew functional cardiomyocytes. However, radioisotope labeling does not reveal the source of new cardiomyocytes, leaving open an important question that could lead to novel insights in therapeutic human heart regeneration.

Kühn and colleagues developed a cardiomyocyte isolation method involving collagenase digestion after formaldehyde fixation of flash-frozen, healthy, nonfibrotic human myocardium samples (Mollova et al., 2013). This enabled accurate and unambiguous quantitation of cardiomyocyte-specific proliferation via immunofluorescence imaging without the need for confocal microscopy. Laser scanning cytometry (LSC) showed that infant hearts (up to 1 year old) contained ~0.04% cardiomyocytes in M-phase (phosphorylated histone 3 (PH3)-positive), which decreased to 0.009% between 10 and 20 year olds. Confocal analysis showed a similar pattern with 0.012% PH3-positive cardiomyocytes in the first year of life, decreasing with age. Both methods showed detectable PH3 staining after 40 years old. The majority of mitotic cardiomyocytes were found to be mononucleate, consistent with observations in rats (Kühn et al., 2007), cats (Chen et al., 2007), and mice (Bersell et al., 2009). Confocal analysis of thick myocardial sections indicated ~0.016% of 1-year-old cardiomyocytes were in cytokinesis (MKLP1-positive), a surprisingly high fraction of the total mitotic cardiomyocytes based on PH3 staining. Cytokinesis declined with age (0.01% and 0.005% for 2–10 and 10–20 years old, respectively) and was undetectable in human samples beyond 20 years of age. Interestingly, Mollova et al. (2013) found that the percentage of mononucleate cardiomyocytes did not change between the first year of life and in adults, both ~65%, consistent with a prior report in adult humans (Olivetti et al., 1996). However, using LSC, they found that ploidy increased in mononucleate cardiomyocytes, consistent with the observation that cycling cardiomyocytes were predominantly mononucleate and the consensus that aging cardiomyocytes are able to undergo genome replication; however, karyokinesis seems to be inhibited. Furthermore, they used optical dissection to determine that between the ages of 0–1 years and 20 years, the number and size of

cardiomyocytes increased by 3.4- and 8.6-fold, respectively, consistent with the preadolescent burst of cardiomyocyte proliferation seen in mice (Naqvi et al., 2014).

Collectively, these results provide strong evidence of cell cycle activity in human cardiomyocytes and the generation of new cardiomyocytes after birth. However, without human lineage tracing experiments, the major source of new cardiomyocytes in humans is still unclear. Considering that neonatal mice and zebrafish both regenerate via cardiomyocyte division, the same mechanism must be considered as a potential major source of cardiomyocyte turnover in humans, but it cannot be ruled out that other sources, i.e. stem/progenitor cells, exist.

Regardless of the source of new cardiomyocytes in humans, the existence of cardiomyocyte renewal and of cardiomyocyte cell cycling are promising findings that support potential for regenerative strategies in humans. To this end, it is interesting to note that several different remodeling modalities are known to exist in humans, underscoring the plasticity of the myocardium for tissue reorganization. For example, in cases of dilated cardiomyopathy (DCM), human myocardium is known to undergo remodeling distinct from that observed in ischemic injury, where DCM is characterized by a reversion to dedifferentiated, fetal-like cardiomyocyte phenotypes, reminiscent of neonatal mouse and zebrafish heart regeneration. Furthermore, some evidence for reverse remodeling and increased cardiomyocyte cell cycling is seen in patients with left ventricular assist devices (Canseco et al., 2015). This could mean that the adult human heart does indeed have the intrinsic ability to regenerate, but that this process is merely inhibited by other processes, such as fibrosis, or the constant mechanical and oxidative demands of the adult human heart. An enormous body of work describes efforts to induce cardiac regeneration in animal models with the ultimate goal of translating findings into human therapy. A summary of recent key findings is presented here.

### 3. Toward induced regeneration

#### 3.1. Undifferentiated cells with cardiac potential

Numerous studies have looked at the potential of injected cells to repair cardiac tissue. Most of these include cells with progenitor and/or stem characteristics. Due to the initial promise of these cellular therapies, an abundance of clinical trials were conducted using various stem and progenitor cells. Many of these trials, however, yielded mixed results and controversial conclusions (Nowbar et al., 2014). We discuss below a small sample of the literature on cardiac stem cell therapy and refer our readers to other available reviews on this well-covered subject (Bollini et al., 2011; Garbern and Lee, 2013; van Berlo and Molkentin, 2014).

The movement for cardiac stem cell therapy was motivated by the thought that stem/progenitor cells might be responsible for natural mechanisms of cardiac repair and renewal. Many earlier works focused on either bone-marrow derived or cardiac resident Lin<sup>-</sup>/c-kit<sup>+</sup> cells. Despite initial promise, their contribution to new cardiomyocytes or cardiac repair in general has been controversial (van Berlo and Molkenkin, 2014).

In pursuit of a possible source of new cardiomyocytes after injury in adult mammals, a study by Hsieh et al. (2007) indirectly implicated potential resident cardiac stem cells. They used  $\alpha$ -MHC-MerCreMer;ZEG transgenic mice to pulse label cardiomyocytes with permanent GFP expression, followed by a chase period to observe the source of turnover. MI resulted in a greater than 100-fold increase in Kit expression in the myocardium (from <0.005% to  $0.75 \pm 0.13\%$ ). In response to MI or pressure overload but not in control mice, the authors observed a reduction in the number of GFP<sup>+</sup> cardiomyocytes, indicating a source of de novo cardiomyocytes other than preexisting cardiomyocytes. Since no off-target GFP expression in bone marrow cells or in KIT<sup>+</sup> or SCA-1<sup>+</sup> cells was found, stem cells were likely not responsible for cardiomyocyte renewal in the normal aging mouse. TUNEL staining showed no enhanced susceptibility to apoptosis within the GFP<sup>+</sup> population compared to GFP<sup>-</sup> cardiomyocytes in response to MI or pressure overload. Although this report provides strong evidence for a lack of proliferative capacity of  $\alpha$ -MHC<sup>+</sup> cardiomyocytes in adult mouse hearts after injury, it does not exclude the possibility that  $\alpha$ -MHC<sup>-</sup> cardiomyocytes could reenter the cell cycle (e.g.,  $\beta$ -MHC<sup>+</sup>, fetal-like cardiomyocytes).

The contribution of injected cells to cardiac repair in general is controversial, but may include some degree of engraftment and/or paracrine effects. Although ES cell-derived cardiac therapies have shown promise, there is concern over the potential for teratoma formation (van Berlo and Molkenkin, 2014). Additionally, Qiao et al. used positron emission tomography and magnetic resonance imaging to show that embryonic stem cells can engraft into cardiac tissue, but at very low rates (Qiao et al., 2009). Along with other studies reporting similarly poor engraftment, this suggests that stem cells may not have a high potential for cardiac regeneration. As mentioned above, bone-marrow derived cells (BMCs) have been widely explored as a potential source of regenerative engraftment in numerous clinical trials. However, Hofmann et al. showed that only a small percentage of BMCs are retained in the human myocardium after intracoronary delivery (Hofmann et al., 2005). A large metaanalysis of clinical trial data illustrated a correlation between discrepancies and ejection fraction improvement, suggesting that BMCs may not provide effective therapy

for cardiac failure. On the other hand, Korf-Klingebiel et al. discovered a paracrine factor (C19orf10), secreted by BMCs, that appears to induce cardiac repair (Korf-Klingebiel et al., 2015), supporting the hypothesis for paracrine-mediated therapeutic effects by BMCs.

Cardiospheres are an in vitro phenomenon, whereby culturing cardiac tissue explants under nonadherent conditions gives rise to proliferative cardiac stem cell niches (Li et al., 2010). Reimplantation of cardiospheres in vivo via intracoronary injection may improve ischemic recovery via paracrine effects. In CAUDUCEUS human clinical trials, cardiosphere treatment safely reduced scar size and increased viable myocardium after MI (Malliaras et al., 2014). However, no definitive improvements in ejection fraction, cardiac output, or quality of life were observed.

Several other works have looked at the role of endogenous cardiac stem cells in cardiac repair and the delivery of exogenous progenitor sources to induce repair in adult mammals. However, there is a growing consensus that progenitor cells do not make a major contribution to regenerating myocardium (reviewed in van Berlo and Molkenkin, 2014).

### 3.2. Proliferation of differentiated cardiomyocytes

Cardiomyocytes are the contractile cells responsible for the pumping function of the heart. Although their significant contribution to myocardial repair in neonatal mice and adult zebrafish has only recently been definitively shown, the regenerative potential of cardiomyocytes has been pondered for decades. As such, much effort has been devoted to stimulating cardiomyocyte proliferation with the hope of replacing lost contractile tissue after injury, such as in myocardial infarction. The generally strong resistance of mammalian cardiomyocytes to cell cycle reentry has been partially attributed to epigenetic silencing of cell cycle regulators (Sdek et al., 2011) through retinoblastoma (Rb) and p130 signaling. Several early reports characterized cell cycle checkpoints and control nodes in cardiomyocytes (reviewed in Pasumarthi, 2002). More recent efforts have focused on the roles of Hippo signaling and the DNA damage response in the regulation of cardiomyocyte proliferation.

Since cardiomyocyte cell division is an important event in heart development and regeneration, there has been significant interest in the development of detection methods to monitor how cardiomyocytes divide. Clonal analysis was performed using a tamoxifen-inducible mosaic analysis with double markers (MADM) system to provide evidence of symmetric division of preexisting MYH6-expressing cardiomyocytes in adult mice (Ali et al., 2014). Analysis of P12 mice (induced P2-P8) revealed that only 10% of labeled cells were single-labeled, showing that cell division during this period was

inefficient. Furthermore, this study showed that daughter cardiomyocytes had a low capacity for renewal, due to the scarcity of clonal expansion.

### 3.2.1. Hypoxia

The impetus for cardiomyocyte cell cycle exit is not well understood, but recent evidence has implicated oxidative stress as a contributing factor (Puente et al., 2014). It was observed that at birth, mice undergo a transition from a hypoxic environment (~30 mmHg) to a relatively hyperoxic environment (~100 mmHg). Furthermore, regenerative zebrafish are adapted to hypoxic conditions, where they can tolerate oxygen pressures down to 15 mmHg. Moreover, there is a shift in metabolism from anaerobic glycolysis to mitochondrial oxidative phosphorylation, where ROS are abundantly generated by the electron transport chain in mitochondria. This led Puente et al. (2014) to identify ROS and the DNA damage response as a stimulus for cardiomyocyte cell cycle exit. Strikingly, hypoxic conditions, ROS scavenging, or DDR inhibition all increased the postnatal window of cardiomyocyte proliferation. Consistently, hyperoxic conditions and ROS generators led to premature cell cycle arrest.

Interestingly, hypoxic signaling may be a generalizable therapeutic strategy, as several recent reports have utilized hypoxic conditions to increase the “stemness” of various progenitor cells ex vivo, including cardiospheres (Tan et al., 2015).

### 3.2.2. Thyroid signaling

Recently, an astute observation of a discrepancy between heart growth and cardiomyocyte size led to the discovery of a burst of predominantly subendocardial cardiomyocyte proliferation around P15 in mice (Naqvi et al., 2014). It was found that thyroid signaling was responsible for this massive and transient mitotic event, potentiated through the IGF-1/AKT signaling axis. The final cardiomyocyte number increased to 140% by P18. Regeneration from MI injury was higher than that of P21 mice, but could not match that of P2 mice. It will be interesting to see how oxidative stress plays a role in the proliferative adolescent burst at P15.

In the above study, the percentage of binucleated cardiomyocytes decreased from ~86% to ~71%, which is inconsistent with a previous report that only a slight decrease of binuclear cells from ~95% to ~88% was observed during this time window (Soonpaa et al., 1996). It would be important for other groups to repeat the same analyses and reconcile the discrepancy. Nevertheless, this event might have been missed in most prior studies, perhaps due to the transient nature and short labeling periods.

### 3.2.3. Meis1

Meis1 is a homeodomain transcription factor that was shown to be critical for heart development (Paige et al., 2012; Wamstad et al., 2012). Recently, Meis1 was shown

to be a negative regulator of the cardiomyocyte cell cycle. Deletion of Meis1 resulted in an extension of the neonatal cardiomyocyte proliferative window in mice, whereas overexpression inhibited neonatal heart regeneration and cardiomyocyte proliferation (Mahmoud et al., 2013). Interestingly, Meis1 overexpression reduced the regenerative response to P1-2 MI (left ventricular ejection fraction (EF) ~69% vs. ~93% in controls at P21), but had no effect on EF in uninfarcted heart. The mechanism could be through transcriptional activation of CDK inhibitors p15, p16, and p21 by Meis1, as measured in isolated cardiomyocytes (Mahmoud et al., 2013). However, the effect of Meis1 deletion on adult cardiac regeneration was not reported.

### 3.2.4. miRNAs

MicroRNAs (miRNAs) have been shown to regulate gene expression in a number of different contexts. miRNA signaling is required for normal cardiac function (Chen et al., 2008; Rao et al., 2009). miR-1 (Zhao et al., 2005, 2007; Eulalio et al., 2012) and miR-33 (Liu et al., 2008) as well as some miR-15 family (Porrello et al., 2011a) miRNAs have been shown to reduce cardiomyocyte proliferation. On the other hand, human miRNA hsa-miR-590 and hsa-miR-199a were found in a large screening to increase cardiomyocyte proliferation in neonatal rats in vivo (Eulalio et al., 2012). Treatment with AAV-miR (hsa-miR-590 or hsa-miR-199a) at day 0 after MI resulted in nearly complete recovery of EF (~52%–58%), whereas control injections experienced drastic reductions (~45%) compared to normal (~58%) up to 60 days after MI. Infarct scar size was 10%–15% in treated mice compared to 27%–32% in control mice with MI (Eulalio et al., 2012). Left ventricular wall thickness also increased to near normal levels with miR treatment (Eulalio et al., 2012).

Belmonte and colleagues identified a conserved miRNA program (miR-99/100 and Let7a/c) that is upregulated in adult zebrafish hearts, but suppressed in early development and during regeneration when cardiomyocytes are actively dividing (Aguirre et al., 2014). miR-99/100 and Let7a/c were found to be inversely correlated with the expression of their targets, *fntβ* and *smarca5*. Knockdown of *fntβ* or *smarca5* or treatment with miR-99/100 mimics reduced ventricle size in development, which could be rescued with modified *fntβ* and *smarca5* mRNA. Furthermore, treatment with miR-99/100 mimics reduced cardiac regeneration in zebrafish following apical resection, with an increase in scarring and a reduction in BrdU staining. The miR-99/100 and Let7a/c program was also found to be upregulated during development in both humans and mice, with a corresponding decrease in *fntβ* and *smarca5*, consistent with a contemporary study of cardiomyogenesis (Coppola et al., 2014). Interestingly, the miR-99/100 program was not activated after MI

in adult mice, potentially contributing to their lack of cardiac regeneration. Knockdown of miR-99/100 and *Let7a/c* in cultured adult murine cardiomyocytes resulted in an increase in *fntB/smarca5*. Elevated GATA4 and PCNA expression was found in response to miR-99/100 in cultured human cardiomyocytes, suggesting potential dedifferentiation and new DNA synthesis. However, no direct evidence of cytokinesis was shown in adult human or mouse cardiomyocytes. Organotypic slice cultures exposed to lentivirus-mediated delivery of miR-99/100 and/or miR-*Let7a/c* showed sarcomeric disassembly and reexpression of GATA4, as well as increased PH3. However, the age of the mouse donors for slice cultures was not reported. An LAD ligation model was used to test the effect of miR-99/100 and *Let7a/c* expression (delivered by an AAV2/9 vector injected immediately following ligation) on healing after myocardial infarction in adult mice. Ejection fraction improved from ~45% (MI+ control) to ~60% (MI+ miRNA) by 14 days post injection and remained stable through 90 days post injection; sham surgery displayed EF of ~70%–75%. This work shows some promising findings in cardiac functional improvement, but the mechanism of recovery from myocardial injury is unclear.

Tian et al. (2015) identified a fetally expressed miRNA controller (miRNA cluster 302-367) of the Hippo pathway that was important in early cardiac development and cardiomyocyte proliferation (Tian et al., 2015). Deletion of miR-302-367 resulted in a lack of proliferation and differentiation during development, whereas overexpression resulted in cardiomegaly and thickened myocardium by embryonic day 18.5. Cell size was significantly lower at P20 with miR-302-367 overexpression using *Nkx2.5<sup>cre</sup>* as a driver. Microarray revealed increased Wnt signaling and a reduction in apoptosis at P14, as well as inhibition of differentiation (e.g., *c-Kit* expression) at P14 and P23. Furthermore, Hippo-negative regulators *Mob1b*, *Lats2*, and *Mst1* were downregulated at E18.5 with miR-302-367 overexpression and upregulated at E12.5 with miR-302-367 deletion. This effectively regulated nuclear localization of Hippo effector YAP, resulting in increased proliferation of neonatal cardiomyocytes with miR-302-367 overexpression. Prolonged expression of miR302-367 in adult hearts compromised heart function, but transient expression aided recovery after MI. Specifically, treatment with miR-302b/c mimics at days 2–8 post MI resulted in a reduction in fibrosis and an increase in EF (~52%) compared to the MI-operated, no-treatment control (~32%), but still short of the sham-operated control (70%). End diastolic volume returned to normal, but end systolic volume of the miR-203b/c mimic-treated group (~28  $\mu$ L) was still higher than that of the sham (~20  $\mu$ L), but significantly lower than that of the no-treatment control (~97  $\mu$ L).

### 3.2.5. Hippo signaling

The importance of Hippo signaling in heart regeneration has recently been reported in several studies (reviewed in Lin and Pu, 2014). The Hippo pathway is one of 2 major pathways (in addition to BMP) known to control organ size in *Drosophila* (Affolter and Basler, 2007; Dong et al., 2007; Pan, 2010). Heallen et al. (2011) found that Hippo also controls heart size and cardiomyocyte proliferation in mice. Conditional deletion (*Tg-Nkx2.5-Cre*) of *Salv* (*Salv* CKO mice) reduced phospho-YAP, a downstream signaling effector of the Hippo pathway, and resulted in cardiomegaly, while maintaining cardiomyocyte size. *Salv* CKO mice demonstrated increased mitotic cardiomyocytes (4% PH3<sup>+</sup> vs. 1% in wt.) and increased nuclear  $\beta$ -catenin (42.5% nuclear vs. 12% in wt) at E12.5. Heterozygosity for  $\beta$ -catenin reduced the effect of CKO *Salv* deletion on cardiomyocyte proliferation, supporting Wnt signaling as the mechanism for Hippo-mediated cell cycle repression. These results clearly demonstrated that Hippo and Wnt signaling play a major role in controlling cardiomyocyte proliferation in the developing mouse, but its influence in adult mice was still unclear.

Martin and colleagues subsequently showed that Hippo signaling could be leveraged to stimulate cardiomyocyte proliferation in healthy adult mice and enhanced recovery from cardiac injury in adult mice (Heallen et al., 2013). The idea that Hippo signaling may be related to regenerative ability was supported by the levels of phosphorylated YAP in heart extracts, which increased nearly 3-fold in mice from P2 to P21, inversely correlating with regenerative ability. Conditional deletion of *Salv* and *Lats 1/2* in healthy mice resulted in enhanced EdU incorporation, as well as Ki67 and Aurora B kinase staining in cardiomyocytes. Furthermore, cardiomyocyte density increased, while size, ploidy, and multinucleation decreased, suggesting that cardiomyocytes were actively dividing when Hippo signaling was blocked (Heallen et al., 2013). Both apical resection and myocardial infarction injury models showed striking regeneration in P8 *Salv* CKO mice, past the postnatal window of 7 days after birth. Interestingly, LAD ligation performed on *Salv* CKO mice of 8–10 weeks old resulted in significant functional recovery by 3 weeks post infarct (~70% EF, similar to controls, whereas *wt* mice with surgery had EF of ~51%). Fibrotic percent cross-sectional area was reduced, but scarring was still evident at 3 weeks post infarct. However, cardiomyocyte proliferation was not evaluated in this adult cardiac injury model.

*Yap1* was shown to regulate heart growth via cardiomyocyte proliferation, independent of hypertrophy (von Gise et al., 2012). Consistent with this and other reports, Olson and colleagues also showed the importance of Hippo signaling in cardiomyocyte proliferation and regeneration (Xin et al., 2013). They showed some

functional redundancy between YAP and TAZ using compound conditional knockout mice, with Yap knockout giving a stronger phenotype of cardiomyopathy, including cardiomyocyte hypoplasia. Yap CKO also severely inhibited neonatal cardiac regeneration in an LAD ligation MI model (performed at P2), whereas controls were able to efficiently regenerate, as previously shown. In contrast, transgenic expression of a constitutively active Yap mutant under the control of a cardiomyocyte-specific promoter ( $\alpha$ MHC-YapS112A) extended the regenerative window to some extent. LAD ligation performed at postnatal day 7 was mostly repaired by postnatal day 28 in transgenic mice, whereas control mice exhibited massive ventricular scarring. Regeneration after MI at postnatal day 28 was also moderately improved in transgenic mice by postnatal day 49, with a reduction in scar size to ~17% compared to ~40% in control mice, and an increase in fractional shortening and ejection fraction (~46% and ~74% compared to ~20% and ~40% in *wt* MI and ~60% and 90% in sham controls, respectively). Furthermore, mitotic marker PH3 was elevated in transgenic  $\alpha$ MHC-YapS112A mice after MI, but strong evidence of cardiomyocyte cytokinesis was not presented. Notably, IGF-1 and phosphorylated AKT were increased in transgenic mice after MI, supporting previous work showing enhanced IGF signaling mediated by Yap (Xin et al., 2011).

Pu and colleagues used doxycycline-inducible cardiac specific expression of constitutively active Yap (*Myh6-Cre; Rosa26<sup>ls,rtTa/ls,rtTa</sup>; TRE-YAP*, referred to as YAP<sup>GOF</sup>) to enhance cardiomyocyte proliferation and cardiac regeneration in an MI model (Lin et al., 2014). YAP<sup>GOF</sup> mice induced between 4–8 weeks of age displayed cardiomyocyte hyperplasia, with equivalent heart volumes and smaller cell size, as well as enhanced BrdU incorporation and PH3 staining at 8 weeks. Surprisingly, sarcomeric disorganization and dedifferentiation was not observed. Mosaic clonal analysis using Brainbow showed that YAP<sup>GOF</sup> induction increases the incidence of single-color clusters, but not multicolor clusters, indicating that YAP<sup>GOF</sup> increases cell division. Interestingly, long-term induction of YAP<sup>GOF</sup> did not result in tumorigenesis, but did cause a small degree of fibrosis. Induction of YAP<sup>GOF</sup> beginning 1 week after LAD ligation (for a total of 4 weeks) resulted in enhanced EdU and PH3 staining near the infarct border, but functional recovery and mitigation of scarring was marginal. Treatment with a cardiac specific AAV9-YAP<sup>GOF</sup> vector at time of infarct resulted in improved survival and enhanced EdU incorporation compared to AAV9-Luc controls, but functional improvement was similarly unstriking.

Although these investigations of Hippo signaling provide a promising new direction in cardiac regeneration research, more work will be required to unravel its complexity. For example, it is interesting that tumorigenesis is not observed with long-term YAP<sup>GOF</sup> expression in the heart (Lin et al., 2014), in contrast to that seen in the

liver (Dong et al., 2007). Additionally, effects of Salvador knockout outside of YAP/TAZ regulation should be explored, considering its superior influence on functional cardiac improvement in response to injury (Heallen et al., 2013). Since Salv CKO mice exhibit higher cardiomyocyte density with greater percent mononucleation even before infarction, it seems possible that the greater regenerative capacity may be due to the enhanced ability of mononucleate cardiomyocytes to proliferate. Alternatively, the effect of Salv knockout may have other downstream effects that contribute to cardiomyocyte proliferation. On the other hand, it also seems feasible that the increased functional recovery of Salv CKO hearts could be due to hypertrophic growth of the elevated pool of cardiomyocytes. Comparison of proliferative markers and cardiomyocyte size of Salv CKO mice before and after injury would be revealing. Finally, failure of cytokinesis has been shown to cause activation of the Hippo tumor suppression pathway (Ganem et al., 2014), potentially explaining how cardiomyocytes transition to the Hippo-regulated cell cycle block between P2 and P10.

#### 4. Conclusion

Collectively, the body of work reviewed here represents significant progress toward achieving heart regeneration in adult mammals. In particular, the regeneration seen with microRNA and Hippo pathway modulation appears robust in younger mice, but the utility in adult mammalian cardiac regeneration will need to be more fully evaluated. Looking forward to translation in humans, it will be revealing to see if manipulation of these molecules and pathways can aid cardiac regeneration in large animal models. Furthermore, since some of the most promising approaches to stimulating heart regeneration involve the upregulation of proliferative pathways (e.g., Hippo signaling), it will be critical to achieve fine spatiotemporal control over pathway modulation, thereby minimizing the risk of neoplasia in the heart or other organs (Moroishi et al., 2015). Ultimately, the ideal therapeutic strategy would be self-limiting with respect to cell proliferation. Therefore, it will be interesting to see how neonatal mice and lower vertebrates are able to control tissue regeneration during cardiac repair. The insights gained in such studies may have a profound impact on therapeutic avenues in human cardiac regeneration.

#### Acknowledgments

This project was funded by the UCSF Program for Breakthrough Biomedical Research (funded in part by the Sandler Foundation), the NIH Pathway to Independence Award (R00HL114738), and the Edward Mallinckrodt Jr. Foundation. JJ was supported by a postdoctoral fellowship from the NIH T32HL007731. The contents of this work are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.



## References

- Affolter M, Basler K (2007). The decapentaplegic morphogen gradient: from pattern formation to growth regulation. *Nat Rev Genet* 8: 663–674.
- Aguirre A, Montserrat N, Zachigga S, Nivet E, Hishida T, Krause MN, Kurian L, Ocampo A, Vazquez-Ferrer E, Rodriguez-Esteban C et al. (2014). In vivo activation of a conserved microRNA program induces mammalian heart regeneration. *Cell Stem Cell* 15: 589–604.
- Ali SR, Hippenmeyer S, Saadat LV, Luo L, Weissman IL, Ardehali R (2014). Existing cardiomyocytes generate cardiomyocytes at a low rate after birth in mice. *P Natl Acad Sci USA* 111: 8850–8855.
- Andersen DC, Jensen CH, Sheikh SP (2015). Comments to the article “A systematic analysis of neonatal mouse heart regeneration after apical resection”. *J Mol Cell Cardiol* 82: 59.
- Aurora AB, Porrello ER, Tan W, Mahmoud AI, Hill JA, Bassel-Duby R, Sadek HA, Olson EN (2014). Macrophages are required for neonatal heart regeneration. *J Clin Invest* 124: 1382–1392.
- Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabé-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H et al. (2009). Evidence for cardiomyocyte renewal in humans. *Science* 324: 98–102.
- Bersell K, Arab S, Haring B, Kühn B. (2009). Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. *Cell* 138: 257–270.
- Bettencourt-Dias M, Mittnacht S, Brockes JP (2003). Heterogeneous proliferative potential in regenerative adult newt cardiomyocytes. *J Cell Sci* 116: 4001–4009.
- Bollini S, Smart N, Riley PR (2011). Resident cardiac progenitor cells: at the heart of regeneration. *J Mol Cell Cardiol* 50: 296–303.
- Cano-Martínez A, Vargas-González A, Guarner-Lans V, Prado-Zayago E, León-Oleida M, Nieto-Lima B (2010). Functional and structural regeneration in the axolotl heart (*Ambystoma mexicanum*) after partial ventricular amputation. *Arch Cardiol Mexico* 80: 79–86.
- Canseco DC, Kimura W, Garg S, Mukherjee S, Bhattacharya S, Abdisalaam S, Das S, Asaithamby A, Mammen PPA, Sadek HA (2015). Human ventricular unloading induces cardiomyocyte proliferation. *J Am Coll Cardiol* 65: 892–900.
- Chen JF, Murchison EP, Tang R, Callis TE, Tatsuguchi M, Deng Z, Rojas M, Hammond SM, Schneider MD, Selzman CH et al. (2008). Targeted deletion of Dicer in the heart leads to dilated cardiomyopathy and heart failure. *P Natl Acad Sci USA* 105: 2111–2116.
- Chen X, Wilson RM, Kubo H, Berretta RM, Harris DM, Zhang X, Jaleel N, MacDonnell SM, Bearzi C, Tillmanns J et al. (2007). Adolescent feline heart contains a population of small, proliferative ventricular myocytes with immature physiological properties. *Circ Res* 100: 536–544.
- Clark LD, Clark RK, Heber-Katz E (1998). A New murine model for mammalian wound repair and regeneration. *Clin Immunol Immunop* 88: 35–45.
- Coppola A, Romito A, Borel C, Gehrig C, Gagnebin M, Falconnet E, Izzo A, Altucci L, Banfi S, Antonarakis SE et al. (2014). Cardiomyogenesis is controlled by the miR-99a/let-7c cluster and epigenetic modifications. *Stem Cell Res* 12: 323–337.
- Dong J, Feldmann G, Huang J, Wu S, Zhang N, Comerford SA, Gayyed MF, Anders RA, Maitra A, Pan D (2007). Elucidation of a universal size-control mechanism in *Drosophila* and mammals. *Cell* 130: 1120–1133.
- Drenckhahn JD, Schwarz QP, Gray S, Laskowski A, Kiriazis H, Ming Z, Harvey RP, Du XJ, Thorburn DR, Cox TC (2008). Compensatory growth of healthy cardiac cells in the presence of diseased cells restores tissue homeostasis during heart development. *Dev Cell* 15: 521–533.
- Eulalio A, Mano M, Dal Ferro M, Zentilin L, Sinagra G, Zacchigna S, Giacca M (2012). Functional screening identifies miRNAs inducing cardiac regeneration. *Nature* 492: 376–381.
- Ganem NJ, Cornils H, Chiu SY, O'Rourke KP, Arnaud J, Yimlamai D, Théry M, Camargo FD, Pellman D (2014). Cytokinesis failure triggers Hippo tumor suppressor pathway activation. *Cell* 158: 833–848.
- Garbern JC, Lee RT (2013). Cardiac stem cell therapy and the promise of heart regeneration. *Cell Stem Cell* 12: 689–698.
- Heallen T, Morikawa Y, Leach J, Tao G, Willerson JT, Johnson RL, Martin JF (2013). Hippo signaling impedes adult heart regeneration. *Development* 140: 4683–4690.
- Heallen T, Zhang M, Wang J, Bonilla-Claudio M, Klysik E, Johnson RL, Martin JF (2011). Hippo pathway inhibits Wnt signaling to restrain cardiomyocyte proliferation and heart size. *Science* 332: 458–461.
- Hofmann M, Wollert KC, Meyer GP, Menke A, Arseniev L, Hertenstein B, Ganser A, Knapp WH, Drexler H (2005). Monitoring of bone marrow cell homing into the infarcted human myocardium. *Circulation* 111: 2198–2202.
- Hsieh PCH, Segers VFM, Davis ME, MacGillivray C, Gannon J, Molkentin JD, Robbins J, Lee RT (2007). Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. *Nat Med* 13: 970–974.
- Jopling C, Sleep E, Raya M, Martí M, Raya A, Izpisua Belmonte JC (2010). Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature* 464: 606–609.
- Kikuchi K, Holdway JE, Major RJ, Blum N, Dahn RD, Begemann G, Poss KD (2011). Retinoic acid production by endocardium and epicardium is an injury response essential for zebrafish heart regeneration. *Dev Cell* 20: 397–404.
- Kikuchi K, Holdway JE, Werdich AA, Anderson RM, Fang Y, Egnaczyk GF, Evans T, Macrae CA, Stainier DYR, Poss KD (2010). Primary contribution to zebrafish heart regeneration by *gata4*<sup>+</sup> cardiomyocytes. *Nature* 464: 601–605.

- Korf-Klingebiel M, Reboll MR, Klede S, Brod T, Pich A, Polten F, Napp LC, Bauersachs J, Ganser A, Brinkmann E et al. (2015). Myeloid-derived growth factor (C19orf10) mediates cardiac repair following myocardial infarction. *Nat Med* 21: 140–149.
- Kragl M, Knapp D, Nacu E, Khattak S, Maden M, Epperlein HH, Tanaka EM (2009). Cells keep a memory of their tissue origin during axolotl limb regeneration. *Nature* 460: 60–65.
- Kühn B, del Monte F, Hajjar RJ, Chang YS, Lebeche D, Arab S, Keating MT (2007). Periostin induces proliferation of differentiated cardiomyocytes and promotes cardiac repair. *Nat Med* 13: 962–969.
- Li TS, Cheng K, Lee ST, Matsushita S, Davis D, Malliaras K, Zhang Y, Matsushita N, Smith RR, Marbán E (2010). Cardiospheres recapitulate a niche-like microenvironment rich in stemness and cell-matrix interactions, rationalizing their enhanced functional potency for myocardial repair. *Stem Cells* 28: 2088–2098.
- Lin Z, Pu WT (2014). Harnessing Hippo in the heart: Hippo/Yap signaling and applications to heart regeneration and rejuvenation. *Stem Cell Res* 13: 571–581.
- Lin Z, von Gise A, Zhou P, Gu F, Ma Q, Jiang J, Yau AL, Buck JN, Gouin KA, van Gorp PRR et al. (2014). Cardiac-specific YAP activation improves cardiac function and survival in an experimental murine MI model. *Circ Res* 115: 354–363.
- Liu N, Bezprozvannaya S, Williams AH, Qi X, Richardson JA, Bassel-Duby R, Olson EN (2008). microRNA-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart. *Gene Dev* 22: 3242–3254.
- Mahmoud AI, Kocabas F, Muralidhar SA, Kimura W, Koura AS, Thet S, Porrello ER, Sadek HA (2013). Meis1 regulates postnatal cardiomyocyte cell cycle arrest. *Nature* 497: 249–253.
- Malliaras K, Makkar RR, Smith RR, Cheng K, Wu E, Bonow RO, Marbán L, Mendizabal A, Cingolani E, Johnston PV et al. (2014). Intracoronary cardiosphere-derived cells after myocardial infarction: evidence of therapeutic regeneration in the final 1-year results of the CADUCEUS trial (CARDiosphere-Derived aUtologous stem CElls to reverse ventricUlar dySfunction). *J Am Coll Cardiol* 63: 110–122.
- Masters M, Riley PR (2014). The epicardium signals the way towards heart regeneration. *Stem Cell Res* 13: 683–692.
- Mollova M, Bersell K, Walsh S, Savla J, Das LT, Park SY, Silberstein LE, Dos Remedios CG, Graham D, Colan S et al. (2013). Cardiomyocyte proliferation contributes to heart growth in young humans. *P Natl Acad Sci USA* 110: 1446–1451.
- Moroishi T, Hansen CG, Guan KL (2015). The emerging roles of YAP and TAZ in cancer. *Nat Rev Cancer* 15: 73–79.
- Naqvi N, Li M, Calvert JW, Tejada T, Lambert JP, Wu J, Kesteven SH, Holman SR, Matsuda T, Lovelock JD et al. (2014). A proliferative burst during preadolescence establishes the final cardiomyocyte number. *Cell* 157: 795–807.
- Nowbar AN, Mielewicz M, Karavassilis M, Dehbi HM, Shun-Shin MJ, Jones S, Howard JP, Cole GD, Francis DP (2014). Discrepancies in autologous bone marrow stem cell trials and enhancement of ejection fraction (DAMASCENE): weighted regression and meta-analysis. *Brit Med J* 348: g2688.
- Oberpriller JO, Oberpriller JC (1974). Response of the adult newt ventricle to injury. *J Exp Zool* 187: 249–253.
- Olivetti G, Cigola E, Maestri R, Corradi D, Lagrasta C, Gambert SR, Anversa P (1996). Aging, cardiac hypertrophy and ischemic cardiomyopathy do not affect the proportion of mononucleated and multinucleated myocytes in the human heart. *J Mol Cell Cardiol* 28: 1463–1477.
- Paige SL, Thomas S, Stoick-Cooper CL, Wang H, Maves L, Sandstrom R, Pabon L, Reinecke H, Pratt G, Keller G et al. (2012). A temporal chromatin signature in human embryonic stem cells identifies regulators of cardiac development. *Cell* 151: 221–232.
- Pan D (2010). The hippo signaling pathway in development and cancer. *Dev Cell* 19: 491–505.
- Pasumarthi KBS (2002). Cardiomyocyte cell cycle regulation. *Circ Res* 90: 1044–1054.
- Porrello ER, Johnson BA, Aurora AB, Simpson E, Nam YJ, Matkovich SJ, Dorn GW, van Rooij E, Olson EN (2011a). MiR-15 family regulates postnatal mitotic arrest of cardiomyocytes. *Circ Res* 109: 670–679.
- Porrello ER, Mahmoud AI, Simpson E, Hill JA, Richardson JA, Olson EN, Sadek HA (2011b). Transient regenerative potential of the neonatal mouse heart. *Science* 331: 1078–1080.
- Porrello ER, Olson EN (2014). A neonatal blueprint for cardiac regeneration. *Stem Cell Res* 13: 556–570.
- Poss KD, Wilson LG, Keating MT (2002). Heart regeneration in zebrafish. *Science* 298: 2188–2190.
- Puente BN, Kimura W, Muralidhar SA, Moon J, Amatruda JF, Phelps KL, Grinsfelder D, Rothermel BA, Chen R, Garcia JA et al. (2014). The oxygen-rich postnatal environment induces cardiomyocyte cell-cycle arrest through DNA damage response. *Cell* 157: 565–579.
- Qiao H, Zhang H, Zheng Y, Ponde DE, Shen D, Gao F, Bakken AB, Schmitz A, Kung HF, Ferrari VA et al. (2009). Embryonic stem cell grafting in normal and infarcted myocardium: serial assessment with MR imaging and PET dual detection. *Radiology* 250: 821–829.
- Rao PK, Toyama Y, Chiang HR, Gupta S, Bauer M, Medvid R, Reinhardt F, Liao R, Krieger M, Jaenisch R et al. (2009). Loss of cardiac microRNA-mediated regulation leads to dilated cardiomyopathy and heart failure. *Circ Res* 105: 585–594.
- Robledo M (1956). Myocardial regeneration in young rats. *Am J Pathol* 32: 1215–1239.
- Rumyantsev PP (1973). Post-injury DNA synthesis, mitosis and ultrastructural reorganization of adult frog cardiac myocytes. An electron microscopic-autoradiographic study. *Z Zellforsch Mik Ana* 139: 431–50.

- Sdek P, Zhao P, Wang Y, Huang CJ, Ko CY, Butler PC, Weiss JN, MacLellan WR (2011). Rb and p130 control cell cycle gene silencing to maintain the postmitotic phenotype in cardiac myocytes. *J Cell Biol* 194: 407–423.
- Senyo SE, Steinhauser ML, Pizzimenti CL, Yang VK, Cai L, Wang M, Wu TD, Guerquin-Kern JL, Lechene CP, Lee RT (2013). Mammalian heart renewal by pre-existing cardiomyocytes. *Nature* 493: 433–436.
- Soonpaa MH, Field LJ (1994). Assessment of cardiomyocyte DNA synthesis during hypertrophy in adult mice. *Am J Physiol* 266: H1439–H1445.
- Soonpaa MH, Kim KK, Pajak L, Franklin M, Field LJ (1996). Cardiomyocyte DNA synthesis and binucleation during murine development. *Am J Physiol* 271: H2183–H2189.
- Tan SC, Gomes RSM, Yeoh KK, Perbellini F, Malandraki-Miller S, Ambrose L, Heather LC, Faggian G, Schofield CJ, Davies KE et al. (2015). Preconditioning of cardiosphere-derived cells with hypoxia or prolyl-4-hydroxylase inhibitors increases stemness and decreases reliance on oxidative metabolism. *Cell Transplant* (in press).
- Tian Y, Liu Y, Wang T, Zhou N, Kong J, Chen L, Snitow M, Morley M, Li D, Petrenko N et al. (2015). A microRNA-Hippo pathway that promotes cardiomyocyte proliferation and cardiac regeneration in mice. *Sci Transl Med* 7: 279ra38.
- Van Berlo JH, Molkenin JD (2014). An emerging consensus on cardiac regeneration. *Nat Med* 20: 1386–1393.
- Vargas-González A, Prado-Zayago E, León-Olea M, Guarner-Lans V, Cano-Martínez A (2005). Regeneración miocárdica en *Ambystoma mexicanum* después de lesión quirúrgica. *Arch Cardiol Mexico* 75: 21–29 (in Spanish).
- Von Gise A, Lin Z, Schlegelmilch K, Honor LB, Pan GM, Buck JN, Ma Q, Ishiwata T, Zhou B, Camargo FD et al. (2012). YAP1, the nuclear target of Hippo signaling, stimulates heart growth through cardiomyocyte proliferation but not hypertrophy. *P Natl Acad Sci USA* 109: 2394–2399.
- Wamstad JA, Alexander JM, Truty RM, Shrikumar A, Li F, Eilertson KE, Ding H, Wylie JN, Pico AR, Capra JA et al. (2012). Dynamic and coordinated epigenetic regulation of developmental transitions in the cardiac lineage. *Cell* 151: 206–220.
- Wills AA, Holdway JE, Major RJ, Poss KD (2008). Regulated addition of new myocardial and epicardial cells fosters homeostatic cardiac growth and maintenance in adult zebrafish. *Development* 135: 183–192.
- Witman N, Murtuza B, Davis B, Arner A, Morrison JI (2011). Recapitulation of developmental cardiogenesis governs the morphological and functional regeneration of adult newt hearts following injury. *Dev Biol* 354: 67–76.
- Wu CH, Huang TY, Chen BS, Chiou LL, Lee HS (2015). Long-duration muscle dedifferentiation during limb regeneration in axolotls. *PLoS One* 10: e0116068.
- Xin M, Kim Y, Sutherland LB, Murakami M, Qi X, McAnally J, Porrello ER, Mahmoud AI, Tan W, Shelton JM et al. (2013). Hippo pathway effector Yap promotes cardiac regeneration. *P Natl Acad Sci USA* 110: 13839–13844.
- Xin M, Kim Y, Sutherland LB, Qi X, McAnally J, Schwartz RJ, Richardson JA, Bassel-Duby R, Olson EN (2011). Regulation of insulin-like growth factor signaling by Yap governs cardiomyocyte proliferation and embryonic heart size. *Sci Signal* 4: ra70.
- Yacoub MH (2015). Bridge to recovery and myocardial cell division: a paradigm shift? *J Am Coll Cardiol* 65: 901–903.
- Zhao Y, Ransom JF, Li A, Vedantham V, von Drehle M, Muth AN, Tsuchihashi T, McManus MT, Schwartz RJ, Srivastava D (2007). Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. *Cell* 129: 303–317.
- Zhao Y, Samal E, Srivastava D (2005). Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. *Nature* 436: 214–220.