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A Neonatal Blueprint for Cardiac Regeneration

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Abstract

Adult mammals undergo minimal regeneration following cardiac injury, which severely compromises cardiac function and contributes to the ongoing burden of heart failure. In contrast, the mammalian heart retains a transient capacity for cardiac regeneration during fetal and early neonatal life. Recent studies have established the importance of several evolutionarily conserved mechanisms for heart regeneration in lower vertebrates and neonatal mammals including induction of cardiomyocyte proliferation, epicardial cell activation, angiogenesis, extracellular matrix deposition and immune cell infiltration. In this review, we provide an up-to-date account of the molecular and cellular basis for cardiac regeneration in lower vertebrates and neonatal mammals. The historical context for these recent findings and their ramifications for the future development of cardiac regenerative therapies are also discussed.

Keywords:

Cardiac regeneration, cardiomyocyte proliferation, cardiac progenitor cells, extracellular matrix, immune system, neonate

1. Introduction

Since the first systematic studies of organ regeneration in the 18th century, countless biologists have interrogated the mechanisms by which animals replace lost body parts. Regenerative phenomena have since been described in hydra, crustaceans, worms, fruit flies, frogs, salamanders, zebrafish, rodents and humans. Organ regeneration involves an astonishing diversity of cellular mechanisms including recruitment and activation of stem cells, as well as de-differentiation, trans-differentiation and proliferation of differentiated cell types (*1*). Despite a rich history of regeneration research spanning several hundreds of years, the first descriptions of cardiac regeneration in lower vertebrates were only reported within the last few decades (*2-4*). The paucity of early studies on heart regeneration, even in highly regenerative lower vertebrates, is likely a consequence of the conceptual constraints that were imposed by the long standing dogma that the adult heart was terminally differentiated and lacked any appreciable regenerative capacity, a view that underpinned mammalian cardiac biology for over a century (*5*).

It is not unreasonable that the heart has been traditionally viewed as a non-regenerative organ. In response to cardiac injury, such as that induced by a myocardial infarction, the adult mammalian heart fails to replace the vast majority of cardiomyocytes that are lost or damaged. In the absence of a robust regenerative response, the heart substitutes the lost cardiomyocytes with scar tissue, which contributes to the contractile demise of the organ and ultimately

leads to heart failure. However, the traditional view of the heart as a terminally differentiated organ without any capacity for cardiomyocyte renewal has been emphatically refuted in recent years by a growing number of studies in humans (*6-8*) and other mammals (*9-11*). The underlying mechanisms for cardiomyocyte regeneration remain highly controversial but the degree of cell turnover is clearly insufficient to replace the large number of cardiomyocytes (up to 1 billion in humans) that are lost following a major ischemic event. As such, regeneration of myocardial tissue following injury remains a major goal of contemporary research in the field. A number of cardiac regenerative strategies have been proposed including cell replacement therapies, activation of endogenous progenitor cell populations, cardiomyocyte cell cycle re-entry and, most recently, direct lineage reprogramming. However, while all of these approaches offer hope to heart failure patients, they all currently suffer from inherent limitations and translational barriers (*12*). An alternative and complimentary approach is to explore, and ultimately attempt to recapitulate, innate mechanisms of cardiac regeneration such as those that have evolved in highly regenerative organisms, such as the zebrafish, as well as those that exist during privileged developmental windows in mammals. In this review, we provide an overview of cardiac regenerative phenomena in lower vertebrates and neonatal mammals, with a particular emphasis on recent advances that are beginning to shed light on the underlying molecular and cellular mechanisms that govern cardiac regenerative capacity during mammalian development.

2. Cardiac regeneration in lower vertebrates

In the 1960's, scientists working in the former Soviet Union conducted the first studies of cardiac regeneration in amphibians (*3, 4, 13*). Mitotic cells were observed following cardiac injury in lower vertebrates, including adult frogs, newts and axolotls (*3, 4, 13, 14*). Later transmission electron microscopy studies following resection of the ventricular apex of adult newts confirmed the presence of mitotic cardiomyocytes in the wounded area but failed to document complete regeneration of the myocardium, which was characterized by the presence of connective scar tissue following injury (*2, 15*). Collectively, these early studies suggested that although amphibians are capable of inducing cardiomyocyte proliferation following injury, the regenerative response is incomplete. However, a more recent study by Witman and colleagues has provided evidence for complete regeneration of the adult newt heart following resection of a small region of heart tissue at the base of the cardiac ventricles (*16*). In contrast to earlier studies where the entire ventricular apex was removed, Witman *et al*. induced a mode of injury that does not breach the ventricular cavity (Figure 1). Resection of the base of the cardiac ventricles in adult newts was associated with gradual restoration of the myocardial tissue over a period of 60 days (*16*). Although these results were at odds with earlier reports of a much more limited regenerative potential of the adult newt heart, the earlier studies may have been prematurely terminated for histological analyses, prior to completion of the reverse remodeling phase, which must take place in order for the heart to regain its normal morphological appearance (*16*). In addition, the anatomical location of

the injury site near the atrioventricular boundary could be important and may reflect a differential regenerative capacity of cardiomyocytes or progenitor cells located in this region compared with the apex of the adult newt heart.

In contrast to the variable descriptions of heart regeneration in amphibians, zebrafish can regenerate following multiple types of cardiac injury including ventricular resection, cryoinjury and genetic cardiomyocyte ablation (*17-19*). Cardiac injury induces a marked increase in cardiomyocyte proliferation in the adult zebrafish heart and genetic lineage tracing experiments suggest that the vast majority of regenerated cardiomyocytes are derived from pre-existing cardiomyocytes rather than from a stem cell population (*20, 21*). Regenerative mechanisms in different adult zebrafish cardiac injury models have been described in detail elsewhere (*22*) and are summarized in Figure 1.

3. Cardiac regeneration in mammals – privileged windows of development

Since Spallanzani's seminal treatise on the age-dependency of tail regeneration in tadpoles and salamanders almost 250 years ago (*23*), the relationship between development and regeneration has received much attention. While the adult human heart does not undergo any appreciable regeneration following injury, the possibility of cardiac regeneration in children and infants has been considered for almost a century. The earliest reports of cardiac regeneration in children involved careful analyses of post-mortem histological specimens from children with diphtheria (*24, 25*). The presence of mitotic figures and splitting of

myocardial fibers in such cases led to the suggestion that cardiac regeneration might be possible during early developmental stages (*24*). There is even some evidence for cardiac regeneration in children undergoing corrective cardiac surgery for congenital heart malformations (*26*). However, direct experimental evidence for this developmental phenomenon has only been provided very recently in different mammalian model systems.

The heart undergoes rapid growth and remodeling during embryogenesis. The heart also retains significant growth plasticity during embryonic and fetal life in the face of different environmental insults including nutrient deprivation, variations in litter size, changes in blood flow, as well as mechanical and volume loading (*27*). Such plasticity is presumably lost during postnatal life, as genetic and environmental insults that lead to excessive myocyte loss during early development are frequently associated with maladaptive compensatory remodeling and cardiac pathologies in adulthood (*28-30*). The most compelling evidence for cardiac regeneration during embryogenesis comes from genetic studies in mice. Similar to genetic cardiomyocyte ablation studies in zebrafish, Drenckhahn *et al*. have provided evidence for compensatory growth of healthy cardiac cells in diseased embryonic mouse hearts (*31*). In response to heterozygous deletion of the X-linked Holocytochrome c synthase (*Hccs*) gene, encoding an enzyme required for mitochondrial respiration, heterozygous female embryos were able to compensate for a 50% deficiency in cardiac cells. Interestingly, the remaining healthy cells gradually replaced the diseased cells

over time, suggesting that the embryonic heart has significant regenerative capacity. The embryonic regenerative response in embryonic mice was associated with robust induction of cardiomyocyte proliferation in healthy cells, which likely underlies the enhanced regenerative capacity at early developmental stages prior to the onset of postnatal mitotic arrest in mammals (*31*). Interestingly, a significant proportion of *Hccs*-deficient cells were still present in the ventricles of neonatal mice (~30% in the interventricular septum), suggesting that this innate cardiac regenerative capacity might extend into the early postnatal period (*31*).

In order to determine whether the neonatal mammalian heart harbors any significant regenerative capacity, we recently developed a surgical model of cardiac injury in 1-day-old neonatal mice (Figure 1) (*32*). Following amputation of a portion of the ventricular apex comprising approximately 15% of the ventricle, neonatal mice mounted a regenerative response that restored the lost myocardial tissue over a period of 3 weeks. Apical resection injury was associated with a series of cellular events highly reminiscent of cardiac regeneration in lower vertebrates, including formation of a blood clot, recruitment of inflammatory cells to the site of injury, epicardial cell activation, early extracellular matrix deposition, cardiomyocyte proliferation and restoration of normal cardiac function (*32*). The ability to efficiently regenerate heart muscle following amputation was lost by postnatal day 7, coinciding with the developmental window when rodent cardiomyocytes begin to lose their proliferative potential (*33, 34*). Genetic lineage

tracing studies confirmed that the vast majority of regenerated cardiomyocytes in 1-day-old mice were derived from pre-existing cardiomyocytes through cell proliferation (*32*). However, a minor contribution of non-myocytes to neonatal cardiac regeneration could not be excluded due to limitations in the cardiomyocyte labeling efficiency that could be achieved using the tamoxifen-inducible *Myh6*-MerCreMer transgenic system (~70-80% in P1 mice) (*32*).

More recent studies by our group and others suggest that 1-day-old neonatal mice are also capable of undergoing cardiac regeneration following myocardial infarction, which is the leading cause of heart failure in humans (*35, 36*). Myocardial ischemia was induced by permanent ligation of the left anterior descending coronary artery (LAD) in 1-day-old mice (Figure 1). LAD ligation was associated with a marked reduction in myocardial viability and a dramatic decline in cardiac function at day 3 following infarction (*36*). However, within 3 weeks, neonatal mice were able to launch a regenerative response that restored 95% of the infarcted myocardium and returned cardiac function to normal. Importantly, cardiac function was still unperturbed at 9 months of age, suggesting that the neonatal regenerative response can sustain cardiac function for several months following injury (*36*). Similar to cryocauterization in the zebrafish, neonatal infarction was associated with early collagen deposition, which later regressed and became marginalized to the periphery of the tissue. Little to no fibrosis was detectable at 3 weeks following infarction, with the exception of a small region of tissue immediately adjacent to the ligature (*36*). Histological and genetic lineage

tracing studies confirmed that the majority of regenerated cardiomyocytes were derived from pre-existing cardiomyocytes through cell proliferation, similar to earlier studies in zebrafish, as well as following apical resection in neonatal mice (*35, 36*). In addition, a robust angiogenic response was associated with heart regeneration in neonatal mice (*36*). Corrosion casting of the coronary vasculature revealed large collateral vessels in the newly regenerated anterior wall of the left ventricle that had originated from the right side of the heart. Consistent with earlier studies using the apical resection model, 7- and 14-day-old mice failed to undergo cardiac regeneration following myocardial infarction, suggesting that cardiac regenerative capacity was lost within the first two weeks after birth in rodents (*36*). These observations in 7-day-old mice were strikingly concordant with earlier studies by Robledo in the 1950's, which noted an incomplete cardiac regenerative response in 4-7 day-old rats following myocardial burn injury using a heated wire (*37*). Collectively, these findings suggest that the neonatal heart has a robust capacity for cardiac regeneration following multiple forms of tissue damage, including myocardial infarction, but this regenerative potential is rapidly silenced after birth.

Adding to the growing repertoire of available cardiac injury models for neonatal mice, Jesty *et al*. recently reported on the cardiomyogenic potential of neonatal mice following cryoinjury (*38*). In response to cryoinfarction at 1-3 days-of-age, neonatal mice underwent a marked cardiomyogenic response and regenerated a large proportion of the infarcted tissue over a period of 94 days. Consistent with

observations in the apical resection and myocardial infarction models, neonatal cryoinjury was associated with robust induction of cardiomyocyte proliferation throughout the heart (Figure 1). However, in addition to cardiomyocyte proliferation, a contribution of c-kit^{+} cardiac progenitor cells to cardiomyogenesis and angiogenesis in the neonate was also noted, suggesting that a pool of cardiac progenitor cells may be capable of supporting cardiac regeneration in neonatal mice (*38*). Although significant scar regression was observed in this model, it should be noted that regeneration was not complete at 94 days following cryoinjury. In contrast, a very recent paper by Strungs et al. has reported complete regeneration of the neonatal heart following cryoinjury at P1 and this regenerative potential is lost by P7, similar to previous findings in apical resection and myocardial infarction models (*39*). However, given that neither Jesty *et al.* nor Strungs *et al*. reported the size or reproducibility of neonatal cryoinfarcts, it is unclear whether the lack of complete regeneration in the Jesty *et al*. study is due to a larger infarct size or the degree of transmurality of the infarcts. Given that cryoablation of only 25% of the zebrafish heart is also associated with a much more protracted regenerative response (*17*), it will be important to assess the regenerative capacity of the neonatal mouse heart following small, large, superficial and transmural infarcts in the future in order to determine the physiological limits of neonatal heart regeneration.

Although studies in rodents offer a powerful experimental approach to the developmental regulation of cardiac regeneration, it is well known that both the

timing of cardiac maturation and the cardiovascular physiology of the rodent heart are different to large animals, such as humans (*40*). Therefore, systematic studies of heart regeneration at different developmental stages in large animal models are important. While the literature in this field is sparse, it has been reported that fetal sheep can undergo regenerative healing following myocardial infarction during early gestation (*41, 42*). Further studies are required to determine the precise timing and cellular mechanisms underlying regenerative arrest in large animal models, including whether this capacity extends into the immediate postnatal period.

4. Cardiomyocyte proliferation contributes to cardiomyocyte replenishment during aging and following injury

An underlying feature of cardiac regeneration in lower vertebrates and neonatal mice is the robust cardiomyocyte proliferative response (*15, 18, 32, 36*), which is absent in non-regenerative adult mammals. In contrast to zebrafish and newt cardiomyocytes, which remain predominantly mononucleated and retain proliferative potential throughout life, most mammalian cardiomyocytes permanently withdraw from the cell cycle before adulthood (*22*). In rodents, cardiomyocytes undergo a final round of DNA synthesis and karyokinesis in the absence of cytokinesis, which results in the binucleation of the vast majority (90-95%) of cardiomyocytes by postnatal day 14 (*33, 43, 44*). In contrast, binucleation in large mammals, such as sheep, is typically completed before birth (*45*). Interestingly, the proportion of binucleated cardiomyocytes is much lower in

humans than other mammals (~30-40%)(*46*). Although the proportion of binucleated cardiomyocytes does not change after birth in humans, there is a significant increase in the number of polyploid cardiomyocytes from birth (~5%) to 40 years of age (~60%)(*46*). Indeed, a recent study by Mollova *et al*. indicates that cardiomyocytes continue to proliferate, albeit at a low rate, during the first 20 years of human life (*46*), suggesting that the "proliferative window" for human cardiomyocytes might extend well into childhood and adolescence.

Over the last decade, a number of studies have proposed that the adult mammalian heart is not a terminally differentiated organ and is capable of turning over cardiomyocytes throughout life and following injury (*47*). The most compelling evidence for this radical concept comes from radiocarbon dating experiments of nuclear DNA from heart samples that were exposed to nuclear fallout during weapons testing during the Cold War. These studies revealed that human cardiomyocytes turn over at a low rate of about 1% per year, which declines to 0.5% per year after the age of 50 (*6*). The cellular source of these regenerated cardiomyocytes in humans remains controversial, but a recent study in mice suggests that cardimoyocyte proliferation is most likely the dominant cellular mechanism (*11*). Therefore, cardiomyocyte proliferation appears to be the primary natural mechanism for cardiomyocyte replenishment in highly regenerative organisms such as zebrafish and neonatal mice, as well as in less regenerative adult mammals. As such, understanding the mechanisms that

govern cardiomyocyte proliferation during development and regeneration is paramount.

5. Cardiomyocyte proliferative signaling during neonatal heart development and regeneration

As with many regenerative processes, cardiac regeneration in adult zebrafish involves the re-engagement of a number of growth factor signaling pathways that guide embryonic heart development, including the Igf, RA, Fgf and Notch signaling pathways (Figure 2) (*48-51*). However, in contrast to the capacity of these growth factors to stimulate myocardial proliferation during embryogenesis and early neonatal life in mammals, adult mammalian cardiomyocytes do not readily re-enter the cell cycle in response to similar developmental cues (*52, 53*). Even in response to mitogens that are associated with some degree of adult cardiomyocyte proliferation, such as neuregulin, periostin, FGF in combination with a p38α MAPK inhibitor or inhibition of glycogen synthase kinase 3β, only a very low percentage of adult cardiomyocytes re-enter the cell cycle and complete cytokinesis, and this effect is thought to be restricted to the mononucleated subset of cardiomyocytes in rodents (*54-58*). Interestingly, even in the highly proliferative adult newt heart, only ~30% of binucleated cardiomyocytes were able to undergo cytokinesis, suggesting that binucleation is a major barrier to cardiomyocyte mitotic progression (*59*). As such, recent attention has focused on unraveling the underlying molecular events that silence the genetic networks required for cardiomyocyte proliferation during neonatal life.

5.1. Chromatin remodeling maintains the post-mitotic cardiac phenotype

Genes are packed in chromatin, which is remodeled into active (euchromatin) and inactive (heterochromatin) states by covalent modifications of DNA and histones. The three primary mechanisms for chromatin-dependent regulation of gene expression are: DNA methylation, ATP-dependent chromatin remodeling, and covalent histone modifications, such as acetylation and methylation. A recent study has demonstrated that heterochromatin (associated with gene silencing) accumulates during postnatal cardiomyocyte maturation and that this is associated with enrichment of the gene-silencing epigenetic mark H3K9me3 at the promoters of cell cycle genes (*60*). Interestingly, both *in vivo* and *in vitro* loss-of-function studies indicated that the recruitment of heterochromatin protein 1-gamma (HP1-γ) to cell cycle gene promoters resulted in their incorporation into heterochromatin and irreversible silencing in adult cardiomyocytes in an Rb/p130-dependent manner (*60*). Although the significance of these findings for cardiac regeneration following ischemic injury is currently unclear, these results indicate that alterations in chromatin structure are associated with the maintenance of the post-mitotic cardiomyocyte phenotype in mammals.

Evidence for a role of epigenetic modifiers in the regulation of cardiomyocyte proliferation also comes from a number of genetic gain- and loss-of-function studies in the mouse. Deletion of the histone deacteylases, *Hdac1* and *Hdac2*, in cardiomyocytes results in neonatal lethality within 2 weeks after birth due to

cardiac arrhythmias and dilated cardiomyopathy (*61*). Combined loss of *Hdac2* and *Hopx* is also associated with increased cardiomyocyte proliferation and myocardial thickening and this effect is mediated through deacetylation of Gata4, which is a transcription factor that is required for cardiomyocyte expansion during embryogenesis (*62*). Hdac3 is also involved in the regulation of cardiomyocyte proliferative capacity, as over-expression of Hdac3 in cardiomyocytes stimulates proliferation by suppressing the expression of a number of cyclin-dependent kinase inhibitors (*63*). In addition, histone methyltransferases and demethylases have been implicated in the regulation of cardiomyocyte mitosis during embryogenesis, with Jumonji and Smyd1 mutants both displaying severe ventricular hypoplasia phenotypes (*64, 65*). Similarly, genetic deletion of the ATP-dependent chromatin-remodeling factor, Brahma-related gene 1 (Brg1), is associated with myocardial proliferative defects in mice (*66*). Taken together, these findings support the contention that epigenetic events underlie the maintenance of the proliferative state and expression of fetal genes during cardiac development. Future studies will be required to determine the relative importance of these epigenetic events for postnatal silencing of cardiac regenerative potential in mammals.

5.2. Regulation of cardiomyocyte proliferation and cardiac regeneration by microRNAs

MiRNAs are now recognized as central modulators of cardiac development and disease. Given their capacity to suppress large collections of proteins

participating in common signaling pathways, miRNAs could be engaged as important biological and therapeutic regulators of cardiomyocyte proliferation (*67*). In an attempt to identify miRNAs involved in postnatal cardiomyocyte mitotic arrest, microarrays were used to profile miRNAs in 1- and 10-day-old mouse hearts (*68*). One of the most highly up-regulated miRNAs during this developmental window was miR-195, a member of the miR-15 family, which was previously identified as a critical tumor suppressor miRNA (*69, 70*). Gain- and loss-of-function studies in the mouse subsequently validated the miR-15 family as an important regulator of postnatal cardiomyocyte mitotic arrest. Over-expression of miR-195 in developing cardiomyocytes was associated with premature cell cycle arrest and a subset of transgenic mice displayed congenital abnormalities including ventricular septal defects (*68*). Following myocardial infarction at P1, miR-195 transgenic mice also failed to regenerate and formed large fibrotic scars with an associated decline in cardiac contractile function (*36*). In contrast, postnatal inhibition of the entire miR-15 family using locked nucleic acid (LNA)-modified antimiRs prolonged the proliferative capacity of neonatal cardiomyocytes beyond the normal window of postnatal cell cycle arrest (*68*). Furthermore, postnatal inhibition of the miR-15 family induced cardiomyocyte proliferation and improved cardiac function in adult mice subjected to ischemia-reperfusion injury (*36*). Importantly, acute inhibition of the miR-15 family in small and large animal models is also associated with an improvement in cardiac function following ischemia-reperfusion injury (*71*), but whether this effect is due in part to cardiomyocyte cell cycle re-entry is currently unclear. Consistent with

the effects of the miR-15 family on cardiomyocyte proliferation, a number of cell cycle genes, including checkpoint kinase 1 (*Chek1*), have been identified as important downstream target genes (*68*). Taken together, these results suggest that the miR-15 family is an important regulator of cardiomyocyte mitosis and cardiac regenerative capacity during the neonatal period.

Another group of miRNAs that is critical for postnatal cardiomyocyte mitotic arrest is the miR-17~92 cluster. The miR-17~92 cluster was originally identified as a human oncogene and genetic loss-of-function studies in mice had previously revealed important functions for this miRNA cluster during embryonic heart development (*72, 73*). A recent study has also assessed the effects of miR-17~92 gain- and loss-of-function on cardiomyocyte proliferation during embryogenesis and postnatal life. Cardiomyocyte-specific deletion of the miR-17~92 cluster demonstrated an essential role during cardiomyocyte proliferation in embryonic and postnatal hearts (*74*). Moreover, transgenic over-expression of miR-17~92 was sufficient to induce cardiomyocyte proliferation in adult hearts and was associated with enhanced cardiac function following myocardial infarction (*74*). miR-17~92 appears to exert its biological actions through regulation of the phosphatase and tensin homolog (PTEN), an important regulator of phosphoinositide 3-kinase (PI3K) signaling. Although further studies are required to determine whether miR-17~92 plays any role during endogenous regeneration of the neonatal heart, these findings suggest that activation of this

miRNA cluster may represent a potentially important strategy towards induction of cardiomyocyte proliferation and cardiac regeneration in adults.

A recent study by Eulalio *et al*. has identified dozens of additional miRNAs with previously unknown roles in cardiomyocyte proliferation (*75*). In this study, cardiomyocytes were systematically screened against a library of 875 miRNA mimics to identify potential candidates capable of inducing cardiomyocyte proliferation. As two of the most potent inducers of cardiomyocyte proliferation, miR-199a and miR-590 were selected for further follow-up studies. In a powerful demonstration of the utility of functional genomic screens, over-expression of miR-199a and miR-590, respectively, was sufficient for induction of cardiomyocyte proliferation *in vivo* in neonatal and adult mice. Moreover, over-expression of these miRNAs was associated with increased cardiomyocyte proliferation rates, cardiac regeneration, reduced fibrotic scarring and improved cardiac function following myocardial infarction (*75*). A number of putative downstream target genes were identified for miR-199a and miR-590, including the Ca^{2+} signaling regulator *Homer1*, the homeodomain protein *Hopx,* and the chloride intracellular channel 5 (*Clic5*), all of which were demonstrated to inhibit cardiomyocyte proliferation (*75*). These findings further highlight the critical role of miRNAs during cardiomyocyte maturation and suggest that miRNAs may prove to be important drug targets for cardiac regeneration in the future.

5.3. Induction of cardiac regeneration through inhibition of Hippo signaling

The Hippo signaling pathway has emerged as a major regulator of organ size in diverse species. The core signaling components of the Hippo pathway in mammals include the mammalian STE20-like protein kinase 1 and 2 (Mst1 and Mst2), the scaffolding protein Salvador (SAV), the large tumor suppressor homologue 1 and 2 (LATS1 and LATS2) protein kinases, Yes-associated protein (YAP) and the transcriptional co-activator with PDZ-binding motif (TAZ). Following activation of this signaling cascade, the transcriptional co-activators Yap and Taz become phosphorylated, which results in their exclusion from the nucleus and disrupts their association with DNA binding transcription factors that normally drive cellular growth. In the heart, genetic deletion of *Salvador* during embryonic development in the mouse is associated with massively overgrown hearts and elevated cardiomyocyte proliferation indices (*76*). Ablation of *Mst1/2* or *Lats2* causes a similar embryonic cardiac phenotype (*76*). Interestingly, *Salvador* deletion was associated with the up-regulation of a number of Wnt target genes and an increase in nuclear β-catenin, suggesting that there is considerable cross-talk between the Hippo and canonical Wnt/ β-catenin signaling pathways. Indeed, reducing the levels of β-catenin rescues the cardiomyocyte proliferation phenotype in *Salvador* knockout mice (*76*).

Recent studies have also established that the transcriptional co-activator Yap is both necessary and sufficient to drive cardiomyocyte proliferation during embryogenesis and neonatal heart regeneration. Genetic loss-of-function studies

have revealed that *Yap* is required for cardiomyocyte proliferation during embryonic heart development (*77-79*). Deletion of Yap in the embryonic mouse heart causes myocardial hypoplasia and early embryonic lethality (*77, 79*). Similarly, deletion of *Yap* in the postnatal heart results in a progressive dilated cardiomyopathy and is associated with a reduced number of mitotic cardiomyocytes during the neonatal period (*78*). Following neonatal myocardial infarction, *Yap* cardiac-knockout mice fail to mount a regenerative response and instead are characterized by cardiac fibrosis and cardiac dilation, reminiscent of pathological cardiac remodeling in adult mice (*78*). Conversely, over-expression of a constitutively active form of Yap in the embryonic heart increases cardiomyocyte number and heart size (*79*). Over-expression of constitutively active Yap in cultured neonatal cardiomyocytes *in vitro* was also associated with induction of cardiomyocyte proliferation and this effect operates through activation of the IGF signaling pathway and downstream inactivation of GSK3β. Moreover, transgenic over-expression of constitutively active Yap in postnatal cardiomyocytes is sufficient to drive cardiomyocyte proliferation, reduce fibrosis and improve cardiac function following myocardial infarction in adult mice (*78*). Similar results were also reported in cardiomyocyte-specific *Salvador* knockout mice, which demonstrated an enhanced regenerative response and diminished fibrotic response to either apical resection injury or myocardial infarction at post-natal day 8 (*80*). These findings demonstrate that the Hippo signaling pathway is a critical regulator of cardiomyocyte proliferation and cardiac regeneration in mammals.

5.4. Meis1 defines a transcriptional hierarchy for cardiomyocyte proliferation

In contrast to the transcriptional networks that govern early embryonic heart development, little is known about the transcriptional regulation of postnatal cardiomyocyte maturation. To this end, a recent study by Mahmoud *et al*. identified a novel role for the transcription factor Meis1 during postnatal cardiomyocyte cell cycle arrest (*81*). Meis1 belongs to the TALE (three amino acid loop extension) family of homeodomain transcription factors and was previously shown to play an important role during embryonic hematopoiesis and heart development (*82, 83*). Recent genome-wide epigenetic mapping studies have revealed important functions for Meis transcription factors during cardiac differentiation (*84, 85*). These studies have shown that Meis transcription factors act independently, as well as in concert with other important cardiac transcription factors such as Gata4, to regulate a plethora of genes with important roles in cardiac development (*84, 85*). However, the functions of Meis transcription factors in the post-natal heart have only recently begun to be elucidated. Constitutive deletion of *Meis1* in the postnatal mouse heart was associated with an increased incidence of cardiomyocyte proliferation (*81*). Importantly, conditional deletion of *Meis1* in the adult heart was sufficient to induce cardiomyocyte cell cycle re-entry in a subset of adult cardiomyocytes. PCR arrays and transcriptional assays subsequently revealed that Meis1 was required for transcriptional activation of the synergistic cyclin-dependent kinase (CDK)

inhibitors p15, p16 and p21, thus identifying Meis1 as an important upstream component of the transcriptional hierarchy that governs cardiomyocyte cell cycle arrest after birth (*81*).

In order to determine the role of Meis1 during neonatal heart regeneration, inducible transgenic mice were subjected to myocardial infarction at P1. In contrast to wild-type mice, Meis1 over-expression in the neonatal heart reduced the number of mitotic cardiomyocytes following injury and was associated with a much more limited regenerative response characterized by increased fibrotic scarring and cardiac contractile dysfunction (*81*). Therefore, similar to miR-195 over-expression or Yap deletion, Meis1 over-expression is sufficient to impair the neonatal cardiac regenerative response through inhibition of cardiomyocyte proliferation (Figure 3).

6. Endogenous cardiac stem and progenitor cells

Resident tissue stem and progenitor cell populations reside in most adult tissues and contribute to cellular homeostasis and repair throughout life. In the heart, several cardiac stem/progenitor populations have been identified at different developmental stages including the embryonic, neonatal and adult heart. Cardiac stem cells have been identified on the basis of cell surface marker expression (e.g. c-kit, Sca-1), transcription factor expression (e.g. Isl-1), physiological dye efflux properties and propensity to form multicellular clusters (e.g. cardiospheres, colony-forming units-fibroblasts (cfu-f)) (*9, 86-90*). Given that mammalian

cardiomyocyte renewal is extremely limited in the adult heart following infarction, it is important to determine whether cardiac stem cells might play an important role in more highly regenerative organisms, such as zebrafish and neonatal mice.

6.1. Epicardial progenitors in the regenerating heart

The epicardium is a single mesothelial layer of cells that envelops the heart. Genetic lineage tracing studies in the mouse using Cre-loxP technology have identified the epicardium as a reservoir of multipotent cardiac progenitor cells that provide an important source of fibroblasts, vascular smooth muscle cells, perivascular cells and cardiomyocytes during embryogenesis (*91-93*). Thus, the role of epicardial cells during cardiac regeneration has also attracted significant interest. However, lineage-tracing studies in adult mice have so far failed to identify a major contribution of the epicardial lineage to cardiomyogenesis following myocardial infarction in adult mice or following cardiac injury in adult zebrafish (*94, 95*).

Epicardial genes are rapidly activated in an organ-wide manner in adult zebrafish and neonatal mice following cardiac injury (*32, 50*). In zebrafish, the epicardium provides an important source of growth factors such as RA and Pdgf, which support cardiomyocyte proliferation and cardiac regeneration (Figure 2) (*49, 96*). In addition, Kikuchi *et al*. and Gonzales-Rosa *et* al. traced the descendants of the *tcf21+* epicardial lineage and identified myofibroblasts and perivascular cells derived from this cell source following apical resection or cryoinjury, respectively

(*94, 97*). Neither study identified any cardiomyocytes derived from the epicardial lineage during cardiac regeneration. However, the vascular and cardiomyogenic potential of epicardial cells could potentially be enhanced in the adult heart following injury by treatment with specific growth factors. For example, the peptide thymosin β4 has been shown to enhance the vascular and cardiomyogenic potential of epicardial progenitor cells *in vivo* following myocardial infarction (*98, 99*). It will be important in the future to determine the relative contribution of epicardial progenitors to different cardiac lineages during neonatal mammalian heart regeneration in order to identify any potential developmental mechanisms that could be exploited to enable cardiac regeneration in adults.

6.2. c-kit progenitor cells and neonatal heart regeneration

To determine whether c-kit+ cells are capable of supporting regeneration of the neonatal heart, Jesty *et al*. subjected neonatal and adult c-kit-EGFP reporter mice to cryoinjury (*38*). Cardiac injury in neonatal mice was associated with robust activation of c-kit around the site of injury but not in remote regions. Interestingly, a substantial proportion of c-kit+ cells were of a cardiac phenotype at day 3 (67%) and day 7 (88%) following cryoinfarction (*38*). The majority of the remaining c-kit+ cells were endothelial cells. *In vivo* pulse-labeling with BrdU also identified a substantial proportion of proliferating cardiomyocytes in the regenerating neonatal heart that were positive for c-kit (42% at day 5 post-cryoinfarction) (*38*). In contrast, adult c-kit+ cells adopted a vascular fate

following cryoinfarction and did not contribute to cardiomyocyte formation, a finding that contradicts other reports of a robust cardiomyogenic potential for this stem cell population in adults (*9, 100*). While this study supports the notion of a differential regenerative capacity of the neonatal and adult heart, which may be in part accounted for by physiological alterations in the properties of c-kit+ cells, some caveats warrant further investigation. In particular, precise lineage tracing studies and ablation of the c-kit+ cell population in neonatal mice, as reported recently for this cell population in an adult isoproterenol-induced heart failure model (*101*), will be required to fully establish the contribution of this stem cell population to neonatal heart regeneration. Furthermore, given that c-kit is expressed by differentiated cardiomyocytes (*102*), de-differentiating cardiomyocytes (*103, 104*) and mast cells (*105*), the relative activity of this reporter construct in these different cell populations will need to be ascertained in order to perform controlled genetic fate mapping studies. It will also be interesting to determine whether cardiac stem cells exert paracrine effects that support heart regeneration during early developmental stages in mammals.

7. Angiogenic and vasculogenic responses during heart regeneration

Re-establishment of a new vasculature following cardiac injury is vital for heart regeneration. In neonatal mice, large epicardial blood vessels are evident in the regenerated heart following apical resection injury (*32*). Recent studies also indicate that cardiac regeneration following neonatal myocardial infarction is associated with a robust vasculogenic response with large collateral vessels

penetrating the infarcted territory during regeneration (*36*). In contrast, a similar vascular injury response is not evident in adult rodents, suggesting that important developmental differences in the reparative potential of vascular cells may contribute to the loss of cardiac regenerative capacity during the neonatal period.

Two of the most important pro-angiogenic factors during zebrafish heart regeneration are Fgf and Pdgf (Figure 2). Following apical resection injury, Fgf ligands, such as *fgf17b*, are up-regulated in the myocardium, while the Fgf receptors *fgfr2* and *fgfr4* are induced in epicardial cells (*50*). Transgenic loss-of-function studies in the zebrafish have established that Fgf receptor expression in epicardial cells is required for epicardial cell invasion into the wound site and neovascularization. In the absence of Fgf receptor-dependent neovascularization, the zebrafish heart fails to regenerate following injury and forms a large fibrotic scar (*50*). Similarly, Pdgf signaling is also required for epicardial cell proliferation and blood vessel formation following cardiac injury in zebrafish. The PDGF receptor alpha (*pdgfr*α) gene is up-regulated in epicardial cells and at the wound site following apical resection injury, while the PDGF-B ligand is expressed locally in the wound site by CD41-positive thrombocytes (*96, 106*). Pharmacological inhibition of Pdgf signaling inhibits neovascularization following apical resection injury (*96*). These findings have established an important myo-epicardial growth factor signaling nexus during cardiac regeneration and it will be important to establish whether these signaling pathways are also activated during neonatal heart regeneration in mammals.

Given the exciting recent developments in modified RNA-based delivery methods for angiogenic growth factors, such as vascular endothelial growth factor (VEGF) (*107*), it may be possible to recapitulate some of these pro-regenerative and pro-angiogenic signaling pathways in the adult heart in the future.

8. The extracellular matrix facilitates cardiac regeneration

The predominant reparative response to cardiac injury in adult mammals involves the replacement of damaged myocardial tissue with a permanent collagenous scar. Cardiac fibroblasts account for up to two thirds of all cardiac cells and are embedded within the extracellular matrix (ECM) where they secrete the collagens required for matrix synthesis, as well as growth factors that support cardiomyocyte function and remodeling (*108*). In contrast to the fibrotic response of the adult heart following infarction, the neonatal heart does not form a stable fibrotic scar. However, there is considerable ECM deposition during the first week following cardiac injury in neonatal mice (*32, 36*). Subsequently, the fibrotic tissue becomes marginalized to the periphery and regresses over several weeks before being almost completely replaced with regenerated cardiomyocytes (*36*). In the absence of an adequate myocyte proliferative response in the neonate, the default repair response is characterized by the presence of a fibrotic scar (*36, 78, 81*). These findings suggest that there is a reciprocal relationship between cardiomyocyte proliferation and fibrosis. Although little is known about the communication between fibroblasts and cardiomyocytes at early developmental stages, there is some evidence that cardiac fibroblasts regulate myocardial

proliferation during embryogenesis. For example, fibronectin, collagen and heparin-binding EGF-like growth factor are all produced by embryonic cardiac fibroblasts and can promote proliferation of immature cardiomyocytes (*109*). These proliferative effects are dependent on the expression of the ECM receptor, β1 integrin, in cardiomycytes. Interestingly, the expression of different integrin subunits (e.g. *Itga1*, *Itgb1*, *Itga6* and *Itga7*), which have varying propensities to bind to different ECM components (e.g. collagen, laminin, fibronectin), changes during cardiomyocyte maturation (*109*). It is therefore possible that postnatal changes in the ECM contribute to cardiomyocyte cell cycle withdrawal during the neonatal period. Furthermore, as re-establishment of the ECM is important for heart regeneration, further studies will be required in order to determine whether ECM components provide integral signals for neonatal heart regeneration.

Results from several recent studies in lower vertebrates are consistent with an important role for ECM-derived signals for heart regeneration (Figure 2). Fibronectin, a major component of the ECM, is secreted by epicardial cells and deposited at the injury site after cardiac injury in zebrafish (*110*). Loss-of-function experiments established that fibronectin is required for cardiac regeneration in zebrafish, although it does not affect cardiomyocyte proliferation in this species (*110*). The mechanism by which ECM-derived fibronectin influences heart regeneration in zebrafish is currently unknown but could be due to a potential role of fibronectin signaling on cardiomyocyte migration or epicardial cell function. On the other hand, transforming growth factor beta (TGFβ) ligands and receptors

(*tgfbr1b*) are induced in fibroblasts and cardiomyocytes following cryoinjury in zebrafish and are required for cardiac regeneration (*111*). Transient scar formation was found to be an essential step in the zebrafish regenerative response following cryoinjury and pharmacological inhibition of TGFβ signaling abolished the regenerative response (*111*). Thus, early ECM deposition and TGFβ-mediated cardiomyocyte proliferative signals are required for zebrafish heart regeneration. Furthermore, a recent study in adult newts has established that early up-regulation of ECM components in the epicardium precedes cardiomyocyte proliferation and migration in this species. Interestingly, the ECM-derived component tenascin-C was shown to be sufficient to induce cardiomyocyte proliferation *in vitro*, suggesting that ECM-derived signals in the adult newt are also required for induction of cardiomyocyte proliferation during cardiac regeneration (*112*).

9. Do neonatal immune responses dictate cardiac regenerative outcomes?

Cardiac injury in lower vertebrates, neonatal mice and adult mammals is associated with inflammation, yet the physiological consequences of injury in neonatal and adult mammals are vastly different. The immune response is important for clearing cellular debris following myocardial infarction in adults but it also facilitates scar formation, which ultimately compromises cardiac function (*113*). Indeed, multiple lines of evidence support the notion that the adult mammalian immune system constrains regenerative capacity and recent studies suggest that the immune response to injury supports regeneration in lower

vertebrates (*114-116*). In mammals, the adaptive and innate immune systems undergo drastic alterations and maturation during the neonatal period, coinciding with the developmental loss of cardiac regenerative capacity (*117-120*).

A recent study by Aurora *et al*. suggests that these postnatal changes in the immune system may also play an important role in dictating regeneration versus repair responses of the neonatal heart following injury (*121*). Through systematic profiling of the cellular immune response to cardiac injury, Aurora *et al*. identified a number of qualitative and quantitative differences in the cellular immune response of 1-day-old (regenerative) and 14-day-old (non-regenerative) mice following myocardial infarction. Differences in the monocyte and macrophage responses to cardiac injury in neonatal mice were particularly prevalent. Subsequent depletion of macrophages in neonatal mice using a clodronate-liposome depletion method revealed that macrophages are required for neonatal heart regeneration following myocardial infarction. In contrast to the pro-fibrotic role of macrophages in the adult heart, neonatal macrophages were found to be required for heart regeneration. Interestingly, macrophages did not influence cardiomyocyte proliferation following infarction but were essential for angiogenesis (Figure 3). Molecular profiling of macrophages in the regenerating (P1) and non-regenerating (P14) heart following infarction revealed a unique transcriptional signature of neonatal macrophages, including the expression of several potentially important pro-angiogenic cytokines. Future studies will be required to uncover the underlying mechanisms that govern these

developmentally distinct macrophage responses to injury. In addition, whether the absence of regeneration in adult mammals is due to differences in the types of inflammatory cells that are recruited, the specific signals secreted from inflammatory cells, or the responsiveness of different cardiac cell types to these inflammatory stimuli remains to be determined.

10. Conclusions and future perspectives

It has been suggested that developmental mechanisms are prisoners of their own phylogenetic histories (*122*). On the basis of recent studies of cardiac regeneration in fetal/neonatal mammals, it would appear that regenerative mechanisms are also prisoners of their developmental histories. These findings raise the following question - what is the evolutionary advantage of shutting down cardiac regenerative pathways after birth? It appears that in the absence of any selective pressure to maintain regenerative capacity in organisms that typically don't succumb to cardiovascular disease until well after reproductive maturity, there may not have been a strong evolutionary drive to maintain regenerative potential after birth in mammals. So, what could be the advantage of arresting cardiomyocyte proliferation after birth? In mammals, the heart undergoes a number of physiological adaptations during the immediate postnatal period to cope with the increased loading and oxygen demands associated with the pulmonary circulation, and the rapid growth demands of postnatal life (*123*). These environmental and physiological stressors are matched by marked structural and metabolic adaptations in the cardiomyocyte, including a switch

from glycolyis to fatty acid oxidation and sarcomere reorganization, both of which are suited to the high-energy demands of the postnatal heart (*124*). Given the requirement to disassemble sarcomeres during cell division (*125*), cardiomyocyte proliferation may not be particularly advantageous during postnatal life in mammals and this adaptation may have come at the expense of cardiac regeneration. An important aspect of future studies that attempt to recapitulate early developmental regenerative mechanisms, including cardiomyocyte cell cycle re-entry, will be to establish whether these approaches have any detrimental effects on adult cardiac physiology. It is our belief that understanding the detailed physiological and molecular mechanisms that drive cardiomyocyte maturation and regenerative arrest during neonatal life will be imperative for establishing the biological basis and clinical utility of future cardiac regenerative therapies.

Much work remains to be done in order to identify the molecular and cellular basis for neonatal heart regeneration. However, recent studies suggest that re-activation of neonatal cardiac regenerative pathways to drive cardiomyocyte proliferation in the adult heart may be possible (*36, 78, 81*). Given the ever-expanding molecular genetic and pharmacological toolkit for studies in mammals and lower vertebrates, it is likely that many more natural cardiac regenerative mechanisms will be identified in the near future. If successful, these studies may indeed uncover a developmental blueprint for cardiac regeneration.

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Figure Legends:

Figure 1: Different injury models for studies of cardiac regeneration in the newt, zebrafish and neonatal mouse. Cardiomyocyte proliferative responses (localized or global) and the outcomes of genetic lineage tracing experiments are highlighted. Green dots denote proliferating cardiomyocytes.

Figure 2: Molecular and cellular mechanisms of cardiac regeneration in adult zebrafish. Retinoic acid (RA) is produced and released by epicardial and endocardial cells shortly after injury and acts on cardiomyocytes to induce proliferation. Other proliferative cues are also received from non-myocyte populations such as fibroblasts, which generate several important pro-regenerative extracellular matrix components such as transforming growth factor β (TGFβ), tenascin-C and fibronectin. Platelet-derived growth factor BB isoform (PDGF-BB) and fibroblast growth factor (FGF) are produced by thrombocytes and myocardial cells, respectively, and act on their cognate receptors (PDGFR and FGFR) in the epicardium to promote angiogenesis.

Figure 3: Molecular and cellular mechanisms of cardiac regeneration in neonatal mice. Cardiomyocyte proliferation and angiogenesis are both required for complete regeneration of the neonatal heart following injury. Cardiomyocyte proliferation in the neonatal heart is activated by Yap and restricted by the miR-15 family and Meis1. Macrophages are also recruited to the injury site where they promote angiogenesis.

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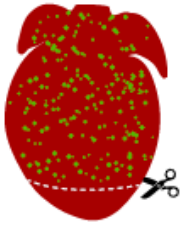
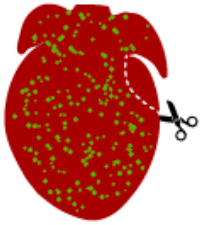
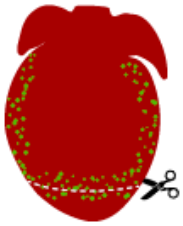
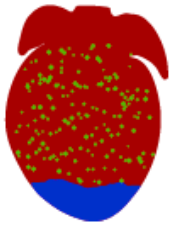
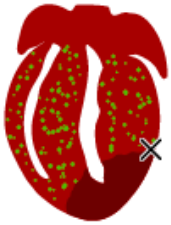
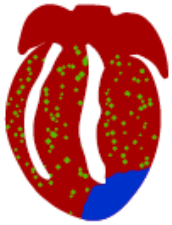
Newt	Apical Resection	Basal Resection	Cryoinjury
			
Regeneration:	Yes (incomplete)	Yes (complete)	N/A
CM Proliferation:	Yes (global)	Yes (global)	N/A
Genetic Lineage:	N/A	N/A	N/A
Zebrafish	**Apical Resection**	**Genetic Ablation**	**Cryoinjury**
			
Regeneration:	Yes	Yes	Yes (protracted)
CM Proliferation:	Yes (sub-epicardial + localized)	Yes	Yes (global)
Genetic Lineage:	Pre-existing CMS	Pre-existing CMS	N/A
Neonatal Mouse	**Apical Resection**	**Myocardial Infarction**	**Cryoinjury**
			
Regeneration:	Yes	Yes	Yes (incomplete)
CM Proliferation:	Yes (global)	Yes (global)	Yes (global)
Genetic Lineage:	Pre-existing CMS	Pre-existing CMS	C-kit+ contribute

Figure 1

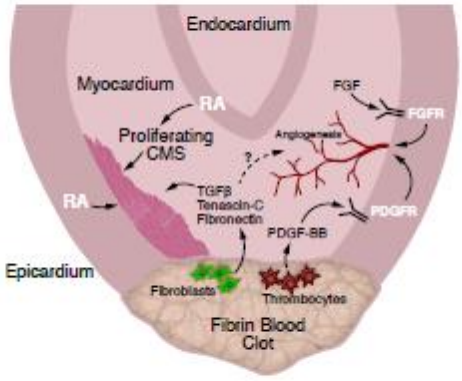


Figure 2

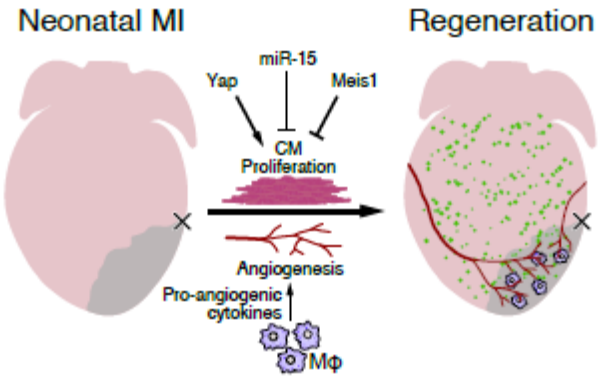


Figure 3

Highlights:

- Neonatal mice regenerate following cardiac injury.
- Neonatal heart regeneration occurs through cardiomyocyte proliferation.
- Cardiomyocyte proliferation is regulated by the miR-15 family, Yap and Meis1.
- Macrophages are required for neonatal heart regeneration.