

# Regeneration and proliferation of cardiomyocytes and its microRNA regulatory mechanisms

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**ABSTRACT** Myocardial regeneration is identified as a concept at histological level. The core content is to increase the number of cardiomyocytes (CMs), so as to maintain the steady state of CMs under pathological or physiological conditions and ensure the normal cardiac function. In this review, we discussed the relevant factors involved in the regeneration of CMs, generalized in mice, large mammals and human. During different development stages of mammalian hearts, CMs showed several controlling and growth modes on the physiological or pathological state: mitosis, hypertrophy, nuclear polyploidy and multinucleation, amitosis and etc. We also discussed the mechanisms of specific microRNAs implicated in the cardiac development, as well as disease-induced apoptosis in CMs and the process of re-entering cell cycle after injury. It is hoped that this review will contribute to a deeper understanding of therapeutic approaches for myocardial regeneration after injury.

**KEYWORDS:** cardiomyocytes, regeneration, proliferation, myocardial infarction, miRNA

## Introduction

The heart comprises parenchyma (cardiac myocytes) and stroma (connective tissue). Connective tissue was considered inert, but some evidence revealed that stroma and its cellular constituents were dynamic and metabolically active to turn over the fibrillar collagen by regulating the composition of self-peptide hormones (Weber *et al.*, 1995). The cardiomyocytes (CMs) are the dominant cell components of myocardium (Sylva *et al.*, 2014). Notably, one gram loss of CMs per year in physiological condition was associated with aging (Olivetti *et al.*, 1991), concomitant with the formation of irreversible fibrotic scar tissue lacking contractile properties (Gamba *et al.*, 2014). Following multiple factors, the decrease in CMs occurred in several pathological processes, including myocardial infarction (MI). In the human left ventricular myocardium, there exists about 2-4 billion CMs, of which 25% slashed in a few hours after MI takes place (Murry *et al.*, 2006). In hearts subjected to pressure overload of the left ventricle induced by hypertension or other cardiovascular diseases, the decreased number of CMs and excessive fibroblast accumulation were responsible for fibrosis of the myocardium, a major clinical issue (Whelan *et al.*, 2010). A critical cause of cardiovascular diseases mortality is the cardiomyocyte's

inability to renew after cardiac injury. The fundamental strategies for cardiac regeneration and repair have kept investigators pursuing them for years. The relevant therapeutic approaches have focused on replacing dead or dying CMs, for instance, by autologous cell transplantation of the orientational differentiation to CMs from different stem cells (Terzic and Behfar, 2016), in parallel with unclear benefit, mode of action, and potential side effects. An alternative target is the stimulation of substantial regeneration after injury through cardiomyocyte proliferation (Mahmoud *et al.*, 2013). mi-

**Abbreviations used in this paper:** CMs, cardiomyocytes; MI, myocardial infarction; miRNA, microRNA; MADM, Mosaic Analysis with Double Markers; RISC, RNA-induced silencing complex; 3'-UTR, 3' untranslated regions; SRF, serum response factor; FSTL1, follistatin-like 1; PDGFs, platelet-derived growth factors; IGF, insulin-like growth factor; ECM, extracellular matrix; MMPs, matrix metalloproteinases; IGF-1, insulin-like growth factor-1; AT1, angiotensin II type 1 receptor; SV40, SV40 large T antigen; pri-miRNA, primary miRNA; pre-miRNA, precursor miRNAs; AGO2, Argonaute proteins; lncRNAs, long noncoding RNAs; 14C, carbon-14; CDK1, cyclin-dependent kinase 1; Myh6, myosin heavy chain 6; Myh7, myosin heavy chain 7; Myh7b, myosin heavy chain 7b; SRF, Serum response factor; PDCD4, Programmed cell death 4; AP-1, Activator protein 1; Drp1, dynamin-related protein-1.

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Submitted: 26 August, 2022; Accepted: 22 December, 2022; Published online: 22 December, 2022.

croRNAs (miRNAs) are a batch of single-stranded noncoding RNAs which play an essential role in regulating the transcription of gene expression level (Ameres and Zamore, 2013). A growing body of evidence indicated that endogenous capacity of regeneration can be achieved by exogenously delivering miRNAs after MI (Eulalio et al., 2012; Migliore et al., 2008). miRNAs light on exploring interventional approaches for preventing heart failure or treating heart failure therapeutically in clinical trials, thus enhancing the heart's function and prolonging the human life span. As a promising strategy to supplement functional myocytes for cardiac repair, there is a pressing need to understand the proliferation modes and relevant miRNA regulatory mechanisms of heart regeneration. Herein, we systematically reviewed the proliferation modes of CMs and well-established and emerging roles of miRNAs in cardiac development, aiming to provide a potential therapeutic target by manipulating the miRNA network for cardiac regeneration.

### An overview of miRNA

miRNAs, a batch of single-stranded noncoding RNAs about 18–22nt long, play an essential role in regulating gene expression at the post-transcriptional level (Ameres and Zamore, 2013). miRNA biogenesis often begins with RNA polymerase II transcription of primary miRNA (pri-miRNA). Unlike protein-coding genes, miRNA transcription start sites can be more than several kilobases upstream of mature miRNAs (Wang et al., 2021). Following the transcription, the long pri-miRNAs are precisely cropped around a hairpin-shaped region by a microprocessor consisting of the enzymes Drosha ribonuclease III and its cofactor protein DGCR8 to generate precursor miRNAs (pre-miRNA) (Wang et al., 2021). Pre-miRNAs are then processed by the enzyme Dicer 1 ribonuclease III, recruiting Argonaute proteins (AGO2) and transactivation response element RNA-binding protein, mediating the assembly of the RNA-induced silencing complex (RISC) where the mature miRNA is then generated following the removed passenger strand of the miRNA duplex (Chekulaeva and Filipowicz, 2009). In combination with the RISC, the mature miRNA bound the 3' untranslated regions of mRNA by forming complementary base pairing and blocked the translation of mRNA or degraded the mRNA to silence its targeted genes by recruiting AGO2 to mRNA targets (Sheu-Gruttadauria and MacRae, 2018). Occasionally, miRNAs constructed a complicated network of interactions by binding to the 5'-UTR, promoter, or the open reading frame regions (Lagos-Quintana et al., 2001). Intriguingly, miRNAs target more than 60 % of protein-coding regions in the human genome, suggesting the widespread effects of miRNAs on transcriptome and proteome diversity as well as function in a context-dependent manner (Lagos-Quintana et al., 2001; Li et al., 2022).

It is of note that miRNA play a pivotal role in modulating the initiation and progression of cardiovascular diseases by regulating different molecular mechanisms. For instance, the up-regulation of miR-29 could reduce lesion size and necrotic zones via targeting Col1A and Col3A (Ulrich et al., 2016). In addition, long noncoding RNAs (lncRNAs), serving as miRNA sponges and the upstream negative regulator of the miRNA/mRNA axis, regulate the biological activities of cardiomyocytes and cardiac fibroblasts. The hypertrophy of CMs may lead to impairment of cardiac function. The lncRNA AK048451, named cardiac hypertrophy-related factor, was identified as an endogenous sponge of miR-489 and promoted

cardiac hypertrophic effects by regulating the miR-489 activity and the expression of its target (Wang et al., 2014a).

Additionally, a myocardial infarction-associated transcript was confirmed as a binding site for miR-150, modulating proliferation, migration, and apoptosis (Shen et al., 2016). Mitochondrial fission and fusion are also related to cardiomyocyte apoptosis. miR-539 could directly interact with lncRNA AK017121, thus reducing cardiomyocyte apoptosis mediated by mitochondrial fission and providing a novel approach for the treating of ischaemic heart disease (Wang et al., 2014b). The miRNAs also participate in the autophagy of cardiomyocytes, an evolutionarily conserved process in response to stress stimuli. miR-188-3p, modulated by lncRNA AK079427, targeted ATG7 to reduce autophagic vesicles formation and cell death in cardiomyocytes and thus viewed as a therapeutic target to preserve cardiac function (Wang et al., 2015b). Cardiomyocyte necrosis mainly contributes to heart failure (HF). The inhibition of miR-103/107 and its target Fas-associated protein with the death domain in response to H2O2 treatment by lncRNA H19 prevented cardiomyocyte necrosis (Wang et al., 2015a). miRNAs also involve in the activation of cardiac fibroblast. The overexpression of GAS5 prevented the growth of cardiac fibroblasts. It inhibited the expressions of Col1A1 and  $\alpha$ -SMA at both the mRNA and protein levels via negatively regulating miR-21 and its target, phosphatase, and tensin homolog (Tao et al., 2017). Expression of miRNAs can be achieved by gene therapy using adeno-associated vectors, which transduce cardiomyocytes with high efficiency (Zacchigna et al., 2014). The clinical application of miRNA required more extensive experimentation in large mammals and rigorous safety assessment.

### Regeneration of mammalian myocardium

Adult zebrafish retain a robust capacity for cardiac regeneration throughout life: the pre-existing CMs in the heart of zebrafish dedifferentiated and entered the cell cycle after injury (Genge et al., 2016; Tahara et al., 2016). The type of cardiac regeneration in zebrafish left no scar even after up to 20% resection of the ventricle (Poss et al., 2002). Did this type of regeneration occur in the mammalian heart?

#### Mouse

To date, several studies have confirmed the regeneration capacity of CMs after injury in mice. An impressive regenerative capacity of fetal heart was shown to compensate for an effective loss of 50% of cardiac tissue (Drenckhahn et al., 2008). Consistently, the hearts of 1-day-old neonatal mice were shown to regenerate after partial surgical resection characterized by minimal hypertrophy or fibrosis. However, this capacity was switched off early by seven days of age. Of note, regenerated tissue originated from pre-existing CMs was detected by genetic fate mapping (Porrello et al., 2011b). In 2012, Haubner et al presented for the first time a mammalian model of complete cardiac regeneration following a severe ischemic cardiac injury (Haubner et al., 2012). Subsequently, Polizzotti et al., presented an alternative model for the study of cardiac regeneration in neonatal mice in which cryoinjury was used to induce heart injury (Polizzotti et al., 2016). Of note, severe systemic hypoxemia induced a robust regenerative response with decreased myocardial fibrosis and improvement of left ventricular systolic function in adult mice (Nakada et al., 2017), lighting on the potential therapeutic role of hypoxia in regenerative medicine.

TABLE 1

## MAMMALIAN MODELS USED IN THE RESEARCH OF CARDIOMYOCYTES

Model	Anatomical Characteristics of Heart	Advantages	Disadvantages
Neonatal Mice	Four chambers and a closed circulation with capillaries (Bettes et al., 2014).	Mice have similarities in evolution with humans making it a more clinical model (Porrello et al., 2013).	1. Mice have a period limitation within the first week after birth (Porrello et al., 2011b). 2. Mice own unclear 'regenerative window' (Uygun and Lee, 2016).
Neonatal Pigs	A classic 'Valentine heart' shape (Crick et al., 1998); Two pulmonary veins into the left atrium (Crick et al., 1998); Aortic-mitral fiber with less continuity than humans (Crick et al., 1998).	The application of large mammals is closer to the clinic (Ye et al., 2018).	1. The use of large mammals makes high expenditure (Ye et al., 2018); 2. Lack of tools to manipulate genetically (Ye et al., 2018). 3. CMs grow primarily by multinucleation and longitudinal hypertrophy in an older pig, distinct from mice and humans (Velayutham et al., 2020).
Neonate	Low pulmonary circulation pressure (Tan and Lewandowski, 2020); High systemic circulation resistance (Tan and Lewandowski, 2020); Foramen ovale and Ductus arteriosus closed (Saker et al., 1997).	Possess the highest value for transfer to clinical practice (Levy et al., 2018).	1. Utilizing neonates as research models have ethical issues (Mackintosh and Armstrong, 2020). 2. Regeneration critically depends on the types of injuries (Sen and Sadek, 2015). 3. There exist various regulators of postnatal cell cycle arrest (Nakada et al., 2015).

So up to now, several studies have revealed the mechanisms required for regenerative program's activation using the mouse neonatal heart regeneration model. The downregulation of low-density lipoprotein receptor-related protein 6 increased cardiomyocyte cell cycle activity in neonatal, juvenile, and adult mice (Wu et al., 2021). Nrf1, a stress-responsive transcription factor, was found to promote heart regeneration and repair by regulating proteostasis and redox balance using the mouse neonatal heart regeneration model (Cui et al., 2021). Remarkably, the loss of regeneration capacity within the first week of life coincided with a metabolic shift from glycolysis to oxidative phosphorylation, thus inducing significantly increased reactive oxygen species production and DNA damage. The utilization of fatty acids exerted adverse effects on cardiomyocyte proliferation in this model, which is emerging as a viable target for cardiac regenerative therapies at the metabolic level (Cardoso et al., 2020). Furthermore, succinate dehydrogenase inhibition in neonatal mice exerted positive effects on adult cardiomyocyte proliferation, revascularization, and heart regeneration via metabolic reprogramming (Bae et al., 2021).

### Large mammals

So far, the large mammal model, including pigs, dogs, and sheep, is an urgent pre-clinical need to advance our knowledge of cardiovascular disease and to explore new approaches to repair the damaged heart (Camacho et al., 2016; Velayutham et al., 2019). Exosomes secreted by cardio-sphere-derived cells were shown to reduce scarring, attenuate adverse remodeling, and improve function in acute and chronic MI in the porcine heart (Gallet et al., 2017). Neonatal porcine hearts were capable of regeneration after MI, likely driven by cardiac myocyte division, but this potential was immediately lost when CMs exited the cell cycle shortly after birth (Ye et al., 2018; Zhu et al., 2018). This regenerative capacity was abrogated by corticosteroid in neonatal pig hearts (Tao et al., 2020). Subsequently, the delivery of human miRNA-199a in infarcted porcine hearts was shown to stimulate cardiac repair with marked improvements in global and regional contractility, increased muscle mass, and reduced scar size (Gabisonia et al., 2019). These results show that achieving cardiac repair through the stimulation of endogenous cardiomyocyte proliferation is attainable in large mammals. Like adult mice, agrin was capable of reducing ischemia-reperfusion injury and improving heart function in a pre-clinical porcine model (Baehr et al., 2020). The relevant application has focused on the transplantation of human CMs

into the pig model, for instance, by transplanting immature human embryonic stem cell-derived CMs in the infarcted porcine heart (Romagnuolo et al., 2019), providing new approaches to testify the mechanistic basis of electrical instability following exogenous CM transplantation in humans.

### Human

Over the past three decades, there was a relatively small number of case reports supporting that patients suffering from MI in the neonatal period, without structural heart diseases, suggests that humans owned the capacity for heart regeneration after birth (Boulton et al., 1991; Cesna et al., 2013; Deutsch et al., 2014; Farooqi et al., 2012; Murugan et al., 2002; Peeters et al., 1993). Saker and colleagues reported a complete myocardial function recovery after massive cardiogenic shock in a neonate within the first day of delivery, without affecting the neurodevelopment of the neonate (Saker et al., 1997). Subsequently, a newborn child having a severe myocardial infarction due to coronary artery occlusion was observed to have functional cardiac recovery, which translated into long-term normal heart function (Haubner et al., 2016). Considering all the evidence, newborn humans, similar to neonatal rodents, might have the intrinsic capacity to repair myocardial damage and recover cardiac function completely. In addition, four adult patients suffering anomalous left coronary artery from the pulmonary artery had no evidence of myocardial scarring after corrective surgery (Fratz et al., 2011), suggesting the possibility of heart regeneration in adults. In addition, endothelial cells in the heart were shown to have turnover rates and, early childhood possessed the highest turnover rate of CMs (Bergmann et al., 2009; Bergmann et al., 2015). The potential regeneration capacity in the adult human heart suggests that it may be rational to work toward the development of therapeutic strategies focused on stimulating this process in cardiac pathologies.

Taken together, fundamental insights gained from cardiac repair in neonatal mice and large mammals generate fresh insights into future cardiac regeneration in humans. Although the observations reported so far hint at the possibilities of cardiac repair in human infants, there still needs to be more cases to map the time window for cardiac functional recovery. Additionally, functional cardiac recovery was observed in infants following a massive infarction, and there remains a lack of evidence on whether it can also occur after permanent coronary vessel occlusion, as in the rodent MI models, or rely on reperfusion. Considering the nature of the

case report, the intrinsic capacity to regenerate cannot directly be proved at a cellular level.

Given all that has been mentioned above, it can be assumed that mammals might have the intrinsic capacity to regenerate their hearts through proliferating and dividing despite doing so at a slow rate. Reliable determination of cardiomyocyte proliferation is challenging. The various models used each have specific shortcomings that undermine definitive conclusions (Table 1). Additionally, the revelation of the proliferation modes of CMs and the related regulatory mechanisms with significant therapeutic value for MI were also discussed below.

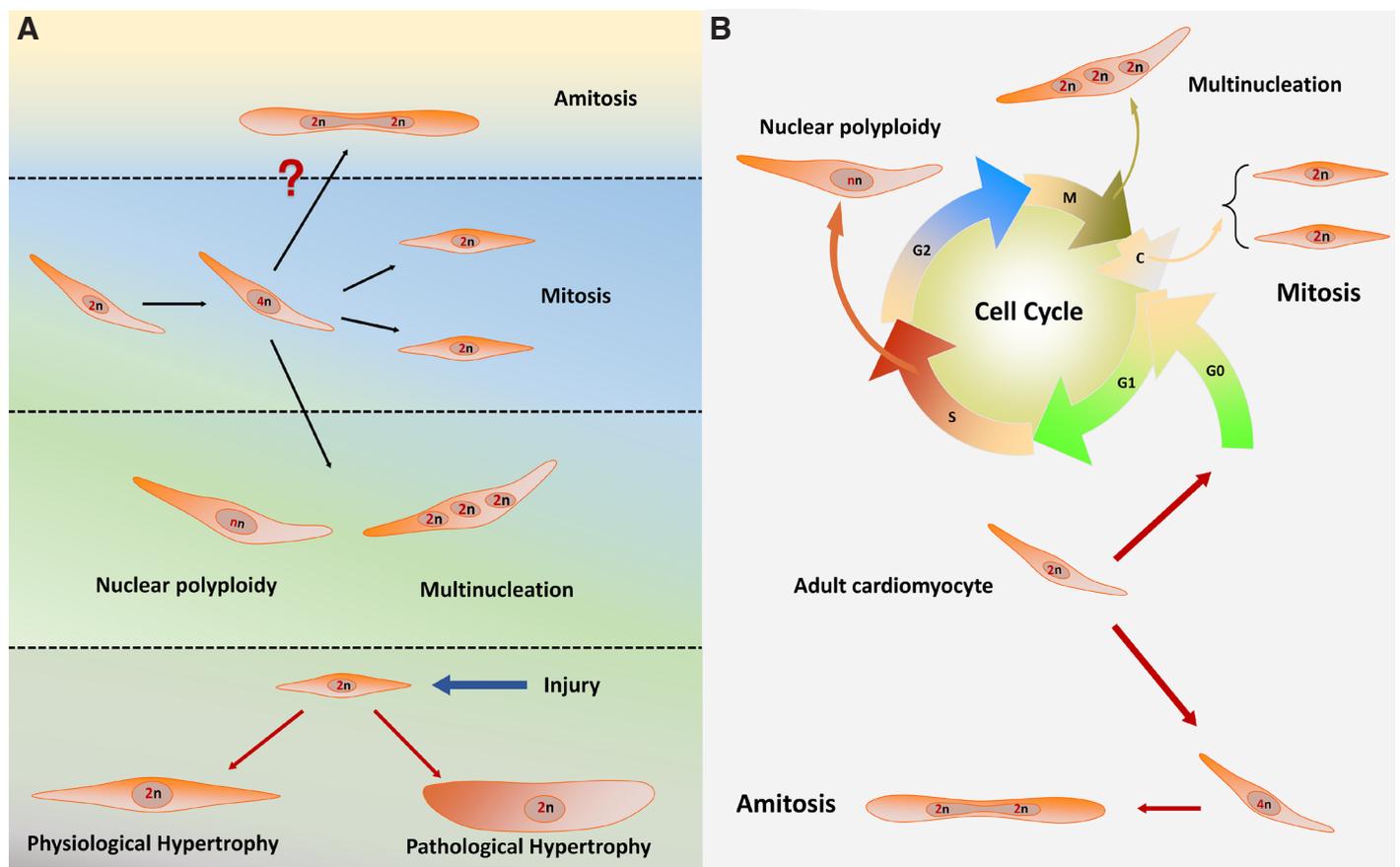
### Proliferation modes of mammalian cardiomyocytes

#### Mitosis

CMs undergo nuclear division at high speed during embryogenesis; however, human heart regeneration becomes limited to very slow cardiomyocyte replacement. Mitosis occupies only ~2% of the cell cycle, making it hard to quantify meaningfully. It is reported that mitotic cells existed in the normal cardiomyocytes in prenatal and postnatal rats but, in parallel, decreased along the developing stages (Guo et al., 2011). Similarly, in a rat model of complete cardiac regeneration following a severe ischemic

cardiac injury, a transcriptional program of significant changes in genes mediating mitosis and cell division between days 1, 3, and 10 postnatal was observed (Haubner et al., 2012). In a study investigating healthy human hearts aged 0-59 years, cardiomyocyte mitosis was detectable throughout life; in contrast, cardiomyocyte cytokinesis was not evident after 20 years, suggesting cardiomyocyte proliferation contributed to developmental heart growth in young humans (Mollova et al., 2013). The overexpression of cyclin-dependent kinase 1 (CDK1), CDK4, cyclin B1, and cyclin D1 induced high CM mitotic activity in post-mitotic mouse, rat, and human cardiomyocytes, which was assessed by co-localization of histone H3 phosphorylation (marking cells that may enter mitosis) staining with cardiac Troponin-T staining (Mohamed et al., 2018). In addition, following activation of E2F1 and E2F3, CMs were induced into DNA synthesis as well as increased apoptosis without actual mitosis and cell division (Ebelt et al., 2005), which indicated that targeting a single factor, either positively or negatively, was not sufficient to achieve complete cell replication.

Various methods were used for the detection of mitotic CMs. The most solid evidence to demonstrate incontrovertibly for the first time that CMs are generated after birth was carbon-14 ( $^{14}\text{C}$ ) dating of cTropT $\beta$  nuclei. The elevated atmospheric  $^{14}\text{C}$  levels were generated by nuclear bomb tests during the Cold War and taken up



**Fig. 1. Different growth and proliferation modes of cardiomyocytes. (A)** The four growth modes of cardiomyocytes and their relevant developing stages are shown. **(B)** The growth modes of cardiomyocytes are divided into the mitosis and amitosis processes. The cell cycle stages when mitotic growth modes happen are shown, and the amitotic process is also exhibited. The "n" in the nucleus of cardiomyocytes means the number of homologous chromosomes. "?" means the indefinite existence of amitosis.

by plants (Levin *et al.*, 2010). After eating these plants, the  $^{14}\text{C}$  was integrated into the synthesized genomic DNA, thus establishing the age of CMs in humans (Spalding *et al.*, 2005a). By correlating levels of  $^{14}\text{C}$  in cardiomyocyte nuclei with atmospheric  $^{14}\text{C}$  levels in different years, Bergmann *et al.*, reported that CMs renewed, with a gradual decrease from 1% turning over annually at the age of 25 to 0.45% at the age of 75. Additionally, less than half of CMs were exchanged in the life cycle (Bergmann *et al.*, 2009). Notably, the resolution of the  $^{14}\text{C}$  dating is about 1-2 years, which makes it unsuitable for species with a short lifespan (Spalding *et al.*, 2005b). Despite this effective method, which indirectly correlated nuclear division with cell division, the analysis of cell division is complicated by cardiomyocyte binucleation shortly after birth and the difficulties in recognizing cardiomyocyte nuclei in conventional histological sections. While the low and discrete rate of cardiomyocyte generation in mice was calculated by combining two different pulse-chase approaches (Senyo *et al.*, 2013), a systematic understanding of the extent of division in the postnatal mammalian hearts was still lacking. Using the mosaic analysis with double markers (MADM) mouse model, Ali *et al.*, provided crucial proof for the differentiated  $\alpha$ -myosin heavy chain-expressing cardiomyocyte as the original cell of postnatal cardiomyogenesis (Ali *et al.*, 2014), suggesting postnatal cardiomyogenesis in a cellular level.

### Hypertrophy

Hypertrophic growth relates to cardiac pathologies with expansion in size and increased amounts of connected sarcomeres. This process maintains the structural integrity and contractile force of the heart wall responding to the changing mechanical workload (Baidyuk *et al.*, 2019). Consistently, cardiac hypertrophy is concomitant with changes in gene expression, thus inducing changes in metabolism, contractility, and cardiomyocyte survival. There are two types of hypertrophy: physiological and pathological. Both types are initially emerging as an adaptive response to stress. Physiology hypertrophy exhibits a mild increase in cardiac mass and growth in both length and width. This process is fully reversible and has preserved or increased contractile function (Nakamura and Sadoshima, 2018). Initially induced as a compensatory response with concentric growth of the ventricle, pathological hypertrophy is irreversibly turned to ventricular chamber dilatation with wall thinning through lengthening of individual cardiomyocytes, contractile dysfunction, and heart failure (Schiattarella and Hill, 2015).

Strikingly, several lines of evidence suggest that the increased rate of cardiomyocyte proliferation is linked to physiological hypertrophy. The inhibition of C/EBP $\beta$  mediated by AKT1 increases CITED4 expression, stimulating cardiomyocyte proliferation and thus promoting physiological hypertrophy rather than pathological hypertrophy (Boström *et al.*, 2010). However, not all experimental models showed similar results. For instance, the overexpression of Parbp1 stimulated cardiac physiology hypertrophy without increased proliferation (Baroldi *et al.*, 1967). Understanding the link between physiological hypertrophy and proliferation will help stimulate CMs regeneration as well as maintain or improve cardiac function in patients with cardiovascular diseases. A growing body of evidence suggests that specific signaling mechanisms might be the primary determinants of hypertrophy types (Riehle *et al.*, 2014; Rose *et al.*, 2010; Takimoto *et al.*, 2009; Tamirisa *et*

*al.*, 1999). For example, calcineurin dephosphorylates the nuclear factor of activated T cells (NFAT) and promotes NFAT nuclear localization. NFAT induces hypertrophy-related gene expression by interacting with transcriptional cofactors (Molkentin *et al.*, 1998). Conversely, the calcineurin–NFAT pathway exerts no effect on physiological hypertrophy during exercise or pregnancy (Wilkins *et al.*, 2004). It may be delightful to work toward the development of therapeutic approaches aimed at selectively inhibiting signaling mechanisms mediating pathological hypertrophy while preserving or even promoting those mediating physiological hypertrophy.

### Nuclear polyploidy and multinucleation

DNA synthesis cannot be equal to cardiomyocyte proliferation, given that the process of polyploidization is typical in the adult heart (Beltrami *et al.*, 2001; Broughton and Sussman, 2019; Hsieh *et al.*, 2007). Mononucleated polyploid cells result from a cell duplicating its DNA during the S phase without undergoing nuclear division (Derks and Bergmann, 2020). Multinucleation in CMs is often the result of failed cytokinesis, concomitant with finished nuclear division (Liu *et al.*, 2010). While nuclear polyploidy and multinucleation are common in mammalian ventricular cardiomyocytes, there is still very little understanding of whether polyploid and multinucleated CMs can undergo cell division, and the related mechanisms. There is a consensus among nuclear ploidy and multinucleation that they have a proliferative advantage, thus inhibiting regeneration (Gan *et al.*, 2020; Patterson *et al.*, 2017; Patterson and Swift, 2019). The evidence reviewed here seems to suggest that therapeutic strategies remain narrow in focus dealing only with cardiomyocyte proliferation without polyploidy.

Polyploid CMs show great diversity among species. The zebrafish's heart mainly contains diploid CMs that proliferate throughout life (González-Rosa *et al.*, 2017). Human adult CMs mostly contain a single polyploid nucleus, while other mammalian species, such as rats and pigs, exhibit predominantly binucleated hearts (Alkass *et al.*, 2015; Bergmann *et al.*, 2015; Gräbner and Pfitzer, 1974). Considering that has been mentioned so far, it can be assumed that high ploidy levels in CMs are associated with the lost ability of the heart to regenerate. In addition, the ploidy levels appear to be related to cardiomyocyte types. While about 80% of CMs in the ventricles are binucleated, the proportion only accounts for 14% in mouse atria (Raulf *et al.*, 2015). Several lines of evidence suggest that mononucleated cells have distinctive electrophysiological characteristics, including a high arrhythmogenicity (Huang *et al.*, 2012); whether the ploidy level results from differences in metabolic activity and electrophysiological properties between cardiomyocytes remains unclear.

The increased cardiomyocyte ploidy is related to disease conditions. Pathological cardiac overload, such as hypertension, led to cardiomyocyte hypertrophy and heart enlargement and, at late stages, resulted in polyploidization in human hearts (Brodsky *et al.*, 1994; Vliegen *et al.*, 1995). Similar observations obviously apply to the mice (Liu *et al.*, 2010). Following MI, except for compensatory hypertrophy in the remaining intact parts of the ventricles, most CMs with positive cell cycle marker Ki-67 in the peri-infarct area exhibited polyploidy in human hearts (Meckert *et al.*, 2005). Though cardiomyocyte hypertrophy accompanied by polyploidization is common in humans and mice, whether polyploidy is a prerequisite or a consequence of hypertrophic growth is unclear.

## Amitosis

Amitosis is recognized as a specialized form of cell division with simple cleavage of the nucleus without the formation of a spindle figure or chromosomes. This process widely exists in prokaryotes and eukaryotes (Vitali et al., 2021), including hepatocytes (David and Uerlings, 1992), pancreatic acinar cells (Nagata, 2003), and macrophages of humans and other mammals (Arkhipov et al., 2008; Il'in et al., 2018; Iljine et al., 2013). So far, little attention has been paid to the role of amitosis in CM proliferation. In the 1980s, Pefitzer et al proposed that the formation of binucleate myocardial cells and occasionally dumbbell-shaped restitution nuclei resulted from mitosis with impeded cellular divisions rather than amitosis (Pfitzer, 1980). However, binuclear cells gradually increased following the decreased mitotic cells during developmental stages. Guo et al., speculated that binuclear cells came from amitosis, which might be a primary division pattern during the adult stage (Guo et al., 2011). Shi and his colleagues also found that the goat CMs proliferated mainly in an amitotic way through nuclear transverse splitting by observing the paraffin sections of the hearts of 12 adult goats. These evident amitotic phases existed in adult goat ventricular muscle cells, including working and conducting cardiac muscle cells (Shi, 2007). Collectively, there still needs a more scientific understanding of amitosis in CM proliferation.

## Regulation of miRNAs in CMs proliferation

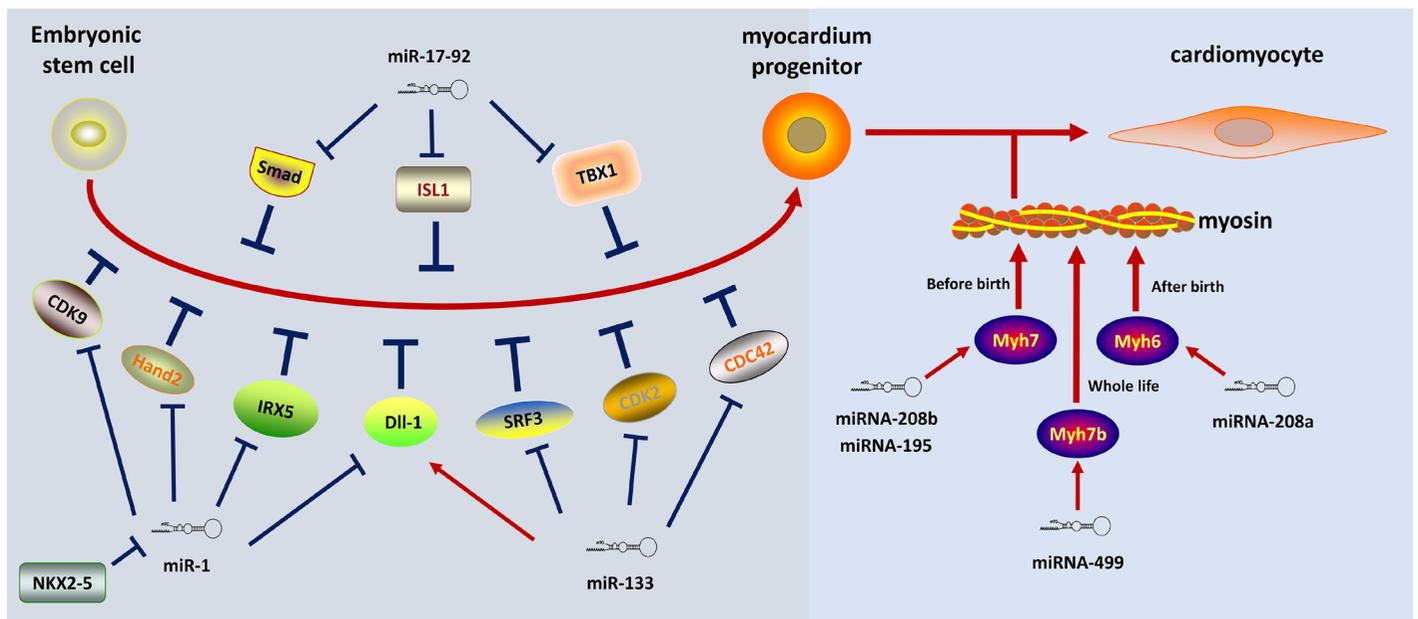
Several of the miRNAs expressed in embryonic stem cells were reported to induce the proliferation of neonatal CMs. It is worth noting that these miRNAs share an identical or almost identical seed sequence, thus binding to a pile of overlapping targets of mRNAs. For instance, miR302-367 clusters were shown to decrease in expression during development and concomitant with the loss of CM proliferation capacity while they were reactivated after MI-induced cardiac proliferation (Tian et al., 2015). They

were encoded by a single polycistronic transcript and produced five precursor miRNAs, including miR-302a, miR-302b, miR-302c, miR-302d, and the unrelated miR-367, the first of which shared the same seed sequence AAGUGCU (Barroso-del Jesus et al., 2009).

Recent advances have identified specific miRNA contributions to cardiac development and disease, as well as cardiomyocyte apoptosis and cell-cycle reentry after injury, which may generate fresh insights into developing miRNA diagnostics and miRNA inhibitors.

## The role of specific miRNAs during cardiac development

Several published studies outline the critical role of miRNAs in myocardial development in the embryo (Fig. 2). miR-1 and miR-133 were the first identified miRNAs necessary for heart development, the former being the most abundant miRNA in the mouse heart (Zhao et al., 2007). While miR-1 and the related miR-133 arose from a common precursor RNA and worked together to promote the differentiation of mesoderm to CMs by targeting Dll-1, miR-1 stimulated, and miR-133 inhibited cardiac formation in the mid-late stage (Porrello, 2013). Serum response factor (SRF) acts as a regulator of cardiac and smooth muscle differentiation genes in cell cycle progression. The mesodermal gene expression of SRF-deficient embryonic stem cells could be promoted by miR-1 and miR-133 (Ivey et al., 2008). The positive effects of miR-1 in cardiac development are mainly mediated by targeting the IRX5 and Hand2 transcription factors (Ono et al., 2011). Conversely, the transcription factor NKX2-5 acted as an upstream inhibitor of miR-1 (Qian et al., 2011). Further, the overexpression of miR-1 mediated the inhibition of myocardial differentiation via targeting cdk9, which induced the activation of cardiac-specific genes (Takaya et al., 2009). In addition, cyclinD2, SRF3, and CDC42 were identified as targets of miR-133, inhibiting cardiomyocyte proliferation in the developing heart (Carè et al., 2007; Liu et al., 2008). Knock-down experiments confirmed that the loss of miR-133a led to lethal ventricular-septal defects in embryos (Liu et al., 2008).



**Fig. 2. The role of specific miRNAs during cardiac development.** The red arrow shows the stimulatory modification while the blue "T" symbol represents the inhibitory modification.

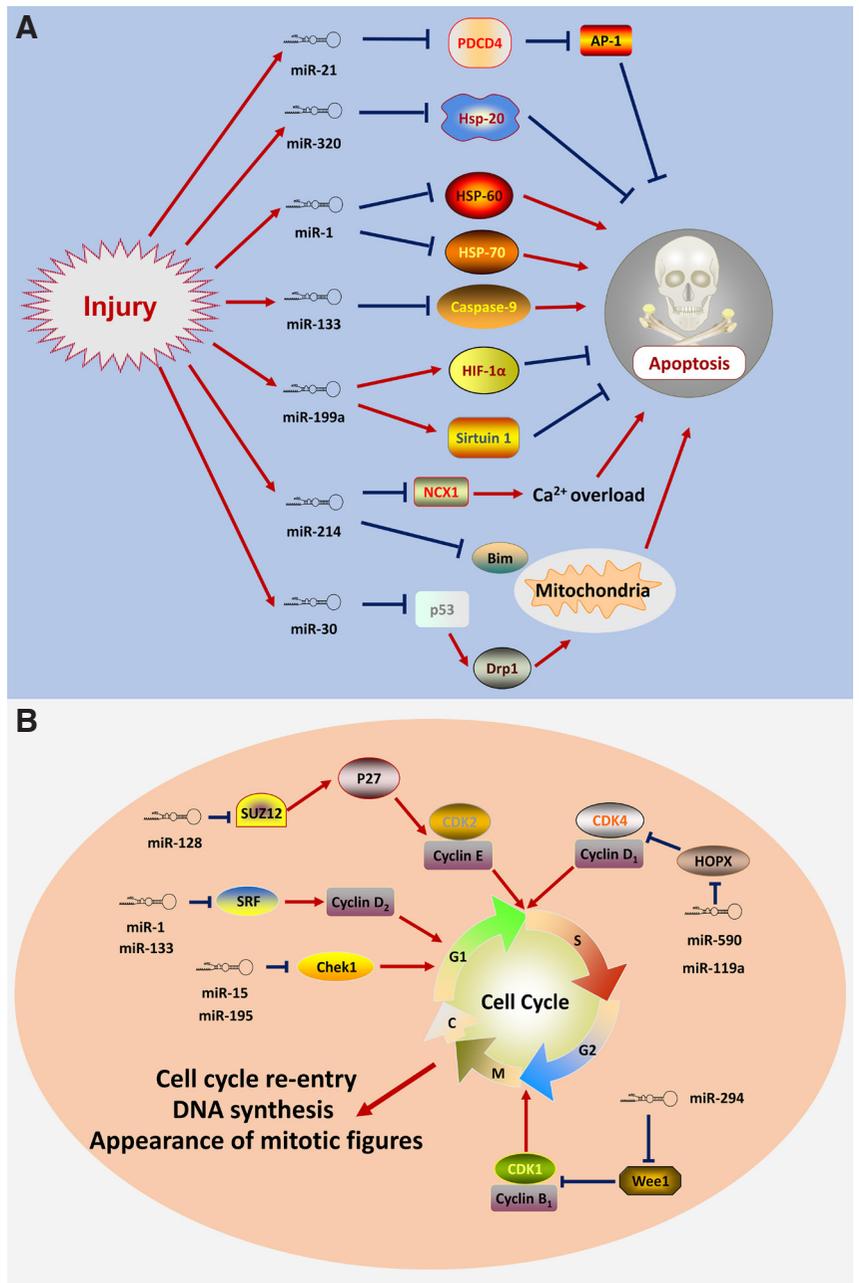
The miR-17-92 cluster is essential for embryonic cardiac development and postnatal cardiogenesis stages. miR-17-92 cluster was indispensable for signaling cascade in the second heart field via targeting conserved Smad-binding sites, as required for the differentiation of the cardiac outflow tract (Wang *et al.*, 2010). Genomic deletion of the miR-17-92 cluster led to ventricular-septum defects and lethal lung hypoplasia (Ventura *et al.*, 2008). Additionally, the downregulation of the cardiac progenitor genes ISL1 and TBX1 appear to be under the control of miR-17-92, thus mediating myocardial differentiation (Wang *et al.*, 2010).

The expression of myosin heavy chain protein of the adult heart was mediated by miR-499, miR-208a, and miR-208b, which expressed in parallel with their respective host genes during development. miR-208a resides in the myosin heavy chain 6 (Myh6) gene encoding the myosin heavy chain protein of the adult heart, whereas miR-208b and miR-499 are co-expressed together with the slow myosin Myh7 and Myh7b (Callis *et al.*, 2009). Following the inhibition of miR-208a expression, fast skeletal markers were shown in the adult heart, suggesting the Myh6 intron-encoded miR-208a guided cardiac differentiation (van Rooij *et al.*, 2007). Thrap1 (a component of the thyroid hormone receptor complex) and myostatin were identified as potential targets of miR-208, mediating effects on miR-208 in the developing and adult heart. Of note, miR-208a/b and miR-499 primarily play a surrogate role during heart development, suggesting the combination of miR-208 and Thrap1 might be nonessential for early cardiac development (Boettger and Braun, 2012). Additionally, miR-208a positively regulates the CX40, HOPX, and GATA4 genes, known as the powerful regulators of heart expression profile and function (Callis *et al.*, 2009). miR-499 promotes the differentiation of cardiac progenitor into CMs via suppressing SOX6 (Pang *et al.*, 2019). Further, upon initiating of cardiac hESC differentiation, miR-499 facilitates ventricular specification (Fu *et al.*, 2011).

miR-195 and miR-497, identified as members of the miR-15 family, were upregulated during early postnatal cardiac development of the mice between postnatal day one and postnatal day ten. In a consistent manner, CMs exited from cell proliferation with subsequent binucleation. In contrast, it has also been shown that miR-195 overexpression in the embryonic heart inhibited the expression of mitotic genes and caused precocious cell cycle arrest (Porrello *et al.*, 2011a).

**Specific miRNAs in cell-cycle reentry after injury**

Cardiomyocyte apoptosis is associated with cardiac disorders, including MI, cardiomyopathy, cardiac hypertrophy, and anthracycline-induced cardiotoxicity. The cardiomyocyte apoptosis cannot be compensated by efficient cell proliferation, thus resulting in contractile dysfunction and the progression of the disease. Recent advances in identifying miRNA that contributed to cardiomyocyte



**Fig. 3. Specific miRNAs in cell-cycle re-entry after injury. (A)** MiRNAs affect the apoptosis process of cardiomyocytes after injury. **(B)** The cell cycle-relevant miRNAs regulate the cell cycle re-entry and DNA synthesis. The red arrow shows the stimulatory modification while the blue “T” symbol represents the inhibitory modification.

apoptosis may provide a mechanism for the pathogenesis after injury (Fig. 3A). Following ischemia/reperfusion, the expression of miR-320 was shown to increase via targeting heat-shock protein 20 (Hsp20) which is known as a cardioprotective protein. Further, knock-down experiments confirmed the defect of miR-320 can attenuate cell death after injury (Ren *et al.*, 2009). Programmed cell death 4 (PDCD4) appears to be the primary target of miR-21, mediating the protective effects on the H<sub>2</sub>O<sub>2</sub>-induced injury. Additionally, Activator protein 1 (AP-1) was identified as the downstream signaling molecule of PDCD4 that is involved in the miR-21-mediated

effect on cardiac myocytes (Cheng *et al.*, 2009). The muscle-specific microRNAs miR-1 and miR-133 produce opposing effects on apoptosis. miR-1 promotes apoptosis by downregulating heat shock protein-60 and heat shock protein-70 levels, while miR-133 participates in the inactivation of apoptosis by reducing caspase-9 expression (Xu *et al.*, 2007). The hypoxia-inducible factor (Hif)-1 $\alpha$  and Sirtuin1 (Sirt-1) were inhibited after the decrease of miR-199a, which recapitulates hypoxia preconditioning in CMs (Rane *et al.*, 2009). The cardioprotective roles of miR-214 after injury were attributed to the repression of the mRNA encoding sodium/calcium exchanger 1 (Ncx1) to attenuate calcium overload, thus repressing several downstream effectors of Ca<sup>2+</sup> signaling that mediated cell death. Moreover, miR-214 promoted cell survival by inhibiting the expression of the pro-apoptotic protein BIM (Aurora *et al.*, 2012). Mitochondrial fission is involved in apoptosis initiation. miR-30 family members contributed to the inhibition of mitochondrial fission and apoptosis by suppressing the expression of p53, which upregulates dynamin-related protein-1 (Drp1) (Li *et al.*, 2010). Considering all of this evidence, it seems that miRNAs are involved in cardiac apoptotic machinery, which might indeed be translatable to future approaches by inhibiting apoptosis after injury.

Following the decreased number of CMs induced by MI, the subject of the following might be focused on facilitating cell cycle re-entry of CMs by manipulating the miRNA network (Fig. 3B). Cyclin D2 and SRF were identified as primary targets of miR-133, mediating the regulation of the retinoblastoma protein phosphorylation during the G1 period (Liu *et al.*, 2008). miRNA-590 and miRNA-119a promoted the cell cycle re-entry of adult CMs by downregulating HOPX, thus promoting the activity of cyclin D1/CDK4 complex and increasing cardiomyocyte proliferation in neonatal and adult animals. This process induced cardiac regeneration after MI based on the high-throughput screening by a whole-genome miRNA library for the significant miRNAs in the process of CMs proliferation (Eulalio *et al.*, 2012). Moreover, the overexpression of miR-294 in adult mice hearts promoted the cell cycle re-entry of CMs by repressing the expression of Wee1, thus increasing the activity of cyclin B1/CDK1 complex (Borden *et al.*, 2019). In addition, the miR-15 family miRNA bound to various components of the cell cycle and DNA damage response machinery, including the checkpoint kinase 1 (Chk1), whose inhibition led to improved cardiac repair after MI (Porrello *et al.*, 2013). The cardiac-specific miR-128 regulated cell cycle controller expression by facilitating the expression of Cyclin E and CDK2 (the positive cell cycle regulators) through the Polycomb Repressive Complex-associated Suz12 protein (Huang *et al.*, 2018).

## Discussion

Humans are not qualified to repair a “broken” heart now, whereas the zebrafish, regarded as the most classical model in heart development and regeneration, showed its excellent ability to regenerate (Sehring *et al.*, 2016). There are remarkable differences in adult hearts between mammals and lower vertebrates in regeneration based on ontogenetic or phylogenetic factors. Acquaintance with similarities in the trigger factors of cardiac regeneration between zebrafish and mammals may help us find more applications in mammals and therapeutic strategies.

The relevant therapeutic approaches have focused on the replacing of dead or dying CMs, for instance, by autologous cell transplantation of muscle progenitor cells or adult stem cells (Hi-

rose *et al.*, 2019), in parallel with unclear benefits, mode of action, and potential side effects. Bergmann *et al.*, showed a high turnover rate of cardiac mesenchymal stem cells (CMSCs) (Bergmann *et al.*, 2015), which signaled cardiomyocytes thus playing a crucial role in regeneration after injury. According to shared functions and commonly expressed biomarkers, Cencioni *et al.*, referred to CMSCs as a general population, including cardiac precursor cells, pericytes, or cardiac fibroblasts (Chiara Cencioni *et al.*, 2017). However, the latter cell population showed a limited differentiation potential under appropriate conditions (Driesen *et al.*, 2014). CMSCs actively participated in the process of cardiac remodeling, emerging as a source of cardiac stem cell reservoir or secreting extracellular matrix to maintain the physical and mechanical integrity (Souders *et al.*, 2009), whose therapeutic effects could also be enhanced by genetic and epigenetic interventions (Ieda *et al.*, 2010; Jayawardena *et al.*, 2015; Qian *et al.*, 2012). CMSCs also contributed to the cardiac electrical conduction system based on the interconnection with cardiomyocyte gap junctions enriched in Cx43 and Cx45 (Zeigler *et al.*, 2016). Additionally, cardiac fibroblasts, which account for a majority of cells in the heart, were reprogrammed into functional cardiac-like myocytes with forced expression of four transcription factors, GATA4, HAND2, MEF2C, and TBX5, improving cardiac function and reducing adverse ventricular remodeling following myocardial infarction (Song *et al.*, 2012). With the plasticity, reduced immunogenicity, and the relative simplicity of isolation and culture *ex vivo* (Gourdie *et al.*, 2016), CMSCs become a strategy for the exogenous replacement of necrotic myocardium.

An alternative target is stimulating the re-entry of mature CM into the cell cycle. Increasing numbers of evidence showed that miRNA controlled this process both positively and negatively after MI. The miRNAs, which were not expressed in the heart but impacted cell proliferation by delivering to CMs exogenously, also had obvious therapeutic implications. Several human miRNAs were identified to induce CM proliferation, including CMs of neonatal rodents and human iPS-derived CMs (Eulalio *et al.*, 2012). One of the most effective pro-proliferation miRNAs is miR-199a-3p. miR-199a-3p, known as a tumor suppressor, directly targeted the regulatory factors in cancer cell proliferation and metastasis, including c-Met (Migliore *et al.*, 2008), mammalian target of rapamycin (Callegari *et al.*, 2018) and CD44 (Gao *et al.*, 2015). In CMs, miR-199a-3p expressed at a relatively low level after birth while its exogenous transfection remarkably induced CMs proliferation in both neonatal and adult heart (Pandey and Ahmed, 2015), lighting on exploring potential therapeutic approaches in humans. The let-7 family, belonging to inhibitory miRNAs, were endogenously expressed in CMs, especially in the transition from fetal to neonatal life, and were highly expressed as CMs exited the cell cycle (Johnson *et al.*, 2007). Invariably, the levels of these miRNA clusters were increased during heart regeneration in zebrafish compared to mammals in which regeneration failed to occur (Aguirre *et al.*, 2014). The regenerative effects in mice by inhibiting these miRNAs after MI light on exploring potential therapeutic approaches of let-7 family miRNAs (Hu *et al.*, 2019). Other inhibitory miRNAs included the muscle tissue-specific miR-1~133 cluster (Zhao *et al.*, 2007; Zhao *et al.*, 2005), particularly miR-133 by inhibiting cardiac regeneration in zebrafish and miR-128 by regulating cell cycle controller expression (Huang *et al.*, 2018; Yin *et al.*, 2012). Altogether, the observations reported so far hint at the possibility of achieving cardiac regeneration by delivering several miRNAs to CMs exogenously or inhibiting other

miRNAs from re-entering the cell cycle. Despite the emergence of the engineered oligonucleotides called “antagomirs” to silence endogenous miRNAs (Lin *et al.*, 2009), there remains a paucity of evidence on a rigorous assessment of the safety and improvement in delivery methods required for setting RNA-based therapeutics as an innovative reliable tool to promote cardiac regeneration in patients.

In addition to cardiomyocytes, many noncardiomyocyte types contribute to the maintenance of cardiac function and regeneration after injury with therapeutic potential. The epicardium also emerges as a source of vascular smooth muscle cells, pericytes, and fibroblasts during heart development and repair. It secretes essential factors for cardiomyocyte proliferation and survival and, therefore, represents an important target for therapeutic interventions (Packer, 2018). Macrophage recruitment is essential for heart regeneration through the secretion of oncostatin M (Li *et al.*, 2020b). Additionally, monoclonal cells resident in the heart and T-regulatory cells during pregnancy played a paracrine regulatory role in CMs proliferation (Lavine *et al.*, 2014; Zacchigna *et al.*, 2018). The regulatory T-cells serve as a source of regenerative factors in a paracrine manner. Their secreted factors, such as chemokine ligand 24, growth arrest-specific 6, or amphiregulin, facilitate CMs proliferation (Li *et al.*, 2019). Subsequently, Li *et al.*, further demonstrated that the specific ablation of CD4+ T-cells potentiated heart regeneration and directly or indirectly facilitated the polarization of macrophages away from the pro-fibrotic M2-like signature in the juvenile heart (Li *et al.*, 2020a).

The stroma signals to CMs, thus playing a crucial role in their proliferation. Lysophosphatidic acid (LPA) acts as a potent extracellular signal in various biological processes, and diseases and LPA3-mediated LPA signaling was shown to play a pivotal role in CMs proliferation (Wang *et al.*, 2020a). The extracellular matrix component was shown to affect the growth and differentiation of CMs in mice, mediated by agrin (Bassat *et al.*, 2017) and myeloid-derived growth factor (Wang *et al.*, 2020b). The extracellular protein reelin (RELN) was required for efficient heart repair and function after neonatal MI. The exogenous delivery of RELN potentiated heart function in adult mice after MI (Liu *et al.*, 2020). Work carried out in the past few years has explored a set of cytokines and growth factors known to stimulate the proliferation of neonatal CMs, including interleukin-6 (Przybyt *et al.*, 2013), follistatin like 1 (Wei *et al.*, 2015), platelet-derived growth factor (Hinrichsen *et al.*, 2007), fibroblast growth factor family (Engel *et al.*, 2006) and neuregulin-1 (Zhao *et al.*, 1998).

## Conclusion

Heart regeneration needs not only the proliferation of a single cell population but also the synergetic effects of different types of cells and extracellular matrix components. Although a growing body of evidence indicates the potential capacity of proliferation in the embryonic and neonatal heart by manipulating the miRNA network that is naturally permissive for regeneration, in parallel, there are less research focused on the stimulation of adult CM proliferation in adult or aging animal hearts. Hopefully, this review will contribute to a deeper understanding of heart regeneration in mammals and its microRNAs regulatory mechanisms that merit further and translational research to possibly one day be able to regenerate a diseased adult human heart.

## Conflicts of interest

The authors declare no conflict of interest associated with this manuscript.

## Author contributions

Shu-ning Guo and Jie-han Li performed the literature search and drafted the manuscript. Both of them contributed equally to this review. Yu-rong Chai, Yi Ding, and Jing Li critically revised the work. All authors read and approved the final manuscript.

## Funding

This research was supported by the Key Scientific Research Project of Higher Education Institution in Henan Province (No: 21A310022).

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