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Binucleation of cardiomyocytes: the transition from a proliferative to a terminally differentiated state

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Abstract

Cardiomyocytes possess a unique ability to transition from mononucleate to the mature binucleate phenotype in late fetal development and around birth. Mononucleate cells are proliferative, whereas binucleate cells exit the cell cycle and no longer proliferate. This crucial period of terminal differentiation dictates cardiomyocyte endowment for life. Adverse early life events can influence development of the heart, affecting cardiomyocyte number and contributing to heart disease late in life. Although much is still unknown about the mechanisms underlying the binucleation process, many studies are focused on molecules involved in cell cycle regulation and cytokinesis as well as epigenetic modifications that can occur during this transition. Better understanding of these mechanisms could provide a basis for recovering the proliferative capacity of cardiomyocytes.

Keywords

Cardiomyocyte; heart development; binucleation; epigenetic; fetal programming

Introduction

Cardiomyocytes are the functional unit of the heart; therefore the number of viable myocytes dictates cardiac function. The total cardiomyocyte population is determined early in life during fetal development and around birth, with negligible increases thereafter [1]. Hence, preservation of cardiomyocyte number will fortify the heart and enable adequate response to stress later in life. It has long been understood that the heart loses proliferative capacity soon after birth in most mammals [2–4]. This timeframe is consistent with the conversion of

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Teaser: The transitional timing of cardiomyocytes from mononucleate to binucleate is crucial in determining cardiomyocyte endowment in the heart and cardiac function throughout life.

Conflicts of interest

The authors declare no conflict of interest.

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cardiomyocytes from a mononucleate to binucleate phenotype. Binucleation is a characteristic of terminally differentiated cells that are unable to proliferate, whereas mononucleate cells continue to cycle. Early in normal fetal development the majority of cardiomyocytes are mononucleate, allowing growth to be achieved by proliferation. In the timeframe surrounding birth, heart maturation occurs where mononucleate cells begin the transition to a binucleate phenotype. The uncoupling of cytokinesis from karyokinesis and ultimate exit of the cell cycle characterize the transition, resulting in binucleation [5]. Subsequent increases in heart size are independent of proliferation and the result of increases in individual cell size is termed hypertrophy.

In humans, the fetal heart consists of mainly mononucleate cardiomyocytes and this is therefore the time at which most proliferation occurs. Just before birth, binucleation begins and can extend into early neonatal life. Similarly, sheep follow this pattern of development, providing a close model for studying the heart. Rodents are another commonly used model however it is to be noted that cardiomyocyte binucleation in rodents begins and ends within the first two weeks after birth [5]. In all these species, the adult heart contains the greatest amount of binucleate cells when compared with the fetal and neonatal stages. However the percentage of binucleate cells within the adult heart varies among species, as reviewed by Botting *et al.* [6]. In humans, there is considerable debate about the amount of binucleate cells present in the adult heart, with values ranging from 25 to 60% [6]. Rodents and sheep, by contrast, have approximately 90% of the cardiomyocyte population binucleated [6].

The physiological importance of binucleation is still poorly understood. A plausible explanation is that multinucleation optimizes cellular response, enhancing cell survival when coping with stress [7]. Another argument is that binucleation occurs to meet the high metabolic demand of cardiomyocytes. As such, binucleation has an advantageous role in enabling the cell to generate twice the amount of RNA to synthesize proteins [3]. This review discusses factors involved in cardiomyocyte transition including alterations to its timing, the underlying molecular mechanisms and the role of epigenetic regulation and therapeutic targets.

Premature transition

The intrauterine environment is highly influential on the health of an individual. Its influence can lead to structural and functional adaptations of several organs, including the heart. Persistence of these adaptations can increase vulnerability later in life to diseases including metabolic syndrome and cardiovascular disease [6,8,9]. Altered cardiomyocyte number could be responsible for this increased susceptibility. In support, animal studies provide evidence that fetal stress caused by hypoxia [10], glucocorticoids [11] or maternal malnutrition [12,13] affects the number of cardiomyocytes and the ability of the heart to cope with stress later in life.

Hypoxia

Hypoxia is a major fetal stressor induced under a variety of conditions including nicotine exposure, high altitude pregnancy, preeclampsia and placental insufficiency. The long-term implications of this adverse environment have been well established [8,14]. Recent studies

have shown that hypoxia directly reduces proliferation in fetal rat cardiomyocytes [15]. In other studies maternal hypoxia was found to result in increased size and percent of binucleate cardiomyocytes [10] along with remodeling of the fetal and neonatal rat heart [16]. Fetal sheep anemia studies by Jonker *et al.* also reported larger and more mature cardiomyocytes [17]. This was marked by increases in the amount of binucleate cardiomyocytes in the right ventricle.

By contrast, studies of hypoxia-inducible factor 1 α (HIF1 α) overexpressed in transgenic mice reveal a possible role in improving cardiac function and reducing infarct size after myocardial infarction (MI) [18]. Moreover, C3orf58, a hypoxia- and Akt-induced stem cell factor (HASF) has been shown to have a positive effect on proliferation of cardiomyocytes in rodents [19]. Similarly, hypoxia was shown to have a role in increasing proliferation of adult zebrafish cardiomyocytes, *in vitro* studies reveal this can be achieved by hypoxia-induced dedifferentiation [20]. These studies indicate a possible dual role of hypoxia in regulating cardiomyocyte proliferation. Altogether demonstrating that hypoxia is involved in cardiac remodeling and can directly affect cardiomyocyte endowment of the heart.

Glucocorticoids

Glucocorticoids are a class of hormones essential to normal lung development and the regulation of the cardiovascular system. Although glucocorticoids are essential to the development and survival of the fetus, excessive exposure has negative implications including delayed maturation of astrocytes [21], reduced birth weights [22] and altered glucocorticoid receptor expression [23].

Evidence exists for a role of glucocorticoids in regulating cardiomyocyte development. Early studies by Rudolph *et al.* reported a reduction in cardiomyocyte proliferation after fetal sheep cortisol infusion, associated with hypertrophic growth [24]. However, more recently, studies in fetal sheep revealed increased proliferation without an increase in cardiomyocyte size after cortisol infusion [11]. In this latter study no differences in length, width and overall percentage binucleation of cardiomyocytes were observed between cortisol-treated and nontreated groups. In addition, the cortisol treatment did not drive the maturation of cardiomyocytes but rather stimulated their entry into the cell cycle suggesting cortisol is associated with hyperplastic growth. These opposing results are probably the result of the different methods of quantification used by the researchers; and are further discussed by Giraud *et al.* [11].

In the fetal rat low-dose dexamethasone, a synthetic glucocorticoid, was found to decrease fetal bodyweight when administered prenatally by Torres *et al.* [25]. In this study the dexamethasone-treated group was found to have increased cardiomyocyte proliferation in the fetal heart as compared with control. In addition, a sex-dependent component of cardiomyocyte proliferation was observed, with females having significantly more DNA synthesis compared with males. Taken together, these findings provide evidence for premature glucocorticoid exposure associated with a developmental delay of heart maturation.

In neonates dexamethasone treatment has been found to decrease total cardiomyocyte number [26] possibly by decreasing proliferation [26,27]. De Vries *et al.* [26] [s1] reported reduced proliferation during neonatal day 2–4 with no subsequent changes. This study also noted no apoptosis, supporting suppressed proliferation as the cause of lower cardiomyocyte number. These reductions in cardiomyocyte number were noted to continue into adulthood, associated with reduced systolic function [28]. It is evident that the effect of glucocorticoid treatment is dependent on the time of exposure. Fetal exposure results in increased cardiomyocyte proliferation, whereas neonatal exposure has an opposite effect. This provides evidence for a time-dependent mechanism of glucocorticoid action, highlighting the importance of monitoring perinatal circulating glucocorticoid levels because of the diverse impact on heart development.

Hypertension

The heart must constantly adapt to the hemodynamic load of the body. In fetal sheep acute hypertension has been found to increase cardiomyocyte proliferation, followed by increases in cell size, whereas longer-term hypertension leads to an increase in binucleate cells [29]. Jonker *et al.* induced fetal hypertension in sheep by intravascular plasma infusion for 4 or 8 days. In both hypertensive groups the heart weight was increased, and the number of mononucleate cardiomyocytes was more than double that of control. In addition, at both time points a significant amount of myocytes was found to be actively cycling. However, after 8 days of infusion the amount of binucleation in the left and right ventricle was significantly elevated. Thus, this suggests there are two physiological responses to hypertension in the fetal heart. Initially hypertension (measured after 4 days) stimulated cell cycle activity, and with extended hypertension treatment (8 days) a marked increase in binucleation of the heart is noted. The initial increase in mononucleate cells could represent a short-term change whereas the later binucleation could reflect long-term premature loss of proliferation [29].

Post-mortem studies of the adult human heart revealed increased ploidy and binucleation after cardiac injury [30]. It is believed that the loss of cardiomyocytes in such pathological conditions increases the hemodynamic load on the remaining cardiomyocytes. In this way the increased workload could stimulate the heart to proliferate in order to regenerate after injury. It has been proposed in the adult heart that cytokinetic machinery loses function, and therefore could be responsible for increased ploidy of the heart after injury. Cytokinetic dysfunction is further discussed in the following section of mechanisms in this review. Taken together, these studies provide a basis for the effect of hypertension short- and long-term on cardiac structure and function.

Intrauterine growth restriction

The *in utero* environment is uniquely designed to protect the developing fetus, and must be maintained to ensure adequate development. Hindrances to growth, termed intrauterine growth restriction (IUGR), can include inadequate nutrition, placental insufficiency, hypoxia or preeclampsia. Implications of IUGR are associated with long-term adverse events including renal disease, metabolic syndrome and cardiac disease [31,32]. In the heart, IUGR has been shown to alter the phenotype of cardiomyocytes. One such change is reducing the

amount of binucleate cells, as noted by Bubb *et al.*, in the left but not the right ventricle in fetal sheep hearts [13].

Louey *et al.* modeled sheep placental insufficiency by umbilicoplacental embolization (UPE) for either 10 or 20 days [33]. In both UPE groups the heart mass and bodyweights of the animals were lower than control with no changes in the heart:bodyweight ratio. After 20 days of UPE the amount of binucleate and proliferating mononucleate cells decreased, with no noted changes after only 10 days. In this study a delay in maturation of cardiomyocytes occurs, noted by altered transition time. Additionally, the different results between 10 and 20 days of UPE suggest the duration of insult is a major contributing factor to the cardiac outcome.

Morrison *et al.* created a sheep placental insufficiency model by removal of a significant portion of uterine caruncles. In this model an increase in mononucleate cardiomyocytes was noted [34]. This finding suggests a delay in the transition from mononucleate to binucleate cells. These results differ from those previously discussed, which could be caused by differences in oxygen levels used by the individual researchers. As Morrison *et al.* noted, a direct link exists between amount of mononucleate cells and hypoxemia in the fetal heart [34]. These observations taken together provide evidence for delayed maturation of the heart in the growth-restricted fetus.

Maternal protein restriction in rats resulted in pups with lower heart mass and bodyweight than control [12]. In this study the percentage of binucleation was unchanged in the protein-restricted group. However, the total number of cardiomyocytes in the restricted group was significantly reduced. It should be noted that myocyte number was also found to be dependent on the litter size. In this model the importance of maternal protein intake is highlighted. Of particular clinical interest is the finding that maternal protein-restricted diet resulted in a reduction in cardiomyocyte number in the developing fetus.

Molecular targets implicated in binucleation of cardiomyocytes

The molecular mechanisms responsible for cardiomyocyte binucleation remain unknown. Considering the distinct characteristics between the two cardiomyocyte phenotypes, it is apparent that a marked change in cell cycle activity must occur to achieve binucleation. The process appears to be tightly associated with regulation of the cell cycle, cytokinesis, hormones and microRNA (miRNA).

Cell cycle regulation

Cell cycle regulators are differentially expressed within the mononucleate versus binucleate state. Cardiomyocytes exit the cell cycle as they become binucleated and as such are terminally differentiated. Cell cycle phases include gap phase 1 (G1), synthesis phase (S), gap phase 2 (G2) and mitosis (M). The G0 phase provides an exit route from the cell cycle in which the cells remain in an indefinite quiescent state. Molecules that determine the rate of growth and proliferation include cyclin-dependent kinases (CDKs) and their inhibitors (CDKIs). CDKs promote the cell cycle whereas CDKIs are known to inhibit the cell cycle [35]. During fetal development, CDKs are highly expressed within the heart and become

downregulated in adulthood. Conversely, the negative regulators of cell cycle, such as CDKIs, are then upregulated in the adult heart [36]. The prominent CDKIs, such as p21, p27 and p57, appear to have a role in the cardiomyocyte arrest of the cell cycle during development, as reviewed in [35]. In neonatal cardiomyocytes, targeting p21 and p27 via siRNA knockdown promoted proliferation and progression of cells into the S phase. Furthermore, the proliferation of adult cardiomyocytes was induced with the knockdown of the three CDKIs: p21, p27 and p57 [37].

A conserved splice variant of cyclin D2, D2SV, has been shown to induce embryonic cardiomyocytes to exit the cell cycle while reducing the capacity for them to enter the cell cycle. D2SV forms micro-aggregates that sequester cell cycle promoting proteins such as CDK4, cyclin D2 and cyclin B1, leading to cell cycle exit [38]. D2SV expression in the embryonic heart is higher than the adult, contrary to expectations. The role of D2SV in negatively regulating proliferation underlines the inherent ability of the heart to autoregulate cell cycle activity. This mechanism appears to be essential in optimizing cardiomyocyte number. Maintenance of the balance between promotion and inhibition of the cell cycle is necessary to obtain the full potential of the heart.

Liu *et al.* found that cyclin G1 expression in the mouse heart was low during fetal (E18) stage and postnatal day 2, and was increased from day 4 onward [39]. The expression of this cell cycle protein corresponds with the polyploidization of cardiomyocytes. This study demonstrated that overexpression of cyclin G1 stimulated S-phase entry but blocked cytokinesis, the latter exhibiting a stronger effect. By knocking out cyclin G1, several proliferative factors such as proliferating cell nuclear antigen, survivin, aurora B and mitotic arrest[s2] deficient (Mad)2 were downregulated, suggesting that cardiomyocytes exited the cell cycle [39]. Altogether, cyclin G1 expression is associated with cardiomyocyte transition and increases multinucleation of these cells.

In rodents, cardiomyocyte transition occurs during the first two weeks of postnatal life. The majority of myocytes are binucleate by postnatal day 7 (P7) [5]. A recent study identified a potential candidate regulator involved in this process: focal adhesion kinase (FAK)-related non-kinase (FRNK) [40]. FRNK is an endogenous inhibitor of a major factor in cardiac growth (i.e. FAK). FRNK expression is increased during the first postnatal week, peaking at P7 through P14. Together with the finding that bromodeoxyuridine uptake is higher in hearts of FRNK null mice these data implicate the role of FRNK in the suppression of cardiac DNA synthesis in postnatal life. The FRNK null mouse hearts from P14 and P21 also showed significantly elevated levels of aurora-B, a protein necessary for cytokinesis [40]. The peak expression of this factor is consistent with the timeframe in which the majority of cardiomyocyte terminal differentiation occurs, providing evidence that FRNK is a regulatory factor in the maturation of postnatal cardiomyocytes.

In addition, Yes-associated protein (YAP)1 is a main target for the Hippo kinase cascade, a key pathway in regulating organ growth. When *Yap1* is inactivated in the fetal heart, lethal hypoplasia and decreased proliferation results [41,42]. In turn, YAP1 activation promotes proliferation of fetal and postnatal cardiomyocytes while activating several cell cycle genes, such as cyclin A2, cyclin B1 and CDK1. Furthermore, the YAP1-induced cardiomyocyte

proliferation requires interaction with TEAD [s3]transcription factors [41]. The targeting of *Yap1* and upstream regulation of the kinase pathway leading to its activation appear to be involved in cardiomyocyte terminal differentiation.

Hypoxia has been shown to downregulate cyclin D2 and upregulate p27 expression, associated with a decrease in proliferation of fetal cardiomyocytes [15]. Under hypoxic conditions, tissue inhibitor metalloproteinase-3 and -4 (TIMP-3, TIMP-4) were upregulated. Upon knockdown, TIMP-3 increased cyclin D2 and the proliferation marker Ki-67 in control cardiomyocytes, whereas TIMP-4 had no effect. However, the hypoxia-mediated effects were blocked completely by TIMP-4 and only partially by TIMP-3 [15].

Oxidative stress can be induced by hypoxia; the role of reactive oxygen species (ROS) on cardiomyocyte transition was evaluated using the scavenger N-acetyl-L-cysteine (NAC). *In vivo* treatment of dams with NAC followed by *in vitro* treatment of isolated cardiomyocytes resulted in increased proliferating cell nuclear antigen expression and decreased binucleation. In addition, these NAC-treated cardiomyocytes had decreased expression of p38 mitogen-activated protein kinase (MAPK) and Connexin43 (Cx43), whereas ROS was shown to activate p38 MAPK and increase expression of Cx43 [43].

Cytokinesis regulation

Cytokinesis is the final step in cell division and is absent during the process of binucleation. Karyokinesis is maintained resulting in multiple nuclei in a single cell. One proposed reason for the lack of cytokinesis during myocyte binucleation is a dysfunctional assembly of the contractile ring. Previous studies suggest that the incomplete disassembly of the contractile ring results in the inability of the cell to fully divide [44–47]. It is possible that miRNA-133 plays a part considering its expression is inversely related to cardiac hypertrophy and sarcomere organization via RhoA expression [48]. However, another study reports complete disassembly and suggests the cause of binucleation is a defect in anillin localization [49]. Anillin is a protein that participates in cytokinesis and the formation of the cleavage furrow. The defect in mid-body localization of anillin during cytokinesis leads to deficient actomyosin ring function and failure to form a cleavage furrow. This defect was rescued by inhibition of p38 MAP kinase (p38), leading to an upregulation of cytokinesis-related genes [49]. These findings suggest that cardiomyocyte binucleation occurs by a mechanism involving p38-regulated cytokinesis.

The regulation of cardiomyocyte transition is intricate and subjected to a complex molecular mechanism. The involvement of numerous factors is necessary in maintaining tight control of this significant event in heart development. The cell cycle and cytokinesis are key processes associated with cellular division. Notably, at any time point, stimulators and inhibitors are modulating the overall cardiomyocyte population. Although myocytes are actively proliferating in the developing heart, it is important to maintain mechanisms that will prevent excessive hyperplasia. As cardiomyocytes become binucleate, a gradual decrease in proliferative factors and simultaneous increase in inhibitors occurs. This extensive regulation illustrates the significance of maintaining optimal cardiomyocyte number.

Hormonal regulation

Cardiomyocyte terminal differentiation is in part regulated by hormonal stimulation. Studies on fetal ovine cardiomyocytes have shown the participation of hormones such as cortisol, thyroid hormone and atrial natriuretic peptide (ANP) in cardiomyocyte maturation [11,24,50–53].

The effect of cortisol on cardiomyocytes is complex as discussed earlier. Thyroid hormone 3,3',5-tri-iodo-L-thyronine (T_3) is shown to inhibit proliferation of mid-gestation and near-term fetal ovine cardiomyocytes *in vitro* [51,52] and *in vivo* [50]. In response to T_3 stimulation, an increase in binucleation and a reduction in proliferation are observed. As well as an increase in protein levels of p21, a cell cycle suppressor, and a decrease in levels of cyclin D1, a cell cycle promoter [50–52], the thyroid hormone T_3 appears to be involved in mitotic suppression and driving cardiomyocytes toward maturation.

ANP has been shown to decrease proliferation of fetal ovine cardiomyocytes. The mechanism occurs through the ability of ANP to inhibit angiotensin II, thereby blocking its ability to promote proliferation via the MAPK and phosphoinositide-3-kinase (PI3K) pathways [53]. Overall hormonal regulation of cardiomyocyte maturation has been investigated with emphasis on cortisol, thyroid hormone and ANP. Cortisol effect on cardiomyocytes is variable and probably age-dependent, as previously discussed, whereas thyroid hormone T_3 and ANP decrease proliferative capacity of cardiomyocytes, leading to maturation of the cells.

Epigenetic regulation

Epigenetic modifications refer to changes in the expression of genes independent of the DNA sequence. Changes in the intrauterine environment have been attributed to long-term adverse effects, known as fetal programming [8]. Initially, an organ can adapt to facilitate immediate survival and functional compensation. However, sustained stress can result in compromised physiology and/or tissue remodeling of an organ.

The epigenetic mechanisms involved in differentiation from progenitor cells to cardiomyocytes have been investigated [54]. However, few studies have focused on the final step (i.e. terminal differentiation). It is known that the heart responds to environmental cues by modifying the epigenome [55,56]; however, the specific details of this regulation of cardiomyocyte maturation are lacking.

The regulation of gene expression via miRNA (miRs) is one such epigenetic mechanism. miRNA profiling during neonatal development revealed a number of differentially regulated miRNAs between neonatal day 1 (P1) and neonatal day 10 (P10) rats. Members of the miRNA-15 (miR-15) family were upregulated during this neonatal period, with miR-195 being the most highly upregulated. When miR-195 is overexpressed in the heart during development, it resulted in heart defects and premature cell cycle arrest. At P1, the hearts overexpressing miR-195 had a threefold higher percentage of multinucleate cardiomyocytes. Cultured neonatal cardiomyocytes with overexpressed miR-195 also had an increased proportion of binucleate myocytes. Between P7–P14, miR-195 expression in the heart is

inversely correlated with the expression of several cell cycle genes such as *Chek1*, *Cdc2a*, *Birc5*, *Nusap1* and *Spag5*. The inhibition of the miR-15 family led to an increase in mitotic entry and progression of cardiomyocytes in the cell cycle [57].

Recently, another study identified a correlation between decreased expression of insulin-like growth factor (IGF)1 and the increased expression of miR-378 in the neonatal heart. The function of this miRNA in targeting the receptor and inhibiting the Akt activation implies a role in postnatal cardiac remodeling [58]. IGF1-mediated Akt activation in the heart is repressed by the action of miR-378, most interestingly this correlates with the timeframe of the cardiomyocyte transition. These findings implicate miRNAs in the regulation of certain factors associated with cellular growth and neonatal heart development.

Polycomb repressive complex (PRC)2 is known to be involved in the trimethylation of histone H3 at lysine 27 (H3K27me3). A component of this complex, enhancer of zeste homolog (Ezh)2 is believed to ensure normal cardiac growth and adult activity [59]. Ezh2 has also been shown to repress negative regulators of the cell cycle such as Ink[s4]4a and Ink4b. He *et al.* inactivated the Ezh2 subunit of PRC2 and noted hypoplasia and upregulation of Ink4a and [s5]b [60]. These data implicate Ezh2 as a regulator of proliferation in the heart potentially via methylation.

Kou and colleagues investigated other epigenetic mechanisms involved in the maturation of cardiomyocytes [61]. As noted before, terminal differentiation and binucleation are inversely correlated to proliferation. A significant increase in global methylation in the heart occurs during the neonatal period [61], the same timeframe when binucleation occurs. Furthermore, expression of DNA methyltransferases involved in *de novo* DNA methylation (DNMT3a and DNMT3b) was significantly increased during the first 90 days of postnatal life. Inhibition of methylation with 5-azacytidine during neonatal day 7 and 10 resulted in a marked increase in DNA synthesis and delayed maturation. Histone modifications were also noted. Altogether these changes are associated with the terminally differentiated form of cardiomyocytes, whereas a DNA methylation inhibitor reverted the myocytes to a less differentiated state. This study provides evidence for a role of methylation in the reduction of proliferation and progression of cardiomyocytes to terminal differentiation. Binucleate myocytes are nonproliferative and terminally differentiated. Therefore, it is plausible to hypothesize that binucleation might be associated with methylation-induced suppression of proliferation.

The discovery of the epigenome has expanded the possibilities of biological regulation. Epigenetic modifications are employed in a variety of biological processes including fetal programming of disease state as well as normal development. This role in terminal differentiation of cardiomyocytes is of particular interest. The studies reviewed here provide evidence for the involvement of the epigenome in regulating cardiomyocyte proliferation and maturation. This complex regulation appears to include miRNAs, DNA methylation and histone modifications. With the observation that epigenetics is key in cardiomyocyte maturation the focus of future studies should be on further elucidating these intricate mechanisms.

Potential therapeutic implications

Although the heart is believed to possess regenerative capacity after damage in mammals [1], such a capacity is far too small to provide adequate repair. The possibility that medical advances might provide insight and therapeutic advantages for heart disease has been the major goal of scientists. Studies in zebrafish reveal regenerative potential in the heart after damage [62] via dedifferentiation and proliferation of existing cardiomyocytes [63,64]. Using genetic fate mapping with multicolor clonal analysis Gupta and Poss traced the origins of cardiomyocytes during regeneration. This study found cortical layer cardiomyocyte clones as the major component of the new wall [65]. In mammals this regeneration was only noted at an early age, before terminal differentiation [66]. Full cardiac recovery was observed after 21 days following left ventricle apex resection in 1-day-old mice. However, cardiac regenerative potential appears to be lost after myocytes exit the cell cycle as seen in day 7 neonates, which show no sign of repair following resection [66].

A major strategy in stimulating the heart to regenerate is by eliminating factors that inhibit the proliferation of terminally differentiated cardiomyocytes, as described by Di Stefano and Martelli [67]. Several studies have focused on manipulation of the cell cycle to promote proliferation of cardiomyocytes. A therapeutic target for this approach is CKIs[s6], inhibitors of the cell cycle as was discussed previously. Knockdown of CKIs[s7] can provide temporary activation of the cell cycle; however, sustained knockdown eventually results in declining proliferation of cardiomyocytes [37,67].

The Notch pathway has also demonstrated therapeutic potential in maintaining cardiomyocyte proliferation. Notch activation in neonatal cardiomyocytes stimulates re-entry into the cell cycle and enhanced cyclin D1 activity, together promoting cell division. Interestingly, activation of Notch led to cell division in younger cardiomyocytes (P2), whereas those from P5 mice were unable to complete the G₂/M phase transition [68]. Activated Notch was also shown to prolong proliferation of myocytes after birth resulting in a larger cardiomyocyte population in adult mice [69].

In the heart key transcription factors and regulatory proteins have been investigated in an attempt to promote proliferation of cardiomyocytes [70,71]. Studies overexpressing adenoviral protein E1A and the transcription factor E2F1 in cardiomyocytes have shown inadequate ability to complete mitosis. However, in co-infection with human CDC[s8]5 (hCDC5), a factor in the pre-mRNA splicing complex, neonatal cardiomyocytes were stimulated to re-enter and progress through the cell cycle. Several cell cycle factors such as CDK1 and cyclin B1 became localized to the nucleus leading to entry into mitosis. Altogether stimulating DNA synthesis, cell division and, ultimately, an increase in number of cardiomyocytes [72].

Meis1, a transcription factor, is a crucial component in cell cycle regulation for the cardiomyocyte and is necessary for normal development of the heart. In the postnatal mouse, deletion of Meis1 prolonged the window of proliferation of cardiomyocytes. The adult heart exhibited cell division reactivation with no adverse effect on heart function in Meis1-deleted

mice. Overexpression of Meis1 in the neonatal cardiomyocyte led to reduced proliferative capacity and prevented heart regeneration [73].

Recent studies have evaluated regenerative possibilities of the heart following MI. One such study utilized transgenic mice overexpressing cyclin D2, a promoter of the cell cycle. Post-MI these hearts exhibited reduced infarct size, formation and syncytium of new myocytes with existing myocardium, and functional improvement, all indicating functional recovery of the heart after injury [74].

miRNAs, as previously discussed, can provide therapeutic targets in promoting regeneration of the heart. Once again, the miR-15 family is a crucial negative regulator of cardiomyocyte proliferation. Overexpression of miR-195 inhibited the regenerative ability of the early neonatal mouse heart. Targeted inhibition of this family of miRNAs in the early neonate and up to adulthood enabled enhanced proliferation of cardiomyocytes in the adult heart as well as improved recovery after MI [75]. Moreover in adult mice, overexpression of miR-17-92 was also found to be protective from MI-induced injury. The miR-17-92 cluster was shown to be required for proliferation of cardiomyocytes and overexpression can stimulate proliferation in hearts of embryonic, postnatal and adult mice [76]. Additionally, a study by Eulalio *et al.* showed that miRNAs, specifically miR-590 and miR-199a, were able to induce proliferation of cardiomyocytes in neonatal and adult animals. Furthermore, these miRNAs promoted cardiac regeneration following myocardial infarction in mice [77]. Lastly, miR-29a is significantly upregulated in 4-week-old rats, a time during which proliferation is not occurring, and its inhibition promoted the proliferation of neonatal cardiomyocytes [78]. Taken together these studies provide potential targets for promoting proliferation in an attempt to regenerate the heart. Although it has been long held that cardiomyocytes lose proliferative capacity in early life, recent findings suggest otherwise. Enhancing proliferation of cardiomyocytes by using therapeutic targets (Figure 1) can enhance the ability of the heart to regenerate. Advances in this field suggest promising opportunities for clinical application.

Concluding remarks

Normal development of the heart is characterized by a transition from mononucleate to binucleate cardiomyocytes. This conversion is associated with a decrease in the proliferative capacity of the heart, as terminal differentiation occurs. Cardiomyocyte number in the adult heart is therefore determined at an early developmental stage; demonstrating the importance of protecting cardiomyocyte number in the fetal and neonatal developmental periods to optimize cardiac function. Stimuli that can alter cardiomyocyte number include but are not limited to hypoxia, glucocorticoids, hypertension and intrauterine growth restriction. These environmental factors have been shown to act by changing the timing and percentages of proliferation and/or binucleation. The mechanisms by which these changes occur probably involve molecules associated with cell cycle, cytokinesis and hormonal regulation. Mechanistic alterations can be induced by epigenetic cues such as miRNAs, DNA methylation and histone modifications. The proposed sequence of events incorporating all these factors is outlined in Figure 2. Integrating these studies enables a better understanding

of the process of binucleation and maturation of cardiomyocytes. Nevertheless, much remains to be investigated to provide a comprehensive overview on this topic.

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Highlights

- Binucleation and terminal differentiation dictate cardiomyocyte endowment for life
- Adverse early life events affect cardiomyocyte number in adulthood
- Epigenetic mechanisms regulate cardiomyocyte binucleation
- Understanding of these mechanisms has significant therapeutic implications in heart injury

Figure 1.
Potential therapeutic targets for promoting proliferation of cardiomyocytes include regulators of the cell cycle, transcription factors, regulatory proteins and microRNA.

Figure 2.

Proposed sequence of events in which an adverse environment might induce epigenetic modifications and, in turn, alter cardiomyocyte number.

Abbreviations: IUGR, intrauterine growth restriction; ROS, reactive oxygen species.