© 2016. Published by The Company of Biologists Ltd | Development (2016) 143, 729-740 doi:10.1242/dev.132910

REVIEW

Building and re-building the heart by cardiomyocyte proliferation

Matthew J. Foglia and Kenneth D. Poss*

ABSTRACT

The adult human heart does not regenerate significant amounts of lost tissue after injury. Rather than making new, functional muscle, human hearts are prone to scarring and hypertrophy, which can often lead to fatal arrhythmias and heart failure. The most-cited basis of this ineffective cardiac regeneration in mammals is the low proliferative capacity of adult cardiomyocytes. However, mammalian cardiomyocytes can avidly proliferate during fetal and neonatal development, and both adult zebrafish and neonatal mice can regenerate cardiac muscle after injury, suggesting that latent regenerative potential exists. Dissecting the cellular and molecular mechanisms that promote cardiomyocyte proliferation throughout life, deciphering why proliferative capacity normally dissipates in adult mammals, and deriving means to boost this capacity are primary goals in cardiovascular research. Here, we review our current understanding of how cardiomyocyte proliferation is regulated during heart development and regeneration.

KEY WORDS: Cardiomyocyte, Heart regeneration, Proliferation

Introduction

Heart failure, which is often a consequence of an initial myocardial infarction (MI) event, is a major cause of morbidity and mortality (Jessup and Brozena, 2003). A primary obstacle to functional recovery of the infarcted or failing human heart is the limited proliferative capacity of cardiac muscle, which, unlike skeletal muscle, has no robust, natural mechanism for regeneration. Instead, the human response to cardiac injury is fibrotic scarring and hypertrophic remodeling of the surviving myocardium (Sutton and Sharpe, 2000). As a result, modern clinical interventions after ischemic cardiac injury primarily focus on rapid re-perfusion to minimize cardiomyocyte death and aim to pharmacologically manage the chronically weakened organ (Gerczuk and Kloner, 2012; McMurray et al., 2012). An enticing and theoretically more permanent solution is the design of therapies for myocardial regeneration or reconstitution.

For decades, the heart was considered a post-mitotic organ (Zak, 1973). Although recent studies have indicated that adult human cardiomyocytes are replaced at a low but detectable rate, this native capacity is insufficient to compensate for the large-scale tissue damage associated with MI (Bergmann et al., 2009, 2015). For this reason, a number of potential approaches for regenerating cardiac muscle have been explored (see Box 1). In the past 5 years, cardiomyocytes themselves have re-emerged as target cells for regenerative intervention. After birth, the mammalian heart grows primarily by cardiomyocyte hypertrophy, a process in which existing cardiomyocytes enlarge but do not divide. With their increased DNA content and contractile structures, most mature cardiomyocytes are poor substrates for cell division and appear to contribute minimal, if any, new muscle after injury. Yet, cardiomyocytes proliferate avidly during fetal development and in the first days of postnatal life, enabling massive cardiac growth (Soonpaa et al., 1996). Moreover, zebrafish and other teleost fish regenerate portions of their hearts after injury via cardiomyocyte division, and some urodele amphibians have also been reported to regenerate to varying degrees (Bader and Oberpriller, 1978; Flink, 2002; Poss et al., 2002; Witman et al., 2011). Even neonatal mice exhibit some cardiac regenerative capacity (Porrello et al., 2011). Although this might simply reflect the varying regenerative capacities of these species (see Box 2), it highlights the fact that cardiomyocytes in various contexts do harbor endogenous proliferative capabilities, albeit to different degrees. Whether, how, and to what extent this endogenous proliferative ability can be enhanced to recover muscle mass and function are now major questions in the field. To address these questions, it is crucial to investigate the regulation of cardiomyocyte proliferation in all accessible contexts and species.

Here, we review how cardiomyocyte proliferation is regulated during heart development, growth and homeostasis. We discuss current insights into the transition from a proliferative to a hypertrophic mode of cardiac growth seen in postnatal mammals, and how studies of naturally regenerative model systems have contributed to our understanding of cardiac regeneration.

Box 1. Approaches to cardiac regeneration

Several strategies for regenerating new cardiac muscle are currently being pursued. Cardiac stem cells – circulating or local non-myocardial progenitors that might differentiate into cardiomyocytes – are an attractive target population in theory to use to increase the cardiomyocyte pool (Beltrami et al., 2003; Ellison et al., 2013). However, objective interpretation of lineage-tracing experiments finds insufficient evidence for stem cell populations harboring significant myocardial repair activity (van Berlo et al., 2014; van Berlo and Molkentin, 2014). This does not preclude applications of stem cells in heart regeneration therapies. Indeed, recent work in developmental and reprogramming biology shows there are in fact many ways to influence cell fate and generate cardiomyocyte-like cells or progenitors *in vitro*. However, to date, transplanted stem cell populations have been found to survive only transiently and provide modest benefits, if any, and might affect endogenous repair mechanisms by paracrine action (Hong et al., 2014; Gnecchi et al., 2008). The use of pluripotent stem cell-derived cardiomyocytes for heart therapy also faces daunting challenges with regard to cell maturation, arrhythmogenesis, immunosuppression, and the need for scaling up (Behfar et al., 2014; Laflamme and Murry, 2011). The ability to stimulate robust endogenous cardiac regeneration without adding exogenous cells would avoid these issues, and remains the field's 'holy grail'. The direct reprogramming of non-muscle cells into cardiomyocyte-like cells by infecting or treating the heart with defined factors would achieve this goal. Although there have been provocative recent demonstrations of the potential of this approach, the low efficiency of reprogramming vectors is a target area for improvement (Chen et al., 2012; Ieda et al., 2010; Qian et al., 2012; Song et al., 2012; reviewed by Sadahiro et al., 2015). The stimulation of cardiomyocyte proliferation, as discussed in this Review, thus remains an attractive approach for regenerating the heart.

Department of Cell Biology, Duke University School of Medicine, Durham, NC 27710, USA.

*Author for correspondence (kenneth.poss@duke.edu)

Development (2016) 143, 729-740 doi:10.1242/dev.132910

Box 2. Regenerative capacity: an evolutionary perspective

Far from a rare talent, the regeneration of injured body parts is a common ability of adult organisms ranging from tiny planarians to large mammals. In humans, hepatocytes increase cell size or divide to replace lost liver mass after surgical resection, and tissues like hair follicles and intestinal epithelium are continually renewed by local stem cell populations. However, animals vary widely in their capacity to regenerate particular tissues. Invertebrates such as planarians and hydra, which can form whole animals from small segments, exhibit the greatest regenerative aptitude (Reddien and Sánchez Alvarado, 2004; Bosch, 2007). Mammals, by contrast, fail to regenerate crucial structures, including limbs, spinal cord and cardiac muscle. However, certain vertebrates, including urodeles (e.g. salamanders) and teleost fish (e.g. zebrafish), retain the ability to regenerate these and other organs. It is thus of significant interest to understand the degree to which fundamental aspects of these organisms' biology, rather than or in addition to cardiac-specific factors, allow them to repair their hearts so effectively. Although we briefly discuss certain non-cardiac influences on cardiomyocyte proliferation in this Review, we refer interested readers to other, more thorough reviews of the comparative biology of regeneration and its mechanisms (Brockes and Kumar, 2008; Poss, 2010).

Shaping cardiac structures by cardiomyocyte proliferation

The first cardiomyocytes to emerge during development arise from the differentiation of progenitor cells located in mesoderm-derived structures called heart fields, and they coalesce to form primitive hollowed chambers (Buckingham et al., 2005). As embryos mature to juvenile and adult stages, the regulated division of cardiomyocytes is responsible for the formation, growth and sculpting of mature cardiac structures, such as the fingerlike ventricular trabeculae and atrial pectinate, and the layered compact muscle of chamber walls (Fig. 1).

Early cardiogenesis

Cardiomyocytes are added to the embryonic heart by two basic mechanisms: (1) the differentiation of cardiac precursors; and (2) the division of existing cardiomyocytes. The first wave of cardiomyocyte precursors forms a crescent near the head folds that subsequently fuses at the midline to form the primitive heart tube (Buckingham et al., 2005). As the heart tube forms, *de novo* cardiomyocyte creation occurs both from the differentiation of these precursors and from a second heart field in the pharyngeal mesoderm (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001; reviewed by Kelly et al., 2014; Paige et al., 2015). Multiple studies in chick have revealed little evidence for cardiomyocyte division occurring during these early stages (Sissman, 1966; van den Berg et al., 2009). However, studies in a variety of species suggest that a transition from progenitor cell differentiation to proliferative myocardial growth occurs subsequently; precisely when this happens is difficult to pin down.

Retrospective lineage-tracing experiments performed in chick and mouse embryos have proposed models for early cardiac growth. Mikawa et al. injected *lacZ*-containing retroviruses into the cardiogenic mesoderm of chick embryos, finding that clonally related cardiomyocytes form vertically arrayed, cone-shaped colonies that often extend transmurally in the mature heart (Mikawa et al., 1992a). Following this, Meilhac et al. used a recombination-dependent *nlaacZ* reporter targeted to the α-cardiac actin (*Actc1*) gene to trace stochastically labeled cardiomyocyte clones in developing mice (Meilhac et al., 2003). They observed rare, large clones at embryonic day (E) 8.5 that extend the length of the heart tube, indicating rostrocaudal dispersion of a cardiac precursor. By contrast, hearts observed at E8.5 or later, at the time of chamber formation, contain coherent clusters of labeled cells more consistent with cardiomyocyte proliferation.

The accessibility of early cardiac development in zebrafish has permitted a more detailed analysis of this process. In zebrafish, evidence indicates that cardiac growth after looping of the heart tube occurs exclusively via the proliferation of existing cardiomyocytes (de Pater et al., 2009; Jopling et al., 2010; Kikuchi et al., 2011a; Qu et al., 2008). Recently, a cardiomyocyte-specific adaptation of Brainbow technology (Livet et al., 2007) was developed in zebrafish to genetically label cardiomyocytes with dozens of unique colors and follow their contributions throughout life (Gupta and Poss, 2012). Among other findings (see below), this method of lineage tracing permitted estimation of the number of cardiomyocytes present at the time of labeling based on the number of individually colored clonal populations. The results of this analysis indicate that ventricular wall growth is driven by the proliferation and coherent clonal expansion of 50-60 (out of a total of ~115) early cardiomyocytes.

Together these findings suggest that, in each of the vertebrate model systems studied, cardiomyocyte division assumes the

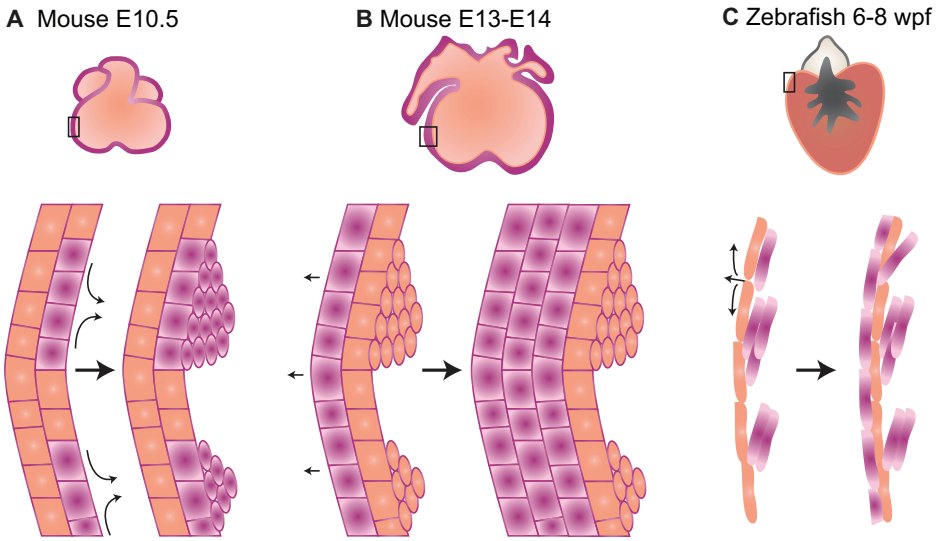

Fig. 1. Cardiomyocyte contributions to the formation, growth and sculpting of mature cardiac structures. (A) Formation of trabecular muscle in mice begins at approximately E10.5, when inductive signals from the endocardium (not shown) stimulate cell division in the myocardium (orange cells). Newly divided cardiomyocytes (pink cells) form muscular ridges known as trabeculae that are thought to strengthen contraction and improve myocardial oxygenation. (B) By E13-E14, the ventricular wall thickens substantially as cardiomyocytes proliferate (pink cells) in the outer layers of the myocardium to form compact muscle. (C) In zebrafish, the ventricular wall does not thicken until 6-8 weeks post-fertilization (wpf), when rare trabecular muscle cardiomyocytes (pink cells) in the chamber lumen emerge through the primordial wall (orange) and proliferate to cover the ventricle in a cortical muscle layer.

Development (2016) 143, 729-740 doi:10.1242/dev.132910

dominant mode of chamber growth early in development. Furthermore, regional variations in proliferation rates suggest that positional cues play a role in chamber morphogenesis, both in experimental models and in humans (Christoffels et al., 2000; Sizarov et al., 2011; Soufan et al., 2006). For instance, a transcriptional program including *Anf* (*Nppa* – Mouse Genome Informatics), *Mlc2v* (*Myl2* – Mouse Genome Informatics), *Irx4* and other genes is activated in the relatively hyperproliferative outer curvature of the looped heart, leading to 'ballooning' of the cardiac chambers (Christoffels et al., 2000). However, the precise cues that generate this spatially heterogeneous pattern of proliferation remain unclear.

Trabeculation

The development of trabecular muscle in ventricular chambers involves the intricate spatiotemporal regulation of cardiomyocyte proliferation (Fig. 1A). In higher vertebrates, trabeculae emerge shortly after looping when regions of myocardium invaginate, forming ridge-like projections along the wall (Challice and Virágh, 1974; Sedmera et al., 1997). This process is driven by signaling between the myocardium and adjacent endocardium, an endothelial lining of the chamber interior. Activation of the Notch pathway has been shown to modulate cardiomyocyte proliferation and hence trabeculation. Endocardial Notch signaling promotes the activity of Bmp10 in the underlying myocardium, and both *Bmp10* and *Notch1* mutants exhibit reduced cardiomyocyte proliferation and impaired trabeculation that can be rescued with exogenous Bmp10 (Chen et al., 2004; Grego-Bessa et al., 2007). Conditional knockout of a Notch1 intracellular domain inhibitor, Fkbp1a, in the endocardium, but not the myocardium, leads to hypertrabeculation (Chen et al., 2013). The extracellular factor neuregulin 1 (Nrg1) is also an indirect target of Notch signaling. This interaction is thought to be mediated by the direct transcriptional regulation of ephrin B2 by Notch. Ephrin B2, in turn, is thought to be required for Nrg1 expression based on analysis of ephrin B2 mutants (Grego-Bessa et al., 2007). Mice lacking functional Nrg1 or its receptors, ErbB2 and ErbB4, die prior to birth and exhibit thin-walled hearts lacking trabeculae (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995), although some evidence suggests that, at this stage, Nrg1 affects trabecular cardiomyocyte differentiation rather than proliferation (Grego-Bessa et al., 2007). A similar phenotype was observed in mice carrying null alleles for ephrin B2 or its receptor ephrin B4 (Gerety et al., 1999; Wang et al., 1998).

In zebrafish, cardiomyocyte division is disrupted during trabeculation in *erbb2* mutant zebrafish hearts, indicating a conserved role for Nrg1/ErbB2 signaling (Liu et al., 2010). Genetic fate-mapping and live-imaging approaches have indicated that trabeculae form by the delamination of cardiomyocytes from the ventricular wall, followed by displacement to a distal trabeculation site, rather than by oriented cell division and *in situ* branching (Gupta and Poss, 2012; Liu et al., 2010; Staudt et al., 2014). Trabeculation in mice is not as accessible to these high-resolution approaches, although the common involvement of molecular factors such as Nrg1 suggests that certain mechanisms of cardiomyocyte proliferation are shared.

Wall maturation

Later during development, the ventricle(s) compensates for increasing hemodynamic demands by developing a thickened, vascularized wall of muscle (Clark et al., 1989; Saiki et al., 1997). In mammals, the myocardium thickens markedly around E13-E14 in mice (Fig. 1B) and between 10 and 12 weeks gestation in humans. The compact muscle, as the maturing wall structure is called, supplants trabecular muscle in both proliferation and contractile force (Sedmera et al., 2000). As previously noted, compact muscle grows as cone- or wedge-shaped clones of proliferating cardiomyocytes that are broadest at the outer layers of the myocardium (Mikawa et al., 1992b). This suggests a gradient of proliferative activity that is highest at the outer layer of the myocardium. This pattern of proliferation is consistent with the presence of some mitogen(s) from the heart's enveloping mesothelial layer, the epicardium. In fact, microsurgical disruption of epicardium formation reduces proliferation in the underlying myocardium, and the co-culture of fetal cardiomyocytes with epicardial cells stimulates their division (Chen et al., 2002; Pennisi et al., 2003; Pérez-Pomares et al., 2002). Mutation of the gene encoding the transcription factor Wilms tumor 1 (Wt1) results in a thin-walled ventricle, as does impaired retinoic acid (RA) or erythropoietin (Epo) signaling (Kreidberg et al., 1993; Merki et al., 2005; Stuckmann et al., 2003; Wu et al., 1999). These released factors are thought to act primarily on the epicardium itself, however, rather than as direct cardiomyocyte mitogens. Epicardium-derived mitogens responsible for the effect of the epicardium on compact muscle proliferation have been challenging to identify. Recent evidence has pointed to Igf2 as one such molecule. *Igf2* is expressed by epicardial cells during mid-gestation murine heart development, and *Igf2*-deficient embryos show reduced cardiomyocyte proliferation in the ventricular wall. Importantly, the cardiomyocyte-specific deletion of the genes encoding the Igf2 receptors Igf1r and Insr replicated this effect (Li et al., 2011). The role of Igf2 is conserved in zebrafish, where expression of a dominant-negative Igf1r or treatment with an Igfr1 inhibitor results in decreased cardiomyocyte number during development (Huang et al., 2013). Brade et al. reported that, in mice, *Igf2* expression by the epicardium is regulated by RA-dependent Epo signaling; however, their data indicated that Epo was secreted by the liver rather than the epicardium (Brade et al., 2011). This provocative finding links ventricular compaction to hepatic development, and further consideration of the relative importance of extra-cardiac influences on cardiomyocyte proliferation is warranted.

Although certain fish, such as tuna species, acquire thick ventricular walls, the zebrafish ventricular wall undergoes only modest expansion during maturation and, like in many fish and amphibians, its trabeculae remain prominent throughout life. Interestingly, and unlike the situation in mammals, zebrafish hearts undergo conspicuous ventricular wall changes quite late in development; multicolor genetic fate-mapping analyses recently identified the origins of the outermost layers of the ventricular wall, called cortical muscle. Notably, a rare group of ~6-12 cardiomyocytes ultimately contributes to building the cortical muscle layer (Fig. 1C). These dominant cardiomyocytes originate from trabecular cardiomyocytes that penetrate an underlying single cardiomyocyte-thick layer of primordial muscle in rare, spatially distinct events at the juvenile stage, emerge on the ventricular surface, and expand to cover the entire chamber in a multicellular layer (Gupta and Poss, 2012). A subsequent study found that experimental micro-injuries, conditions of rapid organismal growth, or heat stress could stimulate precocious cortical muscle formation (Gupta et al., 2013). Ultimately, these studies suggested a model in which cortical layer formation is a developmental response to biomechanical stress, and that the cardiomyocytes that build the final layer arise stochastically.

Development (2016) 143, 729-740 doi:10.1242/dev.132910

Cardiomyocyte proliferation during development thus occurs in a number of phases to construct the various structures of the mature organ. Proliferative dynamics are distinct among species, and this is likely to be the primary basis for variance in cardiac structure. High-resolution proliferation dynamics of cardiomyocytes have yet to be mapped for the later stages of mammalian heart development, and there is much still to learn about the molecular and mechanical signals for cardiomyocyte proliferation that shape the heart.

The growth and homeostasis of established cardiac structures

As we have discussed above, the mammalian heart grows and is shaped primarily by cardiomyocyte division prior to birth. Shortly thereafter, during the second week of life in mice, cardiomyocyte hypertrophy rather than proliferation contributes to increases in myocardial volume. This large-scale transition has been the subject of intense interest; it offers a means of identifying the mechanisms by which cardiomyocytes exit the cell cycle and ways in which they might be induced to re-enter. Indeed, heart growth by cardiomyocyte proliferation, in contrast to hypertrophic expansion, is strongly linked to regeneration. Neonatal mice display an ability to recover with minimal scarring after a severe apical resection injury or an experimental MI in the first day of life (Haubner et al., 2012; Porrello et al., 2011, 2013). This is in addition to experiments indicating that fetal mice have a high capacity for heart regeneration when injured *in utero* (Drenckhahn et al., 2008; Sturzu et al., 2015). Because the postnatal day (P) 0 or P1 heart undergoes massive growth during the 21-day period after injury, it is unclear whether the lost muscle is locally replaced or if compensatory myogenesis from distant areas obscures the injury. Nevertheless, injuries to mice at P7 or later show a higher incidence of scarring, cementing a correlation between the capacity for cardiomyocyte proliferation and regenerative potential.

Early postnatal binucleation and endoreplication in mammals

Studies in the mid-1990s suggested that the mechanism of cardiac growth in mammals shifts abruptly after birth from hyperplasia to hypertrophy, and that postnatal DNA synthesis in mammals is not matched by a concomitant increase in cardiomyocyte number (Li et al., 1996; Soonpaa et al., 1996). Subsequently, it was shown that, in rodents, a burst of DNA replication and karyokinesis takes place without cytokinesis, leading to binucleated cardiomyocytes (Li et al., 1997). This process begins around P4, mirroring the end of the observed window of regenerative potential in neonatal mice, and continues until ∼85-90% of cardiomyocytes are binucleated by P21 (Soonpaa et al., 1996). By contrast, this degree of binucleation is not seen in zebrafish; ∼95% of cardiomyocytes in the adult zebrafish heart are mononucleated (Wills et al., 2008). Findings in newts suggest that mononucleated cardiomyocytes are more likely to undergo cytokinesis than their binucleated counterparts (Matz et al., 1998). This tendency appears to hold true in mammals: in their examination of the effect of Nrg1/ErbB4 signaling on adult mouse cardiomyocytes, Bersell et al. noted that mononuclear cells were more likely to undergo both karyokinesis and cytokinesis after Nrg1 treatment *in vitro* and *in vivo* (Bersell et al., 2009). In humans, only 25% of cardiomyocytes are binucleated, although a major fraction of these and the remaining mononuclear cardiomyocytes are polyploid, the result of endoreplication (Brodsky et al., 1994; Olivetti et al., 1996). Modifications of the canonical cell cycle thus appear to be associated with organisms that exhibit hypertrophic, rather than hyperplastic, injury responses.

Intriguingly, a recent study identified a brief burst of proliferative activity as late as P15 in the mouse, potentially extending the window during which proliferation plays a meaningful role in cardiac growth (Naqvi et al., 2014). The authors of this study reported that the inciting event for this burst is a surge in thyroid hormone that activates the Igf1/Akt pathway. They also noted an increase of ∼500,000 cardiomyocytes during the third week of life that correlates with a marked increase in 5-bromo-2′-deoxyuridine (BrdU)-labeled cardiomyocyte nuclei. To examine cytokinesis, the authors stained cardiomyocytes for aurora B, determining that ∼90% of mitotic cardiomyocytes were binuclear, and that ∼30% of both mono- and binuclear cardiomyocytes were in M phase. As a disproportionate fraction of newborn cardiomyocytes was mononuclear, this suggests that the division of binuclear cells helps to drive the observed increase in cardiomyocyte number. However, this discovery of a preadolescent proliferative burst has been the subject of intense debate. Other groups have not detected evidence of such a burst of cardiomyocyte proliferation, by either cardiomyocyte number assays (via design-based stereology) or proliferation marker assays [via detection of BrdU incorporation, anillin, Ki-67 (Mki67), cyclin B1, cyclin A2, and others] (Alkass et al., 2015; Hirai et al., 2015; Soonpaa et al., 2015). This debate has highlighted the difficulty of obtaining and interpreting cardiomyocyte proliferation data, which can vary significantly based on technical factors, e.g. enzymatic digestion efficiency for cardiomyocyte isolation, sampling area, and cell boundary determination for stereological studies, and the effect of animal husbandry on pre-adolescent development (Naqvi et al., 2015). To achieve a consensus on whether a proliferative burst occurs in pre-adolescent mammals will require either standardization of husbandry, data collection and analytic techniques, or new, more definitive technologies to detect cardiomyocyte cell division. In the meantime, Brainbow-based fate mapping or inducible lineage tracing coupled to cardiomyocyte mitosis, similar to the recently described mosaic analysis with double markers (MADM) experiment (Ali et al., 2014), could be informative if carefully designed to detect cardiomyocyte division during the period in question.

The regulation of cell cycle withdrawal

The regulatory mechanisms behind the shift from hyperplasia to hypertrophy are potentially appealing targets for stimulating or reactivating cardiomyocyte proliferation in adult tissue. Recent studies have therefore aimed to identify and characterize cell intrinsic and extrinsic factors that regulate the cell cycle and control cell cycle arrest in cardiomyocytes (Fig. 2).

Classic cell cycle regulators are prominently involved in the arrest of dividing cardiomyocytes. Cardiomyocyte mitotic arrest is associated with downregulation of G1/S and G2/M cyclins and cyclin-dependent kinases (CDKs) and upregulation of cell cycle inhibitors (Engel et al., 2005; Flink et al., 1998; Poolman and Brooks, 1998). The overexpression of cyclins D1 and D2 and cyclin A2 can stimulate cardiomyocyte DNA synthesis or mitosis, respectively, in adult mice (Chaudhry et al., 2004; Pasumarthi et al., 2005; Soonpaa et al., 1997), and the deletion of p130 (Rbl2) and Rb (Rb1) causes cardiomyocytes to re-enter the cell cycle (Sdek et al., 2011). However, although the manipulation of these direct cell cycle regulators can frequently induce DNA replication, the production of new cardiomyocytes in mature hearts appears to remain inefficient.

More recently, Mahmoud et al. identified the transcription factor Meis1, as a possible key regulator of postnatal arrest, providing

Development (2016) 143, 729-740 doi:10.1242/dev.132910

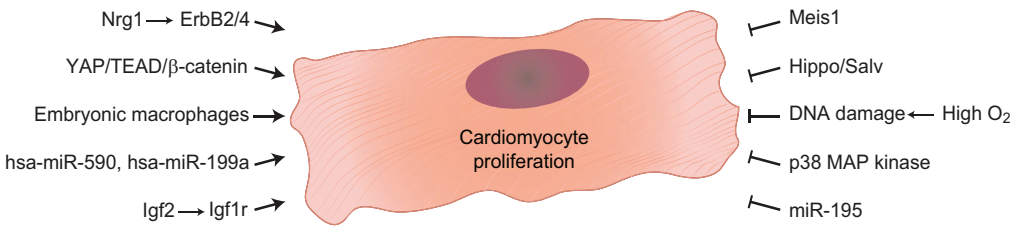

Fig. 2. Cell intrinsic and extrinsic factors that regulate the cardiomyocyte cell cycle. After birth, mammalian cardiomyocytes transition from a primarily proliferative mode of growth to a hypertrophic program characterized by the reduction of cell cycling. Regulatory factors that promote cell division are downregulated whereas inhibitory factors increase in expression. These proposed factors include both cell-intrinsic elements (e.g. Meis1, Yap, miRNAs) and extrinsic signals (e.g. oxygen availability, macrophage-derived factors).

genetic evidence that Meis1 maintains high levels of the cyclin-dependent kinase inhibitors Cdkn1a, Cdkn2a and Cdkn2b and reduces expression of positive cell cycle regulators (Mahmoud et al., 2013). In addition to transcriptional regulation, a number of studies have examined the role of miRNAs in the post-transcriptional regulation of cardiomyocyte proliferation during development and regeneration (reviewed by Giacca and Zacchigna, 2015). One miR-15 family member, miR-195, was identified by microarray analyses of neonatal ventricles as being strongly upregulated after birth and governs the expression of checkpoint kinase 1 and other cell cycle factors (Porrello et al., 2011). The overexpression of miR-195 suppresses cardiomyocyte proliferation in embryonic mice and reduces the regenerative response of the neonatal heart. By contrast, suppression of miR-195 increases the number of mitotic cardiomyocytes in both neonates and adults, albeit modestly in the latter case (Porrello et al., 2011, 2013). A large-scale screen for miRNAs that promote *in vitro* proliferation rates of neonatal rodent cardiomyocytes identified hsa-miR-590 and hsa-miR199a, which were also reported to increase 5-ethynyl-2′-deoxyuridine (EdU) incorporation in adult cardiomyocytes and improve functional recovery after injury (Eulalio et al., 2012). However, intriguing, the efficacy of these molecules at producing new muscle, and their mechanisms of action, are not well understood.

A recent study has highlighted the role of Nrg1/ErbB2 pathway regulation in controlling postnatal cardiac growth (D'Uva et al., 2015). Treatment with Nrg1 causes cardiomyocytes isolated from P1 mice to proliferate avidly; this proliferative effect is markedly reduced by P7. The mechanism for this decrease in Nrg1 sensitivity appears to be the downregulation of the Nrg1 receptor ErbB2 (Fig. 3A). Cardiomyocyte number and proliferation are thus reduced in mice with a cardiac-specific deletion of *Erbb2*; by contrast, inducible expression of constitutively active ErbB2 leads to pathological cardiomegaly (an enlarged heart) with an increase in cardiomyocyte size, cell-cycle activity, mitosis and cytokinesis. Evidence of cardiomyocyte de-differentiation in response to ErbB2 activity was also observed. Furthermore, the induction of constitutively active ErbB2 improved cardiac performance and reduced scarring after MI in adult mice, suggesting that Nrg1/ErbB2 signaling might facilitate heart regeneration through its effects on cardiomyocyte proliferation. Nrg1/ErbB2 signaling has also been implicated in heart regeneration in zebrafish; the induction of endogenous Nrg1 production after injury is associated with cardiomyocyte proliferation (Fig. 3B; discussed below). A key next step is thus to determine whether provision of wild-type ErbB2 after P7 in mice is sufficient to extend the proliferative window, and to identify downstream targets of this pathway in heart muscle.

Another pathway of significant recent interest is the Hippo signaling pathway. Hippo signaling regulates organ size in many species, in part by inhibiting cellular proliferation. Hippo signaling phosphorylates the transcriptional co-activator Yap (Yap1 – Mouse Genome Informatics) to exclude it from the nucleus and restrict heart size during fetal development in mice, and inactivation of this pathway (Fig. 4A) leads to marked cardiomegaly (Heallen et al.,

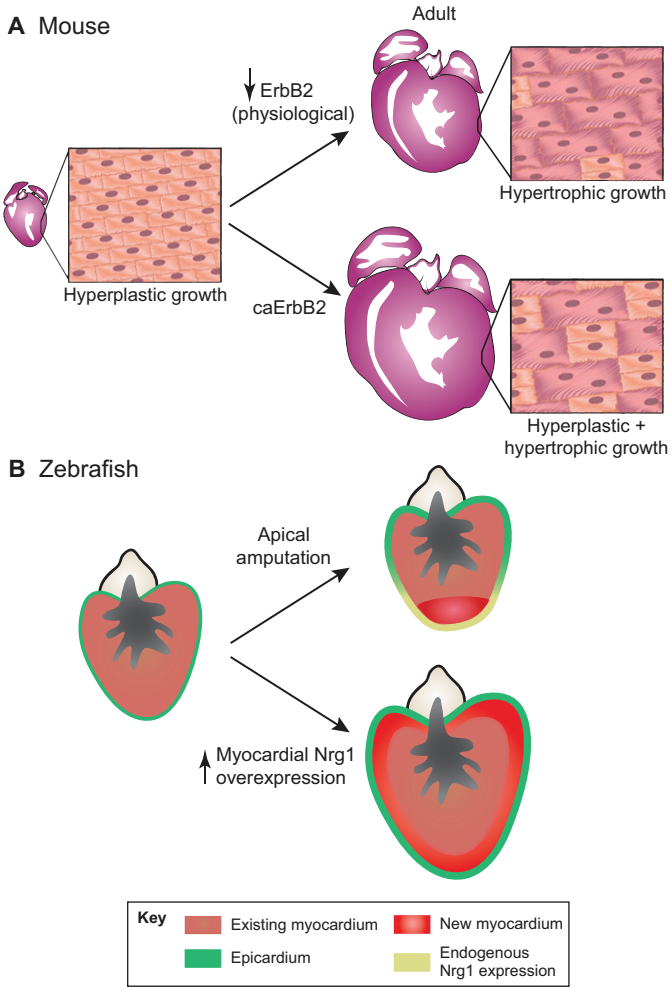

Fig. 3. Neuregulin 1/ErbB2 signaling during cardiac growth and regeneration. (A) In mice, the transition from hyperplastic to hypertrophic growth during the neonatal period correlates with a reduction in levels of the neuregulin 1 co-receptor ErbB2. The expression of constitutively active ErbB2 (caErbB2) in cardiomyocytes can thus extend or re-activate cardiomyocyte cell division and hypertrophy, resulting in cardiomegaly. (B) In zebrafish, the induction of endogenous neuregulin 1 (Nrg1) production after injury (e.g. apical amputation) is associated with cardiomyocyte proliferation and heart regeneration. The transgenic overexpression of Nrg1 in cardiomyocytes causes continuous hyperplastic growth in uninjured adult hearts.

Development (2016) 143, 729-740 doi:10.1242/dev.132910

2011; Xin et al., 2011; von Gise et al., 2012). Heallen et al. showed that hearts from neonatal mice had low levels of phosphorylated Yap (pYap), whereas older mice displayed relatively high levels of cardiac pYap. In addition, hearts in which a Hippo signaling component (Salv; Sav1 – Mouse Genome Informatics) had been deleted specifically in cardiomyocytes showed evidence of regenerated myocardium with reduced scar size after apex resection at P8 (Heallen et al., 2013). A similar phenotype (Fig. 4B) was observed after expressing constitutively active Yap in mouse cardiomyocytes outside of the typical proliferative window (Xin et al., 2013). By contrast, conditional deletion of *Yap* prior to injury led to fibrotic scarring after injury even in very young mice (Fig. 4B). The upstream regulators of the Hippo pathway in neonatal cardiomyocytes are not well described; however, a recent report has implicated the microRNA cluster miR302-367, which targets several Hippo signaling kinases and promotes cardiomyocyte proliferation in adult hearts when overexpressed (Tian et al., 2015). The activity of a wide range of transcription factors, most notably in the TEAD family, is stimulated by Yap co-activation (Zhao et al., 2008; reviewed by Zhou et al., 2015). In the mouse heart, sequential chromatin immunoprecipitation experiments have revealed that Yap/TEAD complexes associate with nuclear β-catenin at pro-proliferative Wnt target genes such as *Sox2* and *Snai2* (Heallen et al., 2011). Expression of the Igf1 receptor has also been shown to increase in hearts expressing the constitutive form of Yap (Xin et al., 2011). This suggests that the Hippo pathway inhibits cardiomyocyte proliferation in a multitude of ways, including by restricting the cell cycle and by reducing cell sensitivity to potential mitogens. Recent evidence indicates that inducible Yap activation in adult cardiomyocytes increases cardiomyocyte proliferation and can potentially improve cardiac function after MI in mice (Lin et al., 2014). Together, these studies suggest that bypassing stage-related changes in Yap activity might be an effective strategy for improving adult heart regeneration.

An extrinsic mechanism of cardiomyocyte cell-cycle arrest has recently been examined, with intriguing results (Puente et al., 2014). The authors found that the generation of reactive oxygen species (ROS) and activation of the DNA damage response increases in postnatal mice. Moreover, hypoxia or conditions that scavenge hydrogen peroxide (H_2O_2) (e.g. catalase overexpression) delay cardiomyocyte arrest, whereas hyperoxic or ROS-generating conditions accelerate it. In the authors' model, mitotic arrest prevents accumulation of DNA damage in mature, aerobic cardiomyocytes. This suggests a physiological mechanism through which the transition at birth to breathing atmospheric oxygen and utilizing aerobic metabolism contributes to the withdrawal of cardiomyocytes from the cell cycle (Fig. 5). The observed differences in cardiomyocyte cytokinesis were relatively small but, in any case, these concepts are intriguing for other groups in the field to examine and potentially extend. In that vein, a recent report by Guimarães-Camboa et al., which examined the regulation of cell stress pathways by Hif1α to promote fetal cardiomyocyte proliferation, provides further contextual support for the role of neonatal oxygen exposure with respect to the exit of cardiomyocytes from the cell cycle (Guimarães-Camboa et al., 2015). Also consistent with this idea is the observation that ventricular resection injury in zebrafish was reported to induce a hypoxia response, with Hif1α implicated as a crucial mediator (Jopling et al., 2012).

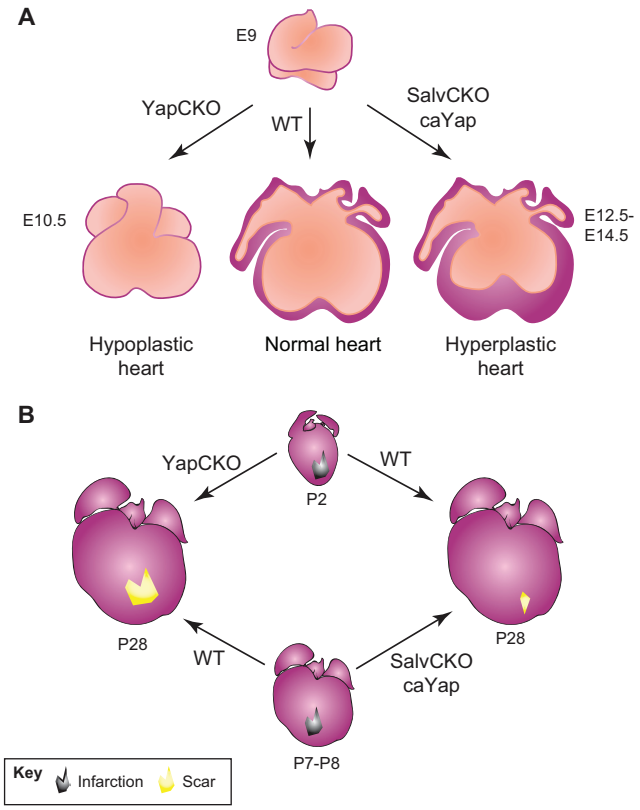

Fig. 4. Hippo signaling during heart development and regeneration. (A) Hippo signaling restricts the ability of Yap to promote cardiomyocyte proliferation in developing mice. Mice with *Nkx2-5* regulatory sequence-driven conditional knockout of *Yap* (YapCKO) have hypoplastic hearts, whereas *Nkx2-5*-driven conditional deletion of the Hippo signaling component *Salv* (SalvCKO) or expression of a constitutively active Yap (caYap) in β-myosin heavy chain-expressing cells causes myocardial hyperplasia. (B) The manipulation of Yap activity after birth can prevent or extend the capacity for compensatory cardiomyocyte proliferation after injury. Wild-type mice that undergo myocardial infarction at P2 exhibit increased cardiomyocyte proliferation with limited scarring, whereas mice infarcted at P7 or P8 show prominent scarring. The α-myosin heavy chain-driven conditional knock-out of Yap (YapCKO) in cardiomyocytes increases scar formation after infarction at P2, whereas α-myosin heavy chain-driven conditional knock-out of Salv (SalvCKO) or the expression of constitutively active Yap (caYap) in α-myosin heavy chain-expressing cells each increase cardiomyocyte division and reduce scarring after apical resection and infarction, respectively, at P7-P8. WT, wild type.

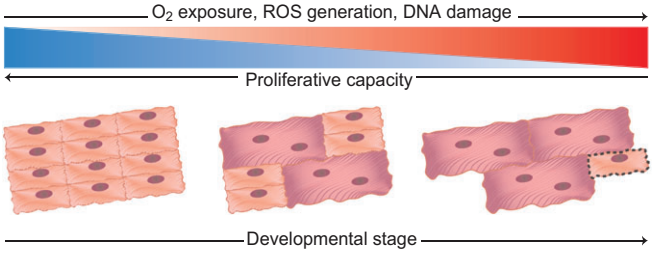

Fig. 5. A model of the effects of oxygen and ROS on cardiomyocyte proliferation. In mice, the transition from relatively hypoxic conditions *in utero* to atmospheric oxygen after birth correlates with the accumulation of reactive oxygen species and DNA damage in cardiomyocytes. One model proposes that cardiomyocyte withdrawal from the cell cycle during the switch from hyperplastic to hypertrophic growth is a response to this damage, with further proliferation in adulthood being retained in only a small pool of hypoxic cardiomyocytes (outlined by dashed line). By contrast, in zebrafish (not shown), which thrive in relatively hypoxic conditions, cardiomyocytes do not lose the capacity for proliferation; this is retained through to adulthood.

Development (2016) 143, 729-740 doi:10.1242/dev.132910

It should be pointed out, however, that, H_2O_2 has been proposed as a positive factor in zebrafish heart regeneration; scavenging of H_2O_2 by transgenic catalase overexpression was shown to impair heart regeneration (Han et al., 2014). Also, H_2O_2 has been shown to stimulate the proliferation of cultured murine stem cell-derived cardiomyocytes (Buggisch et al., 2007), and transgenic mice engineered to produce excess myocardial H_2O_2 were reported to have a prolonged cardiomyocyte cycling period and larger, hyperplastic hearts (Murray et al., 2015). Thus, the level and context of ROS presence might be crucial factors in the nature of effects, and it will be important to determine whether and how hypoxia or ROS scavenging can alter the regenerative response in adult mammals.

Cardiomyocyte turnover in adult mammals

The concept that humans do not generate cardiomyocytes throughout life has been challenged by recent evidence from ^{14}C isotope analysis. These measurements from human cardiac tissue indicate low-level but continuous cardiomyocyte turnover that decreases with age (Bergmann et al., 2009). The authors of this study used these data to establish a mathematical model of cardiomyocyte renewal, predicting a replacement rate of ∼1% per year at age 25, with a steady decline to ∼0.45% by age 75. Stereological analyses of heart tissue from children and adolescents strengthened the assertion that replication is active and ongoing, though steadily decreasing, through the first 20 years of life (Mollova et al., 2013). Despite these findings, the labeling data indicate that the majority of cardiomyocytes are not exchanged during a human lifetime. In addition, newborn cardiomyocytes seem to replace rather than add to existing cardiomyocytes, as the total number remains constant throughout life (Bergmann et al., 2015).

Recent studies in mice have supported these findings, in a way that more directly assesses cardiomyocyte division. Evidence obtained using multiple-isotope imaging mass spectrometry (MIMS) alongside genetic fate mapping suggests that new cardiomyocytes arise from division of existing cardiomyocytes at a low but detectable rate in young adult mice (Senyo et al., 2013). Additionally, Ali et al. identified dividing cardiomyocytes in adult mice using MADM to label dividing cardiomyocytes only after a postnatal dose of tamoxifen (Ali et al., 2014).

Further evidence for a small, persistent pool of proliferation-competent cardiomyocytes arose from a fate-mapping study of hypoxic cells. Interestingly, when hypoxic cardiomyocytes were labeled by tamoxifen administration in adult mice using the oxygen-dependent degradation domain of Hif1α fused to a tamoxifen-inducible Cre recombinase, cardiomyocyte labeling increased between 1 week and 1 month after the initiation of labeling. Although technical factors like tamoxifen persistence could conceivably contribute to this increase, the increase in the incidence over time of apparent clusters of labeled cardiomyocytes (i.e. two or more cells) suggests that some hypoxic cells are actively dividing (Kimura et al., 2015). It remains unclear whether this model describes a distinct, persistent subtype of cells, or rather cells oscillating between normoxic and hypoxic states. The overall number of proliferating cardiomyocytes in the adult mouse is low, so the contribution of hypoxic cardiomyocytes to the myocardium is modest. Although the study did not rule out contributions from non-hypoxic cardiomyocytes, it is an intriguing idea that proliferative homeostasis and regeneration in adult mammals might be driven by a distinct set of hypoxic cardiomyocytes (Fig. 5). Confirmation and extension of this clever approach by other groups, plus further investigation of this putative pool and the means of manipulating it, may help pinpoint target cells for regeneration.

Animal models for injury-induced adult heart regeneration

The goal of regenerating a billion human cardiomyocytes would seem less realistic were it not for discoveries that several species achieve heart regeneration quite successfully as adults. Although only a handful of model systems have been examined, it is now clear that at least certain urodele amphibians and teleost fish can regenerate portions of their hearts in a variety of severe injury models (Flink, 2002; Kikuchi et al., 2010; Lafontant et al., 2012; Poss et al., 2002; Wang et al., 2011). This process can replace most or all of the lost muscle locally in animals that are not concurrently undergoing cardiac growth, with local proliferative and gene expression responses. Some species that have been tested, such as red-spotted newts and medaka fish, show limited regeneration compared with others (Ito et al., 2014; Oberpriller and Oberpriller, 1974). Zebrafish hearts are possibly the most developed model for adult heart regeneration. They can regenerate with limited or no scarring after apical amputation, cryoinjury, or genetic cardiomyocyte ablation (Chablais et al., 2011; González-Rosa et al., 2011; Poss et al., 2002; Schnabel et al., 2011; Wang et al., 2011). Thus, there is value in defining the intrinsic blueprints and extrinsic environmental stimuli that distinguish these highly regenerative organisms.

Cardiomyocyte-intrinsic programs for natural heart regeneration

It is important to discern the source of new tissue when considering any regeneration context. Early studies indicated that adult amphibian and zebrafish cardiomyocytes undergo DNA synthesis and karyokinesis in response to injury at a frequency much greater than that seen in adult mammals (Bettencourt-Dias et al., 2003; Oberpriller and Oberpriller, 1974; Poss et al., 2002). The potential origins of proliferative cardiomyocytes could include undifferentiated progenitor cells, which were implicated in one report (Lepilina et al., 2006), or existing cardiomyocytes. Inducible Cre-based fate mapping of cells expressing a cardiomyocyte-specific promoter provided rigorous evidence that, after apical resection or genetic ablation, newly regenerated zebrafish cardiomyocytes are derived from the existing population (Jopling et al., 2010; Kikuchi et al., 2010; Wang et al., 2011). A similar approach in neonatal mice also supported a cardiomyocyte origin for injury-induced cardiomyogenesis (Porrello et al., 2011). Although some studies in mice have indicated that epicardial cells can transdifferentiate in rare events after injury if provided with certain cues (Smart et al., 2011), lineage tracing of zebrafish epicardial cells revealed no support for this cell type as a muscle source in zebrafish (González-Rosa et al., 2012; Kikuchi et al., 2011a). However, it should be noted that the interpretability of Cre/lox-based lineage tracing is highly dependent on the particular transgenic reagents employed, and the reproduction of these results by multiple groups does strengthen these conclusions considerably.

The extent to which cardiomyocytes alter or reverse their genetic programs to facilitate division is an outstanding question. The existing evidence indicates that cardiomyocytes undergo some level of dedifferentiation; for instance, regenerating zebrafish cardiomyocytes display dissociated sarcomeres and transiently decoupled electrical conductance (Jopling et al., 2010; Kikuchi et al., 2010). Zebrafish compact muscle cardiomyocytes, including those forming the regenerated ventricular apex, also activate regulatory sequences of the transcription factor gene *gata4* after injury (Kikuchi et al., 2010), and they require the activity of this

Development (2016) 143, 729-740 doi:10.1242/dev.132910

factor for myocardial regeneration (Gupta et al., 2013). *Gata4* has a well-studied role during embryonic heart development in mice (Kuo et al., 1997; Molkentin et al., 1997), which suggests that existing cardiomyocytes reactivate embryonic or juvenile developmental programs during regeneration. Increased transcript levels of other transcription factor genes synonymous with embryonic heart development, such as *gata5*, *nkx2.5*, *hand2*, *tbx5* and *tbx20*, have also been reported in zebrafish regenerating heart muscle, but have yet to be tested for function (Kikuchi et al., 2011b; Lepilina et al., 2006). A recent study reported that, after ablation of ventricular cardiomyocytes in the zebrafish embryo, atrial cardiomyocytes re-program to express ventricular markers and repopulate the damaged ventricle (Zhang et al., 2013). This may be associated with increased plasticity of early cardiomyocytes, as there is no evidence of yet that this process occurs in adult animals.

Multiple other transcriptional programs are induced during heart regeneration, as identified by candidate approaches or by whole-heart or cell type-specific profiling approaches (Fang et al., 2013; Lien et al., 2006). With the onset of tools for inducible genetic loss- or gain-of-function experiments in adult zebrafish, several of these programs are beginning to be investigated functionally. One study found that Jak1/Stat3 downstream mediators are induced upon injury. Inhibition of Stat3 signaling in cardiomyocytes, using a dominant-negative transgene, led to fibrotic scarring and reduced cardiomyocyte proliferation. This effect could be partially rescued by administration of recombinant Relaxin 3a, a secreted peptide hormone that appears to be regulated in cardiomyocytes by Stat3. Which of the many potential ligands that regulate Jak1/Stat3 signaling are involved in this context has not been functionally delineated, but the *il11a* gene is known to be induced during regeneration and is thus a good candidate (Fang et al., 2013). Recently, transcriptional regulation by NF-κB was implicated in zebrafish heart regeneration, possibly working through control of *gata4* expression levels (Karra et al., 2015). As an inflammatory response target, NF-κB is well-positioned to couple the injury response to tissue-growth pathways.

Telomere integrity also appears to be important for cardiac regeneration. Using adeno-associated virus to specifically express telomerase reverse transcriptase (Tert) in cardiomyocytes, Bär et al. found that adult mice with hearts expressing Tert show smaller scars and improved ventricular function after MI (Bär et al., 2014). Cardiomyocytes expressing Tert have longer telomeres and more frequently expressed markers of cell cycling. Noting that telomerase is active in adult zebrafish and hyperactivated after injury, Bednarek et al. found that *tert* mutant zebrafish exhibit impaired cardiomyocyte proliferation and regeneration after cryoinjury (Bednarek et al., 2015). Interestingly, this study reported that cardiomyocyte telomeres are transiently elongated in wild-type fish after injury, whereas telomeres are shortened over the same period in *tert* mutant hearts. Moreover, markers of DNA damage accumulate in $tert^{-/-}$ cardiomyocytes, and BrdU incorporation is reverse correlated with DNA damage. The authors proposed a model in which the loss of telomerase activity leads to the accumulation of DNA damage and subsequent senescence of cardiomyocytes, preventing their proliferation after injury. As human telomerase is downregulated shortly after birth (Forsyth et al., 2002), it will be interesting to see whether this contributes to human cardiomyocyte cell cycle withdrawal and ineffective regeneration after injury.

Participation of non-muscle cell types in regeneration

The cardiac regenerative response is multicellular. Following cardiac damage, epicardial cells upregulate embryonic markers, proliferate, and colonize the injury site (Lepilina et al., 2006). In zebrafish, genetic ablation of the epicardium disrupts the proliferative response of cardiomyocytes to injury and delays regeneration (Wang et al., 2015). A recurring theme with induced epicardial factors is pan-epicardial expression immediately after injury that later localizes to the injury site. This effect is also seen after injury in neonatal mouse hearts, although in both organisms the mechanism behind the organ-wide response remains unclear (Porrello et al., 2011). The epicardium has several functions during heart regeneration. Lineage-tracing and functional studies in zebrafish have implicated this cell type in vascularization of regenerating muscle, where it contributes perivascular cells (Kikuchi et al., 2011b; Lepilina et al., 2006; Wang et al., 2015). Signaling by fibroblast growth factors (FGFs) and platelet-derived growth factor (PDGF) also appears to support regeneration via effects on epicardial cell behavior and coronary vessel formation (Kim et al., 2010; Lepilina et al., 2006; Lien et al., 2006). The effect of coronary vessel formation on myocardial regeneration is likely to be multifactorial, including both oxygen delivery and potentially vessel-derived mitogens.

During regeneration, the epicardium is also an apparent source of cardiomyocyte mitogens, as it is during embryonic heart development (Masters and Riley, 2014). For example, the RA-synthesizing enzyme *raldh2* (*aldh1a2* – Zebrafish Information Network) is induced in the zebrafish epicardium after injury, and its activity is required for injury-induced cardiomyocyte proliferation (Kikuchi et al., 2011b). As mentioned earlier, Nrg1 is also induced by cardiac injury in zebrafish at least partly in the epicardial lineage (Gemberling et al., 2015). Inhibition of the Nrg1 co-receptor ErbB2 disrupts the proliferative response, whereas overexpression of Nrg1 from cardiomyocytes enhances cardiomyocyte cell cycling. Interestingly, Nrg1 overexpression without injury has potent mitogenic effects on adult zebrafish cardiac muscle, inducing cardiomyocyte dedifferentiation, myocardial hyperplasia, epicardial activation, and coronary neo-vascularization (Fig. 3B). However, the situation appears to be more complicated in the mouse. It has been reported that Nrg1, through its co-receptors ErbB2 and ErbB4, can drive the division of cardiomyocytes in culture and in adult mice (Zhao et al., 1998; Bersell et al., 2009); as noted above, however, it has been contested that postnatal downregulation of ErbB2 restricts this effect to the neonatal period (D'Uva et al., 2015). A study of cultured human cardiomyocytes similarly showed that Nrg1 loses the ability to promote proliferation in specimens collected from children older than six months of age (Polizzotti et al., 2015). A more recent study has argued that Nrg1 does not stimulate cardiomyocyte proliferation in either normal or injured adult mouse hearts; instead, proliferating cells were found to be non-cardiomyocytes (Reuter et al., 2014). Thus, it might be that in mammals there is a finite developmental window during which Nrg1 stimulates cardiomyocyte division, after which cardiomyocyte hypertrophy is the primary effect. In any case, the upstream factors that induce the expression of Nrg1, and the downstream pathways through which its activity is mediated, require elucidation. Additionally, a recent report indicates that in adult mammals, follistatin-like 1 (Fstl1) might act to improve cardiomyocyte survival and/or proliferation after MI preferentially if produced from the epicardium (Wei et al., 2015).

Endocardial cells are similarly activated following cardiac injury, inducing genes such as *raldh2*, *heart of glass* (*heg1*) and *il11a* (Kikuchi et al., 2011b). It is not clear, however, whether endocardial-specific activation of these factors is required for regeneration. Recent studies have also spotlighted the

Development (2016) 143, 729-740 doi:10.1242/dev.132910

underappreciated influence of additional non-myocardial types on injury-induced heart regeneration. A series of experiments examined the role that nerves play after cardiac injury in both zebrafish and mice (Mahmoud et al., 2015). In these studies it was shown that induced cardiac expression of a known inhibitor of innervation in zebrafish leads to reduced cardiomyocyte proliferation after ventricular injury and disrupts regeneration. Mechanical denervation of the neonatal mouse heart also reduces cardiomyocyte proliferation and prevents compensatory growth after apical resection and MI. Intriguingly, exogenous administration of Nrg1 and nerve growth factor was reported to increase cell cycling in denervated mouse hearts, although the mechanism linking cardiac innervation to these factors is not known. In addition to nervous tissues, the immune system plays an active role in the response of the mammalian heart to injury. Age-related loss of cardiomyocyte proliferation correlates with a shift in monocyte/macrophage gene expression, from pro-angiogenic factors at P1 to pro-fibrotic molecules in the adult (Aurora et al., 2014). The depletion of phagocytic cells from the neonatal mouse results in scar formation, indicating that macrophages – the most prominent phagocytic cell type – are essential for heart regeneration. However, it has been shown that cardiomyocytes enter the cell cycle at a similar rate in macrophage-depleted and control hearts after injury, suggesting that phagocytic cells might primarily affect another process, such as neovascularization of the injured area. A cellular basis for the transition described by Aurora et al. has recently been proposed (Lavine et al., 2014). Using a combination of FMS-related tyrosine kinase 3 (Flt3)-Cre and colony-stimulating factor 1 receptor (Csf1r)-MerCre transgenic mice, the authors of this study distinguished cardiac macrophages derived during embryonic development from those originating from adult hematopoietic stem cells, and suggested that embryonic-derived macrophages provide factors that promote coronary angiogenesis and cardiomyocyte proliferation. By contrast, they found that the adult heart recruits a separate population of pro-inflammatory monocytes and monocyte-derived macrophages that deplete the embryonic-derived lineage. These studies emphasize the importance of taking a broad view of cardiac regeneration in a variety of models, with the idea that regeneration is an orchestration of several cell-specific injury responses and their associated activators and inhibitors.

Conclusions

The belief that adult human cardiomyocytes unequivocally lose their ability to divide has historically frustrated hopes for human heart regeneration. However, recent studies have revealed a previously unappreciated capacity for mammalian cardiomyocyte proliferation that, although limited, raises the prospect for therapeutic stimulation of this endogenous capability after injury. To this end, the field has cultivated insights from a variety of systems, including models of embryonic heart morphogenesis and of natural cardiac regeneration. Mitogens and other participating factors that govern cardiomyocyte proliferation in the developing heart can be re-appropriated to rouse mature cardiomyocytes from quiescence, although it will be important to discern and perhaps guard against the hypertrophic effects associated with such molecules. In addition, regenerative programs demonstrated by lower vertebrates and young mammals have provided inspiring new insights. Given the steadily growing list of associated but sometimes disparate factors that modulate cardiomyocytes, efforts to elucidate the *cis* regulatory elements that coordinate developmental and regenerative cardiomyocyte proliferation are a logical and technologically tractable next step. At the same time, an evolving understanding of the molecular processes that lead to the withdrawal of mature cardiomyocytes from the cell cycle will help identify discrete populations of proliferation-competent cardiomyocytes and generate new targets for therapeutic manipulation. Though caution is warranted to avoid potential negative consequences of hyperstimulated cardiomyocyte proliferation, these approaches constitute a powerful strategy for regenerating the human heart.

Acknowledgements

We thank A. Dickson for artwork, and J. A. Goldman and K. Kikuchi for helpful feedback on this Review. We apologize to our colleagues whose work we could not discuss owing to space limitations.

Competing interests

The authors declare no competing or financial interests.

Funding

M.J.F. was supported by a National Heart, Lung, and Blood Institute (NHLBI) pre-doctoral fellowship [F30 HL126487] and a Medical Scientist Training Program Training Grant [T32 GM007171]. K.D.P. acknowledges grant support from NHLBI [R01 HL081674 and R01 HL131319] and March of Dimes [1-FY14-205]. Deposited in PMC for release after 12 months.

References

Ali, S. R., Hippenmeyer, S., Saadat, L. V., Luo, L., Weissman, I. L. and Ardehali, R. (2014). Existing cardiomyocytes generate cardiomyocytes at a low rate after birth in mice. *Proc. Natl. Acad. Sci. USA* **111**, 8850-8855.

Alkass, K., Panula, J., Westman, M., Wu, T.-D., Guerquin-Kern, J.-L. and Bergmann, O. (2015). No evidence for cardiomyocyte number expansion in preadolescent mice. *Cell* **163**, 1026-1036.

Aurora, A. B., Porrello, E. R., Tan, W., Mahmoud, A. I., Hill, J. A., Bassel-Duby, R., Sadek, H. A. and Olson, E. N. (2014). Macrophages are required for neonatal heart regeneration. *J. Clin. Invest.* **124**, 1382-1392.

Bader, D. and Oberpriller, J. O. (1978). Repair and reorganization of minced cardiac muscle in the adult newt (Notophthalmus viridescens). *J. Morphol.* **155**, 349-357.

Bär, C., de Jesus, B. B., Serrano, R., Tejera, A., Ayuso, E., Jimenez, V., Formentini, I., Bobadilla, M., Mizrahi, J., de Martino, A. et al. (2014). Telomerase expression confers cardioprotection in the adult mouse heart after acute myocardial infarction. *Nat. Commun.* **5**, 5863.

Bednarek, D., González-Rosa, J. M., Guzmán-Martínez, G., Gutiérrez-Gutiérrez, Ó., Aguado, T., Sánchez-Ferrer, C., Marques, I. J., Galardi-Castilla, M., de Diego, I., Gómez, M. J. et al. (2015). Telomerase is essential for zebrafish heart regeneration. *Cell Rep.* **12**, 1691-1703.

Behfar, A., Crespo-Diaz, R., Terzic, A. and Gersh, B. J. (2014). Cell therapy for cardiac repair—lessons from clinical trials. *Nat. Rev. Cardiol.* **11**, 232-246.

Beltrami, A. P., Barlucchi, L., Torella, D., Baker, M., Limana, F., Chimenti, S., Kasahara, H., Rota, M., Musso, E., and Urbanek, K. (2003). Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* **114**, 763-776.

Bergmann, O., Bhardwaj, R. D., Bernard, S., Zdunek, S., Barnabé-Heider, F., Walsh, S., Zupicich, J., Alkass, K., Buchholz, B. A., Druid, H. et al. (2009). Evidence for cardiomyocyte renewal in humans. *Science* **324**, 98-102.

Bergmann, O., Zdunek, S., Felker, A., Salehpour, M., Alkass, K., Bernard, S., Sjostrom, S. L., Szewczykowska, M., Jackowska, T., dos Remedios, C. et al. (2015). Dynamics of cell generation and turnover in the human heart. *Cell* **161**, 1566-1575.

Bersell, K., Arab, S., Haring, B. and Kühn, B. (2009). Neuregulin1/ErbB4 signaling Induces cardiomyocyte proliferation and repair of heart Injury. *Cell* **138**, 257-270.

Bettencourt-Dias, M., Mittnacht, S. and Brockes, J. P. (2003). Heterogeneous proliferative potential in regenerative adult newt cardiomyocytes. *J. Cell Sci.* **116**, 4001-4009.

Bosch, T. C. G. (2007). Why polyps regenerate and we don't: Towards a cellular and molecular framework for Hydra regeneration. *Dev. Biol.* **303**, 421-433.

Brade, T., Kumar, S., Cunningham, T. J., Chatzi, C., Zhao, X., Cavallero, S., Li, P., Sucov, H. M., Ruiz-Lozano, P. and Duester, G. (2011). Retinoic acid stimulates myocardial expansion by induction of hepatic erythropoietin which activates epicardial Igf2. *Development* **138**, 139-148.

Brockes, J. P. and Kumar, A. (2008). Comparative aspects of animal regeneration. *Annu. Rev. Cell Dev. Biol.* **24**, 525-549.

Brodsky, V., Arefyeva, A. M., Gvasava, I. G., Sarkisov, D. S. and Panova, N. W. (1994). Polyploidy in cardiac myocytes of normal and hypertrophic human hearts; range of values. *Virchows Arch.* **424**, 429-435.

Buckingham, M., Meilhac, S. and Zaffran, S. (2005). Building the mammalian heart from two sources of myocardial cells. *Nat. Rev. Genet.* **6**, 826-837.

Buggisch, M., Ateghang, B., Ruhe, C., Strobel, C., Lange, S., Wartenberg, M. and Sauer, H. (2007). Stimulation of ES-cell-derived cardiomyogenesis and

neonatal cardiac cell proliferation by reactive oxygen species and NADPH oxidase. *J. Cell Sci.* **120**, 885-894.
Chablais, F., Veit, J., Rainer, G. and Jaźwińska, A. (2011). The zebrafish heart regenerates after cryoinjury-induced myocardial infarction. *BMC Dev. Biol.* **11**, 21.
Challice, C. E. and Virágh, S. (1974). The architectural development of the early mammalian heart. *Tissue Cell* **6**, 447-462.
Chaudhry, H. W., Dashoush, N. H., Tang, H., Zhang, L., Wang, X., Wu, E. X. and Wolgemuth, D. J. (2004). Cyclin A2 mediates cardiomyocyte mitosis in the postmitotic myocardium. *J. Biol. Chem.* **279**, 35858-35866.
Chen, T. H.-P., Chang, T.-C., Kang, J.-O., Choudhary, B., Makita, T., Tran, C. M., Burch, J. B. E., Eid, H. and Sucov, H. M. (2002). Epicardial induction of fetal cardiomyocyte proliferation via a retinoic acid-inducible trophic factor. *Dev. Biol.* **250**, 198-207.
Chen, H., Shi, S., Acosta, L., Li, W., Lu, J., Bao, S., Chen, Z., Yang, Z., Schneider, M. D., Chien, K. R. et al. (2004). BMP10 is essential for maintaining cardiac growth during murine cardiogenesis. *Development* **131**, 2219-2231.
Chen, J. X., Krane, M., Deutsch, M.-A., Wang, L., Rav-Acha, M., Gregoire, S., Engels, M. C., Rajarajan, K., Karra, R., Abel, E. D. et al. (2012). Inefficient reprogramming of fibroblasts into cardiomyocytes using Gata4, Mef2c, and Tbx5. *Circ. Res.* **111**, 50-55.
Chen, H., Zhang, W., Sun, X., Yoshimoto, M., Chen, Z., Zhu, W., Liu, J., Shen, Y., Yong, W., Li, D. et al. (2013). Fkbp1a controls ventricular myocardium trabeculation and compaction by regulating endocardial Notch1 activity. *Development* **140**, 1946-1957.
Christoffels, V. M., Habets, P. E. M. H., Franco, D., Campione, M., de Jong, F., Lamers, W. H., Bao, Z.-Z., Palmer, S., Biben, C., Harvey, R. P. et al. (2000). Chamber formation and morphogenesis in the developing mammalian heart. *Dev. Biol.* **223**, 266-278.
Clark, E. B., Hu, N., Frommelt, P., Vandekieft, G. K., Dummett, J. L. and Tomanek, R. J. (1989). Effect of increased pressure on ventricular growth in stage 21 chick embryos. *Am. J. Physiol.* **257**, H55-H61.
de Pater, E., Clijsters, L., Marques, S. R., Lin, Y.-F., Garavito-Aguilar, Z. V., Yelon, D. and Bakkers, J. (2009). Distinct phases of cardiomyocyte differentiation regulate growth of the zebrafish heart. *Development* **136**, 1633-1641.
Drenckhahn, J.-D., Schwarz, Q. P., Gray, S., Laskowski, A., Kiriazis, H., Ming, Z., Harvey, R. P., Du, X.-J., Thorburn, D. R. and Cox, T. C. (2008). Compensatory growth of healthy cardiac cells in the presence of diseased cells restores tissue homeostasis during heart development. *Dev. Cell* **15**, 521-533.
D'Uva, G., Aharonov, A., Lauriola, M., Kain, D., Yahalom-Ronen, Y., Carvalho, S., Weisinger, K., Bassat, E., Rajchman, D., Yifa, O. et al. (2015). ERBB2 triggers mammalian heart regeneration by promoting cardiomyocyte dedifferentiation and proliferation. *Nat. Cell Biol.* **17**, 627-638.
Ellison, G. M., Vicinanza, C., Smith, A. J., Aquila, I., Leone, A., Waring, C. D., Henning, B. J., Stirparo, G. G., Papait, R., Scarfo, M. et al. (2013). Adult c-kit (pos) cardiac stem cells are necessary and sufficient for functional cardiac regeneration and repair. *Cell* **154**, 827-842.
Engel, F. B., Schebesta, M., Duong, M. T., Lu, G., Ren, S., Madwed, J. B., Jiang, H., Wang, Y. and Keating, M. T. (2005). p38 MAP kinase inhibition enables proliferation of adult mammalian cardiomyocytes. *Genes Dev.* **19**, 1175-1187.
Eulalio, A., Mano, M., Dal Ferro, M., Zentilin, L., Sinagra, G., Zacchigna, S. and Giacca, M. (2012). Functional screening identifies miRNAs inducing cardiac regeneration. *Nature* **492**, 376-381.
Fang, Y., Gupta, V., Karra, R., Holdway, J. E., Kikuchi, K. and Poss, K. D. (2013). Translational profiling of cardiomyocytes identifies an early Jak1/Stat3 injury response required for zebrafish heart regeneration. *Proc. Natl. Acad. Sci. USA* **110**, 13416-13421.
Flink, I. (2002). Cell cycle reentry of ventricular and atrial cardiomyocytes and cells within the epicardium following amputation of the ventricular apex in the axolotl, Amblystoma mexicanum: confocal microscopic immunofluorescent image analysis of bromodeoxyuridine-labeled nuclei. *Anat. Embryol.* **205**, 235-244.
Flink, I. L., Oana, S., Maitra, N., Bahl, J. J. and Morkin, E. (1998). Changes in E2F complexes containing retinoblastoma protein family members and increased cyclin-dependent kinase inhibitor activities during terminal differentiation of cardiomyocytes. *J. Mol. Cell Cardiol.* **30**, 563-578.
Forsyth, N. R., Wright, W. E. and Shay, J. W. (2002). Telomerase and differentiation in multicellular organisms: turn it off, turn it on, and turn it off again. *Differentiation* **69**, 188-197.
Gassmann, M., Casagranda, F., Orioli, D., Simon, H., Lai, C., Klein, R. and Lemke, G. (1995). Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature* **378**, 390-394.
Gemberling, M., Karra, R., Dickson, A. L. and Poss, K. D. (2015). Nrg1 is an injury-induced cardiomyocyte mitogen for the endogenous heart regeneration program in zebrafish. *eLife* **4**, e05871.
Gerczuk, P. Z. and Kloner, R. A. (2012). An update on cardioprotection: a review of the latest adjunctive therapies to limit myocardial infarction size in clinical trials. *J. Am. Coll. Cardiol.* **59**, 969-978.
Gerety, S. S., Wang, H. U., Chen, Z.-F. and Anderson, D. J. (1999). Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. *Mol. Cell* **4**, 403-414.
Giacca, M. and Zacchigna, S. (2015). Harnessing the microRNA pathway for cardiac regeneration. *J. Mol. Cell Cardiol.* **89A**, 68-74.
Gnecchi, M., Zhang, Z., Ni, A. and Dzau, V. J. (2008). Paracrine mechanisms in adult stem cell signaling and therapy. *Circ. Res.* **103**, 1204-1219.
González-Rosa, J. M., Martín, V., Peralta, M., Torres, M. and Mercader, N. (2011). Extensive scar formation and regression during heart regeneration after cryoinjury in zebrafish. *Development* **138**, 1663-1674.
González-Rosa, J. M., Peralta, M. and Mercader, N. (2012). Pan-epicardial lineage tracing reveals that epicardium derived cells give rise to myofibroblasts and perivascular cells during zebrafish heart regeneration. *Dev. Biol.* **370**, 173-186.
Grego-Bessa, J., Luna-Zurita, L., del Monte, G., Bolós, V., Melgar, P., Arandilla, A., Garratt, A. N., Zang, H., Mukouyama, Y.-s., Chen, H. et al. (2007). Notch signaling is essential for ventricular chamber development. *Dev. Cell* **12**, 415-429.
Guimarães-Camboa, N., Stowe, J., Aneas, I., Sakabe, N., Cattaneo, P., Henderson, L., Kilberg, M. S., Johnson, R. S., Chen, J., McCulloch, A. D. et al. (2015). HIF1α represses cell stress pathways to allow proliferation of hypoxic fetal cardiomyocytes. *Dev. Cell* **33**, 507-521.
Gupta, V. and Poss, K. D. (2012). Clonally dominant cardiomyocytes direct heart morphogenesis. *Nature* **484**, 479-484.
Gupta, V., Gemberling, M., Karra, R., Rosenfeld, G. E., Evans, T. and Poss, K. D. (2013). An injury-responsive gata4 program shapes the zebrafish cardiac ventricle. *Curr. Biol.* **23**, 1221-1227.
Han, P., Zhou, X.-H., Chang, N., Xiao, C.-L., Yan, S., Ren, H., Yang, X.-Z., Zhang, M.-L., Wu, Q., Tang, B. et al. (2014). Hydrogen peroxide primes heart regeneration with a derepression mechanism. *Cell Res.* **24**, 1091-1107.
Haubner, B. J., Adamowicz-Brice, M., Khadayate, S., Tiefenthaler, V., Metzler, B., Aitman, T. and Penninger, J. M. (2012). Complete cardiac regeneration in a mouse model of myocardial infarction. *Aging* **4**, 966-977.
Heallen, T., Zhang, M., Wang, J., Bonilla-Claudio, M., Klysik, E., Johnson, R. L. and Martin, J. F. (2011). Hippo pathway inhibits Wnt signaling to restrain cardiomyocyte proliferation and heart size. *Science* **332**, 458-461.
Heallen, T., Morikawa, Y., Leach, J., Tao, G., Willerson, J. T., Johnson, R. L. and Martin, J. F. (2013). Hippo signaling impedes adult heart regeneration. *Development* **140**, 4683-4690.
Hirai, M., Chen, J. and Evans, S. M. (2015). Tissue-specific cell cycle indicator reveals unexpected findings for cardiac myocyte proliferation. *Circ. Res.* **118**, 20-28.
Hong, K. U., Guo, Y., Li, Q.-H., Cao, P., Al-Maqtari, T., Vajravelu, B. N., Du, J., Book, M. J., Zhu, X., Nong, Y. et al. (2014). c-kit+ cardiac stem cells alleviate post-myocardial infarction left ventricular dysfunction despite poor engraftment and negligible retention in the recipient heart. *PLoS ONE* **9**, e96725.
Huang, Y., Harrison, M. R., Osorio, A., Kim, J., Baugh, A., Duan, C., Sucov, H. M. and Lien, C.-L. (2013). Igf signaling is required for cardiomyocyte proliferation during zebrafish heart development and regeneration. *PLoS ONE* **8**, e67266.
Ieda, M., Fu, J.-D., Delgado-Olguin, P., Vedantham, V., Hayashi, Y., Bruneau, B. G. and Srivastava, D. (2010). Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell* **142**, 375-386.
Ito, K., Morioka, M., Kimura, S., Tasaki, M., Inohaya, K. and Kudo, A. (2014). Differential reparative phenotypes between zebrafish and medaka after cardiac injury. *Dev. Dyn.* **243**, 1106-1115.
Jessup, M. and Brozena, S. (2003). Heart failure. *N. Engl. J. Med.* **348**, 2007-2018.
Jopling, C., Sleep, E., Raya, M., Martí, M., Raya, A. and Belmonte, J. C. I. (2010). Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature* **464**, 606-609.
Jopling, C., Suñè, G., Faucherre, A., Fabregat, C. and Izpisua Belmonte, J. C. (2012). Hypoxia induces myocardial regeneration in zebrafish. *Circulation* **126**, 3017-3027.
Karra, R., Knecht, A. K., Kikuchi, K. and Poss, K. D. (2015). Myocardial NF-κB activation is essential for zebrafish heart regeneration. *Proc. Natl. Acad. Sci. USA* **112**, 13255-13260.
Kelly, R. G., Brown, N. A. and Buckingham, M. E. (2001). The arterial pole of the mouse heart forms from Fgf10-expressing cells in pharyngeal mesoderm. *Dev. Cell* **1**, 435-440.
Kelly, R. G., Buckingham, M. E. and Moorman, A. F. (2014). Heart fields and cardiac morphogenesis. *Cold Spring Harb. Perspect. Med.* **4**, a015750.
Kikuchi, K., Holdway, J. E., Werdich, A. A., Anderson, R. M., Fang, Y., Egnaczyk, G. F., Evans, T., Macrae, C. A., Stainier, D. Y. and Poss, K. D. (2010). Primary contribution to zebrafish heart regeneration by gata4+ cardiomyocytes. *Nature* **464**, 601-605.
Kikuchi, K., Gupta, V., Wang, J., Holdway, J. E., Wills, A. A., Fang, Y. and Poss, K. D. (2011a). tcf21+ epicardial cells adopt non-myocardial fates during zebrafish heart development and regeneration. *Development* **138**, 2895-2902.
Kikuchi, K., Holdway, J. E., Major, R. J., Blum, N., Dahn, R. D., Begemann, G. and Poss, K. D. (2011b). Retinoic acid production by endocardium and epicardium is an injury response essential for zebrafish heart regeneration. *Dev. Cell* **20**, 397-404.
Kim, J., Wu, Q., Zhang, Y., Wiens, K. M., Huang, Y., Rubin, N., Shimada, H., Handin, R. I., Chao, M. Y., Tuan, T.-L. et al. (2010). PDGF signaling is required

for epicardial function and blood vessel formation in regenerating zebrafish hearts. *Proc. Natl. Acad. Sci. USA* **107**, 17206-17210.
Kimura, W., Xiao, F., Canseco, D. C., Muralidhar, S., Thet, S., Zhang, H. M., Abderrahman, Y., Chen, R., Garcia, J. A., Shelton, J. M. et al. (2015). Hypoxia fate mapping identifies cycling cardiomyocytes in the adult heart. *Nature* **523**, 226-230.
Kreidberg, J. A., Sariola, H., Loring, J. M., Maeda, M., Pelletier, J., Housman, D. and Jaenisch, R. (1993). WT-1 is required for early kidney development. *Cell* **74**, 679-691.
Kuo, C. T., Morrisey, E. E., Anandappa, R., Sigrist, K., Lu, M. M., Parmacek, M. S., Soudais, C. and Leiden, J. M. (1997). GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev.* **11**, 1048-1060.
Laflamme, M. A. and Murry, C. E. (2011). Heart regeneration. *Nature* **473**, 326-335.
Lafontant, P. J., Burns, A. R., Grivas, J. A., Lesch, M. A., Lala, T. D., Reuter, S. P., Field, L. J. and Frounfelter, T. D. (2012). The Giant Danio (D. aequipinnatus) as a model of cardiac remodeling and regeneration. *Anat. Rec.* **295**, 234-248.
Lavine, K. J., Epelman, S., Uchida, K., Weber, K. J., Nichols, C. G., Schilling, J. D., Ornitz, D. M., Randolph, G. J. and Mann, D. L. (2014). Distinct macrophage lineages contribute to disparate patterns of cardiac recovery and remodeling in the neonatal and adult heart. *Proc. Nat. Acad. Sci. USA* **111**, 16029-16034.
Lee, K.-F., Simon, H., Chen, H., Bates, B., Hung, M.-C. and Hauser, C. (1995). Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature* **378**, 394-398.
Lepilina, A., Coon, A. N., Kikuchi, K., Holdway, J. E., Roberts, R. W., Burns, C. G. and Poss, K. D. (2006). A dynamic epicardial injury response supports progenitor cell activity during zebrafish heart regeneration. *Cell* **127**, 607-619.
Li, F., Wang, X., Capasso, J. M. and Gerdes, A. M. (1996). Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. *J. Mol. Cell. Cardiol.* **28**, 1737-1746.
Li, F., Wang, X., Bunger, P. C. and Gerdes, A. M. (1997). Formation of binucleated cardiac myocytes in rat heart: I. Role of actin–myosin contractile ring. *J. Mol. Cell. Cardiol.* **29**, 1541-1551.
Li, P., Cavallero, S., Gu, Y., Chen, T. H. P., Hughes, J., Hassan, A. B., Brüning, J. C., Pashmforoush, M. and Sucov, H. M. (2011). IGF signaling directs ventricular cardiomyocyte proliferation during embryonic heart development. *Development* **138**, 1795-1805.
Lien, C.-L., Schebesta, M., Makino, S., Weber, G. J. and Keating, M. T. (2006). Gene expression analysis of zebrafish heart regeneration. *PLoS Biol.* **4**, e260.
Lin, Z., von Gise, A., Zhou, P., Gu, F., Ma, Q., Jiang, J., Yau, A. L., Buck, J. N., Gouin, K. A., van Gorp, P. R. R. et al. (2014). Cardiac-specific YAP activation improves cardiac function and survival in an experimental murine MI model. *Circ. Res.* **115**, 354-363.
Liu, J., Bressan, M., Hassel, D., Huisken, J., Staudt, D., Kikuchi, K., Poss, K. D., Mikawa, T. and Stainier, D. Y. R. (2010). A dual role for ErbB2 signaling in cardiac trabeculation. *Development* **137**, 3867-3875.
Livet, J., Weissman, T. A., Kang, H., Draft, R. W., Lu, J., Bennis, R. A., Sanes, J. R. and Lichtman, J. W. (2007). Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. *Nature* **450**, 56-62.
Mahmoud, A. I., Kocabas, F., Muralidhar, S. A., Kimura, W., Koura, A. S., Thet, S., Porrello, E. R. and Sadek, H. A. (2013). Meis1 regulates postnatal cardiomyocyte cell cycle arrest. *Nature* **497**, 249-253.
Mahmoud, A. I., O'Meara, C. C., Gemberling, M., Zhao, L., Bryant, D. M., Zheng, R., Gannon, J. B., Cai, L., Choi, W.-Y., Egnaczyk, G. F. et al. (2015). Nerves regulate cardiomyocyte proliferation and heart regeneration. *Dev. Cell* **34**, 387-399.
Masters, M. and Riley, P. R. (2014). The epicardium signals the way towards heart regeneration. *Stem Cell Res.* **13**, 683-692.
Matz, D. G., Oberpriller, J. O. and Oberpriller, J. C. (1998). Comparison of mitosis in binucleated and mononucleated newt cardiac myocytes. *Anat. Rec.* **251**, 245-255.
McMurray, J. J. V. C., Adamopoulos, S., Anker, S. D., Auricchio, A., Böhm, M., Dickstein, K., Falk, V., Filippatos, G., Fonseca, C., Gomez-Sanchez, M. A. et al. (2012). ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2012. *Eur. J. Heart Fail.* **14**, 803-869.
Meilhac, S. M., Kelly, R. G., Rocancourt, D., Eloy-Trinquet, S., Nicolas, J.-F. and Buckingham, M. E. (2003). A retrospective clonal analysis of the myocardium reveals two phases of clonal growth in the developing mouse heart. *Development* **130**, 3877-3889.
Merki, E., Zamora, M., Raya, A., Kawakami, Y., Wang, J., Zhang, X., Burch, J., Kubalak, S. W., Kaliman, P., Belmonte, J. C. I. et al. (2005). Epicardial retinoid X receptor α is required for myocardial growth and coronary artery formation. *Proc. Natl. Acad. Sci. USA* **102**, 18455-18460.
Meyer, D. and Birchmeier, C. (1995). Multiple essential functions of neuregulin in development. *Nature* **378**, 386-390.
Mikawa, T., Borisov, A., Brown, A. M. C. and Fischman, D. A. (1992a). Clonal analysis of cardiac morphogenesis in the chicken embryo using a replication-defective retrovirus: I. Formation of the ventricular myocardium. *Dev. Dyn.* **193**, 11-23.
Mikawa, T., Cohen-Gould, L. and Fischman, D. A. (1992b). Clonal analysis of cardiac morphogenesis in the chicken embryo using a replication-defective retrovirus. III: Polyclonal origin of adjacent ventricular myocytes. *Dev. Dyn.* **195**, 133-141.
Mjaatvedt, C. H., Nakaoka, T., Moreno-Rodriguez, R., Norris, R. A., Kern, M. J., Eisenberg, C. A., Turner, D. and Markwald, R. R. (2001). The outflow tract of the heart Is recruited from a novel heart-forming field. *Dev. Biol.* **238**, 97-109.
Molkentin, J. D., Lin, Q., Duncan, S. A. and Olson, E. N. (1997). Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev.* **11**, 1061-1072.
Mollova, M., Bersell, K., Walsh, S., Savla, J., Das, L. T., Park, S.-Y., Silberstein, L. E., dos Remedios, C. G., Graham, D., Colan, S. et al. (2013). Cardiomyocyte proliferation contributes to heart growth in young humans. *Proc. Natl. Acad. Sci. USA* **110**, 1446-1451.
Murray, T. V. A., Smyrnias, I., Schnelle, M., Mistry, R. K., Zhang, M., Beretta, M., Martin, D., Anilkumar, N., de Silva, S. M., Shah, A. M. et al. (2015). Redox regulation of cardiomyocyte cell cycling via an ERK1/2 and c-Myc-dependent activation of cyclin D2 transcription. *J. Mol. Cell Cardiol.* **79**, 54-68.
Naqvi, N., Li, M., Calvert, J. W., Tejada, T., Lambert, J. P., Wu, J., Kesteven, S. H., Holman, S. R., Matsuda, T., Lovelock, J. D. et al. (2014). A proliferative burst during preadolescence establishes the final cardiomyocyte number. *Cell* **157**, 795-807.
Naqvi, N., Singh, R., Iismaa, S. E., Li, M., Calvert, J. W., Martin, D. I. K., Harvey, R. P., Graham, R. M. and Husain, A. (2015). Cardiomyocytes replicate and their numbers increase in young hearts. *Cell* **163**, 783-784.
Oberpriller, J. O. and Oberpriller, J. C. (1974). Response of the adult newt ventricle to injury. *J. Exp. Zool.* **187**, 249-259.
Olivetti, G., Cigola, E., Maestri, R., Corradi, D., Lagrasta, C., Gambert, S. R. and 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, S. L., Plonowska, K., Xu, A. and Wu, S. M. (2015). Molecular regulation of cardiomyocyte differentiation. *Circ. Res.* **116**, 341-353.
Pasumarthi, K. B. S., Nakajima, H., Nakajima, H. O., Soonpaa, M. H. and Field, L. J. (2005). Targeted expression of Cyclin D2 results in cardiomyocyte DNA synthesis and infarct regression in transgenic mice. *Circ. Res.* **96**, 110-118.
Pennisi, D. J., Ballard, V. L. T. and Mikawa, T. (2003). Epicardium is required for the full rate of myocyte proliferation and levels of expression of myocyte mitogenic factors FGF2 and its receptor, FGFR-1, but not for transmural myocardial patterning in the embryonic chick heart. *Dev. Dyn.* **228**, 161-172.
Pérez-Pomares, J. M., Phelps, A., Sedmerova, M., Carmona, R., González-Iriarte, M., Muñoz-Chápuli, R. and Wessels, A. (2002). Experimental studies on the spatiotemporal expression of WT1 and RALDH2 in the embryonic avian heart: a model for the regulation of myocardial and valvuloseptal development by epicardially derived cells (EPDCs). *Dev. Biol.* **247**, 307-326.
Polizzotti, B. D., Ganapathy, B., Walsh, S., Choudhury, S., Ammanamanchi, N., Bennett, D. G., dos Remedios, C. G., Haubner, B. J., Penninger, J. M. and Kuhn, B. (2015). Neuregulin stimulation of cardiomyocyte regeneration in mice and human myocardium reveals a therapeutic window. *Sci. Transl. Med.* **7**, 281ra45.
Poolman, R. A. and Brooks, G. (1998). Expressions and activities of cell cycle regulatory molecules during the transition from myocyte hyperplasia to hypertrophy. *J. Mol. Cell. Cardiol.* **30**, 2121-2135.
Porrello, E. R., Mahmoud, A. I., Simpson, E., Hill, J. A., Richardson, J. A., Olson, E. N. and Sadek, H. A. (2011). Transient regenerative potential of the neonatal mouse heart. *Science* **331**, 1078-1080.
Porrello, E. R., Mahmoud, A. I., Simpson, E., Johnson, B. A., Grinsfelder, D., Canseco, D., Mammen, P. P., Rothermel, B. A., Olson, E. N. and Sadek, H. A. (2013). Regulation of neonatal and adult mammalian heart regeneration by the miR-15 family. *Proc. Natl. Acad. Sci. USA* **110**, 187-192.
Poss, K. D. (2010). Advances in understanding tissue regenerative capacity and mechanisms in animals. *Nat. Rev. Genet.* **11**, 710-722.
Poss, K. D., Wilson, L. G. and Keating, M. T. (2002). Heart regeneration in zebrafish. *Science* **298**, 2188-2190.
Puente, B. N., Kimura, W., Muralidhar, S. A., Moon, J., Amatruda, J. F., Phelps, K. L., Grinsfelder, D., Rothermel, B. A., Chen, R., Garcia, J. A. et al. (2014). The oxygen-rich postnatal environment induces cardiomyocyte cell-cycle arrest through DNA damage response. *Cell* **157**, 565-579.
Qian, L., Huang, Y., Spencer, C. I., Foley, A., Vedantham, V., Liu, L., Conway, S. J., Fu, J.-d. and Srivastava, D. (2012). In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes. *Nature* **485**, 593-598.
Qu, X., Jia, H., Garrity, D. M., Tompkins, K., Batts, L., Appel, B., Zhong, T. P. and Baldwin, H. S. (2008). ndrg4 is required for normal myocyte proliferation during early cardiac development in zebrafish. *Dev. Biol.* **317**, 486-496.
Reddien, P. W. and Sánchez Alvarado, A. (2004). Fundamentals of planarian regeneration. *Annu. Rev. Cell Dev. Biol.* **20**, 725-757.
Reuter, S., Soonpaa, M. H., Firulli, A. B., Chang, A. N. and Field, L. J. (2014). Recombinant neuregulin 1 does not activate cardiomyocyte DNA synthesis in normal or infarcted adult mice. *PLoS ONE* **9**, e115871.

Sadahiro, T., Yamanaka, S. and Ieda, M. (2015). Direct cardiac reprogramming: progress and challenges in basic biology and clinical applications. *Circ. Res.* **116**, 1378-1391.
Saiki, Y., Konig, A., Waddell, J. and Rebeyka, I. M. (1997). Hemodynamic alteration by fetal surgery accelerates myocyte proliferation in fetal guinea pig hearts. *Surgery* **122**, 412-419.
Schnabel, K., Wu, C.-C., Kurth, T. and Weidinger, G. (2011). Regeneration of cryoinjury induced necrotic heart lesions in zebrafish is associated with epicardial activation and cardiomyocyte proliferation. *PLoS ONE* **6**, e18503.
Sdek, P., Zhao, P., Wang, Y., Huang, C.-j., Ko, C. Y., Butler, P. C., Weiss, J. N. and MacLellan, W. R. (2011). Rb and p130 control cell cycle gene silencing to maintain the postmitotic phenotype in cardiac myocytes. *J. Cell Biol.* **194**, 407-423.
Sedmera, D., Pexieder, T., Hu, N. and Clark, E. B. (1997). Developmental changes in the myocardial architecture of the chick. *Anat. Rec.* **248**, 421-432.
Sedmera, D., Pexieder, T., Vuillemin, M., Thompson, R. P. and Anderson, R. H. (2000). Developmental patterning of the myocardium. *Anat. Rec.* **258**, 319-337.
Senyo, S. E., Steinhauser, M. L., Pizzimenti, C. L., Yang, V. K., Cai, L., Wang, M., Wu, T.-D., Guerquin-Kern, J.-L., Lechene, C. P. and Lee, R. T. (2013). Mammalian heart renewal by pre-existing cardiomyocytes. *Nature* **493**, 433-436.
Sissman, N. J. (1966). Cell multiplication rates during development of the primitive cardiac tube in the chick embryo. *Nature* **210**, 504-507.
Sizarov, A., Ya, J., de Boer, B. A., Lamers, W. H., Christoffels, V. M. and Moorman, A. F. M. (2011). Formation of the building plan of the human heart: morphogenesis, growth, and differentiation. *Circulation* **123**, 1125-1135.
Smart, N., Bollini, S., Dubé, K. N., Vieira, J. M., Zhou, B., Davidson, S., Yellon, D., Riegler, J., Price, A. N., Lythgoe, M. F. et al. (2011). De novo cardiomyocytes from within the activated adult heart after injury. *Nature* **474**, 640-644.
Song, K., Nam, Y.-J., Luo, X., Qi, X., Tan, W., Huang, G. N., Acharya, A., Smith, C. L., Tallquist, M. D., Neilson, E. G. et al. (2012). Heart repair by reprogramming non-myocytes with cardiac transcription factors. *Nature* **485**, 599-604.
Soonpaa, M. H., Kim, K. K., Pajak, L., Franklin, M. and Field, L. J. (1996). Cardiomyocyte DNA synthesis and binucleation during murine development. *Am. J. Physiol.* **271**, H2183-H2189.
Soonpaa, M. H., Koh, G. Y., Pajak, L., Jing, S., Wang, H., Franklin, M. T., Kim, K. K. and Field, L. J. (1997). Cyclin D1 overexpression promotes cardiomyocyte DNA synthesis and multinucleation in transgenic mice. *J. Clin. Invest.* **99**, 2644-2654.
Soonpaa, M. H., Zebrowski, D. C., Platt, C., Rosenzweig, A., Engel, F. B. and Field, L. J. (2015). Cardiomyocyte cell-cycle activity during preadolescence. *Cell* **163**, 781-782.
Soufan, A. T., van den Berg, G., Ruijter, J. M., de Boer, P. A. J., van den Hoff, M. J. B. and Moorman, A. F. M. (2006). Regionalized sequence of myocardial cell growth and proliferation characterizes early chamber formation. *Circ. Res.* **99**, 545-552.
Staudt, D. W., Liu, J., Thorn, K. S., Stuurman, N., Liebling, M. and Stainier, D. Y. R. (2014). High-resolution imaging of cardiomyocyte behavior reveals two distinct steps in ventricular trabeculation. *Development* **141**, 585-593.
Stuckmann, I., Evans, S. and Lassar, A. B. (2003). Erythropoietin and retinoic acid, secreted from the epicardium, are required for cardiac myocyte proliferation. *Dev. Biol.* **255**, 334-349.
Sturzu, A. C., Rajarajan, K., Passer, D., Plonowska, K., Riley, A., Tan, T. C., Sharma, A., Xu, A. F., Engels, M. C., Feistritzer, R. et al. (2015). The fetal mammalian heart generates a robust compensatory response to cell loss. *Circulation* **132**, 109-121.
Sutton, M. G. S. J. and Sharpe, N. (2000). Left ventricular remodeling after myocardial infarction: pathophysiology and therapy. *Circulation* **101**, 2981-2988.
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, J. H. and Molkentin, J. D. (2014). An emerging consensus on cardiac regeneration. *Nat. Med.* **20**, 1386-1393.
van Berlo, J. H., Kanisicak, O., Maillet, M., Vagnozzi, R. J., Karch, J., Lin, S.-C. J., Middleton, R. C., Marbán, E. and Molkentin, J. D. (2014). c-kit+ cells minimally contribute cardiomyocytes to the heart. *Nature* **509**, 337-341.
van den Berg, G., Abu-Issa, R., de Boer, B. A., Hutson, M. R., de Boer, P. A. J., Soufan, A. T., Ruijter, J. M., Kirby, M. L., van den Hoff, M. J. B. and Moorman, A. F. M. (2009). A caudal proliferating growth center contributes to both poles of the forming heart tube. *Circ. Res.* **104**, 179-188.
von Gise, A., Lin, Z., Schlegelmilch, K., Honor, L. B., Pan, G. M., Buck, J. N., Ma, Q., Ishiwata, T., Zhou, B., Camargo, F. D. et al. (2012). YAP1, the nuclear target of Hippo signaling, stimulates heart growth through cardiomyocyte proliferation but not hypertrophy. *Proc. Natl. Acad. Sci. USA* **109**, 2394-2399.
Waldo, K. L., Kumiski, D. H., Wallis, K. T., Stadt, H. A., Hutson, M. R., Platt, D. H. and Kirby, M. L. (2001). Conotruncal myocardium arises from a secondary heart field. *Development* **128**, 3179-3188.
Wang, H. U., Chen, Z.-F. and Anderson, D. J. (1998). Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell* **93**, 741-753.
Wang, J., Panáková, D., Kikuchi, K., Holdway, J. E., Gemberling, M., Burris, J. S., Singh, S. P., Dickson, A. L., Lin, Y.-F., Sabeh, M. K. et al. (2011). The regenerative capacity of zebrafish reverses cardiac failure caused by genetic cardiomyocyte depletion. *Development* **138**, 3421-3430.
Wang, J., Cao, J., Dickson, A. L. and Poss, K. D. (2015). Epicardial regeneration is guided by cardiac outflow tract and Hedgehog signalling. *Nature* **522**, 226-230.
Wei, K., Serpooshan, V., Hurtado, C., Diez-Cuñado, M., Zhao, M., Maruyama, S., Zhu, W., Fajardo, G., Noseda, M., Nakamura, K., et al. (2015). Epicardial FSTL1 reconstitution regenerates the adult mammalian heart. *Nature* **525**, 479-485.
Wills, A. A., Holdway, J. E., Major, R. J. and Poss, K. D. (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. and Morrison, J. I. (2011). Recapitulation of developmental cardiogenesis governs the morphological and functional regeneration of adult newt hearts following injury. *Dev. Biol.* **354**, 67-76.
Wu, H., Lee, S. H., Gao, J., Liu, X. and Iruela-Arispe, M. L. (1999). Inactivation of erythropoietin leads to defects in cardiac morphogenesis. *Development* **126**, 3597-3605.
Xin, M., Kim, Y., Sutherland, L. B., Qi, X., McAnally, J., Schwartz, R. J., Richardson, J. A., Bassel-Duby, R. and Olson, E. N. (2011). Regulation of Insulin-like growth factor signaling by Yap governrs cardiomyocyte proliferation and embryonic heart size. *Sci. Signal.* **25**, ra70.
Xin, M., Kim, Y., Sutherland, L. B., Murakami, M., Qi, X., McAnally, J., Porrello, E. R., Mahmoud, A. I., Tan, W., Shelton, J. M. et al. (2013). Hippo pathway effector Yap promotes cardiac regeneration. *Proc. Natl. Acad. Sci. USA* **110**, 13839-13844.
Zak, R. (1973). Cell proliferation during cardiac growth. *Am. J. Cardiol.* **31**, 211-219.
Zhang, R., Han, P., Yang, H., Ouyang, K., Lee, D., Lin, Y.-F., Ocorr, K., Kang, G., Chen, J., Stainier, D. Y. et al. (2013). In vivo cardiac reprogramming contributes to zebrafish heart regeneration. *Nature* **498**, 497-501.
Zhao, Y.-y., Sawyer, D. R., Baliga, R. R., Opel, D. J., Han, X., Marchionni, M. A. and Kelly, R. A. (1998). Neuregulins promote survival and growth of cardiac myocytes: persistence of ErbB2 and ErbB4 expression in neonatal and adult ventricular myocytes. *J. Biol. Chem.* **273**, 10261-10269.
Zhao, B., Ye, X., Yu, J., Li, L., Li, W., Li, S., Yu, J., Lin, J. D., Wang, C. Y., Chinnaiyan, A. M. et al. (2008). TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev.* **22**, 1962-1971.
Zhou, Q., Li, L., Zhao, B. and Guan, K.-L. (2015). The Hippo pathway in heart development, regeneration, and diseases. *Circ. Res.* **116**, 1431-1447.