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Novel strategies to repair the infarcted heart

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Novel strategies to repair the infarcted heart

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Editorial: Straight from the heart: Novel insights and future perspectives for cardiac repair

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Editorial on the Research Topic
Straight from the heart: Novel insights and future perspectives for cardiac repair

As Editors of this Research Topic, we reviewed with great interest several articles and reviews on the relevant strategies for cardiac repair and discussed their translational potential. In this editorial, we will summarize the key aspects and main findings of the accepted articles.

In the last 20 years, cell therapies have initially ignited high expectations as novel strategies to address myocardial regeneration following injury and disease. While bona fide results in terms of the generation of new functional contractile cardiomyocytes using cell therapy have been broadly debated, several independent lines of investigation have reported beneficial effects following the infusion of progenitor cells on other aspects of cardiac disease. Here, Bassetti et al. discuss an updated overview of the impact of cell therapy on the treatment of patients affected by refractory angina (RA). Indeed, RA management denotes an unmet clinical need due to the disease increasing prevalence within the aging population. Nonetheless, progenitor cell-based therapies have been shown in preclinical studies and published trials to be safe and reduce angina symptoms while improving myocardial perfusion and supporting cardiac function in the long term. According to the authors, cell therapy may provide a reliable therapeutic strategy for RA patients as long as it is considered as a range of medicinal products to be developed according to specific regulatory pathways and international guidelines.

Recent progresses in cell biology and tissue engineering have shed new light on innovative strategies to optimize endogenous mechanisms of cardiac repair for ischemic heart disease; yet, cardiac regeneration still represents an extremely challenging goal for translational research. In this Research Topic, several different strategies have been proposed in order to provide cardiomyocyte reconstitution: bioengineering methods to obtain direct reprogramming of cardiac fibroblasts into induced and transdifferentiated cardiomyocytes (iCMs) by Paoletti and Chiono, and rebooting myocardial renewal *via* dedifferentiation, and proliferation of pre-existing cardiomyocytes, by Bongiovanni et al. In the first overview, microRNA (miRNA)-driven reprogramming of fibroblasts into iCMs is comprehensively discussed as a promising translational approach through local injection of the reprogramming molecules into myocardial fibrotic areas. Authors particularly focus on the relevance of biochemical and biophysical factors enhancing direct cardiac reprogramming and the definition of efficient and secure nanocarriers for miRNA delivery. Bongiovanni et al. instead focus their perspective on working strategies addressing direct stimulation of dedifferentiation and proliferation of pre-existing cardiomyocytes for heart regeneration. The authors describe how mammalian cardiomyocyte cell cycle activity is controlled during prenatal and postnatal age. They further discuss how fine-tuning of micro-environmental, extracellular and intrinsic molecular

mechanisms affecting cell cycle checkpoints, cytoskeleton arrangement, and energetic metabolism can re-activate cardiomyocyte proliferative and extend their regenerative potential for future clinical translation.

The review by Lodrini and Goumans falls within a similar scope. However, instead of focusing on the formation of new cardiomyocytes, the authors discuss the cellular processes that ensue after MI, such as apoptosis, necrosis, autophagy, and cellular senescence. Intervening with these processes may result in cardioprotection and increased survival of functional cardiomyocytes after MI.

Also, mitochondrial metabolism has been shown to be crucial for homeostatic processes, including cell proliferation and differentiation and has profound implications during the development and regeneration of the heart. The regenerative capacity of the heart is lost by the first week after birth, partially due to a metabolic shift from glycolysis to fatty acid oxidation. In the review by Bae et al., the authors highlight the mechanisms that regulate cardiac metabolism and could be exploited for future intervention during development, disease, and regeneration.

The era of transcription profile analyses and transcriptional engineering at single-cell resolution is fast evolving. In the review by Schoger et al., the authors discuss how single-cell transcriptomics has extended our knowledge and opened the door for emerging CRISPR/Cas9 technologies in clinical applications. Single-cell transcriptomics can identify changes in the cellular composition of the heart and heterogeneity within the same cell types in healthy and diseased hearts. Integrating this information with the revolutionizing CRISPR/Cas9 technology will greatly advance medical research and open a new chapter of precision and personalized medicine. In the last few years, CRISPR-mediated gene editing has already entered the clinic, making the application of CRISPR/Cas9 approaches a realistic option for more specific treatments of different cardiac diseases.

Streef and Smits offer another perspective on endogenous repair mechanisms by focusing on a cardiac cell type with a specific role in heart development in the embryo: the epicardium. These epicardial cells contribute to formation of cardiac tissue during embryogenesis by contributing cells and by producing paracrine factors. In the adult heart, the epicardium is activated upon injury, but partcipation to cardiac tissue formation is limited. By summarizing data from recent cardiac single-cell studies, Streef and Smits provide insights into the cellular composition of the epicardium during cardiogenesis and in the adult heart including cross-species differences. This information could help optimize the post-MI response of endogenous cell types with a reparative potential.

One of the sequalae of MI is sudden cardiac death (SCD), which is often the result of arrhythmogenesis. There is a potential association between cardiac sympathetic hyperinnervation and SCD. However, the underlying mechanism for hyperinnervation is unclear. Ge et al. investigate how the superior cervical ganglion -which participates in the sympathetic innervation of the heart- and the adjacent carotid body are affected over time after MI. Interestingly, they show that neuronal remodeling occurs within the ganglion toward an adrenergic phenotype and larger neuronal size. This effect is potentially mediated by the carotid body. These data provide a direction for the potential mechanism underlying hyperinnervation of the heart after MI. The authors suggest that the next step is to investigate the functional implications of these findings.

Other strategies to repair the heart after MI include the application of cardio-supportive devices such as cardiac patches. Feng et al. have produced a reduced graphene oxide (rGO)/silk fibroin-modified nanofibrous biomaterial that they sutured onto the infarcted rat heart. These patches can likely improve cardiac systolic function and ventricular remodeling by directly regulating an anti-fibrotic effect in cardiac fibroblasts.

In recent years, growing evidence indicates that non-coding RNAs play essential roles in regulating tissue homeostasis and pathophysiological conditions. Long non-coding RNAs (lncRNAs) are >200-nucleotide long transcripts that can interfere with gene expressions and signaling pathways in different tissues. LncRNAs have been found to be important in the field of cardiovascular medicine in both healthy and diseased conditions. Du et al. demonstrated that overexpression of the lncRNA N1LR improved cardiac function, reduced inflammatory response, and protected from cardiac fibrosis in a mouse model of acute myocardial infarction. This cardioprotective effect was due to decrease TGF-beta and Smad signaling, which activation is associated with cardiac fibrosis, making lncRNA N1LR a promising target for future clinical applications.

As this Research Topic has emphasized, the route to repair after a cardiac injury can be multifactorial, including approaches such as a reduction in damaged tissue (cardioprotection), the induction of cardiomyocyte proliferation, increasing the participation of local regenerative cell types, adjusting metabolism, hyperinnervation, or the fibrotic response. It has been a pleasure to edit this special issue and showcase the multitude of possibilities for cardiac repair that may hopefully contribute to a better quality of life for patients suffering from cardiac disease.

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REVIEW
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The Role of Metabolism in Heart Failure and Regeneration

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Heart failure is the leading cause of death worldwide. The inability of the adult mammalian heart to regenerate following injury results in the development of systolic heart failure. Thus, identifying novel approaches toward regenerating the adult heart has enormous therapeutic potential for adult heart failure. Mitochondrial metabolism is an essential homeostatic process for maintaining growth and survival. The emerging role of mitochondrial metabolism in controlling cell fate and function is beginning to be appreciated. Recent evidence suggests that metabolism controls biological processes including cell proliferation and differentiation, which has profound implications during development and regeneration. The regenerative potential of the mammalian heart is lost by the first week of postnatal development when cardiomyocytes exit the cell cycle and become terminally differentiated. This inability to regenerate following injury is correlated with the metabolic shift from glycolysis to fatty acid oxidation that occurs during heart maturation in the postnatal heart. Thus, understanding the mechanisms that regulate cardiac metabolism is key to unlocking metabolic interventions during development, disease, and regeneration. In this review, we will focus on the emerging role of metabolism in cardiac development and regeneration and discuss the potential of targeting metabolism for treatment of heart failure.

Keywords: heart regeneration, development, metabolism, heart failure, cell cycle

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INTRODUCTION

Heart failure is the leading cause of morbidity and mortality worldwide. In the United States alone, there are over 6,000,000 people with heart failure (1). This is largely due to the inability of the adult mammalian heart to replenish the lost myocardial tissue following injury, which results in the progressive weakening of the heart muscle and the development of heart failure (2). Current therapies are focused on preventing further remodeling of the remaining myocardial tissue. Heart transplantations are the only treatment in patients with severe heart failure (3). Due to the complexity and complications associated with heart transplants they are not always a suitable treatment; therefore, identifying novel therapeutic approaches to promote adult heart regeneration provides immense opportunities to advance heart failure therapy. Endogenous heart regeneration following injury has been demonstrated in some non-mammalian vertebrates (4, 5). Interestingly, neonatal mice are also capable of regenerating their heart tissue following injury, however this regenerative ability is lost within a few days following birth (6, 7). These models of endogenous regeneration provide us with a platform to elucidate the mechanisms that guide heart regeneration to reactivate these processes to promote adult heart regeneration.

Embryonic and neonatal cardiomyocytes produce energy primarily *via* glycolysis, where postnatal maturation is accompanied with a metabolic switch to fatty acid oxidation to meet the energy demands of adult cardiomyocytes (8) (**Figure 1**). This metabolic switch contributes to the postnatal cardiomyocyte cell cycle exit and loss of the regenerative potential of the mammalian heart. This underscores the potential role of cardiac metabolism as a target to promote adult heart regeneration.

In this review, we highlight major studies of cardiac metabolism including fatty acid oxidation, glucose, and amino acid metabolism (**Figure 2**). We also discuss key metabolic targets that may play an important role during cardiomyocyte development and regeneration and their potential as a therapeutic target for adult heart disease.

ENERGY METABOLISM AND HEART REGENERATION

The heart is the most energy-consuming tissue (per gram) in the human body (9), and energy production takes place in the mitochondria. The main function of the mitochondria is generating energy as adenosine triphosphate (ATP); thus, mitochondria play an essential role during development, cellular proliferation, and tissue regeneration, all of which are energy demanding processes (10–12).

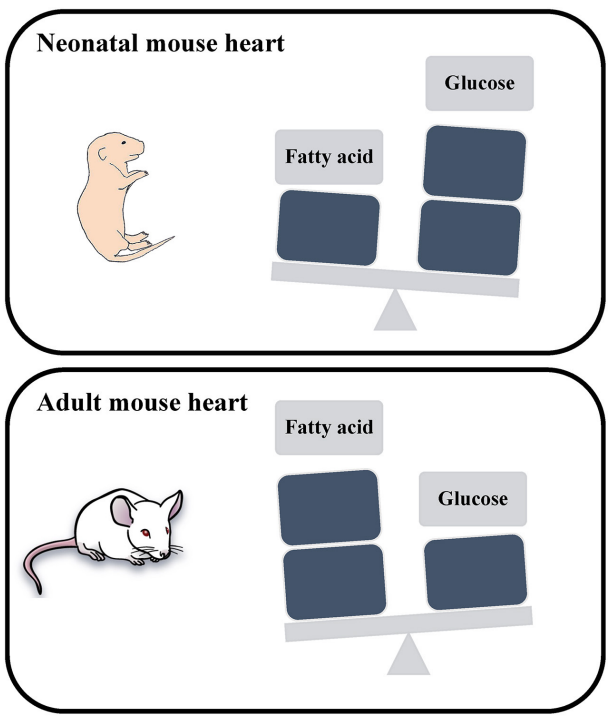

FIGURE 1 | Schematic representation of the energy utilization in neonatal and adult mouse hearts. The neonatal mouse heart generates energy through glucose metabolism, while the adult mouse heart generates energy through fatty acid oxidation.

Heart regenerative capacity differs by model organisms from vertebrates to human. Zebrafish and newts have a remarkable capability to regenerate their hearts after injury. Zebrafish heart regeneration is primarily derived from the proliferation of the pre-existing cardiomyocytes (13, 14). Following injury, cardiomyocyte dedifferentiation and proliferation are required to regenerate the heart following injury. Interestingly, single-cell transcriptional analysis of regenerating zebrafish hearts demonstrate that proliferating border zone cardiomyocytes undergo metabolic reprogramming to glycolysis from oxidative phosphorylation following cryoinjury (15). In contrast, glycolysis inhibitors including 2-deoxyglucose and lonidamine impair cardiomyocyte proliferation and heart regeneration (15). These results suggest that the glycolytic metabolic state mediates cardiomyocyte proliferation and regeneration following injury in zebrafish.

Like zebrafish, embryonic and neonatal mice can regenerate their heart after injury. Both zebrafish and neonatal mouse hearts have lower mitochondrial DNA copy numbers compared to postnatal and adult mice (16). This increase in cardiomyocyte mitochondrial number in the adult heart is due to the switch from glycolytic metabolism in neonatal cardiomyocytes to oxygen-dependent mitochondrial oxidative phosphorylation in adult cardiomyocytes (17). This metabolic switch and increase mitochondrial DNA results in a significant rise in reactive oxygen species (ROS) production from mitochondria which plays an essential role in regulating heart development and regeneration (16). This increase in ROS production contributes to postnatal cardiomyocyte cell cycle arrest. Interestingly, the increased sarcomere contraction in the postnatal heart promotes mitochondrial metabolism, which results in ROS production and DNA damage response activation *via* p53. As a result, inhibition of sarcomeres in cardiac troponin T knockout cardiomyocytes prevents cell cycle arrest and polyploidy resulting in increased cardiomyocyte proliferation (18). Furthermore, ROS scavengers such as N-acetyl cysteine (NAC) prolongs the postnatal window of cardiomyocyte proliferation and regeneration following ischemia reperfusion (I/R) injury (16).

Significant metabolic shifts occur in response to abnormal heart conditions. A healthy adult heart generates energy through fatty acid oxidation, however conditions such as pressure overload, hypertrophy, and ischemia results in a metabolic transition toward anaerobic glycolytic metabolism to be protect against damage (19). A recent study elegantly demonstrates the different metabolite utilization in human hearts by using arterio-venous metabolomics, which is a powerful tool to measure metabolite utilization in humans by measuring the metabolite intake and release in the blood from human hearts. Similar to mouse studies, healthy human hearts mostly uptake fatty acids as a fuel source while they only uptake limited amounts of glucose. Interestingly, the healthy heart releases amino acids, specifically essential amino acids. In contrast, the failing heart utilizes more ketones and lactate, but less fatty acids (20). These results are consistent with previous animal studies demonstrating that ketones and β-hydroxybutyrate are protective in the failing

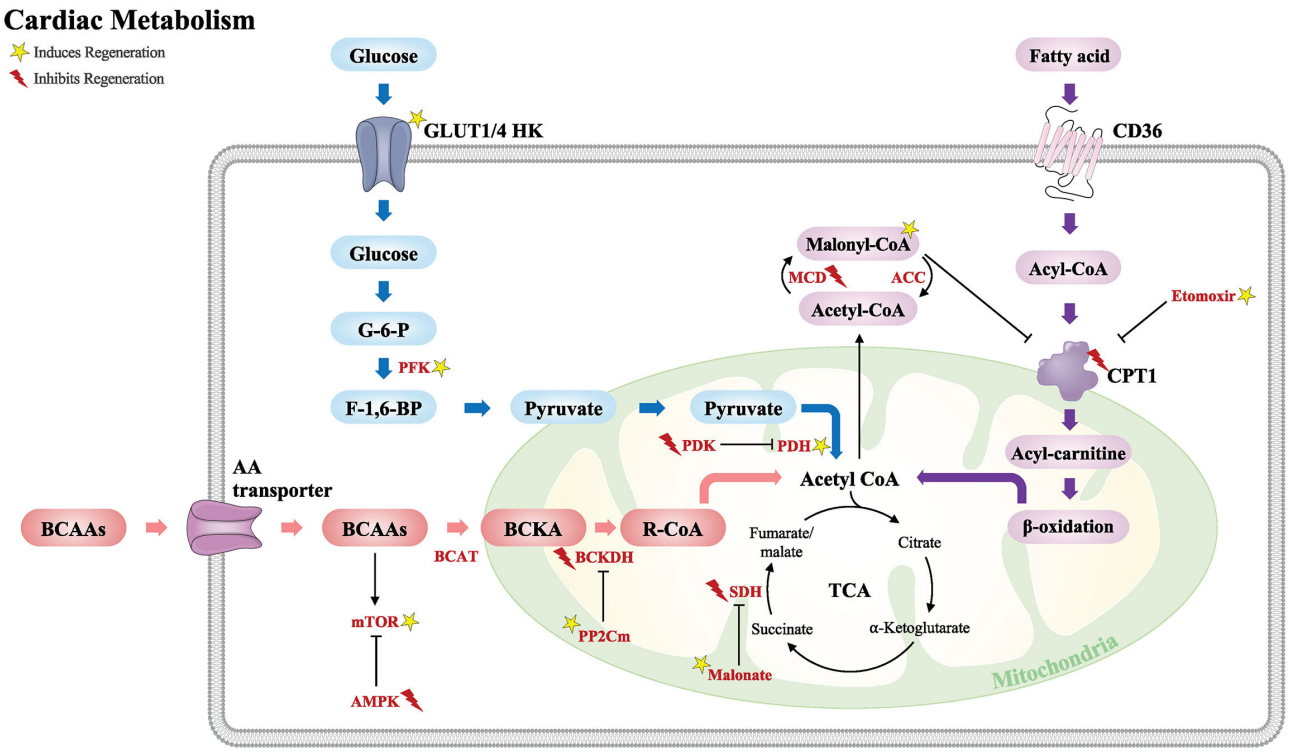

FIGURE 2 | Schematic of the major metabolic pathways that modulate the cardiac regenerative response following injury. Glucose metabolism (blue), fatty acid metabolism (purple), and BCAA metabolism (red). Acetyl CoA from these major metabolic pathways is required for the TCA cycle. GLUT, glucose transporter type; HK, hexokinase; G-6-P, glucose-6-phosphate; F-1,6-BP, fructose-1,6-biphosphate; PFK, phosphofructokinase; PDK, pyruvate dehydrogenase kinase; PDH, pyruvate dehydrogenase; CD36, cluster of differentiation; CPT1, carnitine palmitoyltransferase; MCD, malonyl CoA dehydrogenase; ACC, acetyl CoA carboxylase; BCAAs, branched-chain amino acids; AA, amino acid; BCAT, branched-chain amino-transferase; BCKA, branched-chain alpha keto acids; BCKDH, branched-chain alpha-keto acid dehydrogenase; PP2Cm, protein phosphatase 2Cm; mTOR, the mechanistic target of rapamycin; AMPK, 5′ adenosine monophosphate-activated protein kinase; TCA, tricarboxylic acid cycle; SDH, succinate dehydrogenase. Yellow star induces regeneration and red lightning bolt inhibits regeneration.

heart (21, 22). Collectively, these studies demonstrate that cardiac metabolism is dynamic and can switch to different states during development, disease, and regeneration.

FATTY ACID OXIDATION IN THE HEART

The heart requires high amounts of energy to maintain adult cardiac physiology (9). The adult human heart generates ATP *via* fatty acid oxidation (23–25). Fatty acids are oxidized through the tricarboxylic acid (TCA) cycle in the mitochondria, and the intermediate electrons from the TCA cycle flow through the electron transport chain (ETC) and produce a proton gradient to generate energy through ATP synthesis (26).

The first step for transporting long chain fatty acids from the cytosol into the mitochondria for initiating mitochondrial fatty acid oxidation occurs by carnitine palmitoyltransferase I (CPT1) in the outer mitochondrial membrane. CoA in acyl-CoA, which is derived from fatty acids, is converted to carnitine through CPT1. Thus, CPT1 is a key enzyme in regulating fatty acid oxidation. There are three tissue-specific isoforms of CPT1 that exist in mammalian tissues: CPT1A is expressed in the liver, lung, spleen, pancreas, and kidney; CPT1B is expressed in the heart, skeletal muscle, and adipose tissue; and CPT1C is expressed in the brain (27). Mitochondrial CPT1 activity is very low in the neonatal rat heart. Interestingly, CPT1 level is significantly increased in 7-day-old juvenile mice, which is the timepoint when the majority of mammalian cardiomyocytes have already exited the cell cycle (28). CPT1 expression is increased in adolescent (6 months) sheep hearts compared to fetus (105 days) hearts (29). Thus, CPT1 could be a key regulator of cardiomyocyte proliferation.

CPT1 inhibition reduces fatty acid oxidation due to the blockade of fatty acid transfer into the mitochondria. Inhibition of CPT1 by etomoxir promotes neonatal mouse cardiomyocyte proliferation (30). However, inhibition or activation of CPT1 does not induce cardiomyocyte proliferation in the adult mouse heart (31). Ventricular cardiomyocytes isolated from neonatal mice injected with the CPT1 inhibitor etomoxir show a reduction in fatty acid oxidation genes (30). These results demonstrate that disruption of fatty acid oxidation by inhibition of CPT1 extends neonatal cardiomyocyte proliferation and heart regeneration but is not sufficient to promote adult heart regeneration.

Another metabolite that regulates fatty acid oxidation *via* CPT1 inhibition is malonyl-CoA (32). Inhibition of malonyl-CoA decarboxylase (MCD), which is responsible for malonyl-CoA decarboxylation, results in increased malonyl-CoA levels which reduces fatty acid oxidation and increases glucose

oxidation (33). As a consequence, short-term pharmacological inhibition of MCD increases malonyl-CoA levels in ischemic conditions resulting in improving cardiac function during ischemia/reperfusion (I/R) injury in the swine heart (34) and following myocardial infarction (MI) in the rat heart (35). Genetically MCD deficient mouse hearts show increased glucose oxidation and improved cardiac function following I/R injury (36). These results demonstrate that malonyl-CoA improves cardiac function following injury through CPT1 inhibition.

CPT1 is also regulated by peroxisome proliferator-activated receptors (PPARs), which are lipid receptors that play a critical role in regulating energy metabolism. There are three subtypes of PPAR: PPARα, PPARβ/δ, and PPARγ (37). PPARα, β/δ, γ gene expression levels are lower in the developing mouse heart compared to 14- and 28-day-old mouse hearts (38, 39). The levels of PPARs change during aging, as cardiac PPARα is significantly reduced in aged mice (40). PPARs play multiple roles in cardiac function in several disease states. It has been shown that expression of PPARα and CPT1 is notably reduced in adult mouse hearts following transverse aortic constriction (TAC) injury (41) as well as following I/R injury (42). However, activation of PPARα using the PPARα agonist GW7647 increased CPT1 gene expression which increased fatty acid oxidation and enhanced oxygen consumption rate in the presence of the fatty acid palmitate in isolated mouse cardiomyocytes (30).

However, the role of PPAR in cardiomyocyte proliferation and regeneration remains unclear. The PPARα agonist GW7647 does not promote cardiomyocyte proliferation and cardiac function following MI in adult mouse hearts (31). Furthermore, PPARα activation by agonist WY-14643 reduced cardiac function following I/R injury (42). Moreover, larger infarct size is observed in PPARα knockout mouse heart following I/R injury (43). In contrast, another study showed that PPARα transgenic mouse hearts showed improved cardiac function and reduced left ventricular dilation following TAC injury (41).

Another PPAR family receptor, PPARδ, has been shown to play a role during cardiac injury. The PPARδ ligand, GW501516, has been shown to inhibit cardiac fibroblast proliferation and transdifferentiation to myofibroblasts (44). Furthermore, inhibition of PPARδ reduced cardiomyocyte proliferation following injury in zebrafish hearts, whereas cardiomyocyte-specific PPARδ overexpression induced proliferation and reduced scar size following MI in mouse hearts (45).

Despite the important role of PPAR receptors in a variety of heart disease models, the exact role of these receptors in regulating cardiomyocyte proliferation and heart regeneration remains to be fully defined.

GLUCOSE METABOLISM IN HEART

Although the adult mammalian heart utilizes fatty acids as a main source of energy in the heart, glucose plays an important role as an energy source (46–49). Under healthy conditions the heart mostly uses fatty acids to produce energy, however, it will switch to glucose as an energy source during heart failure (50–52). Glucose metabolism is initiated by glucose uptake. In the heart, glucose enters cardiomyocytes *via* glucose transporters (GLUTs) which are expressed by various cell types. Among 14 members of the GLUT family (53), the most abundant GLUTs in the human heart are the insulin-sensitive glucose transporter GLUT4 (54, 55), and the insulin-independent glucose transporter GLUT1 (54, 56).

Under physiological conditions, GLUT1 is the main glucose transporter in embryonic and neonatal hearts, while GLUT4 is the primary glucose transporter in adult hearts (57, 58). In heart failure, GLUT4 expression is reduced while the levels of GLUT1 increase (59). This results in an increase in GLUT1-mediated glycolysis in heart failure, suggesting that GLUT1 plays an important role in cardiac protection during heart failure. GLUT1 expression is also increased in the heart under hypoxic conditions (60), which is mediated *via* hypoxia-inducible factor-1α (HIF-1α) (61). Cardiac-specific overexpression of GLUT1 results in increased glucose uptake and glycolysis in the mouse heart (62, 63), whereas cardiac-specific GLUT1 deletion reduces glucose uptake and glycolysis in isolated mouse cardiomyocytes following TAC injury (59). Interestingly, GLUT1 overexpression enhanced the regenerative response of neonatal mice following cryoinjury by increasing the levels of glucose metabolites (64). These results provide new evidence that increased GLUT1 expression promotes cardiomyocyte proliferation and heart regeneration through increased glucose metabolism.

Once glucose enters cardiomyocytes through GLUTs, glucose is phosphorylated and metabolized by key glycolytic enzymes such as hexokinase (HK) and phosphofructokinase (PFK) to form two pyruvate molecules (65). Pyruvate is then oxidized to acetyl CoA by pyruvate dehydrogenase (PDH), a key regulator in pyruvate metabolism (66), to enter the TCA cycle in the mitochondria. These glycolytic enzymes have been demonstrated to play a role in cardiac repair and regeneration following injury. In adult zebrafish, increased glycolysis has been shown to promote cardiomyocyte proliferation through increased cell cycle gene expression following injury (67). In addition, inhibition of glycolysis by 2-deoxyglucose reduced cardiomyocyte proliferation in the injured zebrafish heart (15). Thus, key components of glycolysis play an important role during cardiomyocyte proliferation and heart regeneration.

Hexokinase (HK) is the first enzyme of glycolysis that phosphorylates glucose to glucose-6-phosphate. Among the four distinct HK isozymes (HK 1, 2, 3, and 4) (68), HK-1 and -2 are expressed in the heart and regulate cardiac glucose metabolism (69, 70). Cardiac-specific HK-2 overexpression decreased cardiac hypertrophy in isoproterenol-induced mouse hearts and reduced cardiomyocyte size in neonatal rat ventricular cardiomyocytes (71). In addition, HK-2 overexpression reduced ROS accumulation which is upregulated during cardiac hypertrophy (71). In contrast, reduced HK-2 expression in HK-$2^{+/-}$ mice results in increased cardiac dysfunction due to increase in cell death and fibrosis and reduction of angiogenesis following I//R injury (72). Whether HK plays a role during heart regeneration remains to be determined.

Another important enzyme that regulates glycolysis is phosphofructokinase (PFK) which has two isoforms: PFK-1 and PFK-2. PFK-2 regulates PFK-1 activity since PFK-2 regulates the synthesis of fructose-2,6-biphosphate, which activates PFK1 that promotes glycolysis. Thus, PFK-2 is a crucial enzyme that regulates glycolysis (65). PFK-2 is activated upon insulin stimulation which promotes glycolysis, where PFK-2 is reduced in the insulin-deficient streptozotocin-induced diabetic mice and high-fat diet-induced obese mice (73). Glycolysis and insulin sensitivity are decreased in cardiac-specific kinase-deficient PFK-2 mutant mouse hearts (74, 75). As a result, glycolysis is not increased in cardiac-specific kinase-deficient PFK-2 mice in contrast to wild type mice following TAC surgery (75). On the other hand, overexpression of kinase-active PFK-2 enhances contractility in hypoxic mouse cardiomyocytes (76). Thus, PFK-2 regulates glycolysis and may play a role in cardiac protection following injury.

A key glycolysis enzyme is pyruvate dehydrogenase kinase (PDK). There are four PDK isoforms (PDK 1, 2, 3, and 4). PDKs expression is significantly increased during heart development and is further increased in the adult heart (58). PDKs expression is also increased in the infarct zone following cardiac cryoinjury in zebrafish (67). Among the PDK isoforms, cardiac PDK4 is the most significantly upregulated enzyme in 7-day-old mice, where the majority of mammalian cardiomyocytes exit the cell cycle (58). PDKs play in a role in glycolysis *via* inhibition of pyruvate dehydrogenase (PDH), which is a limiting step in glucose oxidation. PDK inhibition by dichloroacetate induces PDH activation which promotes cardiac function following KCl-induced cardiac arrest (77). A recent study demonstrated that cardiac-specific deletion of PDK4 promotes adult cardiomyocyte proliferation and heart regeneration following adult MI (78). In summary, PDK plays an important role in glycolysis *via* inhibition of PDH activity, suggesting that PDKs may be an important therapeutic target to increase glycolysis and promote cardiac repair and regeneration.

Pyruvate kinase muscle isoenzyme 2 (PKM2), a rate-limiting enzyme in the final step of glycolysis, is expressed in embryonic and neonatal mouse hearts; however, it is significantly reduced beyond postnatal day 7 when cardiomyocytes exit the cell cycle (79). Interestingly, overexpression of PKM2 in cardiomyocytes promotes cell cycle and glucose-6-phosphate dehydrogenase expression (79). Cardiomyocyte-specific PKM2 expression by modified RNA (modRNA) promotes adult cardiomyocyte proliferation and cardiac regeneration following adult MI (79). Conversely, loss of PKM2 reduces cardiomyocyte proliferation following injury in zebrafish hearts (67). Moreover, cardiomyocyte-specific deletion of PKM2 impairs heart development as they exhibit smaller heart size and low levels of cardiomyocyte proliferation (79).

Taken together, these studies demonstrate that glycolysis plays an important role in regulating cardiomyocyte proliferation and heart regeneration following injury. Thus, targeting glucose metabolism is a promising approach to promote adult heart regeneration.

AMINO ACID METABOLISM IN THE HEART

Amino acids are key molecules for cell growth and survival. Amino acids are used as the building blocks for protein synthesis as well as inhibiting proteolysis (80). In addition, amino acids serve as precursors to key metabolites (81). Remarkably, amino acids can act as a signaling molecule, such as leucine, which stimulates muscle protein synthesis *via* the mechanistic target of rapamycin (mTOR) signaling pathway (82–84). The levels of cellular amino acids fluctuate throughout development, increasing in postnatal stages until reaching peak levels around P9 and then decreasing into adult stages suggesting a dynamic role for amino acids during development and maturation (85).

A recent study demonstrated that circulating arterial amino acid levels are reduced in patients with heart failure in comparison to healthy patients (86). Decreasing levels of arterial amino acids correlated with reduced heart function, demonstrating the potential use for arterial amino acid levels as a biomarker of heart failure (86). To understand if this reduction of circulating arterial amino acids was the heart reducing its energy consumption of amino acids a recent study aimed to quantify fuel consumption of the failing and non-failing human heart (20). This study demonstrated that energy consumption of amino acids was unchanged between the non-failing and failing hearts (20), suggesting that the role amino acids play in heart failure is not tied to their function as an energy source.

To further understand the role of amino acid metabolism in heart failure, a main focus was placed on a subset of amino acids, the branched chain amino acids (BCAAs), which are utilized differently than the other amino acids. BCAAs consist of leucine, isoleucine, and valine (87). BCAAs account for nearly 5% of total carbon used within the heart, and they also act as regulatory components for other metabolic processes (20, 88). BCAA catabolism has been shown to play a role in heart failure. This is seen in both humans and rodents where all components in BCAA catabolism have altered expression levels in heart failure (87). A study using a mouse model deficient in protein phosphatase 2Cm (PP2Cm), which is a critical component in the conversion of branch chain ketone acids to acyl-CoA derivatives *via* the branched-chain alpha-keto acid dehydrogenase complex (BCKDH), demonstrated that the knockout mice have a higher susceptibility to heart failure in response to pressure overload stress (87). This was due to the higher levels of BCAAs in the PP2Cm deficient mice, which reduced glucose breakdown *via* direct inhibition of pyruvate dehydrogenase (89).

The mechanistic target of rapamycin (mTOR) signaling pathway has been demonstrated to play an important role during heart development and growth (90, 91). Interestingly, BCAAs stimulate mTOR activation which promotes metabolic reprogramming to glycolysis from fatty acid oxidation through HIF-1α (92). In contrast, inhibition of mTOR promotes human iPSC-derived cardiomyocyte maturation and impairs zebrafish heart regeneration following injury (93, 94). mTOR is also inhibited by 5′ adenosine monophosphate-activated protein kinase (AMPK) through tuberous sclerosis complex 2 (TSC2) (95). Pharmacological activation of AMPK by metformin inhibits mTOR pathway activation following TAC injury (96).

In addition, AMPK activation by AICAR promotes human iPSC-derived cardiomyocyte maturation (97). Thus, downstream pathways of BCAAs including mTOR and AMPK can regulate cardiomyocyte proliferation and regeneration.

Conversely, stimulating BCAA catabolism can be protective against heart injury and failure. BCAA catabolism can be activated by inhibition of the branched chain ketoacid dehydrogenase kinase (BCKDK), which results in BCKDH activation (87). BCKDK inhibition increased BCAA catabolism, which increased cardiac function following TAC compared to controls (98). In addition, adenoviral overexpression of PP2Cm in infarcted diabetic mice resulted in a significantly smaller scar size compared to controls (99). These studies demonstrate that enhanced BCAA catabolism can be protective against cardiac injury.

This relationship between BCAA catabolism and heart failure demonstrate that amino acid metabolism plays a role in heart disease and repair. Future studies to dissect the role of amino acids in the heart will establish their role as an important therapeutic target in cardiovascular disease.

TCA CYCLE METABOLITES IN THE HEART

The metabolic switch from glycolysis in neonatal mice to fatty acid oxidation in adult cardiomyocytes is accompanied by a significant increase in mitochondrial number and high levels of ROS production (16). This increase in ROS levels in the postnatal heart induces cardiomyocyte DNA damage, which contributes to cardiomyocyte cell cycle exit in the adult mammalian heart (16). Thus, elucidating the role of mitochondrial metabolites in regulating this metabolic switch is critical to identify metabolic targets to promote adult heart regeneration.

Succinate dehydrogenase (SDH), also known as mitochondrial complex II, is an important enzyme in regulating cell cycle and metabolic reprogramming in cancer because SDH plays a role in both the TCA cycle and the electron transport chain (100). Metabolic reprogramming has been recognized as a hallmark of various cancers due to the unique metabolic signature of cancer (101). In the presence of oxygen, pyruvate is converted to acetyl-CoA which enters the mitochondrial TCA cycle. However, in the absence of oxygen very limited oxidative phosphorylation takes place, instead lactate production increases aerobic glycolysis (101). Interestingly, pyruvate is mostly converted to lactate in cancer cells regardless of the oxygen levels. This metabolic switch promotes cancer cell survival and cell proliferation (100–102).

Recent studies demonstrated that reverse activity of SDH during ischemia results in succinate accumulation (103, 104). The accumulated succinate is then rapidly oxidized following reperfusion and results in a burst of ROS production *via* reverse activity of the mitochondrial complex I (105). These studies suggest that ROS production due to reverse activity of SDH and succinate accumulation is a hallmark of I/R injury (105). Interestingly, SDH inhibition reduces infarct size during ischemia in Langendorff-perfused mouse hearts (106). Furthermore, the SDH competitive inhibitor malonate reduces infarct size during I/R injury in pig hearts (107). These results demonstrate that SDH inhibition during I/R injury blocks the SDH-mediated succinate accumulation, thus protecting the heart against the redox insult during I/R injury. Interestingly, a recent study demonstrated that succinate accumulation in ischemia/reperfusion is not due to the reverse activity of SDH, but rather due to canonical TCA cycle activity (108). Thus, although succinate accumulation during ischemia is conserved across vertebrates, the proposed mechanism of succinate accumulation remains to be further understood.

SDH knockdown induces cell proliferation and migration in human hepatocellular carcinoma cell lines and leads to a metabolic shift to glycolysis as demonstrated by increased level of glycolytic enzymes (109). Interestingly, a recent study demonstrated that metabolic reprogramming to glycolysis promotes cardiomyocyte proliferation and heart regeneration following injury in zebrafish (15). Remarkably, SDH inhibition by malonate promotes adult cardiomyocyte proliferation, revascularization, and heart regeneration following adult myocardial infarction (110). In contrast to the cardioprotective role of malonate during I/R injury in mouse and pig hearts (105, 107); malonate did not protect against infarction post-MI but rather promoted regeneration following infarction (110). Furthermore, SDH inhibition by malonate following adult MI was accompanied by increased succinate levels as a consequence of TCA cycle inhibition, which is distinct from the cardioprotective role of malonate that prevents succinate accumulation during I/R injury (105, 110). Interestingly, metabolic profiling of the adult heart demonstrated an increase in glucose metabolism and a decrease in TCA cycle metabolism following SDH inhibition by malonate, consistent with a metabolic reprogramming from oxidative phosphorylation to glycolysis in the adult heart. These results demonstrate that SDH inhibition by malonate promotes adult heart regeneration *via* metabolic reprogramming (110).

Collectively, these studies demonstrate an important role for mitochondrial metabolites in regulating the cardiac metabolic state, and targeting metabolism has an important therapeutic potential to promote adult heart regeneration.

DISCUSSION

The role of the complex metabolic interactions in the heart and their potential to promote cardiac repair and regeneration are beginning to be appreciated. The shift in metabolism from glycolysis to fatty acid oxidation after birth coincides with the loss of regenerative potential in the neonatal mouse heart. The studies that are highlighted throughout this review demonstrate that manipulation of metabolic pathways is an area of immense potential for identifying new therapeutics to treat heart diseases (**Table 1**). Targeting these metabolic pathways can promote or inhibit regeneration depending upon the specific component that is modulated (**Figure 2**).

Manipulating metabolic components in ways that can stimulate glucose metabolism has been implicated in promoting regeneration, as this shifts the heart's metabolic landscape closer

TABLE 1 | Summary of recent studies demonstrating a central role for metabolism in heart failure and regeneration.

Metabolism	Target gene	Function	Application	Results	References
Fatty acid oxidation	Carnitine palmitoyltransferase 1 (CPT1)	Induces fatty acid oxidation	CPT1 inhibition	Increased proliferation of isolated neonatal cardiomyocytes	(30)
				Reduced in fatty acid oxidation gene expression	
				No change in adult mouse cardiomyocyte proliferation	(31)
	Malonyl-CoA decarboxylase (MCD)	Reduces fatty acid oxidation	MCD inhibition	Increased malonyl-CoA levels in ischemic swine heart	(33, 34)
				Improved cardiac function following rat heart myocardial infarction (MI)	(35)
				Increased glucose oxidation in MCD deficient mouse heart	(36)
				Improved cardiac function in ischemic MCD deficient mouse heart	
	Peroxisome proliferator-activated receptor (PPAR) α	Induces fatty acid oxidation	PPARα activation	Increased CPT1 gene expression and oxygen consumption rate in the presence of the fatty acid palmitate in isolated mouse cardiomyocytes	(30)
				No change in adult cardiomyocyte proliferation and cardiac function following MI	(31)
				Cardiac function decreased following I/R injury	(42)
	PPARδ	Induces fatty acid oxidation	PPARδ activation	Decreased cardiac fibroblast proliferation and myofibroblast transdifferentiation	(44)
				Reduced cardiomyocyte proliferation and increased scar size following MI in mouse heart	(45)
			PPARδ inhibition	Reduced cardiomyocyte proliferation following cardiac injury in zebrafish	(45)
Glucose metabolism	GLUT1	Increases glucose uptake	GLUT1 overexpression	Increased glucose uptake and glycolysis in the mouse heart	(62, 63)
				Increased regenerative response and glucose metabolites in neonatal mouse heart following cryoinjury	(64)
		Decreases glucose uptake	GLUT1 inhibition	Reduced glucose uptake and glycolysis in isolated mouse cardiomyocytes following TAC injury	(59)
	Hexokinase (HK) 2	Increases glycolysis	HK-2 overexpression	Decreased cardiac hypertrophy in isoproterenol-induced mouse hearts	(71)
				Reduced cardiomyocyte size in neonatal rat ventricular cardiomyocytes	
				Reduced ROS accumulation	
		Decreases glycolysis	HK-2 inhibition	Increased cardiac dysfunction and cell death and fibrosis	(72)
				Decreased angiogenesis following I/R injury	
	Phosphofructokinase (PFK) 2	Increases glycolysis	PFK-2 inhibition	Reduced glycolysis and insulin sensitivity in mice	(74, 75)
			PFK-2 overexpression	Increased contractility in hypoxic mouse cardiomyocytes	(76)
	Pyruvate dehydrogenase kinase (PDK)	Increases glycolysis	PDK inhibition	Increased cardiac function following KCl-induced cardiac arrest	(77)
			PDK-4 inhibition	Promoted mouse cardiomyocyte proliferation and heart regeneration following adult MI	(78)
	Pyruvate kinase muscle isoenzyme 2 (PKM2)	Increases glycolysis	PKM2 overexpression	Increased cardiomyocyte proliferation and cardiac regeneration following adult MI	(79)
			PKM2 inhibition	Reduced cardiomyocyte proliferation following injury in zebrafish hearts	(67)
				Impaired heart development and reduced cardiomyocyte proliferation	(79)
Amino acid metabolism	Protein Phosphatase 2cm (PP2 cm)/Protein Phosphatase 1 k (PPM1K)	Reduced BCAA oxidation	PP2cm inhibition	Increased BCAA and BCKA levels	(87)

(Continued)

TABLE 1 | Continued

Metabolism	Target gene	Function	Application	Results	References
				Reduced cardiac function and increased heart failure	(87, 89)
				Decrease in glucose uptake and utilization	(89)
		Increased BCAA oxidation	PP2cm overexpression	Decreased DNA damage and cell death, leading to a smaller scar size post-MI	(99)
	BCKDK	Increased BCAA oxidation	BCKDK inhibition	Decreased free BCAAs, leading to improved heart function post-TAC	(98)
TCA cycle metabolism	Succinate dehydrogenase (SDH)	Reduced succinate accumulation	SDH inhibition	Reduced infarct size during ischemia in I/R mouse hearts	(106)
				Reduced infarct size during I/R injury in pig hearts	(107)
				Induced glucose metabolism in adult mouse hearts	(110)
				Promoted adult cardiomyocyte proliferation, revascularization, and heart regeneration following MI	(110)

to the metabolic state of the regenerative neonatal heart. This was demonstrated with deletion of PDK4, overexpression of PP2cm, as well as SDH inhibition *via* malonate, which promoted regeneration by inducing glucose metabolism *via* modulating their respective metabolic pathways (78, 99, 110).

In contrast, increased fatty acid oxidation has been demonstrated to reduce the cardiac regenerative response following injury. Inducing fatty acid oxidation *via* treatment with the PPARα agonist WY-14643 results in reduced cardiac function after injury (42). Similarly, inhibition of glycolysis exacerbates cardiac injury, as demonstrated by reduced HK-2 expression (72) and PP2cm deletion (87).

The dynamic role of glycolysis and fatty acid oxidation following injury demonstrates a central role for cardiac metabolism during regeneration. Although multiple key components have already been identified that can be targeted therapeutically, these metabolic pathways play an important role in cardiac homeostasis. Thus, elucidating the mechanisms of these pathways during homeostasis, disease, and regeneration is an essential step prior to targeting these pathways for therapeutic development. For example, targeting succinate dehydrogenase post-MI promoted adult heart regeneration, yet the mechanisms by which succinate dehydrogenase inhibition promotes regeneration needs to be fully understood prior to clinical use (110). Furthermore, harnessing the potential of known pharmacological agents that have been demonstrated to target these metabolic pathways needs to be explored as candidates to induce adult heart regeneration.

Elucidating the role of cardiac metabolism in health and disease will provide us with novel avenues with significant therapeutic potential that could aid in promoting heart repair and regeneration. Advancements in this area of research will provide a better understanding of heart disease and regeneration.

AUTHOR CONTRIBUTIONS

JB and AM contributed to conception and design of the manuscript. JB, WP, and AM wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted manuscript.

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Long Non-coding RNA N1LR Protects Against Myocardial Ischemic/Reperfusion Injury Through Regulating the TGF-β Signaling Pathway

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Long non-coding RNAs (lncRNAs) have been shown to play critical roles in various cell biological processes. However, the mechanism of lncRNAs in acute myocardial infarction (AMI) is not fully understood. Previous studies showed that lncRNA N1LR was down-regulated in ischemic cerebral stroke and its up-regulation was protective. The current study was designed to assess the protective effect of N1LR and further to explore potential mechanisms of N1LR in ischemic/reperfusion (I/R) injury after AMI. Male C57BL/6J mice and H9c2 cardiomyocytes were selected to construct *in vivo* and *in vitro* pathological models. In H9c2 cell line, N1LR expression was markedly decreased after H_2O_2 and $CoCl_2$ treatments and N1LR overexpression alleviated apoptosis, inflammation reaction, and LDH release in cardiomyocytes treated with H_2O_2 and $CoCl_2$. Mouse *in vivo* study showed that overexpression of N1LR enhanced cardiac function and suppressed inflammatory response and fibrosis. Mechanistically, we found that the expression of transforming growth factor (TGF)-β1 and smads were significantly decreased in the N1LR overexpression group exposed to H_2O_2. In a summary, our study indicated that N1LR can act as a protective factor against cardiac ischemic-reperfusion injury through regulating the TGF-β/Smads signaling pathway.

Keywords: acute myocardial infarction, LncRNA N1LR, ischemic reperfusion injury, cardiomyocytes, TGF-β pathway

INTRODUCTION

Acute myocardial infarction (AMI), characterized by coronary artery occlusion and myocardial cell necrosis, is the leading cause of death in patients with heart disease (1, 2). In AMI patients, although an early re-established blood flow inside the occluded coronary was essential to protect ischemic cardiomyocytes, this reperfusion can aggravate myocardial cell injury due to artery vascular endothelial cells malfunction and activation of several inflammatory factors (3, 4). It was reported that reperfusion injury can count up to 40% of myocardial cell necrosis and reduce the therapeutic efficacy of active reperfusion therapy (5, 6). Currently, there is a general consensus that myocardial I/R injury is the main cause of cardiac cell death and cardiac dysfunction (4).

The underlying molecular mechanism of I/R injury was complex and multifactorial in which excessive inflammation and apoptosis play the essential roles in the initiation and development of I/R (7). Transforming growth factor-β1 (TGF-β1) is the most critical isotype of TGF-β, and participate in inflammation, apoptosis, and tissue repair (8). Furthermore, a close and solid relationship between the TGF signaling pathway and I/R was found and reported (9). Smad2 and Smad3 are the two major downstream regulators of TGF-β1 signaling, TGF-β1-induced phosphorylation of Smad2 and Smad3 promote cell inflammation and apoptosis and tissue fibrosis (10). Other studies demonstrated that TGF-β/Smads are involved in myocardial pathological process (11). Therefore, TGF-β/Smads signaling pathway might be a potential therapeutic strategy for heart diseases.

In the last decade, non-coding RNAs (ncRNAs), accounting for ∼98% of human genes, have emerged as a hot spot for scientific research (12). Tremendous literature has indicated that ncRNAs play an important role in cell growth, differentiation, immunity and apoptosis (13, 14). Long non-coding RNAs (lncRNA) are a class of lncRNA ribonucleic acid sequences larger than 200nt in length. Previous reports have shown that lncRNAs regulate protein-coding genes at transcriptional and post-transcriptional levels in coronary heart disease (CAD) (15). Furthermore, LncRNA has been reported involved in the molecular mechanism of myocardial I/R injury (16). LncRNA-N1LR, an I/R induced lncRNA, was initially found to be significantly down-regulated in the cerebral I/R rat model. It has shown that lncRNA-N1LR was involved in cell death, angiogenesis and inflammation during ischemic stroke. The infarct size increased significantly with the decrease of N1LR. In addition, N1LR up-regulation can provide neuroprotection against ischemic stroke *via* inhibiting p53 expression (17). All this indicates that lncRNA-N1LR could have a strong protective effect in I/R injury and its potential mechanism is worth to be explored.

In the present study, we aim to test the effect of N1LR on cardiac I/R injury *in vitro* and *in vivo*. Moreover, the underlying mechanism was investigated in the study. We found the N1LR attenuated inflammation and apoptosis of H9c2 cells treated with hypoxia and improved cardiac function in mice. And TGF-β/Smads signaling pathway is one the main mechanisms for N1LR myocardiac protective effects. Taken together, this study provides a potential therapeutic target for I/R-induced cardiac injury.

MATERIALS AND METHODS

Cell Culture and Treatment

The H9c2 cell line derived from rat ventricle was purchased from the Cell Bank of the Chinese Academy of Sciences, Shanghai Institutes for Biological Sciences (Shanghai, China) and was cultured in Dulbecco's modified Eagle medium (DMEM, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS Invitrogen, Gibco, USA) plus 100 mg/ml penicillin/streptomycin (Gibco, CA, USA) at 37°C in the incubator with 95% air and 5% CO_2.H9c2 cells from different groups were then plated in 6-well plates (3×10^5 per well) and treated with 100 μM H_2O_2 for 5h or 800 μM $CoCl_2$ for 20h (Both purchased from Tianjin Kemiou Chemical Reagent Co., Ltd, Tianjin, China) in serum-free DMEM to induce apoptosis and inflammatory response. Treated cells were collected for further experiments.

Adenoviral Transfection

Cardiomyocyte specific vector AAV9-N1LR (AAV9-CTnT-N1LR), Cardiomyocyte specific overexpression vector Adenoviral5-N1LR (Ad5-CTnT- N1LR) and their corresponding control vector (AAV9-CTnT-Control and Ad5-CTnT-Control)were purchased from Shanghai OOBIO Biotechnology Company (Shanghai, China). For *vitro* experiments, 36h after infection with Ad5-CTnT-N1LR or Ad5-CTnT-Control at a multiplicity of infection (MOI) of 25, cells were treated with 100 μM H_2O_2 for 5h or 800 μM $CoCl_2$ for 12h in serum-free DMEM. For *vivo* experiments, each mouse was injected with either AAV9-CTnT-N1LR or AAV9-CTnT-Control (1×10^{12} v.g/ml) *via* tail vein two weeks before I/R operation. Transfection efficiencies were validated by quantitative real-time PCR.

Mouse Model of I/R Injury

The male C57BL/6J mice (6–8 weeks) were purchased from the experimental animal center of Yangzhou University. The study protocol was approved by the ethics committee of the Affiliated Hospital of Yangzhou University and was consistent with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 96-01).

Mice were randomly divided into four groups: NC, N1LR, I/R, I/R+N1LR. The myocardial I/R mouse model was established as previously described (18). Anesthetized (xylazine, 5 mg/kg i.p.) mouse was placed on the operating table, and then was intubated and connected to a rodent ventilator (ALCOTT BIOTECH CO, Shanghai, China). The heart was exposed through a left lateral thoracotomy and the left anterior descending artery (LAD) was ligated by 8-0 nylon suture with a slipknot. After that, the thorax was closed and the mouse was placed on a heating pad to keep the body warm. ST-segment elevation in ECG and regional blanching of the left ventricle were observed to ensure successful myocardial ischemia. After 60-min ischemia, the ligated slipknot was removed to perform consequential 2h or 4 weeks of reperfusion.

Enzyme-Linked Immunosorbent Assay (ELISA)

After various treatments, the blood samples and supernatant of cell culture were collected and then centrifugated to remove cellular debris, the IL-6, IL-1β, and TNF-α levels were quantified using ELISA assay (Elabscience, Wuhan, China) according to the manufacturer's protocol. Analysis of optical density was performed in a standard microplate reader. The concentrations of IL-6, IL-1β, and TNF-α were calculated based on the standard curve.

TABLE 1 | Sequence of primers.

Genes	Forward primer	Reverse primer
GAPDH	CCTTCCGTGTTCCTACCCC	GCCCAAGATGCCCTTCAGT
N1LR	CGCGCTGCCATGACTGACA	CCGCTCTGGTCGGCGTCCT
IL-1β	TCACAGCAGCACATCAACAA	TGTCCTCATCCTGGAAGGT
TNF-α	ACGGCATGGATCTCAAAGAC	GTGGGTGAGGAGCACGTAGT
IL-6	GACTGCGGCAGAATTGCTATC	CGGGCTAATTTCCGTTGCATA
TGF-β1	CGCGGAGATGGAAGCACCGC	CCGCTCACCAAAGCTAAGAC
Colla1	CGCAGCACGTAGCGCACATC	GCCTTTGTGAGCGAACCCGA
Col3a1	GGTTTCCGGGATTGAGGCTG	TGCCCGTCTAATGAATCGGG
a-SMA	TGGGGTACCGGGTATAATCC	ACTGAAGTACGGCCCGTTCA

Cell Death Analysis and Lactate Dehydrogenase (LDH) Release Assay

Cells transfected with different adenoviral vectors were plated in 6-well plates (3×10^5 per well). After adhesion, cells were treated with either H_2O_2 or $CoCl_2$ to induce apoptosis and inflammatory response. To assess the cell death rates, the cells were collected and stained using Trypan Blue (Solarbio, Beijing, China) according to the protocol. The stained cells were observed under a light microscope at ×10 magnification, the numbers of Trypan Blue-positive and Trypan Blue negative were counted using a hemocytometer. Cell death rates (%) were calculated as (number of dead cells/total number of cells) ×100%.

Lactate dehydrogenase (LDH) was a marker of cell injury. The level of LDH released in the serum and cell culture supernatant was evaluated using LDH Release Assay Kit (Beyotime, Nantong, China) according to the manufacturer's protocols. The absorbance of the samples was measured at 490 nm using a microplate reader.

Quantitative Real-Time PCR Analysis

Total RNAs from treated cells or heart samples were extracted using Trizol and treated with DNAase I to remove genomic DNA, and then purified with an RNA purification kit (Invitrogen, USA). cDNA was synthesized using Script cDNA Synthesis Kit (Bio-Rad, Hercules, CA). ABI 7900HT Fast Real-Time PCR System (Applied Biosystems) was used to quantify the expressions of genes. GAPDH was used as an internal normalized reference. The sequences of primers for each gene were shown in **Table 1**. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of different genes.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End-Labeling Assay

H9c2 Cells in different groups were fixed by 4% paraformaldehyde. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling assay (TUNEL) kit (Zhongshan Biotechnology Co, Beijing, China) was used to evaluate apoptosis of cardiomyocytes according to the manufactory's instructions. Images (100×) were taken using a fluorescence microscope (Nikon, Eclipse Ti, Japan).

Echocardiography

4 weeks after I/R operation, mice were anesthetized again with 1.5–2.0% isoflurane, transthoracic M-mode and Color Doppler mode echocardiograms were applied to evaluate the cardiac function using an ultra-high resolution small animal ultrasound Vevo 3100 Imaging System (VisualSonics, Fujifilm) with a 30 MHz transducer. The left ventricular ejection fraction (LVEF), fractional shortening (FS) Left ventricular internal diameter (LVID) and left ventricular posterior wall (LVPW) were calculated through Simpson's measurements.

Heart Histological Analysis

At the end of reperfusion, the mice were anesthetized and body weight (BW) was weighted. Then the heart tissues were harvested and weighed (heart weight HW), To delineate the infarct size of the myocardium, the hearts were cut into 2 mm-slices and the slices were incubated in 1% triphenyl tetrazolium chloride (TTC, Sigma, USA) and fixed with 4% paraformaldehyde and photographed. For general histological analysis, routine hematoxylin and eosin (H&E) staining and Masson's trichrome staining (Solarbio, Beijing, China) were performed. Briefly, after dehydrating with ethanol series, clearing with xylene and mounting with neutral resins, images of the myocardial structure were captured by light microscope. Computer-assisted morphometric analysis of digitized images was performed with image analysis software (Motic Image Advanced, Xiamen, China). The scoring criteria for myocardial injury histoscore were evaluated by two pathologists under blind condition according to a scoring system as described previously (19), i.e.: (4) severe (necrosis with the diffuse inflammatory process), (3) moderate (extensive myofibrillar degeneration and/or diffuse inflammatory process), (2) mild (small multifocal degeneration with a slight degree of the inflammatory process), (1) minimum (focal myocytes damage), and (0) nil (no changes).

Western Blot Analysis

H9c2 cells were lysed for 20 min on ice with RIPA lysis buffer (Solarbio, Beijing, China) containing protease inhibitors and protein phosphatase inhibitor (Both purchased from Roche, Switzerland). BCA protein assay kit (Thermo, Rockford, USA) was used to detect protein concentration. Protein samples were segregated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (EMD,Millipore, USA). After blocking with 5%BSA, the blots were probed with various primary antibodies including TGF-β1, T-Smad2, T-Smad3, p-Smad2, p-Smad3, and GAPDH (1:1000 dilution, All purchased from Cell Signaling Technology, Beverly, USA) at 4°C overnight and then incubated with anti-rabbit IgG H&L (HRP) secondary antibody (1:5000, CST, Beverly, USA) for 1h at room temperature. The bands on PVDF were visualized by use of a Super Western Sensitivity Chemiluminescence Detection System (Thermo, USA). Image-J was used to analyze the band intensities.

Statistical Analysis

Statistical Analysis was performed using SPSS 17.0 software (SPSS Inc., Chicago, IL USA). Continuous variables were shown as "mean ± SD". One-way analysis of Variance (ANOVA) was

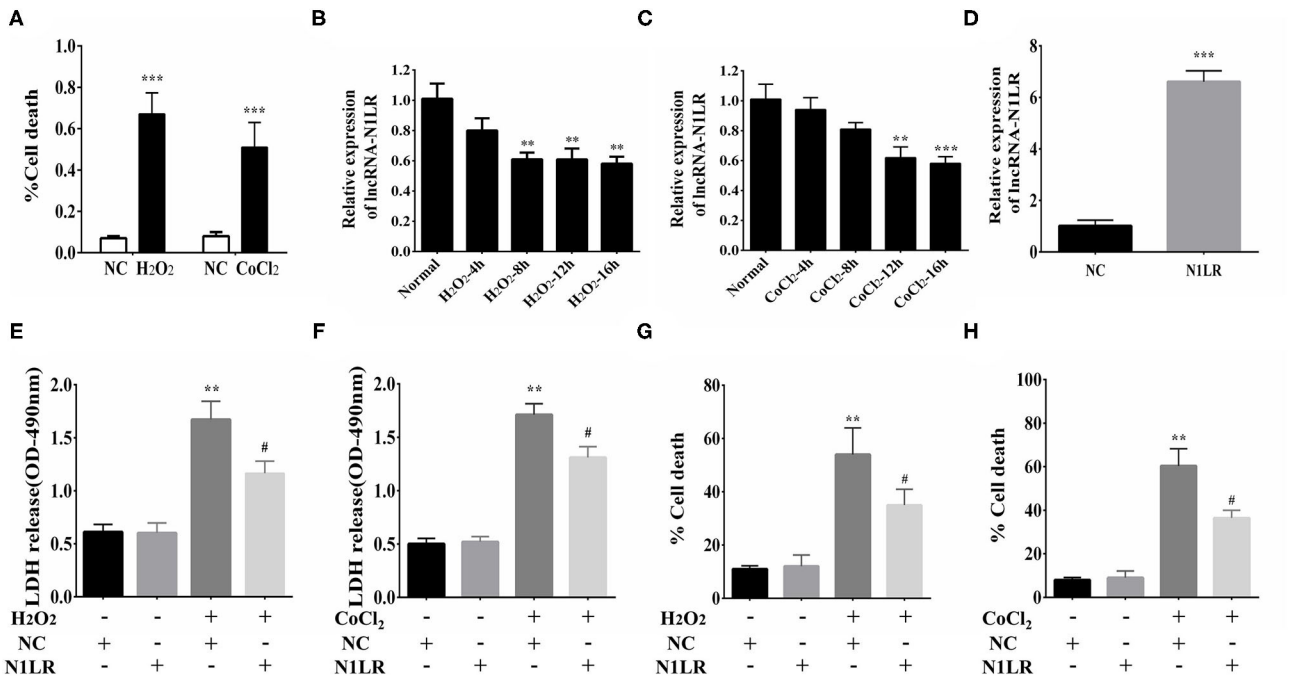

FIGURE 1 | N1LR attenuated the cell death and LDH release of H9c2 cells. **(A)** Cell Death rates were measured to confirm the successful establishment of the hypoxia model. **(B)** The expression of N1LR was down-regulated with continuous exposure to hypoxia-induced by $CoCl_2$. **(C)** The expression of N1LR was down-regulated with continuous exposure to hypoxia-induced by H_2O_2. **(D)** The overexpression of N1LR was verified through qRT-PCR. **(E)** N1LR reduced the release of LDH in H_2O_2-treated H9c2 cells. **(F)** N1LR reduced the release of LDH in $CoCl_2$-treated H9c2 cells. **(G)** The H9c2 cell death was decreased in the N1LR+H_2O_2 group compared with the H_2O_2 group. **(H)** The H9c2 cell death was as well decreased in the N1LR+$CoCl_2$ group compared with the $CoCl_2$ group. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. NC group. $^{\#}p < 0.05$ vs. H_2O_2+NC or $CoCl_2$+NC group. Data were shown as means ± SD.

used to determine the significant differences of each group, and Student's T-test was used for comparisons between two groups. *P*-value < 0.05 was considered statistically significant.

RESULTS

N1LR Attenuated the Death and LDH Release of Cardiomyocytes *in vitro*

H_2O_2 treatment is well known for both cellular apoptosis and necrosis study (20). To determine the effect of N1LR on H9c2 cells, we first treated cells with H_2O_2 (100uM) or $CoCl_2$(800uM) to induce cellular damage. The death rate of cardiomyocytes was increased after H_2O_2 and $CoCl_2$ exposure, as shown by trypan blue staining (**Figure 1A**). Next, the expression of N1LR was detected using qRT-PCR. We found that the expression of N1LR was significantly reduced with continuous exposure to hypoxia in H_2O_2 and $CoCl_2$ treated cardiomyocytes, respectively (**Figures 1B,C**). These results indicated that the level of N1LR was decreased during H_2O_2 or $CoCl_2$-induced cell injury. Transfected with Ad5-CTnT-N1LR or vector, the expression levels of N1LR were about 6 times higher in the N1LR group compared with the NC group, indicating the transfection efficiency of lncRNA N1LR (**Figure 1D**). In addition, LDH release is related to cellular injury and was reduced in N1LR overexpressed group exposed to H_2O_2 and $CoCl_2$ (**Figures 1E,F**). Moreover, it is worth noting that N1LR overexpressed reduced cell death compared with the NC group (**Figures 1G,H**). Thus, these results suggested that N1LR attenuated the cell death rate and LDH release of cardiomyocytes *in vitro*.

N1LR Inhibits the Apoptosis and Inflammatory Response *in vitro*

To investigate the specific effect of N1LR on H_2O_2-induced inflammation and apoptosis *in vitro*. We separately examined inflammatory-related factors (IL-6, TNF-α, and IL-1β) and apoptosis. ELISA results showed that the expression levels of IL-6, TNF-α, and IL-1β were reduced in N1LR overexpressed group (**Figure 2A**). Similar results were detected using qRT-PCR (**Figure 2B**). TUNEL assay was performed to assess the effect of N1LR on H_2O_2-induced apoptotic in H9c2 cells. We observed the tunnel-positive cells were distinctly decreased in the N1LR overexpressed group (**Figures 2C,D**). These results demonstrated N1LR alleviated the apoptosis and inflammatory response *in vitro*.

N1LR Decreased Pro-inflammatory Factor Level and MI Infarction Area in the I/R Mice Model

We then further testified N1LR's effects for I/R injury in mice. One day before I/R operation, N1LR mRNA expression in cardiac

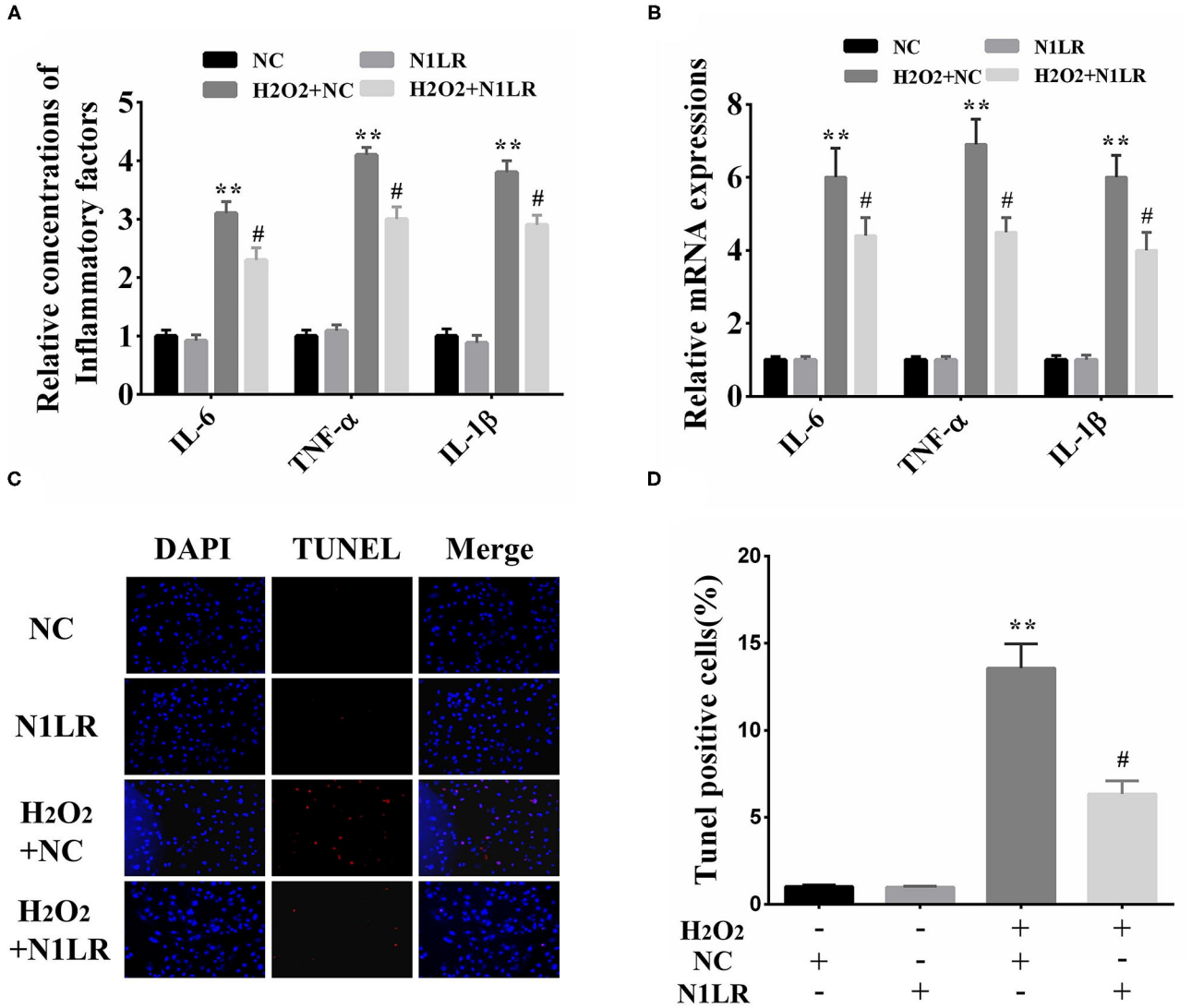

FIGURE 2 | N1LR inhibited the apoptosis and inflammatory response *in vitro*. **(A)** Overexpression of N1LR decreased the levels of IL-6, TNF-α, and IL-1β in the supernatant measured by ELISA. **(B)** Overexpression of N1LR reduced the expressions of IL-6, TNF-α, and IL-1β mRNA. **(C)** A Tunnel assay was performed to measure the apoptotic rates. **(D)** The relative levels of TUNNEL-positive cells were measured *via* Image J software and apoptotic cells distinctly decreased in N1LR overexpressed group. $^{**}p < 0.01$ vs. NC group, $^{\#}p < 0.05$ vs. H_2O_2+NC group. Data were shown as means ± SD.

was verified by RT-qPCR. We can see the expression level of N1LR was about ten times higher than that in the NC group (**Figure 3A**). First, the inflammation effect was tested, after 2h of reperfusion the serum was collected for the detection of inflammatory markers. In the mice model, N1LR overexpression could significantly reduce inflammatory factors (IL-6, TNF-α, IL-1β) level which were caused by I/R jury (**Figure 3B**). LDH, as a cellular injury index, was also decreased by N1LR overexpression (**Figure 3C**). Those results implied that N1LR can reduce inflammation and attenuated the cardiomyocyte death caused by I/R jury *in vivo*. Meanwhile, for the histological analysis, TTC staining results showed that N1LR overexpression significantly diminished MI area (**Figures 3D,E**). In contrast, N1LR overexpression attenuated the morphology of cardiac muscle fibers as compared to the disarrangement in the NC group by using H & E staining (**Figure 3F**), this result was verified by the histological scores through ImageJ software for the N1LR cardiac protective function (**Figure 3G**). These observations revealed a cardioprotective role of N1LR overexpression in I/R induced myocardial injury *in vivo*.

N1LR Ameliorated Fibrosis and Improved the Cardiac Function *in vivo*

After inflammatory tests, we go further to examine the effect of N1LR overexpression on the consequent fibrosis 4 weeks after reperfusion. As shown by Masson Trichrome staining, the fibrosis area was significantly reduced in N1LR overexpressed group compared with the NC group in the mice model (**Figures 4A,B**). TGF-β1 is the most effective

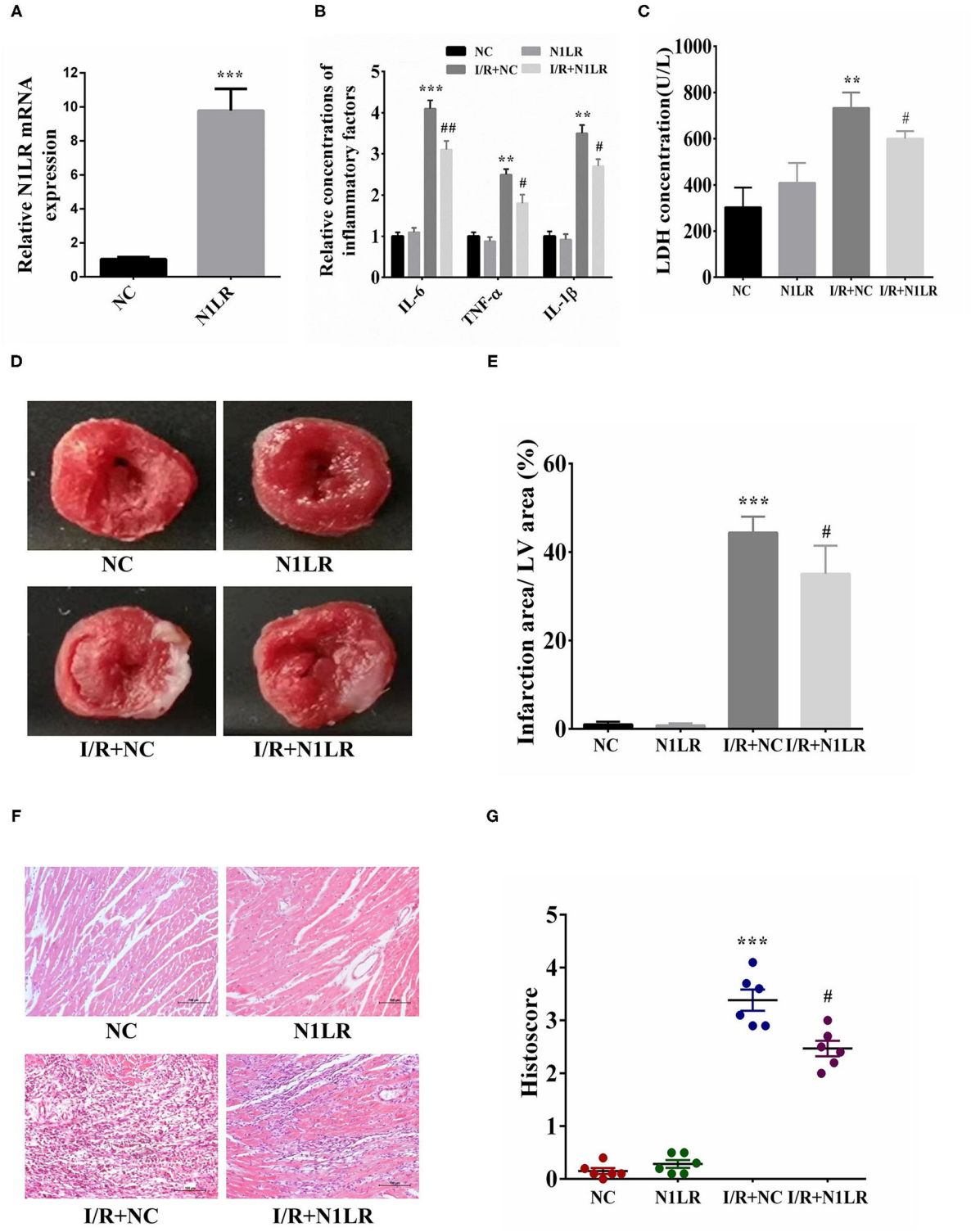

FIGURE 3 | N1LR decreased pro-inflammatory factor level and infarction area in I/R mice model. **(A)** One day before the operation, N1LR mRNA expression in cardiac was verified by RT-qPCR. **(B)** N1LR decreased the IL-6, TNF-α, IL-1β levels in mouse serum. **(C)** N1LR reduced the levels of LDH in mouse serum. **(D)** Infarction area after myocardial I/R injury was detected by TTC staining. **(E)** N1LR decreased infarction area in the I/R mice model. **(F)** HE staining was performed to detect inflammatory infiltration (×200). **(G)** Histological scores were decreased in N1LR overexpressed group. Data are expressed as mean ± SD ($n = 6$–8). $^{**}p < 0.01$, $^{***}p<0.001$ vs. NC group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. I/R+NC group.

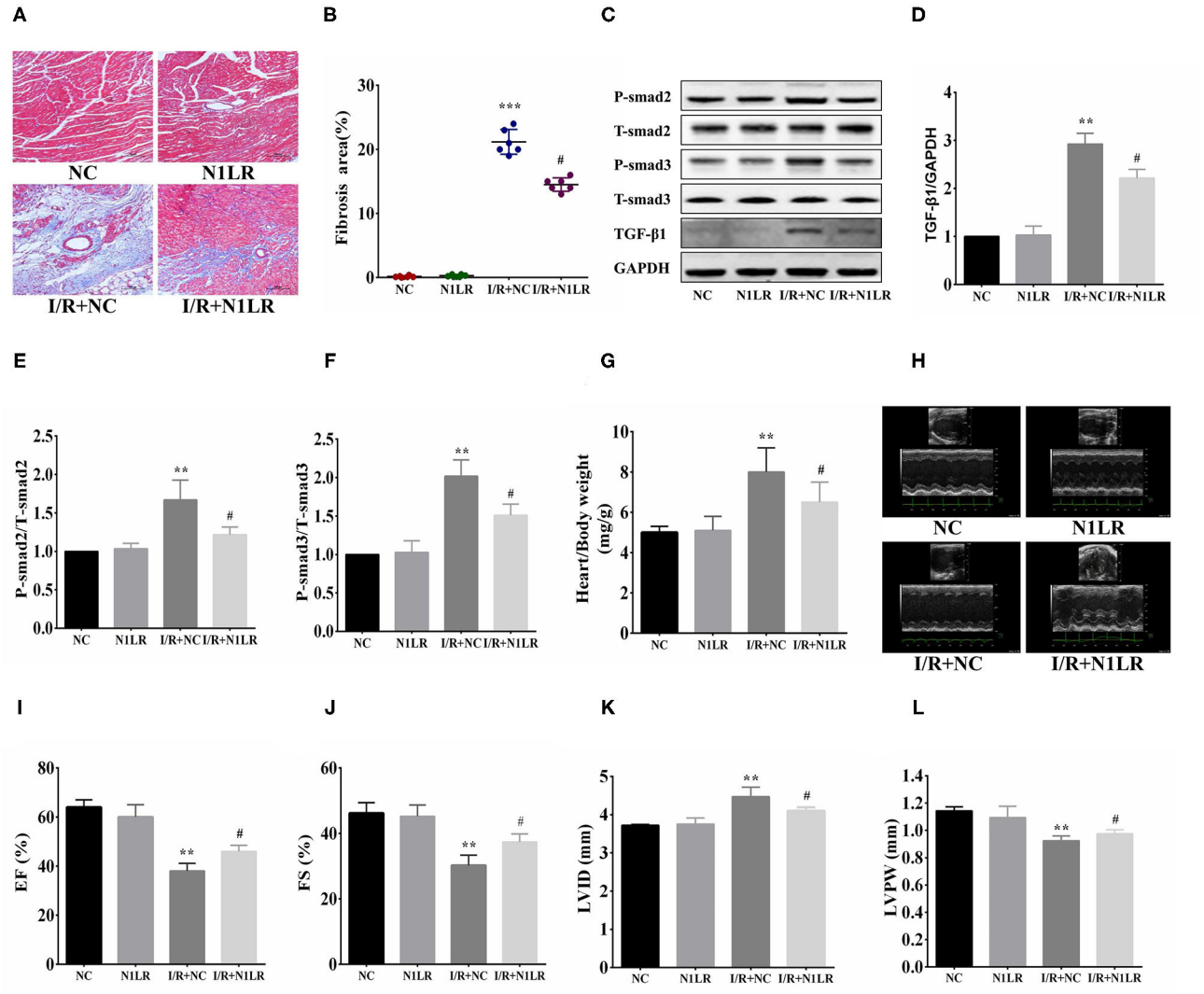

FIGURE 4 | N1LR ameliorated fibrosis and improved cardiac function. **(A)** Masson Trichrome staining was performed to assess fibrosis (×200). **(B)** The fibrotic area was significantly reduced in N1LR overexpressed group. **(C)** TGF-β1, Smad2, P-Smad2, Smad3 and P-Smad3 were detected by Western blot. **(D–F)** Expressions of TGF-β1, P-Smad2 and P-Smad3 were markedly up-regulated in the I/R group. N1LR inhibited TGF-β1, P-smad2 and P-Smad3 expressions. The ratio of p-Smad2/T-smad2 and p-Smad3/T-smad3 were decreased compared to the I/R+NC group **(G)** Overexpression of N1LR decreased the ratio of heart weight to body weight. **(H)** Cardiac function was measured by echocardiography. **(I–L)** EF, FS, LVID and LVPW were improved in N1LR overexpressed group. Data are expressed as mean ± SEM. ($n = 6$–8). $^{**}p < 0.01$, $^{***}p < 0.001$ vs. NC group, $^{\#}p < 0.05$ vs. I/R+ NC group. Data are expressed as mean ± SD. ($n = 6$–8).

cytokine that induces the production of collagens in cardiac fibroblasts. Smad proteins, which are important and necessary for heart development and cardiomyocyte differentiation, are key downstream factors in TGF-β1 signaling pathway (21). TGF-β/Smads pathway plays a mechanical role in myocardial infarction and cardiac fibrosis (22). The effects of N1LR on the TGF-β1 and Smads proteins' expression in the cardiac tissue of I/R mice were investigated. Expressions of TGF-β1, T-Smad2, P-Smad2, T-Smad3, and P-Smad3 were detected by Western blot (**Figure 4C**). Treatment with N1LR could suppress expressions of TGF-β1, p-Smad2, and p-Smad3, the ratio of p-Smad2/T-smad2 and p-Smad3/T-smad3 were significantly lower than the I/R+NC group (**Figures 4D–F**). Worked as an indicator for cardiac hypertrophy, the ratio of heart weight to body weight is also reversed due to I/R jury by N1LR overexpression (**Figure 4G**). For the cardiac function study by echocardiography, both EF and FS were significantly reduced 4 weeks after myocardial I/R compared with baseline. LVID was significantly enlarged while LVPW was decreased after I/R practice. In contrast, cardiac dysfunction demonstrated a manifest improvement in the N1LR overexpressed group (**Figures 4H–L**). These results revealed that N1LR treatment can reduce fibrosis and hypertrophy and further improve cardiac function for a long-term benefit in the mice I/R model.

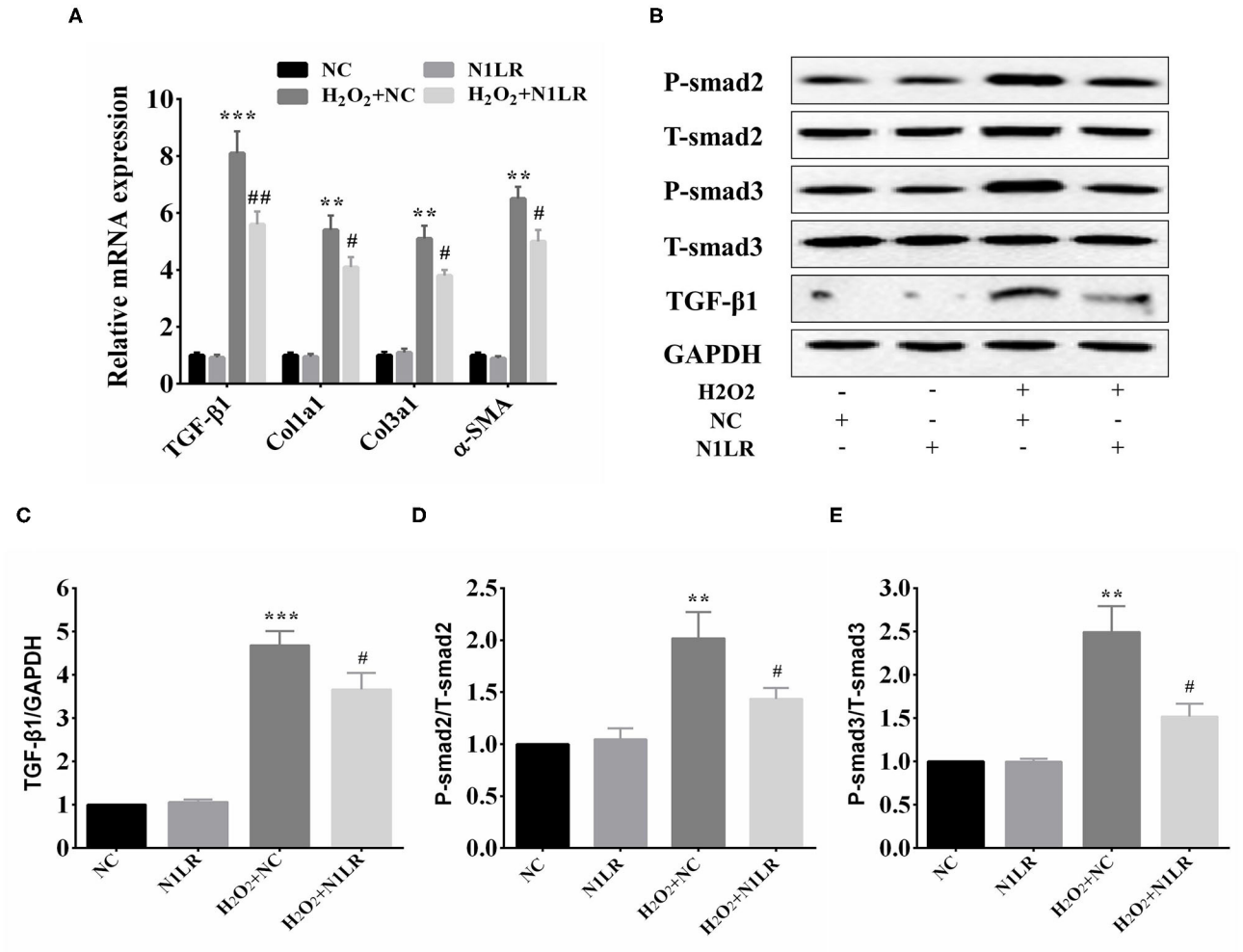

FIGURE 5 | N1LR functioned *via* repressing TGF-β signaling pathway. **(A)** Levels of relevant markers TGF-β1, Col1a1, Col3a1, and α-SMA mRNA were measured by qRT-PCR. The expression of TGF-β1, Col1a1, Col3a1, and α-SMA were markedly decreased in N1LR overexpressed group compared with the H_2O_2+NC group. **(B)** Western blot analysis of TGF-β1, P-smad2 and P-smad3 in H_2O_2 treated H9c2 cells. **(C–E)** The relative expressions of TGF-β1, P-Smad2 and P-Smad3 were measured by using Image J software. Data are expressed as mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. NC group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. H_2O_2+ NC group.

N1LR Functioned *via* Repressing the TGF-β Signaling Pathway

The above results suggest that N1LR can ameliorate myocardial fibrosis after I/R injury by regulating the TGF-β1/Smad pathway. The effects of N1LR on the TGF-β1/Smad3 signaling pathway were further investigated in H_2O_2 treated H9c2 cells. According to the results of qRT-PCR, the mRNA expression levels of TGF-β1, Col1a1, Col3a1, and α-SMA were decreased in N1LR overexpressed group exposed to H_2O_2 (**Figure 5A**). After exposure to H_2O_2, the expressions of TGF-β1/smads also increased similarly to *in vivo* study. In brief, the protein expression levels of TGF-β1, P-Smad2, and P-Smad3 were manifestly increased and partially reversed in the N1LR overexpressed group, with unchanged total Smad2 and Smad3 (**Figures 5B–E**). Thus, our results suggested that lncRNA N1LR protected cardiomyocytes from H_2O_2-induced injury by repressing the TGF-β signaling pathway.

DISCUSSION

Immediate reperfusion could reduce the injury resulting from AMI, but also undermine cardiac function and the structure of myocardial cells (23, 24). The apoptosis and death of cardiomyocytes and the release of inflammation factors are boosted during I/R injury after AMI (25, 26). Myocardial inflammation is considered to be the secondary injury mechanism after I/R (27). Over the last decade, increasing reports have indicated that lncRNAs are involved in various human diseases, including AMI (15, 17, 28). Some lncRNAs are abnormally expressed in the development of CAD and implicated

in cardiac pathophysiology, which suggesting lncRNAs serve as markers of pathogenesis and potential therapeutic targets (29, 30). For example, lncRNA-HOTAIR down-regulation worsens oxidative stress-induced cardiomyocyte injury through sponging miR-125 to inhibit the expression of MMP2 (28). Down-regulating lncRNA MALAT1 improves the outcomes of AMI through miR-320/PTEN axis (31). Specific up-regulation of miR-21 in rat hearts suppresses left ventricular remodeling and myocardial apoptosis induced by I/R injury (32). Studies show that lncRNA Novlnc6 is dramatically down-regulated in dilated cardiomyopathy, and Novlnc6 knockdown leads to the expression decreasing of BMP10 and Nkx2.5, which are two important regulators for cardiomyocytes maturation and differentiation (33). As a novel I/R-induced lncRNA, N1LR was demonstrated to have neuroprotective effects in ischemic mice (16). Overexpression of N1LR could effectively decreased infarct size and neurological deficit *in vivo*, while decreasing N1LR expression increased N2a cell apoptosis induced by Oxygen-glucose deprivation/reoxygenation(OGD/R) treatment *in vitro* (16).

It has been reported that oxidative stress and inflammation caused by hypoxia can induce cardiomyocyte injury and death. In the present study, we tested the effects of N1LR on cardiac I/R injury *in vivo* and H_2O_2-induced cell injury *in vitro*. We found that N1LR was down-regulated in H9c2 cells treated by H_2O_2 and $CoCl_2$. There are many apoptotic cells but not dead cells in the treated cells. Overexpression of N1LR alleviated the H_2O_2-induced cell apoptosis, inflammation response, death, and LDH release in cellular experiments. In addition, N1LR improved cardiac function, ameliorated inflammation and fibrosis *in vivo*. These findings suggested that N1LR is an important regulator of hypoxia-resulted in injury for cardiomyocytes. We next explored the mechanism of N1LR overexpression in H9c2 cells under hypoxia induced by H_2O_2. Mechanistically, overexpression of N1LR significantly inhibited the expression of the TGF-β signaling pathway induced by I/R injury.

TGF-β1, a member of the cytokines family, plays an important role in physiological (like embryonic development, cell growth, and differentiation) and pathological processes (like inflammation, fibrosis, and apoptosis) (34, 35). Smads protein can transduce TGF-β1 family signal from the cell membrane receptor to the nucleus, thus forming TGF-β1/Smads signaling pathway to regulate myocardial fibrosis (36). Moreover, TGF-β1 is overexpressed in the heart in a high-cholesterol-fed porcine model of myocardial infarction, and its downstream Smad2 and Smad3 are activated, thereby increasing collagen synthesis and the levels of Col1a1, Col3a1, and α-SMA (37). In addition, the inflammatory response and apoptosis induced by I/R damage could be regulated by TGF-β1(38). These data support that the TGF-β1/Smads signaling pathway exerts an important effect on heart injury. *In vivo* study, the expression of TGF-β1, p-Smad2, and p-Smad3, were significantly lower than the I/R+NC group. *In vitro* study, the mRNA levels of TGF-β1, Col1a1, Col3a1, and α-SMA were decreased in N1LR overexpressed group exposed to H_2O_2, and the protein expressions of TGF-β1/smads are decreased similarly to *in vivo* study, suggesting that N1LR relies on the TGF-β1 pathway to reduce I/R-induced damage *in vivo* and *vitro*.

In conclusion, the present study suggested that N1LR overexpression represses the TGF-β1 pathway to inhibit H_2O_2-induced apoptosis, inflammatory response, and LDH release in cardiomyocytes after I/R-induced injury, all contributing to the improvement of cardiac function. Our findings provide new insight into the mechanism of I/R injury. The potential roles of N1LR in other cardiac myocyte disease models should be evaluated in subsequent studies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by Athics committee of the Affiliated Hospital of Yangzhou University.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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rGO/Silk Fibroin-Modified Nanofibrous Patches Prevent Ventricular Remodeling *via* Yap/Taz-TGFβ1/Smads Signaling After Myocardial Infarction in Rats

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Objective: After acute myocardial infarction (AMI), the loss of cardiomyocytes and dysregulation of extracellular matrix homeostasis results in impaired cardiac function and eventually heart failure. Cardiac patches have emerged as a potential therapeutic strategy for AMI. In this study, we fabricated and produced reduced graphene oxide (rGO)/silk fibroin-modified nanofibrous biomaterials as a cardiac patch to repair rat heart tissue after AMI and investigated the potential role of rGO/silk patch on reducing myocardial fibrosis and improving cardiac function in the infarcted rats.

Method: rGO/silk nanofibrous biomaterial was prepared by electrospinning and vacuum filtration. A rat model of AMI was used to investigate the ability of patches with rGO/silk to repair the injured heart *in vivo*. Echocardiography and stress–strain analysis of the left ventricular papillary muscles was used to assess the cardiac function and mechanical property of injured hearts treated with this cardiac patch. Masson's trichrome staining and immunohistochemical staining for Col1A1 was used to observe the degree of myocardial fibrosis at 28 days after patch implantation. The potential direct mechanism of the new patch to reduce myocardial fibrosis was explored *in vitro* and *in vivo*.

Results: Both echocardiography and histopathological staining demonstrated improved cardiac systolic function and ventricular remodeling after implantation of the rGO/silk patch. Additionally, cardiac fibrosis and myocardial stiffness of the infarcted area were improved with rGO/silk. On RNA-sequencing, the gene expression of matrix-regulated genes was altered in cardiofibroblasts treated with rGO. Western blot analysis revealed decreased expression of the Yap/Taz-TGFβ1/Smads signaling pathway in heart tissue of the rGO/silk patch group as compared with controls. Furthermore, the rGO directly effect on Col I and Col III expression and Yap/Taz-TGFβ1/Smads signaling was confirmed in isolated cardiofibroblasts *in vitro*.

Conclusion: This study suggested that rGO/silk improved cardiac function and reduced cardiac fibrosis in heart tissue after AMI. The mechanism of the anti-fibrosis effect may involve a direct regulation of rGO on Yap/Taz-TGFβ1/Smads signaling in cardiofibroblasts.

Keywords: reduced graphene oxide, acute myocardial infarction, myocardial fibrosis, cardiofibroblasts, YAP/TAZ-TGFβ1/Smads signaling

INTRODUCTION

Acute myocardial infarction (AMI) is a common cardiac emergency, with potential for substantial morbidity and mortality; more than 7 million people have infarctions each year (1, 2). After AMI, the loss of cardiomyocytes and dysregulation of extracellular matrix (ECM) homeostasis results in impaired cardiac function and leads to heart failure (3, 4). With the development of therapies, the early mortality rate with AMI has declined, but the incidence and prevalence of post-MI heart failure continues to increase (5). Recently, accompanied by the rapid development of cardiac tissue engineering, different kinds of cardio-supportive devices have been manufactured as new strategies for cardiac tissue repair after AMI (6–8). On the basis of the structural and biological properties of myocardial tissue (9, 10), a composite biomaterial with good mechanical support, biocompatibility and excellent biological function is needed for preparing cardiac patches to repair cardiac tissue after AMI.

Silk fibroin (SF) is a natural biopolymer derived from *Bombyx mori* cocoons, it has unique biocompatibility, biodegradability, morphologic flexibility and a number of tangible mechanical properties (11). Many studies have shown promising results for SF-based scaffolds to regenerate cardiovascular tissue both *in vitro* and *in vivo* (12–15). For instance, Chen et al. demonstrated that a Chitosan/SF-modified nanofibrous patch grafted on the infarcted myocardium could be integrated with native cardiac cells and promote cardiac function (14). Recently, reduced graphene oxide (rGO) has been regarded as one of the widely investigated nanomaterials with excellent physical, chemical properties and multiple biological functions (16–20). Furthermore, rGO-based biomaterials including bone, nerves, muscle, and cardiac tissue have been wildly used in tissue engineering technology (21, 22). We previously developed rGO-functionalized nanofibrous biomaterials that showed a great ability to promote cardiomyocyte structure formation and functions *in vitro*, including the expression of cardiac-specific proteins, formation of sarcomeric structures and gap junctions, and spontaneous beating of regenerated cardiac tissues (23). However, further study was needed to explore the role of rGO/silk nanofibrous biomaterials *in vivo*.

In the present study, rGO/silk nanofibrous biomaterials were incorporated in aseptic cardiac patches and used to repair rat heart tissue after AMI. Masson's trichrome staining, immunohistochemistry and myocardial stiffness were used to observe myocardial fibrosis at 28 days after cardiac patch implantation. From these results, we then explored and validated the potential molecular mechanism of rGO reducing cardiac fibrosis in the infarcted zone. Illuminating the role of rGO in ameliorating AMI-induced cardiac remodeling may help in understanding the underlying mechanism and suggest a new therapeutic method.

Abbreviations: AMI, acute myocardial infarction; CFs, cardiofibroblasts; CVF, collagen volume fraction; DEGs, differentially expressed genes; ECM, extracellular matrix; EF, ejection fraction; FS, fractional shortening; GO, gene ontology; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; LVM, left ventricular mass; KEGG, kyoto encyclopedia of genes and genomes; rGO, reduced graphene oxide; RNA-seq, RNA sequencing; SF, Silk fibroin; SD, standard deviation; TAZ, transcriptional coactivator with PDZ-binding motif; TGF-β1, transforming growth factor β1; YAP, yes-associated protein; ΔIVS, ratio of the interventricular septum thickening; ΔLVPW, ratio of left ventricular posterior wall thickness.

MATERIALS AND METHODS

Fabrication of the rGO/Silk Nanofibrous Patches

rGO/silk nanofibrous scaffolds were obtained by coating and reducing the GO membrane with silk nanofibrous mats, which was synthesized as described (23). Briefly, the 8% (wt/v) silk solution and 5% (wt/v) poly (ethylene oxide) (PEO, 900 000 MW, Sigma-Aldrich, St. Louis, MO) was mixed in a ratio of 4:1 and stirred for 15 min at room temperature. Next, the prepared silk solution underwent electrospinning at a voltage of 10 kV to obtain silk nanofibers, and the electrospun nanofibers were collected on a high-speed rotating disc collector. Then the GO solution at 0.02 mg/ml was conglutinated onto the surface of silk nanofibrous mats. Finally, the GO/silk material was immersed in a 1% (wt/v) ascorbic acid (Sigma-Aldrich, St. Louis, MO) solution at 95 °C for 60 min to obtain rGO/silk materials.

Construction of AMI Model and Implantation of rGO/Silk Patches

The experimental protocol used in the present study was approved by the Institutional Animal Care Committee at Xi'an Jiaotong University. A total of 40 male Sprague-Dawley rats (8-week-old male, Laboratory Animal Center of Xi'an Jiaotong University, China) weighing 200–220 g were used. Rats were randomly divided into four groups: sham ($n = 10$), MI ($n = 10$), MI+ silk patch ($n = 10$) and MI+ rGO/silk patch ($n = 10$). Rats were anesthetized with 2% (w/v) sodium pentobarbital (30 mg/kg) by intraperitoneal injection. Then rats underwent endotracheal intubation and assisted ventilation (tidal volume, 3 ml/100 g body weight; ventilation rate, 80/min) and the heart was exposed through a left-sided open thoracotomy. The left anterior descending coronary artery was ligated with 6-0 polypropylene suture. Myocardial ischemia was confirmed by regional cyanosis

and ST-segment elevation on electrocardiography. The rats in the sham group underwent only exposure of the heart, without artery ligation. Silk patches and rGO/silk patches (8*8 mm^2) were sutured onto the left ventricular epicardial surface. Then, the thoracotomy was closed in multiple layers.

Echocardiography for Cardiac Function

Echocardiography was used to evaluate the cardiac function of rats at 28 days after patch implantation. Rats were fixed after anesthesia; ejection fraction (EF), fractional shortening (FS), left ventricular end-diastolic volume (LVEDV) and left ventricular end-systolic volume (LVESV) was measured by standard transthoracic echocardiography (EPIQ 5, Philips, Holland) as recommended by the American Echocardiography Association. Ratio of the interventricular septum thickening (ΔIVS), ratio of left ventricular posterior wall thickness (ΔLVPW) and left ventricular mass (LVM) were calculated. Cardiac ultrasonography was performed by the same researcher to avoid measurement errors. All measurements were repeated 3 times.

Masson's Trichrome Staining

Masson's trichrome staining was used to measure the degree of myocardial fibrosis at 28 days after patch implantation. Rats in each group were anesthetized as above and hearts were exposed and enucleated, fixed with 4% paraformaldehyde and embedded in paraffin. Slices were obtained in the LV transverse direction and Masson's trichrome staining was performed as previously described (14). ImageJ software was used to evaluate percentage collagen volume fraction (CVF%) and left ventricular wall thickness of the infarcted region.

Immunohistochemistry

Immunohistochemistry was used to detect the expression of type I collagen (Col I) and CD68, with anti-Col1A1(Abcam, Cambridge, MA) and CD68(Abcam, Cambridge, MA) antibody, respectively, according to the manufacturer's instructions. Image J software was used to evaluated the level of Col I deposition and CD68 in the infarcted region.

Myocardial Stiffness

Myocardial stiffness was measured with a BOSE Electro Force mechanical tester (type:3200). Myocardial tissue was dissected from the infracted zone and prepared for biomechanical tests. The tensile test involved passively stretching the muscle at a constant strain rate to 1.3 times its initial length, at a rate of 0.01 mm/s. Muscle length, width and thickness were measured by using a micrometer and recorded as l, h and d, respectively. After stretching, a stress-displacement curve was obtained based on the raw data recorded by the mechanical tester, and the slope of this line, k, was calculated. Then myocardial stiffness was calculated as E = kl/hd, where E is myocardial stiffness.

Isolation, Culture, and Identification of Cardiofibroblasts

CFs were isolated from 2- to 3-day-old neonatal rats by using enzyme digestion method. The experimental protocol was approved by the Animals Committee of Xi'an Jiaotong University. In brief, neonatal rat ventricle tissues were separated and fully digested by collagenase II, then purified with differential adhesion method. After 45 min, the supernatant was removed and softly washed with PBS solution twice, then equivalent fresh DMEM/F-12 culture medium containing 10% fetal bovine serum was added and cultured in an incubator with 5% CO_2 and 95% O_2 at 37°C. Cultured CFs were identified by immunofluorescence staining of vimentin as described (24).

Cell Proliferation Assay

Cell counting kit-8 (Abcam, Cambridge, MA) assay was used to observe the cell viability of CFs after treatment with rGO solution. Cultured CFs were seeded in 96-well plates, then exposed to different concentrations of rGO (10, 20, 40, 60, 80, and 100 μg/ml) at 37°C with 5% CO_2 for 6, 12, 24, and 48 h respectively. Then equivalent CCK-8 was added to each well and 96-well plates were incubated at 37°C for 2 h. The absorbance of each well was recorded at 450 nm by using a Thermo Fisher microplate reader.

ELISA Assay for Col I and Co1 III

CFs were seeded in 6-well plates with ∼10^5 cells in each well and randomly divided into 4 groups after 2 days of culture, to which were added different intervention factors: control group, angiotensin II, angiotensin II+ rGO (50 μg/ml), angiotensin II+ rGO (100 μg/ml). The concentration of Col I and Col III in the supernatant was measured by using an ELISA assay kit (Shanghai Enzyme-linked Biotechnology Co, Shanghai, China) ($n = 3$ per group) according to the manufacturer's instructions.

RNA Extraction and Quality Control

Total RNA was extracted from samples of each group by using Trizol reagent (Invitrogen) and the miRNeasy mini kit (Qiagen). RNA quality and quantity were measured by using the NanoDrop spectrophotometer (ND-1000, Nanodrop Technologies, Wilmington, DE, USA) and RNA integrity was determined by gel electrophoresis.

RNA Sequencing (RNA-Seq)

Total RNA was extracted as above mentioned, 1∼2 μg RNA was used to prepare the sequencing library. Total RNA was enriched by oligo magnetic beads; RNA-seq library preparation involved the KAPA Stranded RNA-Seq Library Prep Kit (Illumina), which incorporates dUTP into the second cDNA strand and renders the RNA-Seq library strand-specific. The completed libraries were qualified by using Agilent 2100 Bioanalyzer and quantified by the absolute quantification qPCR method. Before sequencing the libraries, the barcoded libraries were mixed and single-stranded DNA was denatured in 0.1 mM NaOH solution, captured on Illumina flow cell, amplified in situ, and sequenced for 150 cycles for both ends on an Illumina HiSeq instrument. The differentially expressed genes and transcripts were fltered using R package Ballgown. Hierarchical clustering, Gene Ontology and Pathway analysis was performed with the differentially expressed genes in R, Python or shell environment for statistical computing and graphics.

Real Time-PCR

Total RNA was extracted as above, the reverse transcription reaction involved a 50-ng system of total RNA with a high-capacity cDNA archive kit following the instructions. qRT-PCR involved using the SYBR Green qPCR Kit (TaKaRa,Japan) on a Step-One plus PCR system (ABI, USA). The gene-specific primers and annealing temperatures are in **Supplementary Table 1**. The results were normalized to GAPDH level, and relative gene expression was measured with the $2^{-\Delta\Delta Ct}$ method (25).

Western Blot Analysis

CFs were seeded in 6-well plates and incubated for 24 h at 37°C. After the addition of angiotensin II (10 mM) for 2 h, CFs were treated with different concentrations of rGO solution (50 and 100 μg/ml) for 24 h. The treated CFs were lysed with RIPA buffer, and protein concentrations of cell lysates were quantified with a BCA protein assay kit. Equal amounts of protein (50 μg/lane) were separated on SDS-PAGE (different concentration of gel selected depending on the molecular weight of the target protein) and electro-blotted onto PVDF membranes. After blockade with 8% non-fat milk for 2 h, PVDF membranes were incubated overnight at 4°C with primary antibodies against Yes-associated protein (YAP) (CST, Danvers, MA) (1:1,000), Transcriptional coactivator with PDZ-binding motif (TAZ) (CST, Danvers, MA) (1:1,000), transforming growth factor β1 (TGF-β1) (Abcam, Cambridge, MA) (1:1,000), Samd3(Abcam, Cambridge, MA) (1:1,000), Smad4 (Abcam, Cambridge, MA) (1:5,000), and Col1A1(Abcam, Cambridge, MA) (1:1,000). Then, blots were washed three times with TBST and incubated with the corresponding horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Optical density of the bands was scanned by using Tanon-5500 Chemiluminescent Imaging System (Tanon, China). GAPDH was an endogenous control, and data were normalized to GAPDH levels.

Statistical Analysis

All results are expressed as mean ± standard deviation (SD). For multiple comparisons, one-way ANOVA was used with Bonferroni *post hoc* test. All statistical analyses were performed with SPSS 18.0 for Windows (PASW Statistics, SPSS Inc, Chicago, IL). $P < 0.05$ indicated a statistically significant difference.

RESULTS

rGO/Silk Patch Improves Heart Function

Cardiac systolic function was significantly decreased in infarcted rats as compared with the sham group (**Figures 1A–C**). After treatment with silk-alone patches, EF and FS were not improved as compared with the MI group, whereas hearts treated with rGO/silk patches showed a significantly higher recovery of cardiac function than MI-alone rats (EF: 62.53±6.23% vs. 48.11 ± 9.78%, $P = 0.002$; FS: 29.98 ± 4.23% vs. 21.38 ± 5.38%, $P = 0.003$). In addition, both ΔIVS and ΔLVPW were increased in the rGO/silk rats as compared with MI-alone rats (**Figures 1D,E**), we deduced that rGO/silk patches improved the systolic function of infarcted hearts. Furthermore, rGO/silk rats showed improved ventricular remodeling (**Figures 1F–H**): as compared with MI-alone rats, rGO/silk but not silk-alone rats showed decreased EDV (0.94 ± 0.22 vs. 1.27 ± 0.41 ml, $P = 0.071$) and ESV (0.37 ± 0.13 vs. 0.68 ± 0.27 ml, $P = 0.014$). LVM showed similar trends. Therefore, rGO/silk patches had a better effect on improving cardiac systolic function and ventricular remodeling of infarcted rat hearts than did silk-alone patches.

The stiffness of myocardial tissue of the infarcted zone is measured by passively stretching the muscle at a constant strain rate, which offers a direct approach to understanding intrinsic muscle compliance in hypertrophy and failure (26, 27). Young's modulus is a physical quantity describing the resistance of the solid material to deformation, and its magnitude is inversely proportional to elasticity. MI rats had higher Young's modulus both in the long axis and short axis as compared with sham rats (18.61 ± 6.61 vs. 4.74 ± 1.23 kPa, $P = 0.005$; short axis: 28.53 ± 9.71 vs. 6.13 ± 3.31 kPa, $P = 0.02$) (**Figures 1I,J**). In the rGO/silk group, the Young's modulus was significantly decreased in both the long axis and short axis as compared with the MI group (long axis: 7.63 ± 2.05 vs. 18.61 ± 6.61 kPa, $P = 0.02$; short axis: 13.03 ± 4.39 vs. 28.53 ± 9.71 kPa, $P = 0.016$). The silk-alone group showed similar results but not statistically significant in the short axis ($P > 0.05$). Therefore, both rGO/silk and silk-alone patches could decrease the Young's modulus of myocardial tissue in the infarcted zone. We speculated that the rGO/silk patch is more effective than the silk-alone patch perhaps because of the action of rGO. This rGO/silk patch provides direct mechanical support to the infarcted area, improves diastolic compliance and reduces wall stress, preventing chamber dilation.

rGO/Silk Patch Ameliorates Cardiac Fibrosis

After echocardiography evaluation, the hearts of each group were harvested (**Figure 2A**) and Masson's trichrome was used to stain paraffin sections, labeled collagen scar tissue (blue) and cardiac muscle (red). The hearts of the MI group showed the blue color of collagen scar tissue in the infarcted area; the group with rGO/silk patches showed decreased collagen scar and more cardiac muscle (**Figure 2B**). Then we assessed the LV wall thickness of the infarcted region in each group. rGO/silk patches increased LV wall thickness as compared with the MI and silk-alone group (500.64 ± 13.26 vs. 286.53 ± 13.14 μm, $P < 0.0001$; 500.64 ± 13.26 vs. 381.33 ± 18.3 μm, $P < 0.0001$), with no significant difference between the silk-alone and MI groups (**Figure 2D**). We further evaluated the collagen volume fraction (CVF%) of the infarcted region (**Figure 2E**). The rGO/silk patch group had the lowest CVF%, which indicates lower fibrosis of the infarcted zone than the MI group (67.1 ± 6.2 vs. 88.1 ± 5.6, $P = 0.012$), the CVF% for the silk-alone and MI groups was similar (86.5 ± 4.8 vs. 88.1 ± 5.6, $P = 0.0610.05$). These tissue-level histological results suggest that the rGO/silk patches can significantly alleviate the level of cardiac fibrosis, but the mechanism of the rGO/silk patch for ameliorates cardiac fibrosis was unknown.

Type I collagen is the main component of the extracellular matrix of the heart. As compared with the sham group, the MI group showed increased deposition of Col I in the

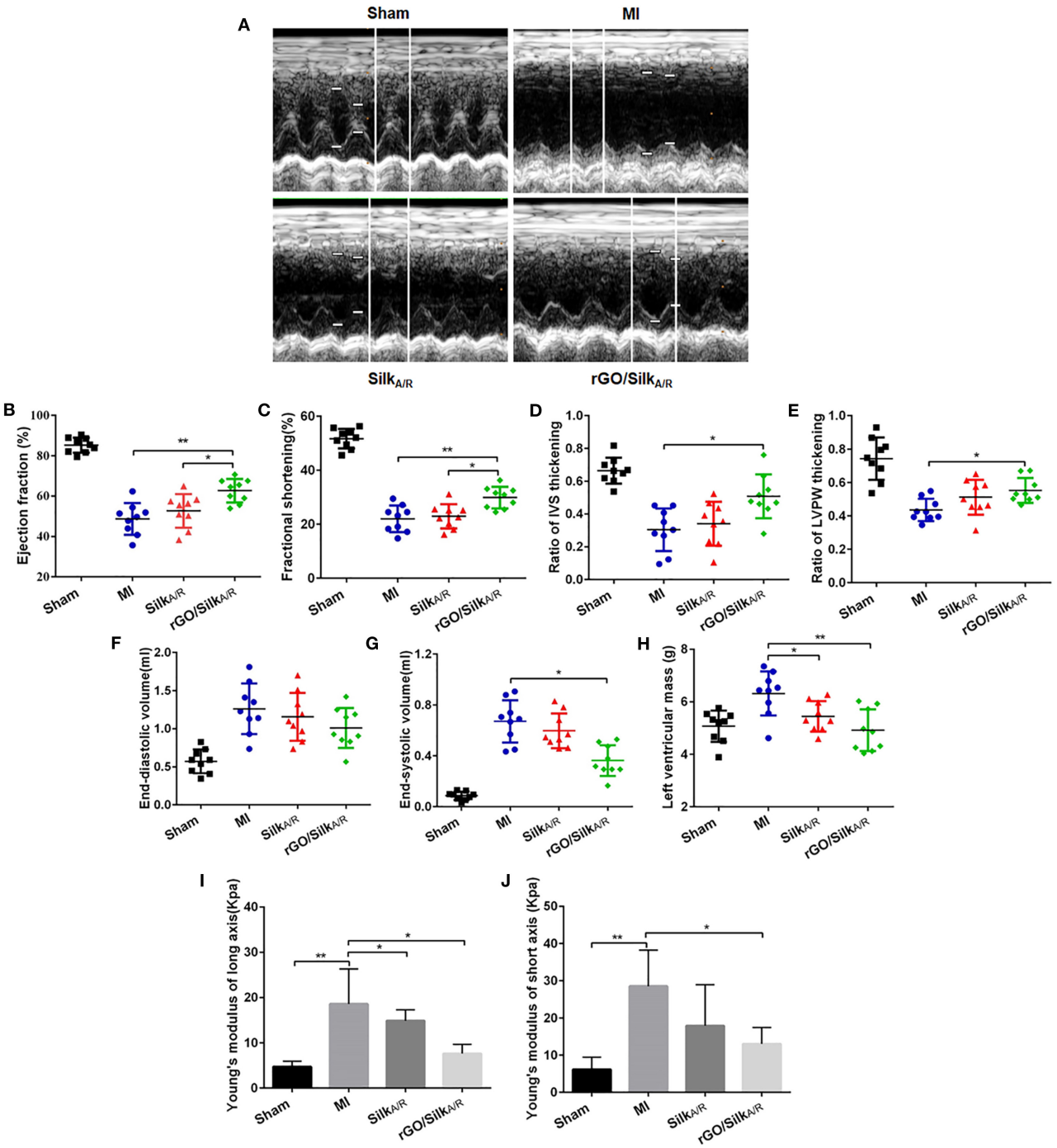

FIGURE 1 | Echocardiographic measurements and biomechanical tests of the effects of rGO/silk cardiac patches on the myocardial functional recovery of infarcted hearts of rats. **(A)** Echocardiography was performed at 4 weeks' post-implantation of patches. Quantification of parameters reflecting blood pumping function, including ejection fraction **(B)**, fractional shortening **(C)**, ratio of interventricular septum (IVS) thickening **(D)** and ratio of left ventricular posterior wall (LVPW) thickening **(E)** at 4 weeks after MI. Quantification of parameters reflecting ventricular remodeling, including left ventricular end-diastolic volume **(F)**, end-systolic volume **(G)** and left ventricular mass **(H)**. Young's modulus both in long axis **(I)** and short axis **(J)** were gauged by an BOSE Electro Force mechanical tester. Data are mean ± SEM from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

cardiac interstitial space (**Figures 2C,F**). With rGO/silk patches, deposition of Col I was decreased. Col I area fraction was significantly reduced in the rGO/silk group as compared with MI group (6.73 ± 0.79 vs. 10.22 ± 1.12, $P < 0.0001$), with no difference between the silk patch alone and MI groups, which suggests the potential role of rGO/silk cardiac patches

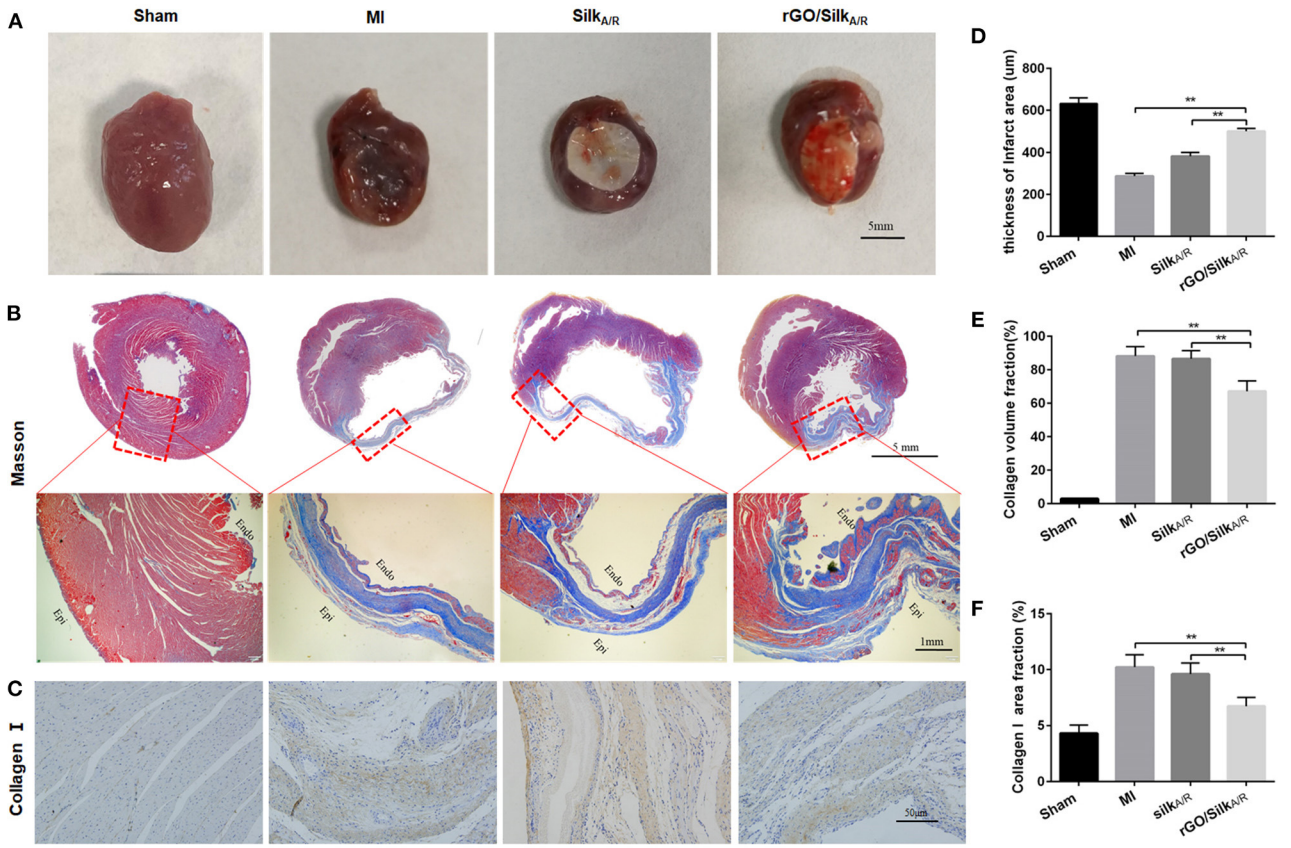

FIGURE 2 | Histological characterization of infarcted hearts. **(A)** Photographs of rat hearts harvested 28 days after implantation. **(B)** Masson's trichrome staining of the heart transections of rats. **(C–F)** Immunohistochemical staining and quantitative analysis of Col1a1 to detect cardiac fibrosis of myocardial tissues. Left ventricle wall thickness **(D)** and collagen volume fraction (CVF%) **(E)** of infarcted area. Data are mean ± SEM from three independent experiments. $^{**}P < 0.01$.

in diminishing the deposition of Col I in the infarcted area. Moreover, all these improvements were more obvious in the rGO/silk than silk-alone group. In addition, both the rGO/silk and silk-alone group showed very good histocompatibility, as measured by immunohistochemical staining with CD68, the specific marker of macrophages to observe the level of chronic inflammation (**Supplementary Figure 1**).

rGO Altered CF Cell Viability and Dysregulated Gene Expression

To observe the direct effect and potential anti-fibrosis mechanism of rGO on CFs, we used CF viability assay and RNA-seq. CF viability was measured after treatment with rGO at different concentrations (10, 20, 40, 60, 80, and 100 μg/ml). rGO inhibited CF proliferation dose-dependently (**Figure 3A**). Cell viability was sharply decreased with 40 and 60 μg/ml rGO, and more than 50% of CFs died. Therefore, 50 μg/ml was used as the 50% inhibitory concentration of rGO in the following experiments to investigate its effect on the function of CFs.

To explore the differential expression of genes involved in CFs after rGO treatment, we used RNA-seq to map and analyze the regulatory elements of the genome. A total of 13214 mRNAs were differentially expressed (**Figure 3B**). At fold change ≥1.5 and $P < 0.05$, 903 differentially expressed genes (DEGs) were detected, including 450 upregulated and 453 downregulated. The functions of the DEGs were predicted by using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. At the cellular component level, for the 453 downregulated DEGs, GO terms were mainly related to extracellular region, ECM and cell surface (**Figure 3C**). Hence, the function change of CFs after intervention of rGO focused on the extracellular region including ECM. Moreover, KEGG pathway analysis showed that those DEGs were involved in signal transduction pathways (**Supplementary Figure 2**), including IL-17 signaling pathway (PATH: rno04657), cytokine receptor interaction (PATH: rno04060) and hypertrophic cardiomyopathy (PATH: rno05410).

The Mechanism of the rGO/Silk Patch in Ventricular Fibrosis After MI

To validate the potential mechanism of the rGO/silk patch in reducing the cardiac fibrosis of the infarcted zone, we observed the protein expression of the canonical TGF-β1/Smads signaling pathway and mechanical stress-related signaling pathway, the

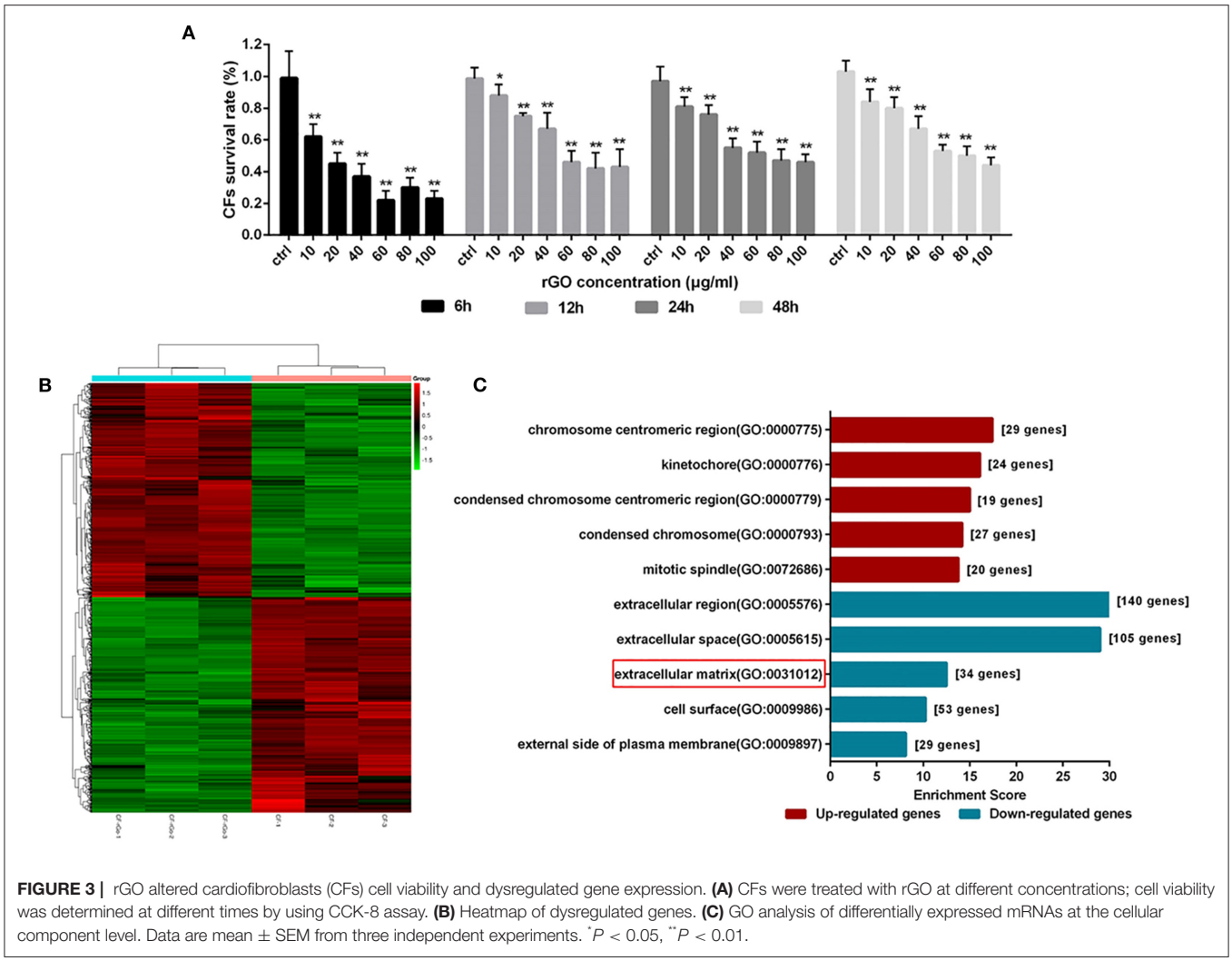

FIGURE 3 | rGO altered cardiofibroblasts (CFs) cell viability and dysregulated gene expression. **(A)** CFs were treated with rGO at different concentrations; cell viability was determined at different times by using CCK-8 assay. **(B)** Heatmap of dysregulated genes. **(C)** GO analysis of differentially expressed mRNAs at the cellular component level. Data are mean ± SEM from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

Hippo pathway (specifically including YAP/TAZ), by western blot analysis. The expression of the TGF-β1/Smads signaling pathway and YAP/TAZ genes in the silk-alone group was downregulated as compared with the MI group (**Figures 4A–C**), which suggests that the silk patch may activate Hippo signaling via mechanical support, then downregulate the expression of the TGF-β1/Smads signaling pathway. Furthermore, the rGO/silk patch significantly reduced the expression of YAP/TAZ and myocardial fibrosis biomarkers TGF-β1, Smad3, and Smad4 in the infarcted rat myocardium as compared with silk alone (**Figures 4A–F**), so the rGO/silk patch had a better effect on reducing the expression of the TGF-β1/Smads signaling pathway than silk alone. rGO may have direct effect on the TGF-β1/Smads and YAP/TAZ pathways.

To further test this hypothesis, we observed the angiotensin-induced expression of Col I and Co1 III by ELISA and real-time PCR after rGO intervention. As compared with the angiotensin II control group, after treatment with different concentrations of rGO (50 and 100 μg/ml), the secretion was decreased but not dose-dependently for Col I (9.32 ± 0.18 vs. 7.41 ± 0.45 ng/ml, $P < 0.001$; 9.32 ± 0.18 vs. 8.04 ± 0.29 ng/ml, $P < 0.001$) and Col III (34.65 ± 2.46 vs. 28.68 ± 1.01 ng/ml, $P < 0.001$; 34.65 ± 2.46 vs. 30.47 ± 3.10 ng/ml, $P = 0.006$) (**Figures 5A,B**). Angiotensin II upregulated the mRNA level of Col I, Col III and TGF-β1 ($P < 0.01$) (**Figures 5C–E**). After treatment with rGO (50 and 100 μg/ml), the mRNA levels showed similar decreased levels as with ELISA: Col I (4.55 ± 1.47 vs. 0.74 ± 0.23, $P < 0.0001$; 4.55 ± 1.47 vs. 0.81 ± 0.12, $P < 0.0001$), Col III (2.43 ± 0.81 vs. 0.81 ± 0.28, $P = 0.001$; 2.43 ± 0.81 vs. 1.61 ± 0.23, $P = 0.063$). Moreover, the mRNA level of TGF-β1 was decreased (1.1 ± 0.1 vs. 0.79 ± 0.13, $P = 0.001$; 1.1 ± 0.1 vs. 0.88 ± 0.12, $P = 0.009$) as compared with the angiotensin II control.

To confirm the molecular mechanism of rGO reducing cardiac fibrosis, we compared the protein levels of YAP/TAZ and canonical TGF-β1/Smads signaling pathway at the cytological level. The protein levels of YAP/TAZ, TGF-β1, Smad3, Smad4 and Col I was increased after angiotensin II treatment ($P < 0.05$) (**Figures 5F–K**). With rGO treatment (50 and 100 μg/ml), the protein levels of the above genes were significantly decreased but not dose-dependently, and the differences were

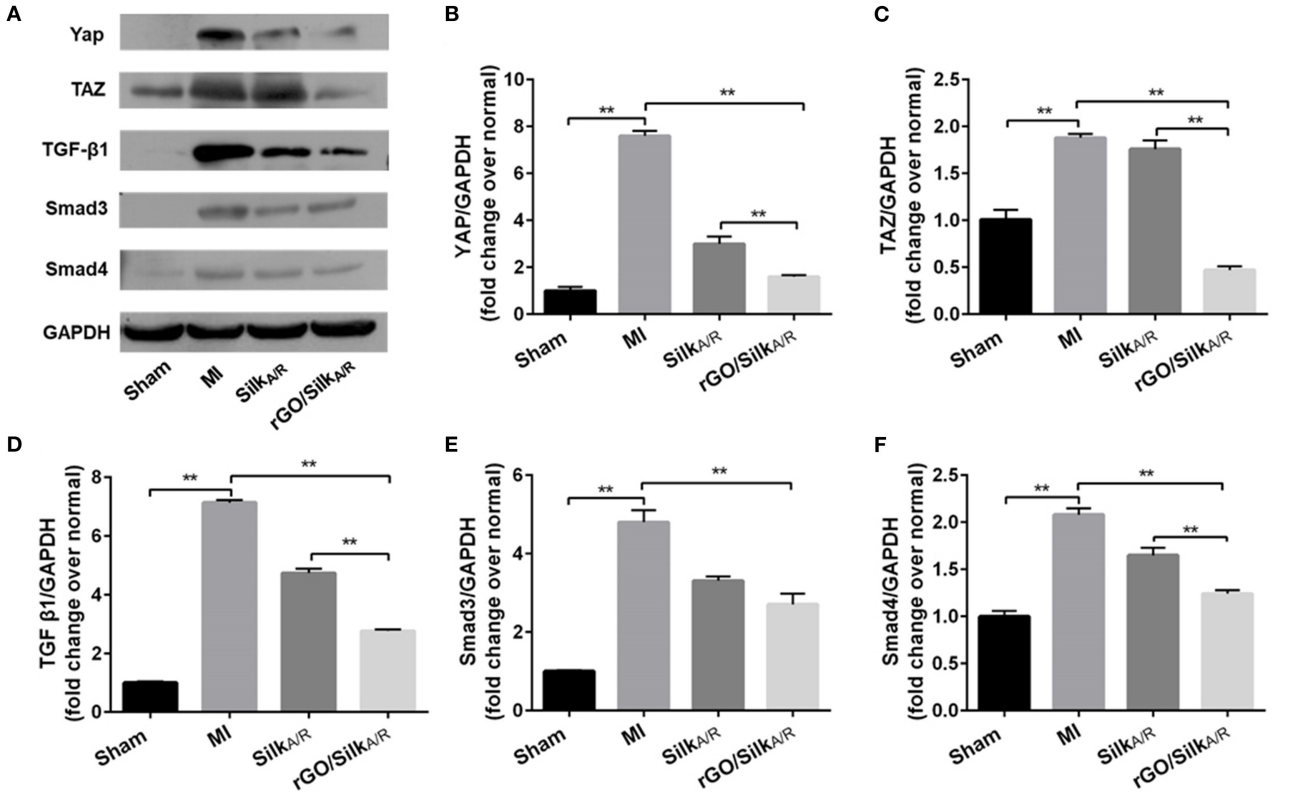

FIGURE 4 | Western blot analysis of the expression of YAP/TAZ and canonical TGF-β1/Smads signaling pathway in infarcted region. Myocardial fibrosis (YAP, TAZ, TGF-b1, Smad3 and Smad4) in rat myocardium was examined at 28 days' post-surgery. Data are mean ± SEM from three independent experiments. Data are mean ± SEM from three independent experiments. $^{**}P < 0.01$.

statically significant ($P < 0.05$). Thus, rGO reduced the cardiac fibrosis possibly through YAP/TAZ and the TGF-β1/Smads signaling pathway. Our data suggested that the addition of rGO regulated the expression of collagen. The introduction of rGO could promote the nuclear translocation of YAP/TAZ, which subsequently regulated the transcription of the fibrosis-related gene TGF-β. The proteins downstream of TGF-β, Smad proteins, activated the fibrotic genetic program to regulate collagen synthesis and ECM deposition (**Figure 6**).

DISCUSSION

In the present study, we constructed a silk-derived nanofiber material incorporating rGO and used this rGO/silk cardiac patch to repair rat hearts after AMI. This reduced GO functionalized silk biomaterial showed important therapeutic effects by improving cardiac function and attenuating cardiac fibrosis of infarcted hearts. We further revealed the roles of rGO/silk in reducing fibrosis and inhibiting the secretion of Col I and Co1 III, which provides new insights into the potential molecular mechanism of the rGO/silk cardiac patch but also helps explain the phenomenon of myocardial functional recovery in infarcted hearts.

Cardiac patches based on synthetic scaffolds are becoming a new strategy for treating AMI (28, 29). Previous studies have investigated implantation of different types of cardiac patches in AMI animal hearts, with positive results including improved cardiac function, neovascularization and attenuated fibrosis (30–32). The biology and mechanics of healing infarcted hearts are complex; mechanical support is the most common explanation for cardiac patches improving cardiac function and ventricle remodeling *in vivo*. Previous studies showed that treating MI rat hearts with appropriate mechanical supports, such as a woven nylon cardiac restraints or poly(l-lactide-co-ε-caprolactone) patches, can also reduce LV diameter and improve some cardiac functions (12). By mechanically reinforcing the infarcted area, cardiac patches decrease wall stress in the infarct and adjacent border zone, which can improve pump function and reduce LV dilation (2). Our rGO/silk patch had not only excellent mechanical but also special biological properties, which could restore cardiac function and reduce fibrosis of the rat heart after AMI.

Silk fibroin is one of the major proteins forming the silkworm cocoon, which has shown excellent biocompatibility, biodegradability and mechanical properties, thus silk fibroin is regard as a promising biomaterial in cardiac tissue engineering (33, 34). SF has been applied in various forms

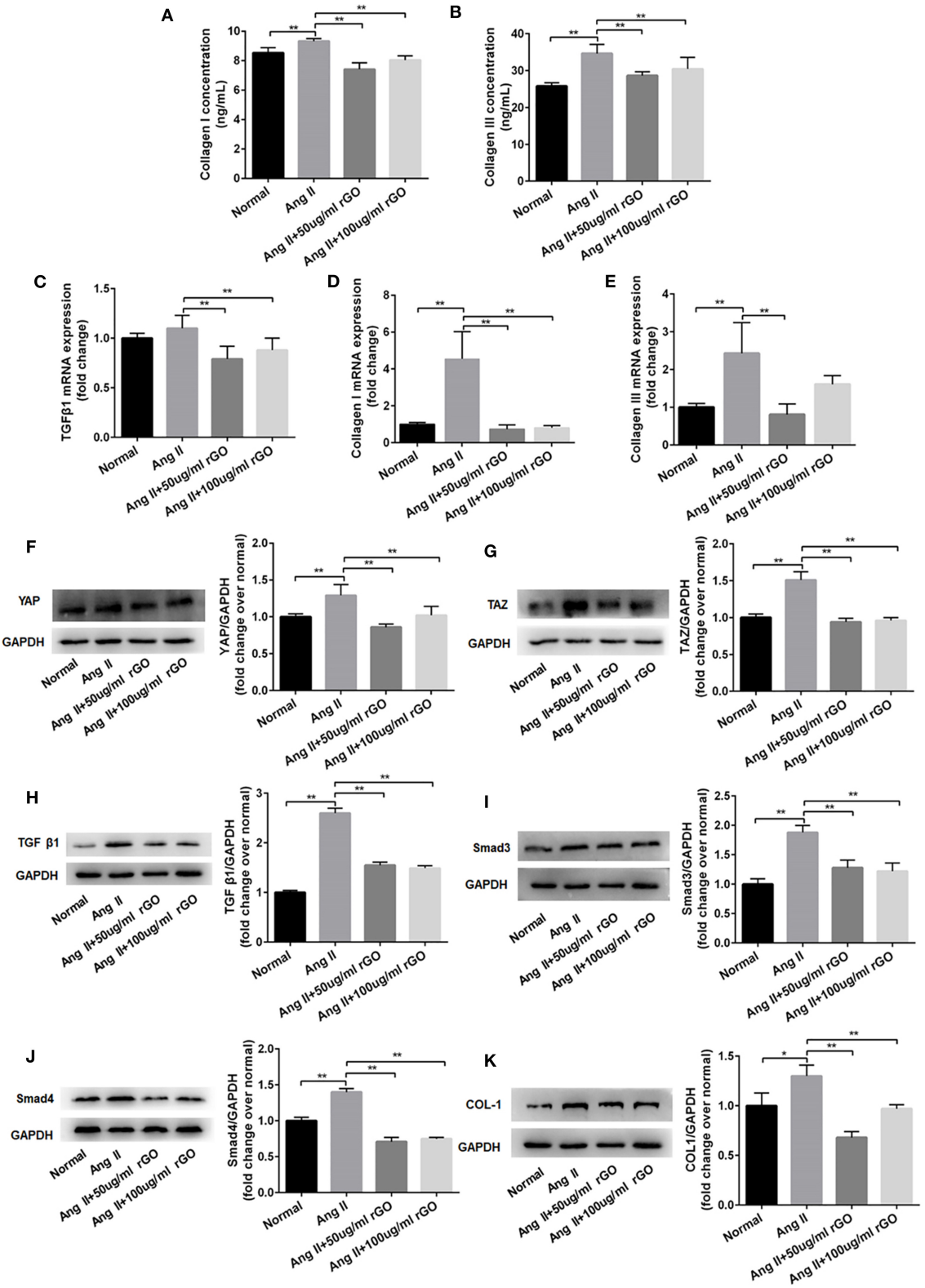

FIGURE 5 | rGO decreased the secretion of Col I and Co1 III in cardiofibroblasts (CFs) through Yap/Taz-TGFβ1/Smads signaling. **(A,B)** Effect of rGO on the secretion of Col I and Co1 III by CFs after angiotensin II (Ang II) treatment; CFs were treated with Ang II and rGO at 50 or 100 μg/ml for 24 h. **(C–E)** Effect of rGO on the mRNA expression of Col I, Co1 III and TGF-β1. **(F–K)** Western blot analysis of the signaling pathway (YAP, TAZ, TGF-b1, Smad3, Smad4 and Col I) in CFs stimulated with Ang II and with rGO. Data are mean ± SEM from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

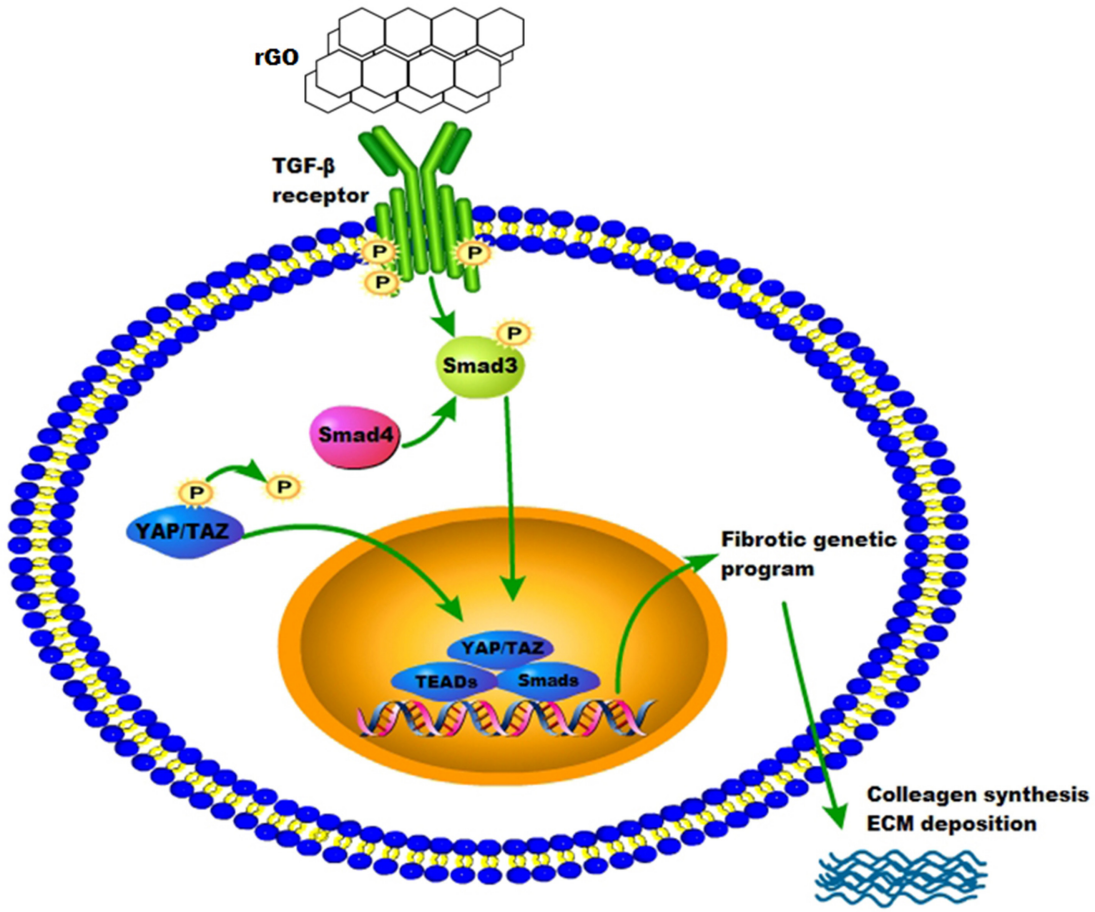

FIGURE 6 | Proposed model of the possible mechanisms of rGO in reducing myocardial fibrosis.

of biopolymer-based composites as cardiac patches and has demonstrated ideal efficacy (14). These scaffolds fabricated by SF can mimic the natural extracellular matrix (ECM) of hearts and provide mechanical support for the recovery after myocardial infarction. However, simple SF scaffolds are limited by poor biological functions Therefore, we incorporate the rGO/silk fibroin-modified nanofibrous patches. The rGO/silk patch is a bi-layered scaffold with isotropic mechanics, incorporating an epicardial-facing cardiac rGO-enriched layer. With the addition of rGO, the beneficial effects were significant in rat hearts with a rGO/silk patch vs. a silk-alone patch. However, as we previously showed, Young's moduli showed no significant change between silk alone and rGO/silk biomaterials (23), so the beneficial effects are not due to different mechanical characteristics. We speculated that rGO may magnify the effect of silk, which reduced cardiac fibrosis by mechanical support.

rGO has attracted great attention in tissue engineering because of its excellent physical and chemical properties including electrical conductivity and mechanical properties and also multiple biological functions to promote the adhesion, growth, proliferation and differentiation of various cells including neural, embryonic, pluripotent and mesenchymal stem cells (35–37). Lee et al. found that rGO/hydroxyapatite matrixes promoted osteogenesis of MC3T3-E1 pre-osteoblasts and induced new bone formation, which has potential application in future bone regenerative medicine (38). A similar repair effect of rGO was found in nerve regeneration and skin tissue engineering (37, 39, 40). In a related study, rGO and its derivatives were used in cardiac tissue engineering, which showed good electric connection between healthy myocardium and cardiomyocytes in the infarcted zone and enhanced cell–cell coupling (41–45). Furthermore, Norahan and co-workers demonstrated that rGO-incorporated collagen scaffolds could improve mechanical properties and cell viability with increasing cardiac gene expression. Also, rGO coating showed antibacterial activity for cardiac patch application (46). In our study, the rGO/silk patch had effects on macrophage, which reflects chronic inflammation by immunohistochemical staining with CD68. Both expression level of CD68 and investigation of differentially expressed mRNAs in cardiac fibroblasts indicated that rGO/silk patch induced infection/immunity and pro-inflammation. A related study also found that the biological application of GO in timely

modulation of the immune environment in MI for cardiac repair (47), so the anti-inflammation role of rGO maybe another reason for myocardial functional recovery in infarcted hearts.. Additionally, the rGO/silk patch significantly decreased CVF% at 28 days post-MI in a rat model, similar effects as with angiotensin-converting enzyme 1, recognized as a good inhibitor of reverse ventricular remodeling (48). However, the molecular mechanism of rGO reducing fibrosis was still unknown.

After AMI, CFs play a critical role in cardiac remodeling by synthesizing and depositing ECM, communicating with myocytes and other cells. Activation of the renin-angiotensin aldosterone system and release of TGF-β induces conversion of fibroblasts into myofibroblasts, promoting deposition of ECM proteins (4). Related studies revealed that fibroblast differentiation arrest was mediated by the Hippo–YAP pathway, which regulated ECM composition and vascular remodeling during heart development and restore cardiac function (49, 50). Recent studies suggested that rGO and its derivatives could regulate other cell biological functions through TGF-β signaling. Li et al. found that graphene could trigger apoptosis of macrophages by activating the mitochondrial pathway via mitogen-activated protein kinase and TGF-β signaling (51). Also, rGO triggers specific biochemical and biological responses via TGF-β signaling. rGO induced neuronal differentiation by affecting YAP/TAZ localization outside the nuclei and increasing the level of neuronal differentiation marker (52). In this study, the expression of the canonical TGF-β1/Smads signaling pathway and YAP/TAZ in the infarcted rat myocardium was markedly decreased with the rGO/silk patch as compared with the MI and silk patch group. On RNA-seq, the expression of the TGF-β1/Smads signaling pathway in CFs was decreased after rGO treatment. Furthermore, the protein expression of the TGF-β1/Smads signaling pathway and YAP/TAZ in CFs stimulated by angiotensin II and with rGO treatment was reduced. Thus, rGO may play a major role in reducing infarct fibrosis via YAP/TAZ genes and the TGF-β1/Smads signaling pathway, but further study is needed to determine the mechanism of rGO regulating the expression of YAP/TAZ genes.

In this study, we have shown the importance of rGO/silk patch to repair the infarcted myocardium and its potential mechanism of the anti-fibrosis effect, there still exist some limitations that require further investigations. First, the cardiac patch was applied on the epicardium through suturing, which limits clinical translation and applications. second, combining the rGO/silk patch with some stem cells which has shown good therapeutic effects for MI may promote the therapeutic effectiveness. Third, the mechanisms for the observed effects of rGO/silk patch in this study are not fully understood and deserve further investigations.

CONCLUSION

rGO/silk, magnifying the effect of the silk-alone patch, improved cardiac function and reduced cardiac fibrosis in heart tissue after AMI. The mechanism of the anti-fibrosis effect may be rGO regulating the Yap/Taz-TGFβ1/Smads signaling pathway in CFs. rGO could be a potential candidate in cardiac tissue engineering and cardiac patch therapeutic strategies.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the GEO repository, accession number GSE179878.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care Committee at Xi'an Jiaotong University.

AUTHOR CONTRIBUTIONS

DG and XZ led the project and supervised the experiments. YF, GZ, MX, XX, and LY conducted experiments and fulfilled data analysis and performed the bioinformatics work. YF, YM, and MQ discussed the results. YF wrote the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.718055/full#supplementary-material

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REVIEW
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Son of a Lesser God: The Case of Cell Therapy for Refractory Angina

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In the last decades, various non-pharmacological solutions have been tested on top of medical therapy for the treatment of patients affected by refractory angina (RA). Among these therapeutics, neuromodulation, external counter-pulsation and coronary sinus constriction have been recently introduced in the guidelines for the management of RA in United States and Europe. Notably and paradoxically, although a consistent body of evidence has proposed cell-based therapies (CT) as safe and salutary for RA outcome, CT has not been conversely incorporated into current international guidelines yet. As a matter of fact, published randomized controlled trials (RCT) and meta-analyses (MTA) cumulatively indicated that CT can effectively increase perfusion, physical function and well-being, thus reducing angina symptoms and drug assumption in RA patients. In this review, we (i) provide an updated overview of novel non-pharmacological therapeutics included in current guidelines for the management of patients with RA, (ii) discuss the Level of Evidence stemmed from available clinical trials for each recommended treatment, and (iii) focus on evidence-based CT application for the management of RA.

Keywords: refractory angina, cell therapy, meta-analyses, recommendation, guidelines

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INTRODUCTION

Refractory angina (RA) is a clinical condition defined by the presence of persistent (≥3 months) symptoms of angina, according to the Canadian Cardiovascular Society (CCS) class, which is caused by untreatable coronary artery disease with objective evidence of reversible myocardial ischemia (1, 2). Clinical data Registry of the prevalence and incidence of RA remain limited and geographically clustered, making difficult a comprehensive evaluation of this clinical problem worldwide. One of the most widely recognized critical issues for the lack of epidemiologic data is the heterogeneous phenotype of patients labeled with a diagnosis of RA, which encompasses those with incomplete revascularization, unsuitable coronary anatomy, comorbidities, and other coronary disorders (3). The global prevalence of RA is increasing due to the growing prevalence of advanced aged population with coronary artery disease. According to the Heart Disease and Stroke Statistics-2019 (4), 9,400,000 patients are estimated to live with chronic angina and the proportion of these patients meeting the RA criteria lies in the esteem of about 7.5% (1, 5). On the basis of 1 million cardiac angiograms performed *per* year in the USA (6), the incidence of RA is then estimated to be 67,000 new cases/year (7).

The successful management of RA is often extremely challenging. At present, therapeutic options span from lifestyle modifications, state-of-the-art pharmacological therapy up to the most advanced mechanical revascularization solutions, with the main goal of improving prognosis, minimizing or abolishing symptoms and preventing episodes of angina (8–10). Nevertheless, it is important to underline that patients with persistent or recurrent chest pain despite optimal medical therapy frequently attend the general practitioner and/or the outpatient referrals and revisit hospital emergency departments, often undergoing repeated angiographic investigations. In this context, the social and economic burden for National Health Systems remains considerable due to the high rates of hospitalizations and multiple medications despite a limited quality of life. A recent Ontario-based study conservatively estimated the annual costs of angina-related disability (from a societal perspective including direct, indirect, and system costs) at $19,209 *per* patient (11).

Given these premises, novel treatments for RA in patients nonresponsive to standard pharmacologic therapies and not amenable to mechanical revascularization procedures are evermore needed. Notably, the one and only anti-anginal drug approved by the US Food and Drug Administration (FDA) in the last 20 years ranolazine (a selective inhibitor of the late sodium current—INaL—in cardiomyocytes) (12), was recently questioned in light of the uncertain evidence related to the safety and efficacy in reducing cardiovascular mortality, all-cause mortality, non-fatal acute myocardial infarction and frequency of angina (13).

It is worth to highlight that the long-term mortality of patients with RA is not as high as previously thought, reaching a 9-year rate of 28.4% (13). Therefore, the goal of novel therapies is primarily aimed at improving quality-of-life and chest pain relief rather than extending lifespan.

GUIDELINES AND RECOMMENDATIONS FOR REFRACTORY ANGINA

Several innovative therapeutics have been developed to specifically address anginal symptoms. As suggested by Gallone et al. (3), these therapeutics can be classified as treatments targeting myocardial perfusion by (i) invasive/non-invasive interventions or treatments addressing neural processing and by (ii) chemical, mechanical or electrical means to interfere with pain signal. The former includes enhanced external counterpulsation (EECP) (14), coronary sinus reducer (CSR) (15), transmyocardial laser revascularization (TMLR) (16), extracorporeal shockwave myocardial revascularization (17), and cell-based applications (18). The latter comprises spinal cord stimulation (SCS) (19), cervico-thoracic stellate ganglion blockade/sympathectomy (20), and subcutaneous or transcutaneous electrical nerve stimulation (21).

TABLE 1 | Level of evidence of non-pharmacological treatment options in the 2019 ESC and 2012–2014 ACC/AHA guidelines for refractory angina.

Treatment strategy	2019 ESC guidelines		2012–2014 ACC/AHA guidelines	
	Class	Level of evidence	Class	Level of evidence
Enhanced external counterpulsation	IIb	B	IIb	B
Spinal cord stimulation	IIb	B	IIb	C
Coronary sinus reducer	IIb	B	–	–
Transmyocardial laser revascularization	III	A	IIb	B

Noteworthy, some of the above-mentioned technologies have already provided evidence of feasibility and clinically efficacy for RA patients qualified as "no option." Notably, as the Level of Evidence supporting such advanced therapeutic strategies differs significantly and is constantly evolving as new evidence becomes available, guidelines are needed to incorporate such information. The most updated clinical practice guidelines for RA have been issued by the task forces of the American College of Cardiology/American Heart Association (ACC/AHA) in 2012 (22) and 2014 (23) and the European Society of Cardiology (ESC) in 2019 (2), respectively. In essence, these guidelines provide recommendations related to the treatment options available for RA based on a systematic review of the up-to-date evidence at the time of their publication. As usual, these recommendations are rely on Level of Evidence (from A to C) and class of recommendation (I, IIa, IIb, and III). Among the emerging non-pharmacological technologies, those listed in current United States (US) and Europe (EU) guidelines (**Table 1**) are the following: (1) EECP, (2) SCS, (3) CSR, and (4) TMLR. Cumulatively, EECP, are considered as treatments recommended for RA, even if with a relatively weak effectiveness level (class of recommendation IIb/Level of Evidence B). Conversely, TMLR is currently not recommended in EU (class of recommendation III/Level of Evidence A) (2).

Enhanced External Counterpulsation

The EECP is a non-invasive FDA approved therapy for patients with RA. The first model of external counterpulsation dates back to the 60's. The modern EECP, developed in 1983 (24), consists of three pairs of external cuffs compressing the calves, lower and upper thighs, which are inflated/deflated from distal to proximal according to the cardiac cycle. While in diastole the device aims to increase the retrograde aortic flow, improve coronary perfusion and venous return, in systole it reduces systemic vascular resistance, improve cardiac workload and systemic perfusion. The standard treatment protocol includes a total of 35 1-h sessions (5 days/week for 7 weeks). Two different and complementary mechanisms of action have been associated with

Abbreviations: ACC, American College of Cardiology; AHA, American Heart Association; BM, Bone marrow; CABG, Coronary artery bypass grafting; CCS, Canadian Cardiovascular Society; CSR, Coronary Sinus Reducer; EECP, Enhanced external counterpulsation; ESC, European Society of Cardiology; FDA, Food and Drug Administration; MACE, Major adverse cardiac events; MNC, Mononuclear cells; MTA, Meta-analysis; RA, Refractory Angina; RCT, Randomized controlled trial; SCS, Spinal Cord Stimulation; TMLR, Transmyocardial laser revascularization.

the beneficial anti-ischemic effects of EECP therapy. Firstly, it was supported the concept that EECP, akin to a circulatory support, exerts central hemodynamic effects by improving coronary collateral growth and fractional flow reserve (i.e., oxygen supply) (25, 26). Secondly and more recently, researchers have focused on the direct and durable effect of EECP on the peripheral vasculature (i.e., oxygen demand). In particular, EECP has been shown to reduce arterial wall stiffness, promote peripheral artery flow-mediated dilation and improve shear stress, thus modulating the release of endothelial-derived vasoactive agents, pro-inflammatory cytokines, endothelial adhesion molecules and markers of lipid peroxidation (27–29).

The largest randomized controlled trial (RCT) aimed at evaluating the efficacy of EECP therapy in patients with RA (MUST-EECP trial) indicated that the application of EECP, when compared with a sham protocol ($n = 59$ treated patients vs. $n = 65$ controls), is safe with minor adverse events and provides clinical improvements in relation to the frequency of angina episodes, use of nitrates and time to exercise-induced ischemia. A number of smaller observational and randomized clinical trials (27–36) have generated three relevant meta-analyses (MTA) reporting positive results with regard to objective and subjective outcomes of angina (37–39). In particular, Qin et al. (37) showed a significant increase in myocardial perfusion, particularly in those patients who completed the entire 35 EECP sessions (pooled weighted mean difference from pre- to post-EECP: −0.19, 95% CI: −0.38 to 0.00, $p = 0.049$). However, as also declared by the authors, this study presented some limitations including the small sample size ($n = 109$ patients) and the high variability among imaging techniques applied (37). Other MTA found a reduction of at least 1 CCS functional class in 85% of patients treated with EECP (38, 39). Notably, some investigators confirmed the sustained benefit of EECP therapy for up to 5 years in relation to the frequency of angina episodes and major adverse cardiac events (MACE), although the results mainly stemmed from uncontrolled studies (34, 36, 40).

Based on these premises, the ACC/AHA and ESC guidelines concordantly recommend a class IIb/Level of Evidence B for EECP. However, it is important to highlight that, despite substantial evidence in its favor, EECP application has still not widely entered clinical practice since a number of critical issues and limitations remain unresolved, including the time-consuming protocol (1 h for 35 days), minor and major contraindications (e.g., coagulopathy, arrhythmias, peripheral artery, and venous disease), reimbursement issues and the lack of specialized centers.

Spinal Cord Stimulation

Spinal cord stimulation (SCS) is a FDA-approved device conceived to alleviate chronic pain derived from various pathological conditions including chronic RA. The device consists in a programmable pulse-generator placed subcutaneously, below the left costal arch, and multipolar leads which are introduced under fluoroscopic guidance into the epidural space between the C7 and T4 level to obtain precordial pain relief. Although the standard protocol requires generally 1-h session, 3 times a day, the SCS device allows the modulation and the self-control of the stimulation based on the intensity of angina attacks. The precise mechanism by which SCS acts is still not fully understood. Its use was proposed for the first time on the basis of the "pain gate control" theory according to which impulses are transmitted in the nociceptive C-fibers of the central nervous system (41, 42). In patients with RA, SCS can provide dual beneficial effects: an analgesic effect by reduction of cardiac neuron activity following an ischemic attack, and a more debated anti-ischemic effect by adenosine-mediated coronary vasodilation and reduction of sympathetic tone (43–48). For example, the implantation of SCS device in RA patients has been associated with the improvement of myocardial ischemia tolerance, myocardial blood flow, and endothelium-mediated vasomotor function (48).

In the clinical setting of RA, spinal cord stimulation has been widely investigated in uncontrolled studies (48–54) or in comparison with various control treatments such as mechanical revascularization, standard-of-care or inactivated device (46, 55–57). Most of them reported positive results as regard to angina symptoms, quality of life, and acute hospital admissions. In particular, the ESBY trial, in which 53 RA patients receiving SCS were compared with 51 controls receiving coronary artery bypass grafting (CABG) for symptomatic indication "only", demonstrated an equivalent effect of both treatments in terms of angina relief at 6 months ($p < 0.0001$); although the CABG group experienced higher exercise capacity and decreased ST-segment depression at follow-up (56). Moreover, the analysis of 121 patients enrolled in the European Angina Registry Link Study indicated a long-term efficacy of SCS implantation (mean 12.1 months) (49). Unfortunately, the STARTSTIM trial, which was designed to enroll a sufficient number of patients to support regulatory approval in the United States (by measuring the time to angina onset on standard exercise treadmill test at 6 months as primary endpoint), was prematurely stopped due to low recruitment rate (58). By merging the results of multiple clinical studies, five MTA and systematic reviews have been published so far (59–63). The comprehensive analysis of 14 studies which includes a total of 518 participants demonstrated that patients receiving SCS have longer exercise resistance (1.90 min, 95% CI: 1.71, 2.06), lower angina frequency (1.55 less daily; 95% CI: −1.75, −1.33), reduced nitrate consumption (1.54 less daily; 95% CI: −1.81, −1.26) and improved quality of life (95% CI: 10.76, 32.81; $p < 0.0001$) (59). These encouraging results were mitigated by other MTA which reported mild or small angina improvements (60, 63), also arising the problem of study interpretation due to the great variability in clinical trial designs (62). Although the safety profile appears to be satisfactory, a number of complications strictly related to the device implantation were reported and includes implant failure (49), lead displacement and superficial infections at the side of electrode insertion or pulse-generator (54). In essence, SCS in this clinical context does not seem to be an attractive area of investigation anymore if we look at the number of ongoing registered studies on clinicaltrials.gov. Consistently, the most recent ACC/AHA and ESC guidelines for the management of chronic stable angina made no change to recommendation for the use of SCS which remains Class IIb/Level of

Evidence C in US (22) and Class IIb/Level of Evidence B in EU (2).

Coronary Sinus Reducer

The coronary sinus reducer (CSR) is a relatively novel CE marked device designed to reduce disabling symptoms and improve quality-of-life of patients dealing with RA (15). It follows a long-standing concept of surgical narrowing of the coronary sinus proposed by Beck and colleagues between 1950's and 1960's (64). Basically, it is a balloon expandable stainless-steel mesh with the shape of an hourglass that is implanted percutaneously *via* the right jugular vein and works by creating a focal narrowing of the coronary sinus lumen. The subsequent elevated backward pressure in the coronary venous system leads to redistribution of blood flow from the less ischaemic subepicardium to the more ischaemic subendocardium. As therapy for "no option" RA patients, CSR was proposed for the first time in 2007 (65). Although based on registries and open-label/uncontrolled trials (66–70), the majority of published studies provided evidence of angina relief showing a 70–80% rate of treatment-responders (15). In this context, the largest available study is the COSIRA trial (COronary SInus Reducer for treatment of refractory Angina) in which 52 RA patients were allocated to CSR implantation and 52 to a sham procedure (71). After 6 months from the device implantation the 71% of treated patients experienced an improvement of at least 1 CCS class as compared with 42% of controls ($p = 0.003$). In addition, a *post-hoc* efficacy analysis revealed a significant between-group differences in exercise time improvement (+27.9, 95% CrI = 2.8–59.8%) and quality of life (stability +11.2 points, 95% CrI = 3.3–19.1; perception +11.0, 95% CrI = 3.3–18.7) (72). Consistently, a systemic review, by combining the results of six studies and 196 patients, showed that CSR significantly improves CCS angina class (from 3.2 at baseline to 1.9 after a mean follow-up of 8.6 months) (73). On the other hand, this work provides some interesting insights about the CSR safety profile. Indeed, a 2% implantation failure rate (e.g., unsuitable coronary sinus or valvular anatomy) as well as a 2.6% of short-term complications (e.g., migration, hematoma, non-ST elevation myocardial infarction) were documented (73). It is worth to highlight that 20–30% of patients are still deemed non-responders for reasons still not fully elucidated. In the attempt to predict responsiveness to CSR implantation, Baldetti et al. (74) measured the differential pressure between baseline right atrial pressure and coronary sinus systolic pressure in the context of coronary sinus balloon occlusion showing that the patient group having a developed accessory venous drainage systems had lower anti-ischemic effects due to preserved alternative coronary venous outflow.

Interestingly, a health technology analysis on CSR device for RA patients was recently made available (75). Results confirmed the positive impact of CSR regarding both objective and subjective endpoints of ischemia (i.e., Seattle Angina Questionnaire score, dobutamine echocardiography, thalium single-photon emission computed tomography perfusion studies, and 6-min-walk test and myocardial perfusion reserve index). Yet, these findings should be considered with caution since the lack of internal validity of included studies may have undermined the positive results. More definitive indications will likely come from the on-going clinical investigations evaluating (i) the long-term safety and benefit of CSR therapy (NCT02710435), (ii) the objective improvement of CSR implantation in terms of exertional capacity and myocardial reversible ischemia (NCT04121845). According to the abovementioned evidence, CSR device received class IIb recommendation and Level of Evidence B from the 2019 ESC guidelines. In US, CSR was granted with a "Breackthrough Designation" by the FDA in 2018 based on the "orphan" need of this population but additional data are required to enter into US guidelines.

Transmyocardial Laser Revascularization

The transmyocardial laser revascularization (TMLR) technique uses FDA approved laser ablation (i.e., carbon dioxide, holmium: yttrium-aluminum-garnet [Ho:YAG] or XeCL excimer) to create transmural channels in targeted ischemic regions of myocardium to restore myocardial perfusion. The beneficial effect of TMLR has been ascribed to two principal mechanisms; sympathetic denervation that acts for the acute clinical benefits and angiogenesis responsible for the long-term benefits. The procedure was performed either surgically or percutaneously.

The surgical approach *via* thoracotomy or sternotomy allows direct position of a laser device on the epicardial surface of the left beating ventricle and the delivery of ∼1 mm transmural laser channels from the epicardium to the endocardium. In the past years surgical TMLR for RA was investigated either as a stand-alone therapy for patients not suitable to further revascularization procedures (76–81) or in combination with CABG for those patients who would be incompletely revascularized with CABG alone (82–86). In particular, Allen et al. (80) demonstrated the superiority of sole TMLR vs. best medical treatment in improving classes of angina ($p < 0.001$), survival free from cardiac events ($p < 0.001$), exercise tolerance ($p = 0.05$), and quality-of-life scores ($p = 0.003$). However, a similar study design did not demonstrate objective difference in exercise time and walking distance, although improvements in angina were showed (81). Regarding TMLR combined with CABG, a multicenter, randomized, prospective study enrolling 266 RA patients blinded to treatment arm indicated that CABG *plus* TMLR is more effective in lowering operative mortality, post-operative inotropic support and short-term MACE compared to CABG alone (86). Furthermore, these results were confirmed after a 5-year follow-up, showing a sustained reduction of recurrent severe angina in the CABG *plus* TMLR group, although the survival rate was not different (82).

The percutaneous approach has been proposed as a less invasive strategy taking the advantage of commercialized catheters designed for positioning an optical fiber coupled to a laser. This application was tested in multiple unblinded studies with discordant results (77, 87–90). Of note, the "DMR In Regeneration of Endomyocardial Channels—DIRECT" Trial, which was the first and only RCT study with blinded patients and outcome assessors, reported essentially negative results in terms of exercise duration, angina symptoms, and myocardial perfusion scores (91).

In a limited number of pilot experiences, TMLR was used, either surgically or percutaneously, as an adjunctive therapy to cell therapy with the rationale to boost the angiogenic response (92–96).

By combining all these important studies, the Cochrane reviewers provided evidence of higher early post-operative mortality in patients treated with TMLR compared to standard medical therapy (pooled OR was 3.76, 95% CI: 1.63–8.66) (97).

On these bases, surgical TMLR and percutaneous TMLR are not recommended in EU (Class III recommendation) while in US a Class IIb/Level of Evidence B recommendation was given in the last 2012 ACC/AHA guideline.

THE CASE OF CELL THERAPY

Cell-based therapies (CT) for heart diseases have been extensively investigated over the last 20 years and, despite a number of methodological limitations regarding both cell therapeutics and patient profile might have influenced clinical outcomes (98), RA appears the cardiac conditions in which CT has shown the most promising results. Indeed, a consistent body of evidence (RCT and MTA) cumulatively indicated that CT is safe and can effectively increase physical function and well-being by reducing angina symptoms and drug assumption in the absence of relevant side effects (18). Different pro-angiogenic cells were administered in an autologous setting, including unfractioned bone marrow (BM)-derived mononuclear cells (MNC) (99, 100), selected endothelial progenitors (i.e., $CD34^+$ and $CD133^+$ cells) derived from BM or peripheral blood (101–104) or mesenchymal stem cells derived from BM (105) or adipose tissue (106–108) (**Figure 1**). In addition to pilot and proof-of-concept clinical studies, a significant proportion of published trials may be categorized as phase II RCT (100, 101, 109–111). In particular, the ACT34-CMI trial, by enrolling 167 RA patients to receive intramyocardial injection of BM-derived $CD34^+$ cells (0.1×10^6 or 0.5×10^6 cells/Kg) or placebo, demonstrated the superiority of $CD34^+$ cells vs. placebo in improving exercise tolerance ($p = 0.01$) and weekly angina frequency ($p = 0.02$), especially for the group that received 0.1×10^6 $CD34^+$ cells/Kg (111). The 2-year follow-up confirmed the persistence of clinical effects along with a trend of reduction in MACE (112). Similarly, positive results were observed in the study of van Ramshorst and coworkers in which the treatment with 100×10^6 autologous BM-derived MNC is associated with a significant improvement of myocardial perfusion at single-photon emission computed tomography ($p = 0.001$) and CCS class ($p = 0.001$), in parallel with a modest LVEF amelioration at MRI (~3%) after 6 months of follow-up (100).

These favorable results encouraged the initiation of three large phase III RCT. However, none of them can be considered conclusive due to early termination for (i) sponsor strategic reasons (RENEW study (102)), slow recruitment rate (REGENT-VSEL trial (103)), and procedure-related issues (ATHENA trial (113)). More in details, the phase III RENEW trial was designed to definitely assess the efficacy of intramyocardial injection of autologous $CD34^+$ cells in 444 "no option" RA patients. Unfortunately, results were available for only 112 patients suggesting, in accordance with earlier phase studies, a greater exercise capacity and a dramatic reduction in angina frequency in CT treated patients (102). Conversely, the recent sub-analysis of the REGENT-VSEL trial did not demonstrate a

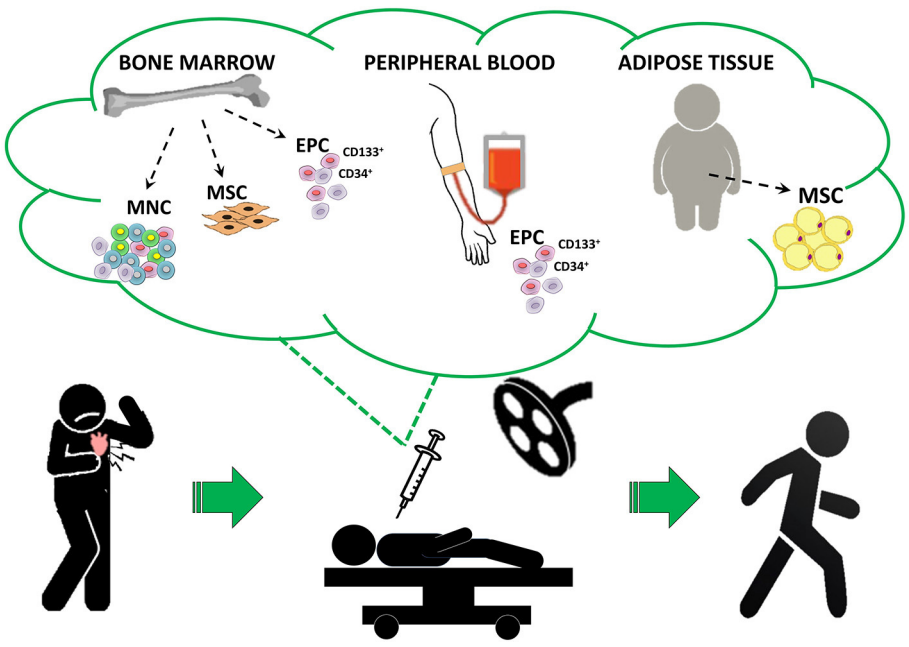

FIGURE 1 | Proposed cell therapy approach for refractory angina patients. The figure represents the ideal in-hospital protocol of different cell-based therapies for RA following a gold standard approach. EPC, endothelial progenitor cells; MNC, mononuclear cells; MSC, mesenchymal stem cells.

statistical difference of quality of life and clinical symptoms in patients receiving $CD133^+$ cells compared with those receiving placebo (114).

To combine multiple clinical research results, six MTA were conducted so far and the cumulative results on CT safety and efficacy have been shown (115–120), among which the most updated are herein presented. The work of Shah et al. (115), based on 10 RCT including 658 patients with 6- to 24-month follow-up, represent the most comprehensive MTA on this topic. In particular, CT in RA patients determined an improvement in CCS class (risk ratio (RR) [95%CI]: 1.53 [1.09, 2.15], $p = 0.013$), exercise capacity (standard mean difference (SMD) [95%CI]: 0.56 [0.23, 0.88], $p = 0.001$), and a reduction in angina frequency (SMD [95%CI]: −1.21 [−2.40, −0.02], $p = 0.045$). Moreover, authors highlighted that CT has positive effects on myocardium by reducing perfusion defects (SMD [95%CI]: −0.70 [−1.11, −0.29], $p = 0.001$) and improving LVEF (SMD [95%CI]: 0.64 [0.27, 1.00], $p = 0.001$). The risk of all-cause mortality was similar in patients treated with CT or placebo ($p = 0.121$). It is important to point out that such results, although promising, derived from the pooled effect of different cell products and, thus, cannot be deemed conclusive but only hypothesis-generating.

In this regard, a less comprehensive, but more focused, MTA published by Velagapudi et al. (117) provided strong evidence supporting beneficial effect of intramyocardial delivery of $CD34^+$ cell-based therapy in RA and a rationale for a definitive Phase III RCT. As for safety, the risk of MI and stroke did not differ in patients treated with $CD34^+$ cells with respect to placebo (odd ratio (OR) [95%CI]: 0.77 [0.36, 1.63] and 0.50 [0.08, 3.06], respectively), but, in return, the overall risk of mortality was significantly lower in $CD34^+$ cell than in placebo group (0.24 [0.08, 0.73], $p = 0.01$) (117). Finally, the most updated systematic review further confirmed that CT in RA patients entails lower incidence of MACE (OR [95%CI]: 0.41 [0.25, 0.70], $p < 0.0001$) and all-cause mortality (0.24 [0.10, 0.60], $p = 0.002$) respect to placebo/controls (116). Interestingly, the subgroup analysis revealed that the favorable outcome in the pooled analysis is primarily driven by data derived from clinical studies with

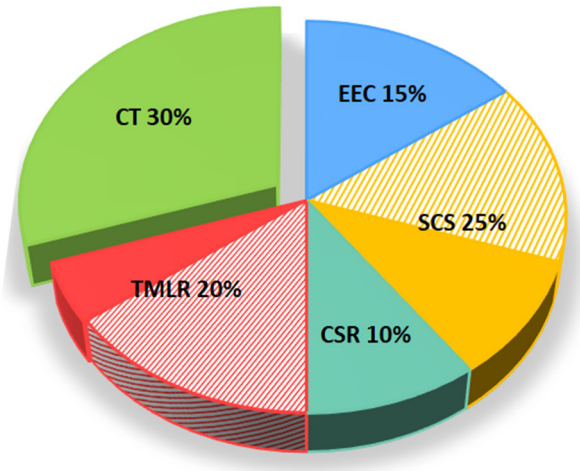

FIGURE 2 | Meta-analyses and outcomes of non-pharmacological treatment options for refractory angina as per guidelines. The figure depicts the number and outcomes of available MTA for each non-pharmacological treatment options for RA including EEC, SCS, and CSR, in addition to CT. Positive MTA are represented with full color while those negatives are depicted with stripes. CSR, coronary sinus reducer; CT, cell-based therapy; EECP, enhanced external counterpulsation; MTA, meta-analysis; RA, refractory angina; SCS, spinal cord stimulation.

TABLE 2 | Non-pharmacological treatment options in the 2019 ESC and 2012–2014 ACC/AHA guidelines for refractory angina vs. cell therapy.

Treatment strategy		Proposed principle of action	Effectiveness	
			Pros	Cons
Recommended	Enhanced external counterpulsation	Improved venous return and coronary perfusion in diastole, decreased workload in systole	+++ Improved indices of angina, myocardial perfusion, exercise capacity, and MACE	Time-consuming protocol, minor, and major contraindications (e.g., coagulopathy, arrhythmias, peripheral artery, and venous disease)
	Spinal cord stimulation	Reduction of cardiac neuron activity and sympathetic tone, anti-ischemic effect by adenosine-mediated coronary vasodilation	+/– Improved indices of angina and exercise capacity	Invasive, surgical complications (e.g., implant failure, lead displacement, and infections)
	Coronary sinus reducer	Coronary blood flow redistribution	+++ Improved indices of angina, myocardial perfusion, and exercise capacity	Invasive, surgical complications (e.g., implant failure, migration, hematoma, NSTEMI)
	Transmyocardial laser revascularization	Sympathetic denervation and angiogenesis	No effect	Invasive, post-procedural higher mortality
Not yet recommended	Cell therapy	Angiogenesis and cardioprotection	+++ Improved indices of angina, myocardial perfusion, exercise capacity, and MACE	Invasive, surgical complications (e.g., hematoma, bleeding, and arrhythmias)

$CD34^+$ cells which embody the largest patient cohort (74%) (116). Recently, the retrospective analysis of phase I/IIa, phase II ACT-34 and phase III RENEW was published showing that RA patients who received $CD34^+$ cell therapy experienced the reduction of hospitalizations, cardiac procedures, and health care expenditures in the first year following treatment compared to the year prior (121).

DISCUSSION AND CONCLUSIONS

The management of RA patients is still challenging, as demonstrated by the most recent reviews on the topic (122–124). After exhausting traditional medical therapies, the options for RA are very limited with EECP, SCS and CSR being the only recommended approaches (2, 23). In essence, to date we do not have a definitive answer on the best non-pharmacological treatment strategy for RA because, as shown in **Table 2**, each comes with its own advantages and disadvantages. Nevertheless, it is clearly evident that CT for this specific cardiac condition has all the features to be ultimately considered in the international guidelines. Indeed, a substantial body of clinical evidence, by means of RCT and MTA, indicates CT as a viable therapeutic option for RA, which appears a favorable target for the first introduction of CT in the clinical arena. As depicted in **Figure 2**, a number of MTA have been conducted in the past years to address the efficacy and safety of emerging non-pharmacological treatment options for RA, of which three for EECP, five for SCS, two for CSR, four for TMLR, and six for CT (see **Supplementary Table 1** for evidence supporting **Figure 2**). As for SCS and TMLR, evidence arising from MTA is mixed. On the contrary, MTA for CT are the most represented and yielded 100% positive outcomes. Despite this, CT for RA has not yet been incorporated into current guidelines and relegated as a "potential treatment option."

On top of guidelines, the introduction of CT into the therapeutic armamentarium of cardiologists needs to match the regulatory framework of advanced medicinal products. In addition, it is important to point out that CT (differently from other non-pharmacological technologies described above) cannot be conceived as a unique therapeutic agent, but as a wide spectrum of highly innovative products which have to ensue specific development plans and regulatory pathways.

In this perspective, promising developments are expected from the $CD34^+$ cell technology which has recently received a "Regenerative Medicine Advanced Therapy Designation" by the FDA to expedite the approval for use in no-option RA (125). In summary, we believe that the scientific and clinical framework is mature enough for the introduction in the international guidelines of the first biological product to cure RA.

AUTHOR CONTRIBUTIONS

BB, ER, and EG analyzed the studies and wrote the manuscript. GP conceived and wrote the manuscript. All authors have read and approved the final manuscript and agreed to be personally accountable for the author's own contributions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.709795/full#supplementary-material

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REVIEW
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Epicardial Contribution to the Developing and Injured Heart: Exploring the Cellular Composition of the Epicardium

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The epicardium is an essential cell population during cardiac development. It contributes different cell types to the developing heart through epithelial-to-mesenchymal transition (EMT) and it secretes paracrine factors that support cardiac tissue formation. In the adult heart the epicardium is a quiescent layer of cells which can be reactivated upon ischemic injury, initiating an embryonic-like response in the epicardium that contributes to post-injury repair processes. Therefore, the epicardial layer is considered an interesting target population to stimulate endogenous repair mechanisms. To date it is still not clear whether there are distinct cell populations in the epicardium that contribute to specific lineages or aid in cardiac repair, or that the epicardium functions as a whole. To address this putative heterogeneity, novel techniques such as single cell RNA sequencing (scRNA seq) are being applied. In this review, we summarize the role of the epicardium during development and after injury and provide an overview of the most recent insights into the cellular composition and diversity of the epicardium.

Keywords: epicardium, heterogeneity, development, cardiac repair, single-cell RNA sequencing

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INTRODUCTION

Ischemic heart disease, and especially myocardial infarction (MI) remains a major cause of death globally (1). MI is primarily caused by obstruction of the coronary vasculature, and the resulting sudden loss of oxygen supply to the cardiac muscle leads to massive cell death. Cardiomyocytes lack the ability to sufficiently self-renew and therefore they are unable to replenish the lost muscle. Instead, dead cells are replaced by a fibrotic scar (2, 3). While this non-contractile scar protects the damaged myocardial wall from rupture, it also impairs proper cardiac contraction. This persistent loss of cardiac pump function eventually results in heart failure (HF), a disease for which a cardiac transplant is the only curative therapy. Since intrinsic repair mechanisms are insufficient to restore cardiac function after injury, the focus shifted to inducing cardiac repair through other means. These procedures include the direct injection of various (stem) cell populations to generate new tissue, or the delivery of exosomes or paracrine factors to induce vascularization and prevent apoptosis. Many of these approaches resulted in some degree of improved heart function after MI in pre-clinical studies (4–7). However, the anticipated promise of cell-therapy was not upheld after transition to clinical trials: the results of cell injections on cardiac function in patients have been inconclusive (8). This leaves the mechanisms underlying the observed positive effect in pre-clinical studies unclear (4), but it demonstrates that cardiac regeneration requires more than solely the injection of cells. Another approach that is currently under investigation to achieve cardiac

regeneration is through the stimulation of endogenous cell populations that eventually replace the lost cells and stimulate repair. This includes stimulating the local endothelium to increase vascularization (9), as well as the option to induce proliferation in pre-existing cardiomyocytes to create new contractile units (10–12). In this regard, an interesting candidate for endogenous repair that has seen increasing attention is the epicardium.

The Epicardium as an Endogenous Cell Population for Cardiac Repair

The epicardium is a single-cell layer of mesothelial origin located on the outside of the heart. Intriguingly, this cell type is of crucial importance during cardiac development. In brief, the epicardium contributes cardiac cell types to the developing heart (13, 14), it facilitates the formation of the coronary vasculature (15, 16), it can induce the proliferation of cardiomyocytes through the secretion of paracrine factors (17–19), and derivatives of the epicardium can modulate the extracellular matrix (20). All these processes are also essential to repair the heart after injury.

In the healthy adult heart, the epicardium is a quiescent layer. However, it is reactivated after certain types of injury and subsequently it recapitulates several of its developmental processes. In animal models that display the potential for cardiac regeneration such as zebrafish and neonatal mammals (21, 22), the epicardium has been shown to play an important role in facilitating and regulating processes involved in repair, including modulation of inflammatory responses and of the composition of the extracellular matrix, secreting paracrine factors, and contributing cells to the damaged heart (23–25). These observations prompted researchers to attempt to stimulate the adult epicardium in mammals to increase its participation in repair (26).

To optimize the post-injury response, it is important to understand the processes underlying the activation of the epicardium and the regulation of its differentiation into cardiac cell types. An unresolved question in this context has been whether the whole epicardial population can participate, or whether distinct cell types reside within the epicardial layer that have specific abilities within the reparative response. With the advent of single cell sequencing, we are gaining more insight into the composition and the potential contribution of endogenous cells in the heart. Here, we will highlight the role of the epicardium during development and cardiac repair and discuss novel insights on the composition of this cell layer based on single cell RNA sequencing (scRNA seq) data.

THE EPICARDIUM IN HEART DEVELOPMENT

The Proepicardium and the Formation of the Epicardium

As stated above, the epicardium has an important function in the formation of the heart during embryogenesis. The developmental origin of the epicardium lies within the proepicardial organ (PEO). The PEO is an evolutionary conserved cluster of cells that develops from the lateral plate mesoderm and is located at the venous pole of the heart near the septum transversum. In mice, the PEO becomes visible around embryonic day 8.5 (E8.5) (27), a stage when the developing heart is still a primitive tube-like structure. After E9.5, when the heart tube has started to loop and form distinguishable segments such as the primitive left ventricle and outflow tract, cells from the PEO start to translocate and attach to the outside of myocardium, where they will ultimately form the epicardium. In mammalian development this proepicardial translocation has been described to occur *via* the formation of free-floating cell aggregates or *via* direct contact with the myocardium (28–30), while in avian and zebrafish models cells from the PEO are likely to migrate toward the heart *via* a "bridge" consisting of extracellular matrix components such as heparan sulfate and fibronectin (31, 32). Upon reaching the bare myocardium, cells from the PEO flatten and form a continuous epithelial layer that will completely cover the heart around E12.5 in mice and week 5 in human cardiac development (33, 34).

Identification of the PEO as a transient structure has relied on scanning electron microscopy (SEM) (27) and staining with specific antibodies. The most commonly used markers to identify the PEO include for example transcription factor 21 (TCF21), T-box transcription factor 18 (Tbx18), Wilms' Tumor-1 (Wt1), Scleraxis (Scx), Semaphorin3D (SEMA3D), and GATA5 (31, 35–39). The expression of some of these markers persists after the epicardium is formed and they are therefore often also used to identify the epicardial layer in later developmental stages. But as will become clear, these markers have a heterogeneous spatiotemporal expression in the PEO and in the epicardium throughout development. This could suggest the existence of subtypes of cells that have distinct roles in cardiogenesis, or even in regeneration of the injured heart.

Cellular Contributions of the Epicardium During Development

The vital role of the epicardium for cardiac development was highlighted by studies in an avian model where epicardial outgrowth from the PEO was physically inhibited. This led to the formation of a thin myocardium and malformation of the coronary vasculature, amongst other developmental defects (13, 40), indicating that the epicardium is more than a static epithelial cell layer enveloping the heart. Indeed, once the epicardium is fully formed a subset of the epicardial cells will undergo a process called epithelial-to-mesenchymal transition (EMT), thereby forming epicardium-derived cells (EPDCs) (41, 42). EMT is a well-described process which is crucial in embryonic development but also observed in diseases such as metastatic cancer and fibrosis (43). During EMT, epithelial cells lose their apical-basal polarity and cell-cell adhesions, and acquire a mesenchymal phenotype that allows the migration and invasion of cells into tissue (44). EMT-derived mesenchymal cells have the potential to differentiate into various mesenchymal cell lineages, such as adipocytes, chondrocytes, and osteoblasts (45). A similar feature is observed in cells derived from the epicardium; EPDCs have been reported to differentiate into various cell types,

including fibroblasts, pericytes and smooth muscle cells (SMCs) (14, 46–49). Other reports claim that the epicardium upon EMT also contributes cells to endothelial cell (EC) lineages and to the cardiomyocyte (CM) population. However, these findings are under debate and an epicardial contribution to these tissues is likely very limited at best (49–52).

A possible explanation for these discrepancies in differentiation capacity is because analysis of cell fate is mainly based on lineage-trace models where Cre-recombinase (Cre) is driven by promoters that are considered specific to epicardial cells. By crossing these mice with transgenic reporter lines containing a lox-flanked stop-codon followed by a reporter gene, cell specific Cre expression results in indefinite expression of a reporter protein like Green Fluorescent Protein (GFP) or β-galactosidase. Several promoters of epicardial related genes such as *Wt1*, *Tbx18*, *Tcf21*, *GATA5*, *Scx*, and *Sema3D* have been used to trace the fate of epicardial cells based on transgene expression. An even better controlled lineage trace system can be achieved by fusing Cre to a mutated ligand-binding domain of the human estrogen receptor, in which recombination relies on the presence of tamoxifen. This provides lineage tracing with a temporal control (53), as demonstrated in mice by using promoters of *Tbx18, Wt1,* and *Tcf21* in zebrafish (37, 54, 55). Unfortunately, most of the promoters used in epicardial lineage-tracing models are not uniformly expressed in the epicardium and have a dynamic temporal expression pattern in the epicardium and its derivatives. Additionally, they can also be expressed in the PEO, and in various other cell types of the developing heart, such as ECs and CMs (31, 56–58). As a result, lineage trace models can potentially label cells that not necessarily originate from the epicardium. This problem was highlighted by a study comparing various lineage-tracing models and the contribution of different lineages to the EC population in the heart. Carmona et al. showed that Wt1 lineage-trace models should not be used after E13.5, since *de novo* expression of Wt1 in other tissues (i.e., endothelium) arises as well as through recruitment of extracardiac progenitors (51). However, the authors found that EPDCs contribute roughly 4% of the coronary endothelium using $GATA5^{Cre}$ mice (51). Others reported that coronary endothelium expressed Wt1 as early as E11.5 in a $Wt1^{CreERT2}$ model, meaning that tamoxifen should be administered at E9.5 to prevent labeling of coronary ECs (59). Interestingly, it has been argued that since epicardial markers *Wt1, Sema3d, Tbx18, Scx,* and *Tcf21* overlap, and $Tbx18^{Cre}$ and $Tcf21^{CreERT2}$ show no endothelial contribution, that this applies to the entire (pro)epicardium (59). Nevertheless, carefully controlled lineage-trace models have still provided valuable insight into the cell fate of EPDCs and the mechanisms steering epicardial differentiation, and the current consensus is that EPDCs have the capacity to differentiate into SMCs, fibroblasts and pericytes, and potentially ECs (**Figure 1**). Additionally, these models have shown that the epicardial EMT is crucial for cardiac development.

Regulation of Epicardial EMT and Differentiation

Epicardial EMT is a defining process for the contribution of epicardial cells to tissue formation as it grants cells the capacity to migrate and differentiate. In general, epithelial cells preserve their phenotype *via* expression of epithelial cadherin (CDH1, E-Cadherin), which is responsible for maintaining cell-cell adhesion and adherens junctions. The EMT-inducing transcription factors, including Snail Family Transcriptional Repressors 1 and 2 (SNAI1/2), zinc finger E-box binding family members 1 and 2 (ZEB1/2), and twist-related protein 1 (TWIST1) (44) can all repress CDH1 and simultaneously activate the expression of mesenchymal genes. Interestingly, the most commonly used markers to identify the epicardium like Wt1, Tcf21, and Tbx18 also appear to have a role in the regulation of EMT upstream of these factors (60). Wt1 is a zinc-finger protein, initially recognized for its role in the formation of Wilms' tumor, that was found to be expressed in both the PEO and the epicardium, and during EMT (33, 61, 62). Wt1 regulates epicardial EMT through transcriptional activation of Snai1 as well as a direct repression of E-cadherin (63, 64). However, in mouse embryos the removal of *Snai1* specifically in Wt1- or Tbx18-positive epicardial cells did not affect cardiogenesis, and embryos displayed normal epicardial EMT (65). This suggests that SNAI1 may not be the sole inducer of EMT, and that compensatory mechanisms are in place. In contrast, embryos that lack *Wt1* were found to have severe epicardial defects with an absence of EPDCs in the subepicardial mesenchyme and impaired cardiac morphogenesis, resulting in embryonic lethality at E13.5 due to pericardial bleeding (16, 62). Additionally, *Wt1* knockout mice revealed a role for Wnt/β-catenin and retinoic acid signaling pathways downstream of WT1 (16). Indeed, in additional studies epicardial β-catenin signaling was found to be crucial for epicardial EMT, myocardial invasion, and differentiation into coronary smooth muscle of EPDCs (66).

Tbx18 is a marker commonly used to identify the epicardium and that is also expressed in the PEO (36). *In vitro*, using mouse primary epicardial cells, a bi-directional role for WT1 and Tbx18 was reported. *Wt1* knockdown induced epicardial EMT through expression of *Snai2*, which could be reversed by knockdown of Tbx18, thus acting as a regulator of EMT (67). *Tbx18*- lineage-tracing models have shown that *Tbx18*-positive cells differentiate into SMCs and fibroblasts (46), and this was corroborated by a study in which an activating form of Tbx18 induced EPDCs to undergo a pre-mature differentiation into SMCs mediated by Notch and transforming growth factor β (TGFβ) (68). In contrast, *Tbx18* null embryos survive until birth and die due to skeletal malformations, indicating that Tbx18 is dispensable for epicardial development (68).

Tcf21 (also known as Pod1/epicardin/capsulin), a class II basic helix-loop-helix (bHLH) transcription factor, is another epicardially expressed protein that is also involved in the regulation of EMT and the differentiation into various cellular lineages. Depletion of Tcf21 during early stages in *Xenopus* development led to incomplete formation of a mature epithelial epicardium. Additionally, Tcf21 depletion resulted in the epicardial cells retaining a migratory phenotype, as they maintained their PEO cell-like phenotype (69). In *Tcf21* null mice, epicardial cells lacked the ability to become mesenchymal cells, indicating that Tcf21 is required for EMT. In the same study, it was shown that $Tcf21^{+}$ cells were committed to a cardiac

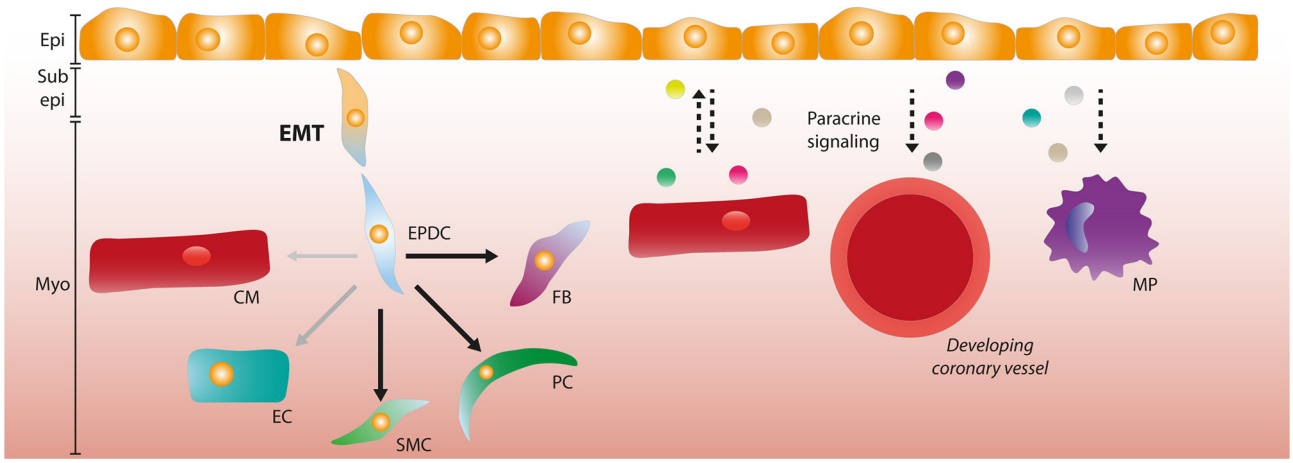

FIGURE 1 | The role of the epicardium during development. Epicardial cells can undergo epithelial-to-mesenchymal transition (EMT) and form epicardium-derived cells (EPDCs) that migrate through the sub-epicardial space into the myocardium. EPDCs can differentiate into various cardiac cell types such as fibroblasts (FBs), pericytes (PCs), and smooth muscle cells (SMCs). The contribution to the endothelial cell (EC) lineage is limited (as depicted by opaque and smaller arrow), and the capacity to differentiate into cardiomyocytes (CMs) is debated. Paracrine signaling interactions (depicted by dashed arrows) occur between the epicardial layer and CMs, and epicardial signaling is involved in coronary vessel formation and macrophage (MP) recruitment. Epi, Epicardium; Sub epi, Subepicardium; Myo, Myocardium.

fibroblast fate, supporting the importance of Tcf21 in epicardial differentiation (48). The regulation and downstream effects of Tcf21 may be more intricate, as another study showed that Tcf21 was regulated by retinoic acid signaling and that absence of Tcf21 led to an increased smooth muscle cell differentiation, but that EMT was unaffected (57). These studies showed a complex regulatory role for Tcf21 in differentiation of EPDCs, and further investigation into these cell fate decisions is needed to elucidate the mechanisms behind it.

Besides the "classical" markers of the epicardium, other epicardial transcription factors have also been described to regulate epicardial characteristics beyond EMT, like migration and invasion into the myocardium. These include for instance the myocardin-related transcription factors (MRTFs), nuclear factor of activated T-cells 1 (NFATC1) and protein arginine methyltransferase 1 (PRMT1). The MRTF serum-response factor (SRF) regulatory network modulates epicardial migration and invasion. Cell motility is driven by interactions between SRF and MRTF-A/B, modulating the expression of regulators of actin dynamics (70). In an *ex vivo* heart culture model, deletion of *Mrtfa* and *Mrtfb* reduced EPDC migration. Additionally, in embryos lacking both *Mrtfa* and *Mrtfb,* epicardial integrity was compromised, as well as the coronary angiogenesis due to reduced epicardium-derived pericytes (71). NFATC1, a transcription factor involved in extracellular matrix (ECM) remodeling during valve maturation (72), is expressed in a subset of epicardial cells within the epicardial layer and in EPDCs in the subepicardial space (73). It was found to influence the invasion of EPDCs into the myocardium through enhancing cathepsin K expression, an ECM degrading enzyme. *Wt1-Cre* mediated deletion of NFATC1 in mice led to a reduction in the number of α-smooth muscle actin-expressing EPDCs in the myocardium, as well as a reduced intramyocardiac vessel penetration and fibrous matrix synthesis (73). However, in this model, the initial stages of epicardial formation and EMT were not affected, indicating that modulation of the ECM required for EPDC invasion is affected in part by NFATC1.

Molecular regulation of epicardial behavior beyond transcription factors also occurs. A newly identified regulator of epicardial EMT is PMRT1, an arginine methyltransferase responsible for post-translational modifications. PRMT1 knockout mice showed a reduced migration of EPDCs and an attenuated formation of EPDC-derived lineages such as fibroblasts, SMCs, and pericytes. The mechanism of these processes is likely the stabilization of p53 due to the loss of PRMT1, and higher levels of p53 lowered the expression SNAI2, and thereby blocked epicardial EMT, confirming a role for *Snai2* in epicardial EMT in mice. Interestingly, the reduction of p53 levels in Tbx18-mediated PRMT1 knockout mice normalized the disrupted invasion, as well as the formation of epicardium-derived mesenchymal lineages (74). In short, the epicardial contribution to various cell lineages is of great importance for proper development of the heart. The genes that are involves in these processes are in several cases also used to identify the epicardium, although their expression may not be uniform due to spatiotemporal control.

Paracrine Signaling During Development

Besides a cellular contribution, the epicardium is a rich source of growth factors and cytokines and as such it provides essential cues for cardiac development including factors that support cardiomyocyte proliferation and vessel formation. The epicardium expresses various members of the fibroblast growth factor (FGF) family and its receptors (FGFR). For example, FGFR1 is expressed both in the PEO and in the epicardium, and loss of FGFR1 in quail embryos was shown to reduce

the myocardial invasion of epicardial cells (75). In contrast, in a *Tbx18*^{Cre} mediated deletion of *Fgfr1* and *Fgfr2* in mice no differences in myocardial fibroblast numbers were observed, and importantly cardiac development was not affected (76), indicating that in Tbx18^{+} cells FGFR signaling is not required for fibroblast invasion into the myocardium.

FGF9 is another family member related to the epicardium. Epicardial FGF9 is induced by retinoic acid (RA) produced by the epicardium and promotes proliferation and differentiation of cardiomyocytes *via* receptor splice variants FGFR1c and FGFR2c (17). Additionally, FGF9 signaling plays a role in the formation of the coronary vasculature (77, 78). Besides inducing expression of FGF9, RA signaling leads to expression of *Wt1* and *Tcf21* (57), two TFs that regulate epicardial EMT, and it stimulates myocardial expansion *via* IGF2 (79). RA also has an epicardium-specific role, since epicardial specific knockout of RA receptor Retinoid X Receptor a (RXRa) mediated by *Gata5*^{Cre} led to reduced EMT, cardiac compaction, and defects in coronary arteriogenesis *via* impaired FGF2 signaling (80). Conversely, signaling from the myocardium to the epicardium also occurs. Myocardial signaling to the epicardium is for instance mediated by FGF10. FGF10 is expressed by cardiomyocytes during development and it stimulates invasion of EPDCs into the myocardium, and their differentiation to fibroblasts *via* FGFR2b (81).

The epicardium also interacts with the developing coronary vasculature. It was found that *Wt1*-KO mice have deficient epicardial expression of angiogenic factors *Vegfa* and *Angpt1*, suggesting a contribution to abnormal coronary vessel development (16). C-X-C motif chemokine 12 (CXCL12) is expressed by the epicardium and by mesenchymal cells derived from the epicardium, it was shown to be crucial for the maturation of the coronary vasculature *via* C-X-C motif receptor 4 (CXCR4) on nearby endothelial cells (82). A single factor was found using single-cell sequencing of developing mouse hearts at E10.5. The authors found that *Rspo1* is expressed by epicardial cells and which was hypothesized to promote proliferation of compact myocardium (83). Another important epicardial signaling family comprises the platelet-derived growth factors (PDGFs). PDGFA and PDGFB are both expressed by various cardiac cell types, including the epicardium, and mediate various aspects of cardiac development (84–87). Its receptors, PDGFRα and β, are both expressed in the epicardium (84, 88), and loss of these receptors led to defective epicardial EMT and migration *in vivo. Ex vivo* these hearts displayed decreased epicardial migration also in the presence of EMT-inducing growth factors TGFβ1 and FGF2 (89). *In vitro,* expression of *Sox9* in PDGFR-deficient epicardial cells partially rescued the deficient EMT, implicating a signaling pathway downstream of PDGFR-signaling regulating this transcription factor (89). Moreover, epicardial loss of PDGFRα and PDGFRβ resulted in a reduction in myocardial fibroblasts and SMCs, respectively, indicating that these receptors likely play a role in epicardial cell differentiation and migration (76, 88).

Besides signaling to and from other cardiac cell types, the epicardium also secretes factors that can function in an autocrine fashion. TGFβ is a well-established inducer of EMT, and its isoforms are present during (pro)epicardial development (90, 91). TGFβ1 and TGFβ2 induce loss of epithelial morphology and the differentiation into SMCs through ALK5 signaling, the TGFβ type I receptor (92). Concordantly, a *Gata5* mediated *Alk5* knockout prevented EMT upon TGFβ3 stimulation and reduced the number of proliferating cardiomyocytes. Furthermore, it impaired adherence of the epicardial layer to the myocardium, and diminished differentiation into SMCs due to a lack of epicardial EMT (93). Moreover, mice embryos lacking ß-glycan, also known as *Tgfbr3,* have a diminished coronary vessel development and hyperplasia of the subepicardial layer due to decreased proliferation and invasion of EPDCs (94–97). Overall, these studies highlight the importance and complexity of the regulation of paracrine signaling in epicardial and cardiac development (**Figure 1**).

THE ADULT EPICARDIUM

Cellular Contributions From the Adult Epicardium

In contrast to the developing epicardium, in the adult heart the epicardium displays limited Wt1 expression and under homeostatic conditions it does not actively contribute cells to the myocardium (98). Other genes that are expressed in embryonic, active epicardium, such as *Tbx18* and *Raldh2,* are merely expressed at low levels, indicating that the epicardium is in an inactive state in the healthy adult heart (99, 100). However, after ischemic insults like MI the epicardium covering the injured area is lost and the remaining epicardium will start to proliferate and migrate to re-cover the heart (100). This wound healing process leads to a thickening of the epicardial layer near the site of injury, instead of a single-cell layer in a normal heart (98, 100, 101). This reactivation seems to be specific to ischemic injury and is not observed in cardiac hypertrophy models such as transverse aortic constriction (102). Importantly, after ischemic injury, the expression of the epicardial genes *Wt1, Tbx18, Raldh1*, and *Raldh2* is reactivated, peaking 3 days after injury and subsiding after 2 weeks (100). Interestingly, this re-expression occurs throughout the entire epicardium and is not restricted to the site of injury (98). *Wt1* was found to be induced by hypoxia-inducible factor 1a (HIF1a) in ECs (103), which could explain its reactivation after cardiac ischemia, but not after pressure-overload. Furthermore, HIF1a regulates epicardial invasion during development (104), indicating that HIF1a could be a central regulator of the epicardial post-injury response.

Much effort has been put into identifying the regulatory elements that activate the epicardium after injury. In the embryonic heart and after injury, the CCAAT/enhancer binding protein (C/EBP) family of transcription factors was identified as a regulator of *Wt1* and *Raldh2* by binding to their enhancer elements (105). More recently, the transcription activator BRG1 was found to be recruited to conserved regulatory elements in the *Wt1* locus by C/EBPβ and thereby induced *Wt1* expression (106).

Based on knowledge gained from cardiac development, it was anticipated that autonomic recapitulation of an embryonic gene

program would result in epicardial EMT and subsequently to a contribution of the adult epicardial derived cells to various cardiac cell types after injury. Several groups have addressed this using lineage tracing, but the results have varied based on the mouse model that was used (107). When using a Bacterial Artificial Chromosome (BAC)-Wt1^{Cre} lineage-trace model, cells derived from the epicardium were reported to contribute to fibroblast, EC and CM lineages (100). Moreover, using a Wt1^{Cre}/R26R^{LacZ} lineage-tracing model, Duan et al. reported that cells expressing *Wt1* adopt a fibroblast fate, but other differentiation trajectories were not investigated (101). However, in a similar study using Wt1$^{CreERT2/+}$;Rosa26$^{mTmG/+}$ mice, the differentiation into EC and CM was not observed after MI, decreasing the likelihood of these differentiation trajectories to occur (98). Indeed, as shown by various lineage tracing experiments, the contribution of the epicardium to EC and CM is likely very limited at best after MI (108, 109), and the current conception is that ECs and CMs that arise after injury derive from resident populations in the heart (110–112). Although the multipotency of epicardial cells during development is still under debate, it was established that after injury there appears to be a limited multipotency of epicardial cells in the postnatal heart (113). It is important to note that in the adult, epicardial markers often used in lineage tracing experiments, similar to developmental studies, are not specific enough to label the entire epicardium and cells derived from the epicardium could be missed (114). Other approaches to labeling EPDCs such as MRI-based molecular imaging are being developed, but their specificity *in vivo* has not been determined yet (115, 116).

Although the intrinsic cellular contribution of adult epicardium after injury may be limited, migration of the re-activated epicardial cells appears to be a part of the epicardial injury response. Epicardial reactivation after injury has mostly been shown to have a beneficial effect on cardiac function after MI (98, 99, 101). Therefore, considering the marginal cellular contribution to the injured heart it could be relevant to promote epicardial proliferation and migration *via* external stimuli. One approach to stimulate cardiac repair *via* the epicardium is by treating mice with thymosin ß4 (Tß4), a peptide secreted by endothelial cells and the epicardium during development and after injury (117–119). Importantly, treatment post-MI resulted in an increase in proliferating EPDCs and neovascularization of the injured heart (120). Somatic and cardiomyocyte- and endothelium-specific knockout of Tß4 did not lead to impaired cardiac development or function (121, 122), while shRNA knockdown of Tß4 in CMs and ECs resulted in cardiac defects (123, 124). The discrepancy between these two models could be due to genetic compensatory mechanisms in complete knockouts, while shRNA induced knockdown does not induce a similar compensation (125). Although the mechanism is incompletely understood, a likely explanation is that Tß4 interacts with BRG1, a transcriptional regulator of *Wt1* expression, and that exposure to Tß4 before injury increased the expression of *Wt1* (106). In line with these findings, systemic Tß4 injections prior to injury in the adult mouse resulted in a recapitulation of an embryonic gene program in both healthy and injured hearts and differentiation into cardiomyocytes (99). However, when Tß4 was given post-injury, differentiation of EPDCs into cardiomyocytes was not found (108).

In parallel to the developing heart, several studies imply that besides a cellular contribution there is an important role for paracrine factors secreted by the activated epicardium. These paracrine factors can be used to increase the regenerative potential of the epicardium and of the heart.

Paracrine Signaling After Injury

The reactivation of the epicardium coincides with the secretion of paracrine factors that can contribute to cardiac repair (98). In a study using lineage tracing in EPDCs in Wt1$^{CreERT2/+}$;Rosa26$^{mTmG/+}$ mice after MI, the authors observed a higher localization of vessels near the GFP^{+} cells. Using fluorescence activated cell sorting (FACS) to isolate these cells, they found that the EPDCs secrete pro-angiogenic factors *in vitro*. Further analysis revealed that FGF2 and VEGFA were in large part responsible for these effects (98). Injection of EPDC conditioned medium after MI increased vessel density and reduced adverse remodeling in both the long and short term. Treatment with a single injection of conditioned medium immediately post-MI also displayed a beneficial effect on cardiac function 1 week after injury, although this effect was not sustained after 9 weeks (98).

In a study in adult zebrafish using cardiac cryoinjury, epicardial Cxcl12b-Cxcr4a signaling was found to guide coronary revascularization. Moreover, the expression of Cxcr12b was induced by hypoxia through Hif1a, again underlining the importance of this factor in regulating the cellular response to ischemic injury (126). Interestingly, these newly formed coronary vessels also functioned as a scaffold for regenerating cardiomyocytes, indicating a new function for the vasculature besides facilitating exchange of nutrients and oxygen. A comparable paracrine pro-angiogenic effect was observed in MI hearts that were treated by transplanting human EPDCs into the borderzone of the injury. Since the injected EPDCs were not found in the vessel lining while there was an increase in vessel density throughout the entire left ventricle regardless of number of engrafted EPDCs, this pointed to a predominantly paracrine effect of the injected cells (127). Human adult EPDCs were also shown to stimulate neurite outgrowth *in vitro* (128), indicating that EPDCs could have an effect on multiple cell types after injury.

Besides a mixture of secreted factors by the epicardial cell layer after injury, single components identified in the epicardium can also be used to enhance cardiac repair. For instance, Follistatin-like 1 (FSTL1), a factor present within the secretome of adult rat epicardial cells *in vitro*, was found to induce cardiomyocyte proliferation *in vivo* when locally applied onto the infarcted area in mouse and swine (129). Interestingly, when investigating the endogenous *in vivo* expression of FSTL1, it was apparent in the epicardium during development and in the adult, but after MI the expression of FSTL1 shifts to the myocardium (129). This finding was confirmed in another study, where the authors established that FSTL1 expression after MI is localized to activated cardiac fibroblasts (110). Nevertheless, increasing local levels of FSTL1 may provide a way to positively affect cardiac function. In a study

where modified RNA coding for VEGF-A, a paracrine factor also produced by EPDCs (98), was injected into the infarct zone of the myocardium, an increase in proliferating epicardial cells was observed. Also, an improved migration of EPDCs into the myocardium, and a contribution to EC and SMC populations (130). Similarly, upon the injection of brain natriuretic peptide (BNP) post-MI an increase in proliferation and migration of Wt1-positive cells was observed in $Wt1^{CreERT2}$;$Rosa26^{mTmG}$ mice, together with an increased contribution to the EC lineage (131). In both studies, the contribution to the EC population should be carefully interpreted due to native expression of Wt1 in endothelial cells after injury, which could lead to labeling of ECs in lineage-tracing experiments (114). In a mouse neonatal heart regeneration model epicardial cells were shown to secrete RSPO1, a factor that promotes angiogenesis *in vitro*, suggesting that this factor can promote revascularization after injury (132). Moreover, as this factor was shown to induce cardiomyocyte proliferation in the developing heart (83), its expression after injury may even have more potential to influence the regenerative response after injury.

Modulation of the Extracellular Matrix by the Adult Epicardium

The ECM is a cell-free three-dimensional scaffold secreted by cells that provides structural integrity and biochemical and biomechanical signaling cues to surrounding cells (133). Epicardial-derived fibroblasts are an important source of ECM producing cells in the adult heart (14). Interestingly, the adult epicardium itself is also encased by ECM components which are lost after MI and subsequently re-formed (134). Not only does the epicardium rebuild its own ECM components, but also that of the regenerating heart. In newt, an organism that has comparable regenerative potential to zebrafish, resection injury induced epicardial enrichment of tenascin C (TSC), fibronectin (FN) and hyaluronic acid (HA) preceding the migration of progenitor cells, suggesting that the matrix directs progenitor cells toward the wound site (135). Similarly, in a zebrafish regeneration model it was found that FN is induced in the epicardium after cardiac damage. One of its receptors, *itgb3,* is upgregulated on cardiomyocytes near the injury site (25). Initially, *fn1* is expressed in the entire heart before becoming expressed in the epicardium near the injury site. Loss of FN expression disrupted cardiac regeneration, indicating that FN is required for this process in the zebrafish heart (25). Another study in zebrafish indicated a potential role for HA in cardiac regeneration. HA and its receptor hyaluronan-mediated motility receptor (Hmmr) were found to be essential for epicardial EMT and for migration of EPDCs into the ventricle. In rats, in the first few days after damage, both HA and HMMR were induced in the infarct area, indicating that this pathway may also be involved in cardiac repair in mammals (136). Concordantly, in embryonic mouse epicardial cells, TGFβ2 induced the production of HA and was partially required for the induction of epicardial cell differentiation and invasion *in vitro* (137), indicating a recapitulation of embryonic gene programs during injury on the level of ECM components. In a cryoinjury model in zebrafish, the ECM component collagen XII (ColXII) was found to be induced in the epicardial layer. Interestingly, ColXII in the epicardium and in fibrotic tissue had a heterogeneous cell source, being the epicardium, EPDCs, and cardiac fibroblasts. Additionally, the authors described that ColXII partially co-localizes with TSC and FN, two ECM components that were previously implicated in cardiac regeneration. The authors hypothesized that TGFβ signaling coordinates formation of a transient collagen network which contributes to an ECM conductive to cardiac regeneration (138). Recently, other ECM factors have been identified that may play a role in the cell-cycle of cardiomyocytes. Mass-spectrometry on the ECM of embryonic and postnatal hearts revealed that embryonic cardiac fibroblasts, which are derived from the epicardium, secrete SLIT2 and nephronectin (NPNT). Injections of these ECM proteins *in vivo* in postnatal mouse hearts promoted cardiomyocyte cytokinesis, indicating that the ECM composition could play an essential role in cardiac regeneration by inducing proliferation of cardiomyocytes (139). Agrin, a neonatal ECM protein found to regulate epicardial EMT during development (140), was shown to promote cardiac regeneration after MI *in vivo* in adult mice (141). Intriguingly, a single injection of recombinant human agrin into the pig hearts after MI was sufficient to improve cardiac function, which could be the result of cardioprotection, enhanced vascularization and cardiomyocyte proliferation (142). Overall, these studies show that components of the ECM, especially those produced by the epicardium or by EPDCs, can provide a target for inducing cardiac regeneration (**Figure 2**).

Immunomodulation by the Adult Epicardium

The initial immune response after MI is mainly triggered by signaling from necrotic cells and is aimed at removing cell debris, ECM and dead cells. Subsequently, this inflammatory phase is repressed and followed by a reparative phase that allows for the deposition of ECM and the formation of a fibrotic scar that maintains integrity of the ventricular myocardium (143). The importance of a precisely regulated spatiotemporal response after MI is highlighted by reports that chronic unresolved inflammation enhances fibrosis and has a negative effect on function (143). The epicardium is involved in regulating the immune response through various routes. Already during development, hematopoietic cells ($CD45^+$) cells are recruited to the epicardium that are distinct from $Wt1^+$ cells, and after MI these cells are activated and migrate into the subepicardial space (134). During development, *Wt1* is required for the recruitment of epicardial macrophages (144) and in zebrafish, *wt1b* is expressed in a regenerative subset of macrophages after cryoinjury (145). Surprisingly, macrophages also contribute collagen to scar formation during heart regeneration in zebrafish and during cardiac repair in mouse, which was considered to be derived primarily from myofibroblasts (146). Inhibition of C/EBP mediated activation of *Wt1* and *Raldh2* after MI caused a significant reduction in neutrophil count and resulted in improved function, indicating a role for C/EBP mediated epicardial activation and subsequent leukocyte recruitment and

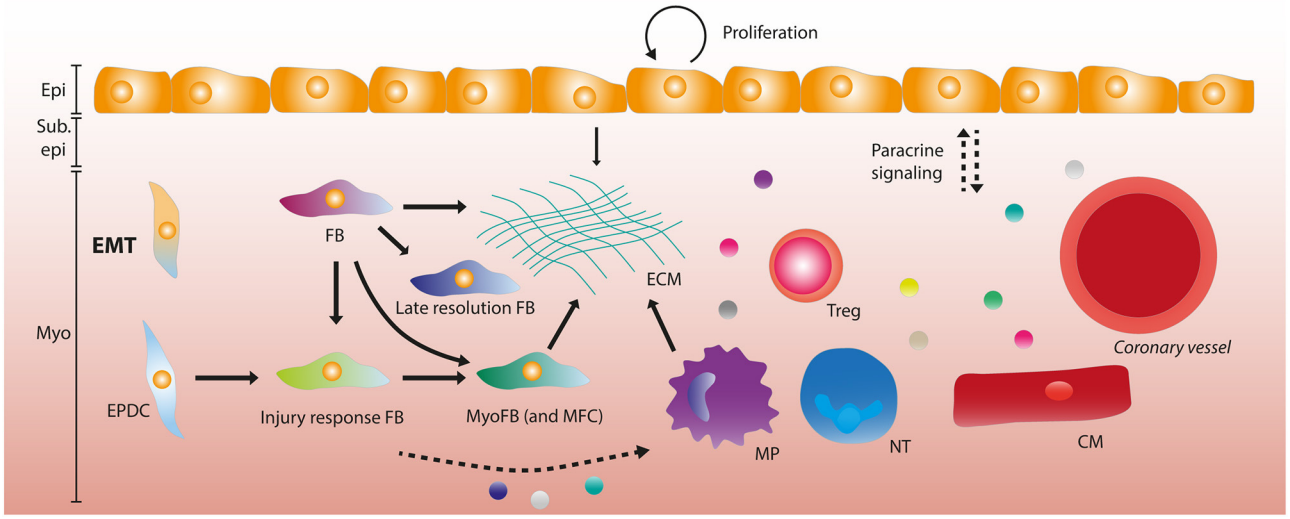

FIGURE 2 | The adult epicardium and stromal heterogeneity after myocardial infarction. After myocardial infarction (MI), the epicardial layer starts proliferating in order to regenerate the lost cells. A subset of epicardial cells undergo epithelial-to-mesenchymal transition (EMT) forming epicardium-derived cells (EPDCs). EPDCs and resident fibroblasts (FBs) can form various stromal cell subtypes, being late resolution FBs and injury response FBs that differentiate into myofibroblasts (MyoFBs) and matrifibocytes (MFCs). Several of these FB subgroups can contribute to extracellular matrix (ECM) deposition. Macrophages (MPs) and the epicardial layer also contribute to ECM formation. Paracrine signaling (depicted by dashed arrows) occurs between the epicardial layer and leukocytes (MPs, neutrophils (NTs) and regulatory T cells (Tregs), vessels and cardiomyocytes. Some stromal subsets have been implied to interact with MPs and NTs through paracrine signaling. Epi, Epicardium; Sub epi, Subepicardium; Myo, Myocardium.

inflammatory processes (105). Wt1 is reactivated in epicardial cells after injury (100), and yes-associated protein (YAP) and WW domain–containing transcription regulator 1 (TAZ) expression in $Wt1^{+}$ cells is an important immunomodulator after injury (24). After MI, mice with a $Wt1^{CreERT2/+}$ mediated deletion of YAP/TAZ had reduced expression of interferon-γ leading to impaired regulatory T-cell (Treg) recruitment to the myocardium, causing increased fibrosis, cardiomyopathy and death (24). In a zebrafish regeneration model, prostaglandin E_2 (PGE_2) and its receptor *ptger2* were shown to be upregulated after injury. COX enzymes catalyze the rate limiting step in the syntheses of prostanoids such as PGE_2, and epicardial cells were observed to have a higher expression of *cox2a* in their model compared to macrophages and other cardiac cell types (147). Additionally, small molecule inhibitors of Cox2 activity led to decreased PGE_2 concentrations and cardiomyocyte proliferation, indicating that Cox2 drives PGE_2 synthesis and CM proliferation during heart regeneration (147). Interestingly, PGE_2 has also been associated with YAP activation and Treg recruitment, indicating a potential interaction within the epicardium that modulates the inflammatory response (147). In conclusion, the epicardium is an important mediator of the inflammatory response after MI (**Figure 2**), which can potentially be modulated to improve cardiac repair.

THE COMPOSITION OF THE EPICARDIUM

As we have described, the adult epicardium recapitulates several of its embryonic capacities, such as proliferation, EMT, and migration to contribute to cardiac repair. However, in the absence of external stimuli like Tβ4, these processes appear to be less efficient in the adult epicardium compared to its developing counterpart. A potential difference in capacity could derive from differences in the cellular composition of the epicardium in development or in the adult heart. It remains unknown if epicardial cells have a uniform function during development and after injury, or if subsets of cells exist within the layer that contribute more to cardiac development and repair. Therefore, it is vital to know the composition of the epicardium during development and in the adult heart in order to optimize the post-injury response, below we will address the most recent knowledge.

Cellular Heterogeneity in the Developing Epicardium

Since epicardial cells have the potential to differentiate into various cardiac cell types, it is hypothesized that the epicardial layer is not composed of one specific cell type. This concept was supported by the notion that the source of the epicardium, the PEO, is a heterogeneous cell cluster consisting of endothelial cells (ECs) (148) within a mesenchymal core, covered by an epithelial outer layer, which can all be characterized by the expression of specific markers or combinations thereof. Analysis of the ECs in the PEO revealed that these cells have a heterogeneous origin from the PEO itself, the liver bud and the sinus venosus. The ECs in the PEO are immature and likely provide nutritional support (148, 149). However, there has been no indication that these ECs contribute to the developing heart (149). The previously mentioned observation that well-established epicardial markers Wt1, Tcf21, and Tbx18 are heterogeneously expressed in both the

PEO and the developing chick and mouse epicardium (35, 57) supports the hypothesis of a heterogenous epicardium. However, through lineage tracing experiments in mice it was shown that proepicardial cells from the mesenchymal core expressing SEMA3D or Scx may comprise a proepicardial subcompartment that specifically contributes to the formation of the coronary vasculature (35). In contrast, a recent study showed that the expression of *Sema3d* and *Scx* overlaps with other epicardial markers in the PEO. Exclusive expression of *Sema3d* and *Scx* was observed in the septum transversum, but these cells did not appear to contribute to the developing heart (59). Unfortunately, the ability to definitively study cell fate of EPDCs has thus far been limited due to the non-uniform expression of transcription factors in epicardium, and to the use of Cre-based lineage tracing models that are either not sufficiently specific to the epicardium or that fail to label all cells within the entire layer. However, the recent advent of single cell RNA sequencing, with which the transcriptome of individual cells can be identified, has greatly simplified the identification and composition of cell populations within a tissue, as well as their differentiation trajectories. Another suggestion of proepicardial heterogeneity was established by Tyser et al. A novel source of proepicardial cells was identified dubbed the juxta-cardiac field (JCF), through a combination of single-cell RNA sequencing and genetic lineage tracing from early cardiac development (E7.5) onwards. When tracing the fate of these JCF-derived cells, they were found to contribute to both the PEO and subsequently to the epicardium and/or to cardiomyocytes in the developing mouse heart (150). These data suggest that there are populations contributing to the PEO that may have specific abilities to differentiate into various other cell types besides ECs. The question remains whether or not these cells can differentiate into both cell types, generating epicardial cells that can continue to become cardiomyocytes or if they are bipotent cells from the onset that can become either epicardial cells or cardiomyocytes (150). When looking beyond the PEO, in the developing epicardium there are also several indications supporting the hypothesis of heterogeneity. The human ventricular epicardium has been described to be formed as a multiple-cell layered epicardium while the atria have a single-cell layer epicardium, suggesting that localization of epicardial cells could influence their behavior (34). Concordantly, when unraveling the role of ECM components in the developing mouse heart, a morphologically heterogeneous epicardium was observed related to the EMT-status of epicardial cells. Epicardial cells undergoing EMT were located near regions with a distinct ECM composition, composed of less integrin α4 and laminin and more agrin puncta. Conversely, loss of agrin resulted in fewer Wt1-positive cells in the epicardium and the myocardium and an increase in β-catenin, suggesting more cell-cell adhesion and thereby a decreased ability to undergo EMT (140). These data suggest that epicardial differentiation is affected not only by the transcriptome and secretome of epicardial cells but also by the ECM that is formed by epicardial cells and its derivatives during cardiac development. Interestingly, ECM components were also found to influence epicardial EMT and were upregulated in EPDCs during EMT and migration (118). Additionally, bone-marrow derived $CD45^{+}$ cells were found within the epicardium, indicating that there are other cell populations besides the PEO that contribute to the cellularity of the epicardial layer (134). Various studies have recently tried to deconvolute the composition of the developing epicardium using scRNA seq. Weinberger et al. identified three functional subpopulations within the developing zebrafish epicardium at 5 days post-fertilization (5 dpf) by sequencing cells from reporter fish lines for *tcf21*, *wt1b*, and *tbx18*. Analysis of transcriptomes of these cells showed three distinct epicardial clusters. The function of these subpopulations was confirmed using newly generated knockout zebrafish for the markers found therein. One of the subpopulations expressed *transglutaminase 2b* (*tgm2b*), and both transient and stable somatic knockdown of this gene led to defects in the epicardial layer. This suggests *tgm2b* plays a crucial role in maintaining the integrity of the epicardial sheet during its formation. By creating somatic knock-out animals for genes found in the other subpopulations, the authors observed that *sema3fb* and *cxcl12a* had distinct effects on epicardial migration and composition, respectively. *Sema3fb* was strongly expressed within the bulbous arteriosus (BA), a part of the outflow tract. *Sema3fb* knockout regulated the number of *tbx18*-positive cells contributing to SMCs covering the outflow tract. The third population, which was enriched for *cxcl12a,* was spatially restricted to an area between the BA and atrium. Knockout of this gene revealed that this cell population was involved in homing of leukocytes to the developing heart (151), establishing a mechanism for the contribution of CD45+ cells of non-PEO origin to the developing epicardium (134). The finding of these three subpopulations suggests that the epicardium could be heterogenous in zebrafish during development, and that these epicardial subpopulations are spatially and functionally distinct. A potential regulator of epicardial heterogeneity has been identified by a scRNA seq study on epicardial cells derived from human pluripotent stem cells. Gambardella et al. found that basonuclin (BNC1) can modulate the expression of essential epicardial transcription factors Wt1 and Tcf21. In the absence of BNC1 cells have an increased expression of Tcf21 and a reduced expression of Wt1 (152), indicating that BNC1 can influence the phenotype of epicardial cells. It is important to note that these two studies described above were performed in different organisms and models and are confined to a limited developmental timeframe. Additionally, it is not clear whether these cells are veritable epicardial cells that are located on the outside of the heart, or EPDCs that are undergoing EMT and initiated differentiation (59). Also, studies using cell culture models could have a bias toward certain cell states due to the culture conditions, a lack of interactions with surrounding tissues and a proper developmental progression. Therefore, more evidence on the epicardial cellular composition based on other models is still necessary.

Another source of information regarding the epicardial heterogenicity could derive from cardiac cell atlases that have been generated using scRNA seq to identify rare cell populations and interactions within the developing heart. In these studies, the epicardium is often annotated but its potential heterogeneity is often overlooked due to low epicardial cells numbers relative to the total number of cardiac cells, or

heterogeneity is ascribed to developmental progression (153–156). Another possible explanation is that in these studies epicardial cells are characterized based on known markers and not further scrutinized, thus novel (sub)populations are potentially not identified.

New insights regarding the composition of the developing epicardium have come from several studies focusing on spatiotemporal analysis. Contrary to previous studies (151, 152), a larger developmental timeframe is studied by performing sequencing at several timepoints. Liu et al. used scRNA seq at three developmental stages (early to late septation) of the developing outflow tract in mice, a transient structure that gives rise to the aorta and the pulmonary trunk. Although they revealed two epicardial populations that were heterogenous in their composition, this was most likely the result from developmental progression, rather than different subpopulations (157). In the human heart, a combination of spatial transcriptomics and scRNA seq at various timepoints during development [4.5–9 weeks post-conception (PCW)] was able to identify the epicardium in all stages. In this dataset there appeared to be no heterogeneity in the epicardial cells, but mesenchymal cells showed heterogeneity based on their expression of marker genes. However, due to the low number of cells sequenced (3,717 in total) at the intermediate stage (6.5–7 PCW), the low resolution of their spatial transcriptomic approach (∼30 cells per spot), and the limited number of genes used in validation through *in situ sequencing*, it is difficult to draw a concrete conclusion about epicardial composition (158). In a similar study in which spatiotemporal analysis of the heart was performed during key developmental stages in chicken, 5,621 epicardial cells from the ventricular free wall were clustered. In their analysis, the epicardial cells and its derivatives (EPDCs) clustered based on their position within the differentiation process. The data were able to confirm that epicardial cells follow the anticipated trajectory where they undergo EMT and migrate into the myocardium before committing to either fibroblast or mural cell fate (118). Although no functional epicardial heterogeneity was observed, cells that have started EMT might reside in the epicardial layer during later stages of development (day 7) and may continue to stay in the epicardium in an undifferentiated intermediate phenotype until day 10. This variation in differentiation state could give the impression of heterogeneity in the epicardial layer if observed at a singular timepoint, but these data suggest that this heterogeneity does not stem from a difference in initial cell population. The finding that the epicardium lacks functional heterogeneity was corroborated in a study where the developmental stages from the formation of the PEO (E9.5), the establishment of the epicardium (E13.5) up until the differentiation of EPDCs (E15.5) were investigated. Here, established markers such as *Wt1, Tbx18, Tcf21, Scx,* and *Sema3d* did not demarcate functional subpopulations. Moreover, they showed that expression of these markers did not influence the differentiation trajectory of EPDCs to either mural cells or fibroblasts, and that EPDCs lost expression of these markers upon the induction of EMT (with the exception of *Tcf21,* which goes up after EMT until differentiation) (59). This further illustrates that subpopulations (see **Table 1**) are more likely to be a result of developmental progression and that reported heterogeneity is rather a reflection of transcriptional changes after EMT. Additionally, it has been suggested that the various differentiation trajectories of EPDCs might be due to extrinsic cues such as paracrine factors and location relative to cardiac cell types (e.g., ECs) and ECM rather than intrinsic expression of transcription factors (59).

Heterogeneity in the Postnatal Epicardium

The composition of the fetal epicardium, although subject to debate, has been researched intensively (see section Cellular heterogeneity in the developing epicardium and **Table 1**), but very little is known about the composition of the epicardium in the adult heart. In its quiescent state, very few cells express markers such as Wt1 that denote activated epicardium and its functional heterogeneity is likely limited. However, since ischemic injury induces re-activation of the epicardial layer, identifying subpopulations that participate in the wound healing process either through cellular contributions, or *via* paracrine signaling, could result in the identification of mechanisms that aid in cardiac repair. A mouse model of cardiac ischemia/reperfusion (I/R) injury revealed that after 7 days Wt1, Tcf21, and Tbx18 were expressed in distinct as well as in overlapping populations within the subepicardial mesenchyme (102). In the same study, reactivation of Tcf21 and Wt1 was found in interstitial fibroblasts and not myofibroblasts after I/R, indicating that these markers are present in differentiated fibroblasts, but not activated myofibroblasts. This suggests that the expression of these markers coincides with the induction of fibrogenesis after I/R (102). To establish whether injury mediated activation of the epicardium results in similar effects as during development, a direct comparison between embryonic EPDCs (E12.5) and adult EDPCs after Tß4 priming and MI (2, 4, and 7 days post-MI) was performed. A majority of Wt1-positive cells in the adult cells expressed stem-cell antigen-1 (Sca-1) compared to their embryonic counterpart (159). Sca-1$^+$ cells are considered a progenitor cell population for various cell populations, such as CMs, ECs, SMC and fibroblasts, although their adoption of CM fate is debated (160, 161). Wt1$^+$Sca-1$^+$ displayed increased expression of mesenchymal markers CD105, CD44, Thy-1, and PDGFRb compared to embryonic EPDCs, and a heterogenous expression of these markers (159). Although this reactivation is not autonomous, i.e., it is stimulated by Tß4, it does indicate that subpopulations in the activated adult epicardium may have distinct functions. Interestingly, a similar degree of heterogeneity was observed in epicardial cells 5 days post-MI without external activation (162). However, from these studies it is unclear if the different populations arose from a common ancestor cell in the epicardium or if heterogeneity pre-existed within the inactive epicardial layer.

Using scRNA seq in *tcf21*$^+$ epicardial cells of adult zebrafish, Cao et al. report three subpopulations with a distinct gene expression signature in the uninjured heart and found that this heterogeneity persisted after injury (163). In this study, only a few dozen cells were sequenced which may make the interpretation more challenging. When comparing transcriptional changes between a model for mitochondrial cardiomyopathy and

TABLE 1 | Overview of findings in search of heterogeneity in developing epicardium.

Model	Technique	Timepoint	Sequenced tissue	Finding	References
Zebrafish reporter/knockout lines	scRNA seq, hybridization chain reaction	5 dpf	*Wt1b* (47 cells), *tcf21* (137 cells), *tbx18* (52 cells) from reporter lines	Distinct functions for *tgm2b, cxcl12a, sema3fb* in epicardial development	(151)
hPSC-epicardium	scRNA seq/BNC1 knockdown	-	232 hPSC-epi single cells	BNC1 drives heterogeneity	(152)
Human embryonic hearts	Spatial transcriptomics (ST), scRNA seq, *in situ* sequencing, smFISH	4.5–9 PCW	ST: 3115 spots containing ∼30 cells. scRNA seq: 3717 cells from 6.5 to 7 PCW	Epicardial cells displayed no heterogeneity. Heterogeneity was observed in mesenchymal cells	(158)
Chicken embryonic hearts	Spatial transcriptomics (ST), scRNA seq, smFISH	4–14 days (HH21–HH40)	ST: 6,800 barcoded spots (10–20 cells per spot). scRNA seq: 22,315 cells	No functional heterogeneity: post-EMT cells residing in epicardium	(118)
$Wt1^{CreERT2}$;$Rosa26^{tdTom}$	ISH, scRNA seq	E9.25–E15.5	Published datasets (E9.25–E10.5) - 276 tdTom+ cells at E15.5	Epicardial cells displayed no heterogeneity. Heterogeneity was observed in mesenchymal cells at E15.5	(59)

healthy postnatal mouse hearts the epicardium displayed no heterogeneity, although this may be due to the disease model used (164). Recently, the epicardial layer and subepicardial mesenchyme (or epicardial stromal cells – epiSCs) were subjected to single cell sequencing 5 days post-MI by Hesse et al. (119). This approach provided a higher resolution by focusing on the epicardial layer specifically. Interestingly, they described heterogeneity in the epicardial layer which was partly due to a proliferative phenotype and a high degree of protein synthesis. The function of the identified subpopulations was not assessed in mice, nor whether these populations resulted from differential progression, as observed during development. Nevertheless, a high degree of heterogeneity was detected in stromal and ECM producing cells (119) (see below). Strikingly, there was some conservation between these data and functionally heterogenous epicardial subpopulations in zebrafish as described by Weinberger et al. (119, 151). Since a tamoxifen-inducible Wt1 reporter line was used to obtain the scRNA seq data, the contribution of $Wt1^+$-derived cells to the identified populations could be analyzed. In this set-up no contribution of traced cells to $Wt1^-$ populations was observed within the 5 days post-MI (119). In a subset of the $Wt1^+$ population there was a high expression of *Tmbs4x*, the gene coding for Tß4 which can induce cardiomyogenesis *in vivo* and induce cardiac repair when given prior to injury (99, 120). A similar cellular subset has been reported in the chicken epicardium during development (118). Based on their data, Hesse et al. also hypothesized that a subset of the $Wt1^+$ cells may have cardiomyogenic potential due to their high expression of cardiac specification markers and sarcomere proteins. Additionally, they found that all stromal cells expressed paracrine factors previously observed in $Wt1^+$ cells, and that this was not exclusive to epicardial stromal cells but also in myocardial stromal cells (119). Although this study investigates the epicardial composition post-MI in a high resolution, it does not address the function and differentiation trajectory of observed subpopulations. Since only a singular timepoint is analyzed (5 days post-MI), a conclusion whether or not these subpopulations are a reflection of functional differences or of a varying differentiation state cannot be made. In a study where scar formation was analyzed over multiple timepoints, a similar degree of heterogeneity was observed in stromal cells compared to the study by Hesse et al. Using $Wt1^{Cre}$;$RosaZsGreen^{f/+}$ mice to label epicardial derivates at day 0 (d0), d1, d3-5,7, d14, and d28, the evolution of mesenchymal cells was identified. A novel subpopulation was identified dubbed injury response (IR) cells at d1. The IR subpopulation had a high expression of monocyte-macrophage chemoattractants *Ccl2, Ccl7*, and *Csf1* and neutrophil activators *Cxcl1* and *Cxcl5*. Additionally, it also expressed pro-inflammatory and pro-fibrotic factors *Il33*, *Cxcl12*, and *Tgfb12*. These IR cells transitioned to myofibroblasts at d3, and myofibroblasts also displayed heterogeneity. A subpopulation of myofibroblasts was similar to recently identified matrifibrocytes (MFCs) in the mature scar at d14-d28. MFCs were identified as cells that express high levels of ECM genes and support the integrity of the mature scar in injured hearts (165). At this stage, late-resolution (LR) fibroblasts were also found, expressing genes associated with differentiation and regulation of matrix remodeling and deposition (166). In general, while more information regarding the adult epicardium and its composition is becoming available, the current studies indicate the need for further analysis at multiple timepoints of mesenchymal subpopulations and their function in cardiac repair and regeneration. The epicardial layer seems to lack functional heterogeneity in the adult heart after injury, but scRNA seq analysis could shed more light on the role of paracrine factors and cellular contributions of the epicardium and its derivatives post-injury (**Figure 2**).

CONCLUSIONS

The epicardium has been unequivocally shown to be essential during cardiac development, both *via* the contribution of cells and through the secretion of paracrine factors. Since these processes are also required to repair the heart after injury, the epicardium has been considered a very appealing source for endogenous cardiac repair. It has become clear that

most processes that occur within the epicardial setting during development are recapitulated in the adult epicardium after injury, albeit less efficient. Therefore, there has been an interest in deconvoluting the epicardial composition to identify targets to optimize the post-injury response. High-resolution analysis of the epicardial layer in the developing heart has suggested heterogeneity within the layer. However, this is mostly the case in studies where a limited timeframe has been studied. In more elaborate approaches where development through time has been investigated, the heterogeneity seems to be explained by developmental progression, i.e., cells are in different stages of EMT or are in the process of differentiating into a specific cell type. There is very limited evidence that the heterogeneity derives from distinct subsets of cells. This is also true for the PEO; while there is a certain degree of heterogeneity this does not account for the different cell types, apart from perhaps cells from the JCF. In the adult heart, the epicardium is in an inactive state and likely has very little heterogeneity, but analysis of the epicardium after injury has suggested he presence of subpopulations in epicardial-derived mesenchymal cells during cardiac repair. Overall, our knowledge on the composition of this intriguing cell population is steadily increasing through the advent of novel techniques such as single RNA seq. Moreover, besides knowledge on its cellular composition, a vast amount of data has been accrued regarding novel proteins, signaling pathways or paracrine factors produced by epicardial cells in regenerative and non-regenerative species. Therefore, whether or not a specific population can be identified and targeted to stimulate repair, other approaches such as delivery of epicardium related paracrine factors, specific modulation of the ECM or the immune response can provide additional means to further enhance the development of novel therapies to repair the injured heart.

AUTHOR CONTRIBUTIONS

TS and AS contributed to the conception and design of the manuscript. TS wrote the first draft of the manuscript. AS wrote sections of the manuscript. Both authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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Reawakening the Intrinsic Cardiac Regenerative Potential: Molecular Strategies to Boost Dedifferentiation and Proliferation of Endogenous Cardiomyocytes

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Despite considerable efforts carried out to develop stem/progenitor cell-based technologies aiming at replacing and restoring the cardiac tissue following severe damages, thus far no strategies based on adult stem cell transplantation have been demonstrated to efficiently generate new cardiac muscle cells. Intriguingly, dedifferentiation, and proliferation of pre-existing cardiomyocytes and not stem cell differentiation represent the preponderant cellular mechanism by which lower vertebrates spontaneously regenerate the injured heart. Mammals can also regenerate their heart up to the early neonatal period, even in this case by activating the proliferation of endogenous cardiomyocytes. However, the mammalian cardiac regenerative potential is dramatically reduced soon after birth, when most cardiomyocytes exit from the cell cycle, undergo further maturation, and continue to grow in size. Although a slow rate of cardiomyocyte turnover has also been documented in adult mammals, both in mice and humans, this is not enough to sustain a robust regenerative process. Nevertheless, these remarkable findings opened the door to a branch of novel regenerative approaches aiming at reactivating the endogenous cardiac regenerative potential by triggering a partial dedifferentiation process and cell cycle re-entry in endogenous cardiomyocytes. Several adaptations from intrauterine to extrauterine life starting at birth and continuing in the immediate neonatal period concur to the loss of the mammalian cardiac regenerative ability. A wide range of systemic and microenvironmental factors or cell-intrinsic molecular players proved to regulate cardiomyocyte proliferation and their manipulation has been explored as a therapeutic strategy to boost cardiac function after injuries. We here review the scientific knowledge gained thus far in this novel and flourishing field of research, elucidating the key biological and molecular mechanisms whose modulation may represent a viable approach for regenerating the human damaged myocardium.

Keywords: heart regeneration, direct cardiogenesis, cardiomyocyte proliferation, cardiomyocyte dedifferentiation, heart development, endogenous cardiac repair

TOWARD THE DIRECT STIMULATION OF CARDIOMYOCYTE PROLIFERATION FOR HEART REGENERATION

Heart failure, consisting in the inability of the heart to pump enough blood to meet the body's needs, is a prominent cause of death worldwide and often occurs as a result of severe cardiac injuries, such as those induced by myocardial infarction [reviewed by Savarese and colleagues (1)]. Besides left ventricular assist devices and heart transplant, which is the most curative approach, yet with severe limitations (scarcity of donors, extremely high costs, immune response, and organ rejection, etc.), currently available therapies are mainly based on pharmacological treatments for slowing down disease progression and reducing symptoms. However, none of these treatments can reverse the progression of the disease or cope with the underlying conspicuous loss of cardiac muscle cells (cardiomyocytes) that are replaced by fibrotic scar tissue. During the last decades, scientific studies based on transplantation of adult stem cells, isolated from skeletal muscle, bone marrow, blood, or fat tissue, have been carried out with the hope to replenish lost or damaged cardiomyocytes, restoring cardiac function. Unfortunately, these approaches demonstrated modest beneficial effects on heart function most probably attributable to paracrine factors rather than the generation of new cardiac muscle cells [reviewed by Tzahor and Poss (2) and Sadek and Olson (3)]. Moreover, although a population of lineage negative c-kit+ cardiac stem cells was initially reported to give rise to all major cardiac cell types, including cardiomyocytes (4), more recent lineage tracing studies based on tamoxifen-inducible Cre-LoxP technology unveiled that newly cardiomyocytes generated from c-kit+ cells are extremely rare, irrelevant in terms of cardiomyocyte regeneration, despite abundantly contributing to the generation of endothelial cells (5) [reviewed by Passier and colleagues (6) and Chien and colleagues (7)].

During the last two decades, the attention of many research groups has shifted toward the possibility to regenerate the damaged heart by reawakening the intrinsic regenerative potential. Indeed, studies of the animal kingdom have enlightened the amazing ability of some animals to regenerate themselves. Hydra, planarians, and lower vertebrates, such as salamanders, frogs, and fishes, can trigger complex repair mechanisms, totally or partially restoring missing or damaged tissues and organs, such as limbs, retinas, eye lenses, spinal cords, tails, and even the heart. These astonishing observations have led to intense scientific investigations in cardiac regenerative medicine, aiming at developing innovative therapeutic strategies suitable for humans. Specifically, studies in the zebrafish model at the adult stage unveiled its ability to efficiently regenerate the damaged cardiac tissue, achieving complete scar resolution and regeneration of lost cardiomyocytes within 2 months after surgical resection of 20% of the ventricular myocardium (8). This striking self-healing property emerges even after more severe cardiac damages, such as cardiomyocyte-specific depletion of 60% of the ventricular myocardium (9), and cryoinjury-induced lesions (10, 11). Interestingly, genetic labeling of differentiated cardiomyocytes with fluorescent markers highlighted that cardiac muscle cells generated post-injury derive from the proliferation of endogenous cardiomyocytes. In this process, transient and partial dedifferentiation of cardiomyocytes has been documented, as manifested by cardiomyocyte detachment from one another, sarcomere disassembly, loss of Z-line structure, and expression of fetal genes (12, 13). Unlike zebrafish, for a long-time, the mammalian heart has been considered non-regenerative because of its injury-induced replacement of dead muscle cells with fibrotic tissue and its inability to restore the reduced contractile function after major injuries. Despite adult mammals fail in regenerating their heart, cardiac regeneration appears to be quite robust during prenatal and early postnatal stages. Indeed, mammalian fetuses can compensate for a loss of about half of cardiomyocytes (14, 15). Newborn mice can robustly regenerate their heart following resection of 15% of the ventricular apex within 2 months, by inducing the proliferation of pre-existing cardiomyocytes, as assessed by lineage tracing analyses and staining of cell cycle markers (16, 17). A complete cardiac regeneration process has also been documented in newborn mice following induction of myocardial infarction by ligation of the left anterior descending artery (18). The cardiac regenerative ability at the neonatal stage has also been documented in large mammals. For example, myocardial infarction in 1 or 2-days-old swine, is followed by cardiac tissue replacement achieved by dedifferentiation and proliferation of pre-existing cardiomyocytes in the border zone (19, 20). It has also been reported the astonishing clinical case of a newborn child undergoing a rapid functional cardiac recovery after myocardial infarction, although it was not possible to assess if the observed recovery was due to bona fide regeneration or reversible functional impairment (21). Importantly, cardiomyocyte regenerative potential in the mouse model dramatically decreases during the first week of postnatal life; consequently, severe cardiac injuries evolve in permanent scarring and impair heart function (16). This decline was suggested to start already 2 days after birth (22). Similar observations were documented in larger mammals during the early postnatal period. For example, swine begin losing cardiomyocyte regenerative ability at postnatal day 3 and more pronouncedly at later developmental stages (postnatal day 7 and 14), undergoing extensive cardiac fibrosis and not recovering cardiac function after injury (19, 20).

In this review we first describe how mammalian cardiomyocyte cell cycle activity is regulated during prenatal and postnatal life, with particular emphasis on the early postnatal period, when most cardiomyocytes become bi/multi-nucleated or polyploid, withdraw from the cell cycle, and continue to grow in size (hypertrophic growth), consequently losing the regenerative potential. Then, we review the major changes occurring at birth and in the immediate postnatal period, along with systemic, micro-environmental, intracellular stimuli influencing the proliferative ability of endogenous cardiomyocytes, whose manipulation is a promise for enhancing cardiomyocyte regeneration and boosting cardiac function in heart failure patients.

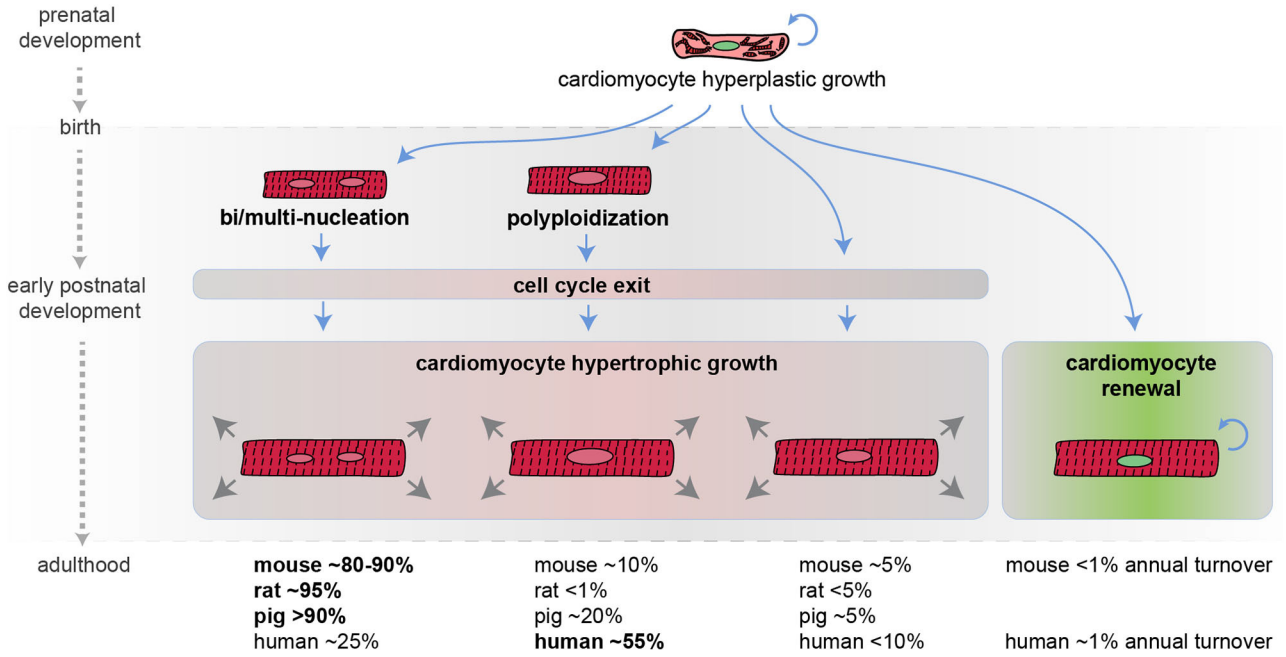

FIGURE 1 | Developmental regulation of cardiomyocyte cell cycle activity in mammals. Schematic representation of mammalian cardiomyocyte growth in prenatal and postnatal life. Most cardiomyocytes during the early postnatal period become bi/multi-nucleated and/or polyploid, withdraw from the cell cycle, and continue to grow in size (hypertrophic growth). An approximate percentage of bi/multi-nucleated, polyploidy, and diploid cardiomyocytes in different mammalian species at the adult stage is provided (it may not add up to 100% because cardiomyocytes can have several polyploid nuclei and because these values are derived from different reports) along with the estimated cardiomyocyte annual turnover.

DEVELOPMENTAL REGULATION OF CARDIOMYOCYTE CELL CYCLE ACTIVITY

In zebrafish, cardiomyocytes are predominantly mononucleated and diploid throughout life and retain pronounced proliferative capacity (23, 24).

In contrast, cardiomyocyte cell cycle activity and nucleation in mammals are strictly connected to the developmental stage (**Figure 1**). During embryonic and fetal development heart growth in mammals is characterized by the increase in the number of cardiomyocytes. Importantly, genetic fate mapping in the mouse model, allowing the identification of the temporal sequence during which the lineage segregation between cardiomyocytes and non-myocytes takes place, unveiled that non-myocytes, which include stem cell populations, contribute to new cardiomyocyte generation exclusively in the early embryonic development (25). Starting from mid-gestation, pre-existing cardiomyocytes become the predominant source of cardiomyocyte replacement in physiological mammalian cardiac development (25). As further detailed later in this review, multiple signaling pathways were shown to play a key role in cardiomyocyte proliferation during prenatal life and, in some cases, their manipulation can partially reactivate the cardiac regenerative ability in the adult stage.

In the mouse model, during the first week after birth, the majority of cardiomyocytes undergo DNA synthesis and karyokinesis (nuclear division), without proceeding to cytokinesis (cytoplasm division), thus resulting in binucleation (two diploid nuclei per single cell) (26, 27) [reviewed by Soonpaa and Field (28)]. Specifically, on postnatal day 2 most mouse cardiomyocytes are mononucleated. On postnatal day 3 mouse binucleated cardiomyocytes raise to ∼17% and reach the adult level of ∼80–90% by day 11 (26) [reviewed by Derks and Bergmann (29)]. Similarly, in rats, the percentage of binucleated cardiomyocytes, which is around 3–4% in the first 3 days of postnatal life, increases at ∼17% on postnatal day 4 and reaches the adult level of ∼90% by day 12 (30) [reviewed by Derks and Bergmann (29)].

In humans, during the early postnatal period, the majority of cardiomyocytes undergo DNA synthesis without karyokinesis, resulting in polyploidization (single tetraploid nuclei) (31). Other large mammals, such as swine, undergo primarily multinucleation and to a less extent polyploidization (32) [reviewed by Derks and Bergmann (29)].

The time at which cardiomyocytes become bi/multi-nucleated or polyploid is coincident with the time when mammals lose their regenerative potential [reviewed by Derks and Bergmann (29) and Gan and colleagues (33)]. In support of a causal relationship between multinucleation/polyploidy and loss of cardiac regenerative ability, enforced cardiomyocyte polyploidization has been demonstrated to reduce cardiomyocyte proliferation and to represent a barrier to heart regeneration in the zebrafish model (24). Importantly, during the early postnatal development, the vast majority of mammalian cardiomyocytes

also exit from the cell cycle. As a consequence, the number of postnatal cardiomyocytes does not increase in mammals during postnatal life (34), and further growth of the heart is achieved by increasing cardiomyocyte size, a phenomenon known as hypertrophic growth.

Historically, adult human cardiomyocytes were considered completely unable to divide. However, this belief has been disproved in 2009. Indeed, the analysis of the integration of ^{14}C generated by nuclear bomb tests during the Cold War allowed to precisely estimate cardiomyocyte renewal in adult humans. This study detected 1% annual cardiomyocyte turnover at the age of 25, declining to 0.3% at the age of 75. Based on these data, it is therefore estimated that fewer than 50% of cardiomyocytes are physiologically exchanged during the course of life (34). Even though the adult cardiomyocyte renewal rate is extremely low, definitely insufficient to pursue a successful regenerative process after major injuries, this remarkable observation suggests that increasing the rate of adult cardiomyocyte proliferation may represent a novel strategy for cardiac regeneration.

MOLECULAR STRATEGIES FOR CARDIOMYOCYTE REGENERATION

Immediately after birth, a complex reorganization of the cardiovascular system occurs. Recent studies have unveiled that the adaptation from intrauterine to extrauterine life driven by the sudden lack of exposure to circulating maternal factors, the increase in oxygen levels, the increase in heart workload, as well as changes of systemic, microenvironmental, and intracellular stimuli, lead to maturation of cardiomyocyte cytoarchitecture, switch in energetic metabolism from glycolysis to fatty acid oxidation and cell cycle withdrawal during the early postnatal period, concurring to postnatal loss of cardiac regenerative ability. Importantly, the manipulation of specific molecular mechanisms has been demonstrated to be sufficient for inducing cardiomyocyte proliferation and heart regeneration upon injury (**Figure 2**).

Cell Cycle Checkpoints

Multiple regulators of cell cycle checkpoints, including cyclins, cyclin-dependent protein kinases (CDKs), CDK-activating kinases (CAKs), and CDK inhibitors (CKIs) were documented to regulate cardiomyocyte cell cycle activity during prenatal and postnatal development. Cyclin/CDK function is mainly regulated by post-transcriptional or post-translational modifications. However, cardiac mRNA and protein levels of several cyclins/CDKs were documented to decrease during postnatal development (35–38) (bioinformatic analysis in **Figure 3A**) and, in several cases, their overexpression was sufficient to induce postnatal cardiomyocyte cell cycle activity (**Figure 3B**). Thus, the decline in expression levels of specific cyclins/CDKs contributes to mammalian cardiomyocyte cell cycle blockage in postnatal life.

D-type cyclins, when complexed with CDK4 or CDK6, drive cell cycle re-entry (transition from G0 to G1 phase). High protein levels of D-type cyclins, CDK4 and CDK6, have been reported in the fetal heart, dramatically declining in the early postnatal and adult stage (26). In agreement, we observed that cardiac mRNA levels of cyclin D3 (Ccnd3) and CDK4 significantly decline in the early postnatal period (postnatal day 9–P9), whereas cardiac mRNA levels of cyclin D1 (Ccnd1), cyclin D2 (Ccnd2), and CDK6 decline in the subsequent postnatal developmental step (postnatal day 23–P23) (see **Figures 3A,B**). **Cyclin D1** overexpression has been reported to induce abnormal multinucleation (35). The impairment of its nuclear import in differentiated cardiomyocytes, in part due to the accumulation of CDK inhibitor p27, has emerged as a barrier that prevents postnatal cardiomyocyte proliferation (40). Indeed, overexpression of Skp2 ubiquitin ligase, which triggers the degradation of p27, enhances the mitogenic effect mediated by nuclear-targeted cyclin D1 (D1NL)/**CDK4** and improves cardiac function after myocardial infarction (40). Cardiac-specific overexpression of cyclin D1, **cyclin D2** or **cyclin D3** results in increased DNA synthesis of mammalian cardiomyocytes in adult mice (38). However, myocardial damage reduces the pro-proliferative effect of transgene-encoded cyclin D1 and D3 by inducing their cytoplasmatic accumulation. Importantly, the cardiac injury does not induce cytoplasmatic accumulation of transgene-encoded cyclin D2, which indeed has been documented to maintain persistent cell cycle activity in cardiomyocytes and to trigger infarct regression (38).

E-type cyclins, in association with CDK2, control the G1 phase of the cell cycle and are known to initiate the assembly of the pre-replication complex. Cardiac protein levels of CDK2 are drastically reduced from fetal to adult stage (38). In this regard, we observed that cardiac mRNA levels of CDK2 significantly decline in the early postnatal period (P9) (see **Figures 3A,B**). Chemical inhibition of **CDK2** suppresses DNA synthesis of neonatal cardiomyocytes (41), whereas its overexpression increases the number of smaller mononuclear cardiomyocytes in adult mice (42). We also noticed that cardiac mRNA levels of cyclin E1 (Ccne1) and cyclin E2 (Ccne2) significantly decline in the early postnatal period (P9) and the subsequent developmental step (P23) (see **Figures 3A,B**). However, the role of E cyclins in cardiomyocyte proliferative and regenerative ability is currently unexplored.

A-type cyclins are required for entry into S phase (in association with CDK2) or into M phase (in association with CDK1). Interestingly, cardiac protein levels of cyclin A1 and A2 were shown to decline in postnatal development, and very low levels of CDK1 have been reported in the adult heart (37, 43). Consistently, we observed that mRNA levels of cyclin A2 (Ccna2) and CDK1 significantly decline during the early postnatal period (P9, see **Figures 3A,B)**, whereas cyclin A1 (Ccna1) mRNA was generally poorly expressed in postnatal life (P1-P4-P9 and P23, **data not shown**). Adenoviral overexpression of **cyclin A2** has been documented to enhance the endogenous regenerative mechanism after myocardial infarction by the generation of new cardiomyocytes in the infarct and border zones, along with improved cardiac function and reduced collagen/muscle density ratio (37, 44, 45). The injection of adenovirus encoding cyclin A2 into the peri-infarct myocardium has been reported to induce

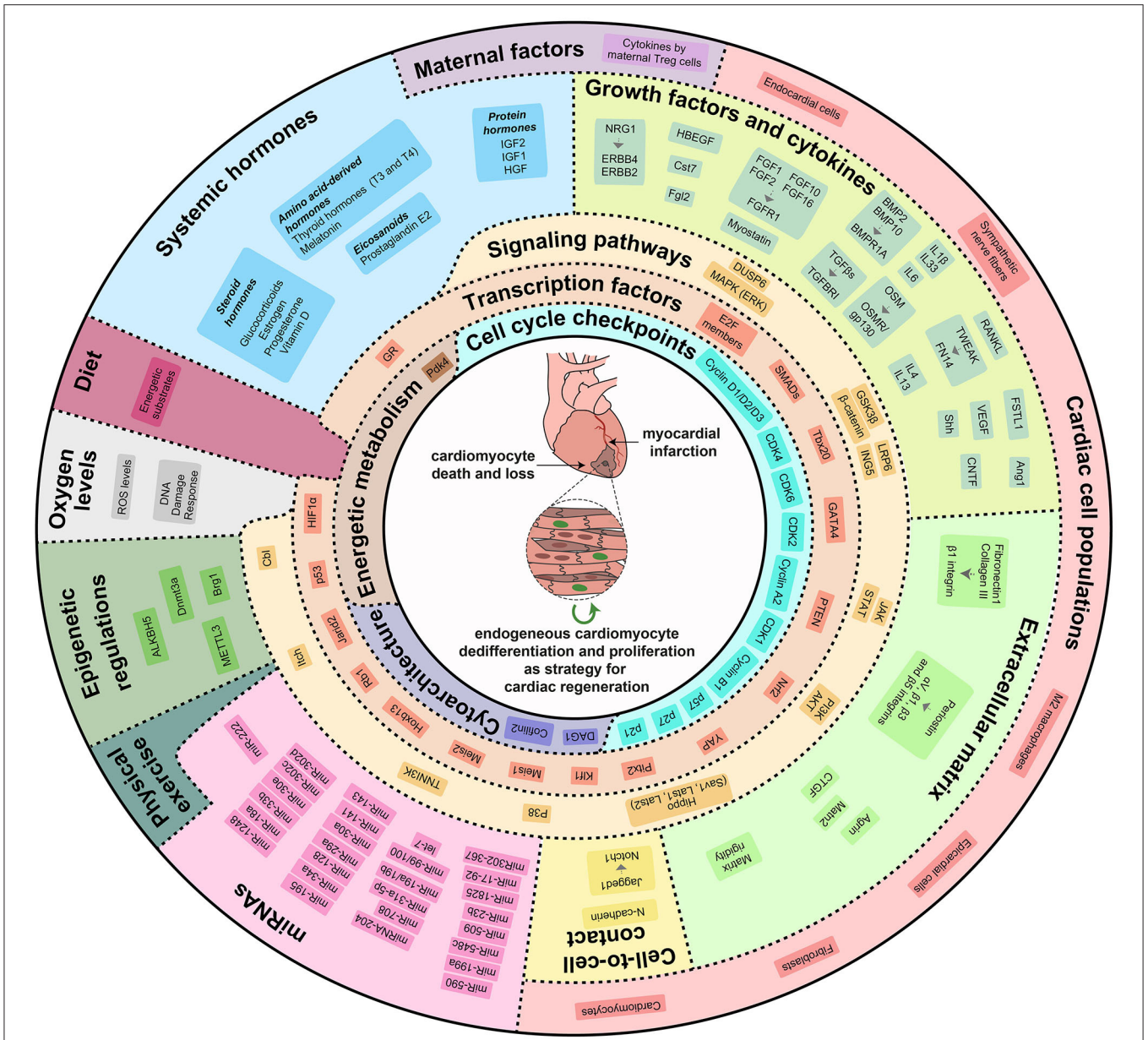

FIGURE 2 | Cardiac regenerative strategies based on direct stimulation of cardiomyocyte dedifferentiation and proliferation. Modulation of external, systemic, micro-environmental, and intrinsic molecular mechanisms can re-activate cardiomyocyte proliferative and regenerative potential. Locally produced growth factors and cytokines, extracellular matrix rigidity and components, direct cell-to-cell contacts, maternal factors, systemic hormones, oxygen levels, physical exercise, miRNAs and epigenetic regulations modulate a variety of signaling pathways and transcription factors that control cardiomyocyte dedifferentiation and proliferation by regulating cell cycle checkpoints, cytoarchitectural organization and energetic metabolism.

cardiomyocyte mitosis, to decrease fibrosis and to boost cardiac function in larger preclinical models (swine) (46).

B-type cyclins in association with CDK1 positively regulate the transition from G2 to M phase. Interestingly, cardiac levels of cyclin B1 and CDK1 protein were documented to be dramatically reduced from fetal to adult stage (36). In line, we observed that cyclin B1 (Ccnb1) mRNA levels significantly decline in the early postnatal period (P9), whereas cardiac mRNA levels of cyclin B2 (Ccnb2) and cyclin B3 (Ccnb3) decline later on during postnatal development (P23) (see **Figures 3A,B**). Forced expression of **cyclin B1** and CDC2 (human homolog of **CDK1**) increases the number of neonatal and adult rat cardiomyocytes *in vitro* (36).

Interestingly, overexpression of a combination of **CDK4-cyclin D1** and **CDK1-cyclin B1** complexes in adult cardiomyocytes has been documented to promote a high rate (∼15%) of cardiomyocyte proliferation and to contribute to heart regeneration after coronary artery ligation in adult mice (47).

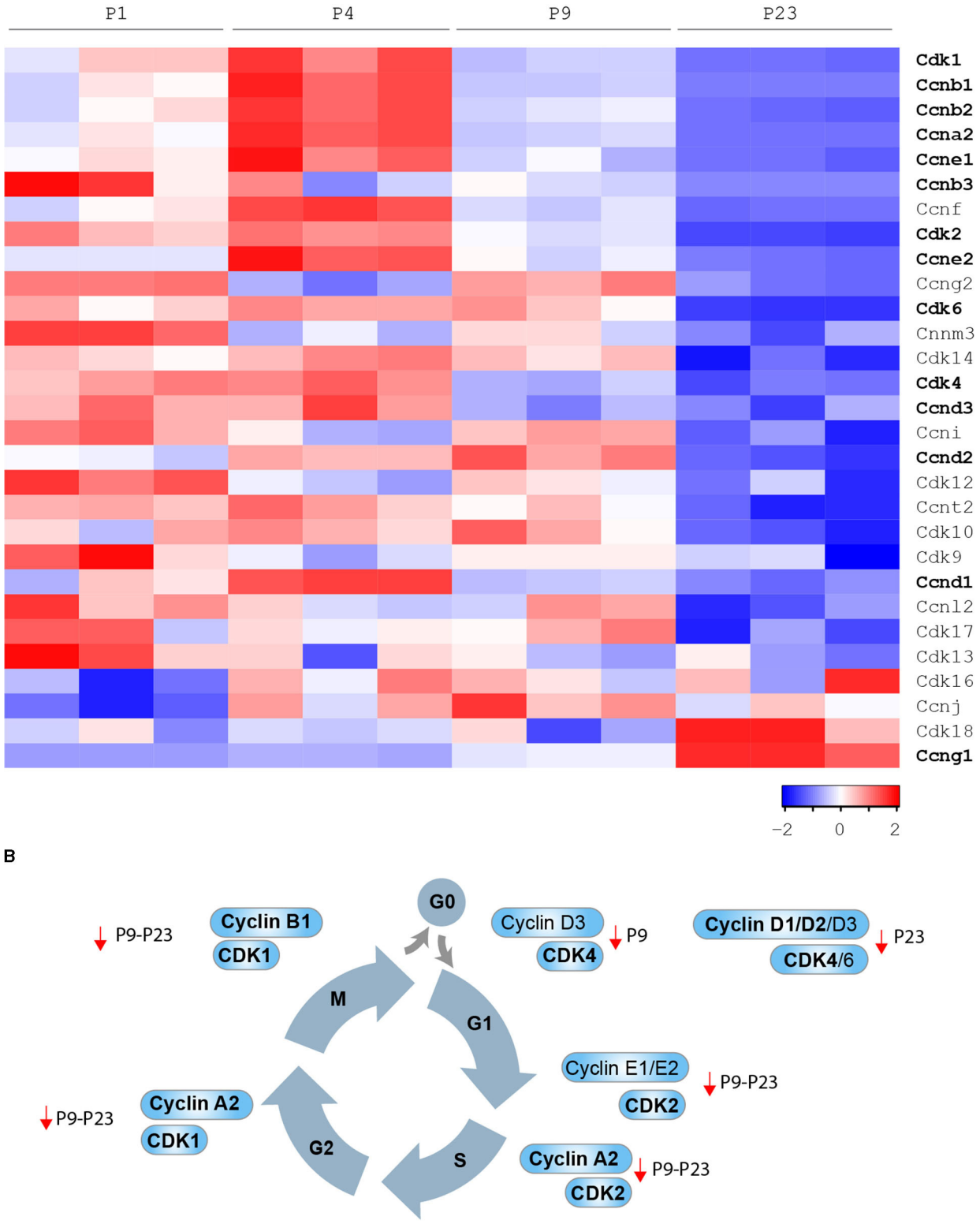

FIGURE 3 | Developmental regulation of cardiomyocyte cell cycle in mammals. **(A)** Cyclins and Cdks expression levels by bioinformatic analysis of the gene expression profile of the mouse heart at different developmental stages [P1, P4, P9, and P23 from Talman et al. (39)]; **(B)** Cyclins and CDKs whose modulation has been demonstrated to be sufficient to induce postnatal cardiomyocyte cell cycle progression (in bold cell cycle factors that were reported to induce adult cardiomyocyte regeneration after major injuries).

The replacement of CDK1-cyclin B1 overexpression through a pharmacological approach, based on the administration of inhibitors of Wee1 (CDK1-inhibitor) and transforming growth factor-β (TGF-β), proved to be an alternative way to unlock adult cardiomyocyte replicative ability (47).

Oppositely, cardiac mRNA and protein levels of cyclin G1 (Ccng1) increase during the early postnanal development and in the adult stage (48) (see **Figure 3A**). However, unlike the other cyclins described above, cyclin G1 has been linked to the onset of postnatal cardiomyocyte polyploidization and multinucleation. Indeed, its overexpression in primary neonatal rat cardiomyocytes promotes entry in S-phase (uptake of ^{3}H-thymidine), however reducing the number of cytokinetic events (Aurora B immunostaining), and thus resulting in an increase of polynucleated cells. In contrast, the knockout of cyclin G1 prevents the increase of cardiomyocyte multinucleation in response to pressure overload and hypertrophy (48).

Cyclin-dependent Kinase Inhibitors (CKIs), including **p21**, **p27**, and **p57**, were suggested to contribute to cardiomyocyte cell cycle withdrawal as the heart ages. Manipulation of their physiological expression by siRNA delivery has been documented to stimulate cytokinesis of neonatal cardiomyocytes and progression to the S phase of post-mitotic cells without DNA damage or apoptosis (49).

Maternal Factors

In mammals, during fetal life, the placenta supplies all physiological needs. A major change occurring after birth, besides major hemodynamic and biochemical events, is the sudden lack of exposure to the maternal circulation. Intriguingly, exposure to the serum of pregnant animals has been reported to promote neonatal cardiomyocyte proliferation (50), suggesting that mother's serum factors might be involved. Further analyses unveiled a role for **regulatory T cells** (**Tregs**) in this process (50). Tregs are physiologically expanded during pregnancy and they are crucial for the suppression of allogenic responses toward the fetus (51). Endogenous Tregs have been found able to support cardiomyocyte hyperplasia and the increase in heart size physiologically occurring during pregnancy (50). Furthermore, Treg injection at the site of myocardial infarction has been documented to promote cardiomyocyte replication and heart regeneration (50). The effect appears mediated by a group of six cytokines secreted by Tregs, namely **TNF superfamily member 11 (Tnfsf11 or RANKL)**, **Interleukin-33 (IL33)**, **Insulin-like growth factor 2 (IGF2)**, **Cystatin F (Cst7)**, **Fibrinogen-like 2 (Fgl2)**, and **Matrilin2 (Matn2)** (50). Indeed, the production of the six factors by adenoviral vectors is sufficient to induce neonatal cardiomyocyte proliferation *in vitro*, as well as cardiomyocyte proliferation and heart regeneration *in vivo* in adult mice (50).

Oxygen Levels

During fetal stages, the oxygenated maternal blood mixes with poorly oxygenated blood within the placental space. Thus, the oxygen content supplied to the fetus is lower than the maternal uterine arterial blood, resulting in the fetus living in a more hypoxemic environment. One of the major adaptations that mammals must face during the transition from fetus to newborn, when pulmonary circulation starts, is the exposure to a more oxygenated environment. Importantly, the change in oxygen concentration has been demonstrated to impact on cardiomyocyte proliferative and regenerative ability. In contrast to anoxia, which is reported to impair cardiomyocyte proliferation (52), the exposure to mild hypoxic conditions (15% O2) in neonatal mice is sufficient to enhance cardiomyocyte mitogenesis, protecting cells from oxidative stress (53). In line, hypoxemia exposure to 2-months-old mice, by a gradual reduction in inspired oxygen until 7%, is sufficient to facilitate the proliferation of pre-existing cardiomyocytes and heart regeneration after myocardial infarction, thus improving left ventricular systolic function (54). Importantly, intermittent hypoxia-hyperoxia appears to facilitate the rehabilitation of patients with coronary artery disease (55). In contrast, hyper-oxidative (100% O2) exposure is responsible for oxidative DNA damage and decreased cytokinesis in mouse models (53).

During the first week of postnatal life, the increase in oxygen levels contributes to the decline in heart regenerative ability by triggering oxidative energetic mitochondrial metabolism (further described in the "*Energetic metabolism*" section) and by inducing **reactive oxygen species (ROS), oxidative DNA damage,** and **DNA damage response (DDR)** (53). Indeed, ROS scavenging, or inhibition of DDR is sufficient to extend the postnatal proliferative window of cardiomyocytes, whereas ROS production shortens it (53).

Clinical studies on cyanotic congenital heart disease infants suggest that the hypoxic condition reflects an increased mitotic potential of cardiomyocytes (56). Ablation of **Hypoxia-inducible factor 1-alpha (HIF1α)**, a major mediator of the hypoxic response, reduces fetal cardiomyocyte proliferation and results in ventricular hypoplasia (57). Moreover, by lineage-tracing studies employing a tamoxifen-inducible Cre fused to the oxygen-dependent degradation domain of HIF1α, it has been unveiled that a population of hypoxic cycling cardiomyocytes contributes to the slow cardiomyocyte turnover occurring in the adult mammalian heart (58). Interestingly, a downstream target of HIF1α, named Zinc finger E-box-binding homeobox 2 (ZEB2), has been recently demonstrated to be enriched in injured cardiomyocytes of zebrafish models. Its overexpression improves cardiomyocyte survival and cardiac function, as well as angiogenesis following cardiac damage (59), however, the role of ZEB2 on cardiomyocyte proliferation has not been explored.

Energetic Metabolism

In zebrafish, proliferating cardiomyocytes in the border zone of the wounded heart, where cardiomyocyte dedifferentiation mainly occurs, switch their metabolism from oxidative phosphorylation to glycolysis, as manifested by reduced mitochondrial genes and increased glycolytic genes (60). This process was reported to be induced by Neuregulin-1/Erbb2 signaling (60). Importantly, inhibition of glycolysis after cardiac injury impairs cardiomyocyte proliferation in adult zebrafish (60).

In mammals, during the prenatal period, glucose is the main source of energy for cardiomyocytes, and anaerobic

glycolysis is the primary energetic route. With the transition to extrauterine life, the low-fat and high glucose supply in the umbilical blood is replaced by the high fat, low glucose diet of the mother's milk. As a consequence of the increase in oxygen levels (due to the opening of the pulmonary circulation) and the shift in substrate utilization (from glucose to fatty acids), cardiomyocytes experience a profound change in the energetic metabolism during the early postnatal development, with a rewiring from anaerobic cytoplasmic glycolysis to mitochondrial-dependent oxidative phosphorylation [reviewed by Piquereau and Ventura-Clapier (61)]. This transition is driven by the upregulation of **genes involved in fatty acid metabolism and oxidative phosphorylation**, and the downregulation of **glycolytic genes** (62). The maturation of cardiomyocyte cytoarchitecture occurring in the early postnatal development (further described in the "*Cytoarchitectural organization*" section), is coupled with a transition from sparse to dense and well-organized mitochondrial clusters and a more efficient energy transfer system from mitochondria to sarcomere structures [reviewed by Piquereau and Ventura-Clapier (61)]. Although mitochondrial oxidative metabolism is a more efficient energy production to face the increasing cardiomyocyte needs of the postnatal heart, recent insights have demonstrated that the glycolysis-to-fatty-acid-oxidation metabolic switch concurs to the postnatal loss of cardiomyocyte proliferative and regenerative ability. In this regard, mitochondrial maturation has been suggested as a mediator of cardiomyocyte cell cycle arrest (53). Further, fetal cardiomyocytes were found more mitotic and with delayed maturation when exposed to maternal hyperglycemia (63). Administration of a fat deficient diet is sufficient to increase the generation of new cardiomyocytes in young mice, even though no differences were then observed after 10 weeks of age (64). In addition, cardiac-specific ablation, or pharmacological inhibition of **pyruvate dehydrogenase kinase 4 (PDK4)**, which physiologically inhibits mitochondrial pyruvate dehydrogenase thus improving cardiac fatty acid oxidation, induces cardiomyocyte proliferation and improves cardiac function after myocardial infarction (64).

Cytoarchitectural Organization

During the early postnatal heart development in mammals, cardiomyocytes experience a profound maturation of the **cytoarchitecture organization** that, along with an increase in matrix rigidity (described in "*Extracellular matrix*" section), is essential to adequately respond to the increased workload of the extrauterine life [reviewed by Guo and Pu (65)]. Specifically, the loss of cardiac regenerative potential is coupled with an increase in cardiomyocyte cell size, and a shift of the cardiomyocyte cytoarchitectural structure from loose spatial organization to highly organized and efficient sarcomere units, characterized by the alignment of Z-lines, distinguishable M-lines and switch from fetal to adult sarcomere isoforms [reviewed by Guo and Pu (65)]. Importantly, the sarcomere apparatus occupies a large proportion of the cell, and the rigid sarcomere structure of adult cardiomyocytes makes them more refractory to cytokinesis. Interestingly, spontaneous heart regeneration occurring in injured zebrafish and neonatal mice appears coupled with sarcomere disassembly (12, 13, 16). Some regulators of the remodeling of the cardiomyocyte architecture have been demonstrated to affect cardiomyocyte proliferative and regenerative ability [reviewed by Ali and colleagues (66)], including actin-depolymerizing factor **Cofilin 2** (67) (further described in the "*miRNA*" section) and dystroglycan **DAG1** (68) (further described in the "*Extracellular matrix*" section), which anchors the cardiomyocyte cytoskeleton to the extracellular matrix. Furthermore, unlike zebrafish and newts, which preserve intact centrosomes throughout life, **centrosome integrity** is lost shortly after birth in mammals and has been described to contribute to postnatal cardiomyocyte G0-G1 cell cycle arrest (69).

Cardiac Cell Populations

After cardiac injuries in lower vertebrates or neonatal mammals, a series of cellular events take place to trigger the regeneration of the damaged tissue. An inflammatory phase driven by recruited leukocytes starts immediately after the injury. In this regard, endogenous **macrophages** have emerged as essential players for heart regeneration in lower vertebrates and neonatal mice. Indeed, macrophage depletion impairs myocardium regeneration following injuries, leading to scar formation in zebrafish (70) and neonatal mice (71, 72). Secretion of Oncostatin M (OSM, described in the "*Growth factors and cytokines*" section) by macrophages/monocytes appears to be essential for cardiomyocyte proliferation during neonatal heart regeneration (73). In addition, the positive effect exerted by hypoxia exposure on cardiomyocyte proliferation (discussed in the "*Oxygen levels*" section), has been suggested to be dependent on an increase in the number of resident macrophages (56). In contrast to the neonatal stage, adult mammalian hearts mainly undergo repair processes based on scarring and fibrosis, mostly as a result of the interaction between infiltrating immune cells (including macrophages) and fibroblasts [reviewed by Chen and colleagues (74)]. The paradoxical role of macrophages, triggering cardiac regeneration in lower vertebrates and neonatal mammals, and maladaptive remodeling in injured adult mammals, has been a matter of investigation. In this regard, neonatal mice in response to cardiac injuries have been shown to expand a population of embryonic-derived resident cardiac macrophages with a pro-reparative (**M2**) polarization phenotype, which generates minimal inflammation and secretes numerous soluble factors that facilitate cardiomyocyte proliferation (71, 72). In contrast, adult mice in response to cardiac injuries expand monocyte-derived macrophages with an inflammatory (M1) phenotype, which lack regenerative properties (71, 72). Inline, M2 compared to M1 macrophage-conditioned media has been shown to upregulate neonatal cardiomyocyte proliferation and to suppress myofibroblast-induced differentiation via secretion of the anti-inflammatory cytokine **IL4 (Interleukin 4)** *in vitro* (75). However, the potential cardiac regenerative role of IL4 administration has not been further explored thus far. Moreover, administration of the anti-inflammatory cytokine IL10 (76) or BMP7 (bone morphogenetic protein 7) [reviewed by Aluganti Narasimhulu and Singla (77)] has been reported to improve cardiac remodeling after myocardial infarction by stimulating

M2 macrophage polarization, although their potential impact on cardiomyocyte proliferation has not been analyzed. Thus, manipulation of macrophage lineages and/or polarization, or their secreted factors, may represent a viable strategy for cardiac regeneration.

The initial injury-induced inflammatory response in zebrafish and neonatal mice is accompanied by the activation of the endocardium and epicardium, which together with cardiac fibroblasts, repair the tissue and support its regeneration by inducing cardiomyocyte proliferation. **Endothelial cells** migrate into the apical thrombus early after cardiac damage, develop into functional arteries, and precede cardiomyocyte ingrowth during mammalian heart regeneration (78). The pro-proliferative and pro-regenerative effect of endothelial cells is likely due to paracrine factors, such as NRG1 (further described in the "*Growth factors and cytokines*" section). Activated **epicardial cells** also secrete signals with the potential to influence cardiomyocyte proliferation and heart regeneration, including BMPs, TGFbs, SHH and IGFs [reviewed by Cao and Poss (79)] (further described in the "*Growth factors and cytokines*" section). In contrast to adult cardiac fibroblasts that are known to promote myocyte hypertrophy, embryonic **cardiac fibroblasts** have been reported to enhance cardiomyocyte replication in co-culture experiments (80). This effect appears to reside in fibroblast-secreted factors, such as the extracellular matrix components fibronectin1 and collagen III as well as the growth factor HBEGF (80) (further info available in the "*Extracellular matrix*" and "*Growth factors*" sections).

Innervation also plays a key role in cardiac regeneration. In this regard, **sympathetic nerve fibers** re-grow and fully reinnervate during the spontaneous cardiac regeneration in neonatal mice (81). Importantly, denervation, achieved by pharmacological ablation of cholinergic signaling, restrains heart regeneration by inhibiting cardiomyocyte cell cycle activity in zebrafish and neonatal mice (81, 82). Interestingly, neonatal sympathetic lesions result in increased expression of Meis1 (83), a transcription factor involved in postnatal cardiomyocyte cell cycle arrest (further described in the "*Transcription factors*" section). Administration of Neuregulin-1 (NRG1) and Nerve Growth Factor (NGF) have been shown to partially rescue denervated hearts, enhancing cardiac regeneration post-injury. However, unlike NRG1, NGF is not able to directly promote the proliferation of cultured cardiomyocytes (82).

Growth Factors and Cytokines

A wide spectrum of mitogens sustains cardiomyocyte proliferation during prenatal development. Administration of these factors has been investigated as a strategy to restore cardiomyocyte mitogenic potential, reminiscent of the embryonic stage. Nowadays, different growth factor ligands and receptors have been found able to induce adult cardiomyocyte cell cycle re-entry and proliferation and to achieve substantial improvements in terms of cardiac tissue regeneration.

Fibroblast growth factors (FGFs) act as paracrine or endocrine signals in heart development, health, and disease, exerting biological activities by binding to cell surface FGF receptors (FGFRs) [reviewed by Itoh and colleagues (84) and Khosravi and colleagues (85)]. Several FGF members, including FGF9, FGF10, FGF16, and FGF20 were shown to induce cardiomyocyte proliferation during embryonic/fetal development (86–88). Importantly, the ability of some FGFs in inducing postnatal cardiomyocyte replication and cardiac regeneration has also been documented. Administration of **FGF1**, alone and more pronouncedly in combination with an inhibitor of mitogen-activated protein kinase (p38), has also been demonstrated to induce neonatal and adult rat cardiomyocyte proliferation *in vitro* (89) as well as *in vivo* after myocardial infarction in adult rats, resulting in reduced scar formation and improved cardiac function (90). **FGF10** has been reported to trigger cell cycle re-entry of adult cardiomyocytes (86); however, its delivery as a strategy for adult cardiac regeneration has not been evaluated thus far. The role of **FGF16** in the regulation of postnatal cardiomyocyte replication is currently debated. Cardiac levels of FGF16 have been shown to increase in early postnatal life (91). However, in contrast to the documented positive role on cardiomyocyte proliferation during heart development (87, 88), FGF16 administration to neonatal cardiomyocytes does not influence their proliferation and even abrogate FGF2-induced cell cycle re-entry (91). Nevertheless, cardiac-specific FGF16 overexpression has been shown to improve cardiac function and cardiomyocyte replication after cryoinjury in a GATA4-knockout mouse model (92). Intriguingly, a decrease in expression levels and an isoform switching of type 1 fibroblast growth factor receptor **FGFR-1** have been reported in early postnatal life. Consistently, FGFR-1 overexpression has been shown to enhance the proliferation of postnatal rat cardiac myocytes, which appears to be dependent on **FGF2**, since its neutralization with antibodies inhibits the proliferative response (93).

Neuregulin-1 (NRG1) is a growth factor, mainly produced by endothelial cells and acting in cardiomyocytes via its tyrosine kinase receptors **ERBB4** and **ERBB2**. NRG1/ERBB4/ERBB2 signaling axis is essential for heart development (94–96). In zebrafish, NRG1 is sharply induced in perivascular cells after cardiac damage and inhibition of its co-receptor ERBB2 disrupts cardiomyocyte proliferation in response to injury (97). In mice, administration of NRG1 has been shown to induce adult cardiomyocyte proliferation and heart regeneration (98). Administration of NRG1 moderately improved cardiac function in heart failure patients in phase I and phase II trials (99–101). However, it has been observed that its mitogenic effect in mammals is more pronounced during the neonatal period than in later postnatal development and in adulthood (102, 103), due to the decline in cardiac levels of ERBB2, which is necessary to transduce the mitogenic signaling of NRG1 (102). Thus, combinatorial strategies of NRG1 with ERBB2 overexpression or ERBB2 inducing factors should be further explored. Indeed, transient induction of ERBB2 signaling in cardiac muscle cells of juvenile and adult mice is sufficient to robustly induce cardiomyocyte dedifferentiation and proliferation and to trigger heart regeneration following myocardial infarction (102). Analysis of ERBB2 downstream players mediating these effects in cardiomyocytes revealed the involvement of ERK, AKT, and GSK3β/β-catenin pathways (102). More recently, ERBB2 signaling has been shown to lead to phosphorylation of

YAP in ERK-dependent and Hippo-independent manner (104). Interestingly, **HBEGF**, a growth factor that activates ERBB4 and the cognate EGF receptor (EGFR), has been shown to induce mammalian cardiomyocyte proliferation (80) (described in the "*Cardiac cell populations*" section).

Bone morphogenetic proteins (BMPs) are multi-functional growth factors belonging to the transforming growth factor beta (TGFβ) superfamily. BMPs play a key role in multiple steps of cardiac development, including differentiation of cardiomyocytes from mesoderm, cardiomyocyte growth and ventricular trabeculation [reviewed by Vanwijk and colleagues (105)]. Spatially resolved RNA sequencing of regenerating zebrafish heart unveiled that BMP signaling is activated in the border zone of the damaged myocardium, as manifested by expression of BMP ligands (BMP2 and BMP7), receptors (Bmpr1aa), and activation of downstream **SMAD** players (Smad 1, 5, and 8) (106). Importantly, BMP signaling is essential for injury-induced cardiomyocyte proliferation in zebrafish (106). In particular, a loss-of-function mutation in Bmpr1aa reduces cardiomyocyte proliferation and heart regeneration (106). Furthermore, **BMP2** overexpression appears sufficient to boost cardiac regeneration in zebrafish (106). Nevertheless, the ability of BMP2 in inducing cycle re-entry of mammalian neonatal cardiomyocytes (rat model) is currently controversial (106, 107). BMP2 administration in adult infarcted mice reduces cardiomyocyte apoptosis and scar size, protecting cardiomyocytes from oxidative stress and hypoxia, although potential effects on cardiomyocyte proliferation were not evaluated and deserve further investigations (108, 109).

BMP10 is essential for maintaining cardiac growth during cardiogenesis in murine models (110). Mechanistically, BMP10 promotes the production of the transcription factor **Tbx20** by inducing its promoter activity through a Smad binding site (111). In turn, cardiomyocyte-specific Tbx20 gain of function, beginning in fetal development, maintains cardiomyocytes in an immature proliferative status, characterized by fetal gene expression and smaller, cycling, mononucleated cells, by inducing BMP2/pSmad1/5/8 and to a lesser extent PI3K/AKT/GSK3β/β-catenin (107). Importantly, intramyocardial injection of BMP10 increases cell cycle activity of adult cardiomyocytes, and its delivery by a sponge scaffold for 12-weeks in an infarcted rat model enhances cardiomyocyte progression to the S-phase, cell re-entry and cytokinesis, and improves cardiac function (112). Other BMP ligands, such as BMP14 and BMP7, were suggested as positive regulators of cardiac repair, even though the documented effects are independent of cardiomyocyte proliferation. *In vivo* ablation of BMP14 (also known as GDF5) results in increased cardiomyocyte apoptosis, fibrosis and adverse cardiac remodeling after myocardial infarction in adult mice (113). BMP7 exerts anti-inflammatory and anti-fibrotic properties (114, 115) [reviewed by Aluganti Narasimhulu and Singla (77)]. Conversely, other BMPs appear to exert an opposite role. It is the case of **BMP4**, which induces hypertrophy and apoptosis in cultured cardiomyocytes (116).

TGFβ1, TGFβ2, and TGFβ3, pleiotropic factors belonging to the transforming growth factor beta (**TGFβ**) superfamily, have emerged as crucial mediators of multiple cellular responses in the infarcted myocardium, including cardiac reparative, inflammatory, angiogenic, and fibrotic responses [reviewed by Frangogiannis (117), Hanna and Frangogiannis (118) and Sorensen and colleagues (119)]. The inhibition of TGFβ receptor 1 (**TGFBR1**) activity by SB-431542 was reported to reduce the number of proliferating cardiomyocytes in zebrafish embryos (120). Intriguingly, robust activation of TGFβ/SMAD3 signaling has been documented during zebrafish heart regeneration, as evidenced by upregulation of TGF ligands (tgfb1a, tgfb1b, tgfb2, and tgfb3), receptors (alk5a known as Tgfbr1, and alk5b known as Tgfbr1b) and the downstream effector SMAD3 (10, 121). Furthermore, inhibition of TGFβ/SMAD3 signaling reduces cardiomyocyte cell cycle activity and abolishes heart regeneration in adult zebrafish upon cardiac injury (10, 121). Interestingly, opposite results have been obtained in mammals. Indeed, the administration of TGFβ has been documented to inhibit the proliferation of neonatal rat cardiomyocytes and suppress the mitogenic effect of growth factors such as bFGF or IGFs (122). Transgenic mice overexpressing TGFβ1 display increased cardiomyocyte size and cardiac hypertrophy accompanied by interstitial fibrosis (123). Moreover, administration of TGFβ inhibitor (SB-431542) robustly induces the proliferation of human iPS-derived cardiomyocytes, if combined with a Wee1 inhibitor, and overexpression of CDK4 and cyclin D1 (47). Another member of the TGFβ superfamily, known as **Myostatin**, was found able to inhibit proliferation of dividing fetal and neonatal rat cardiomyocyte by blocking the G1-S phase transition (124). The opposite role of TGFβ signaling in the modulation of cardiomyocyte replication of lower vertebrates versus mammals deserves further investigation.

Sonic hedgehog (Shh) is a ligand of the hedgehog family, which has been mainly implicated in the formation of coronary vasculature and reported to modulate cardiac regeneration and repair [reviewed by Wang and colleagues (125)]. In zebrafish embryos, administration of Shh agonist (SAG) or antagonists (CyA), respectively increases or decreases the number of proliferating cardiomyocytes (120). Moreover, hedgehog signaling is required for myocardial regeneration in zebrafish, further increasing cardiomyocyte proliferation (120). Shh ligand expression and activation of downstream pathways are observed during heart regeneration after cardiac injury in neonatal mice, but not 1 week after birth, when mice are no longer able to regenerate their hearts (126). Finally, genetic or pharmacological augmentation of Shh signaling within the first week of postnatal life in the mouse model has been shown to improve heart regeneration, whereas its inhibition impairs the regenerative response (126). Even if cardiomyocyte proliferation has not been analyzed, gene therapy with Shh after acute and chronic myocardial ischemia in adult mammals results in enhanced neovascularization, reduced fibrosis, and augmented cardiac function (127).

Several **pro-inflammatory cytokines**, such as Interleukin-1β (IL1β), Interleukin-33 (IL33), Interleukin-6 (IL6), Oncostatin (OSM) and TNF-related weak inducer of apoptosis (TWEAK), can induce cardiomyocyte dedifferentiation and/or proliferation, in most cases promoting a beneficial effect in the short run. Indeed, cardiomyocyte dedifferentiation physiologically protects

the heart after acute damage. However, in the long run, pro-inflammatory cytokines lead to chronic inflammation, fibrotic disorders, adverse remodeling and/or heart failure. For example, the administration of **IL1β**, which is upregulated upon cardiac injury in neonatal mice (128), induces neonatal cardiomyocyte proliferation (89, 129). However, IL1β is also responsible for profibrotic signaling and cardiomyocyte apoptosis. Its blockage, through platelet microparticles armed with selective antibodies, prevents adverse cardiac remodeling inhibiting cardiomyocyte apoptosis (130). In contrast, **IL33,** another member of the IL-1 superfamily, is among the pro-regenerative factors produced by Treg cells (described in the "*Maternal factors*" section).

In addition, pro-inflammatory cytokines of the IL6 family, including **IL6** and **Oncostatin M (OSM)**, were found elevated in the acute response to cardiac injury. OSM triggers dedifferentiation of cardiomyocytes, as demonstrated by the reduction of sarcomere structure and reactivation of fetal sarcomere components (such as alpha-SMA) and stem cell markers (such as Runx1 and Dab2) (131), physiologically protecting the heart after acute damage. However, in the long run, OSM-induced cardiomyocyte dedifferentiation leads to adverse remodeling and heart failure (131) [reviewed by Fontes and colleagues (132)]. Intriguingly, OSM administration, acting through its receptor (OSMR) and the co-receptor **gp130 (glycoprotein 130)**, also induces cardiomyocyte proliferation in neonatal mice, and synergizes with other mitogenic stimuli such as fibroblast growth factor 2 (FGF2) or adenovirus-induced E2F2 (131). Conditional overexpression of gp130, triggers cardiomyocyte replication and heart regeneration in juvenile and adult mice, via Src-mediated YAP activation (73). IL6 knock out neonatal mice fail to regenerate the heart, whereas IL6 overexpression results in enhanced proliferation of neonatal cardiomyocytes (128, 133). Mechanistically, the pro-regenerative effect of IL6 appears mediated by STAT3 signaling, which also is required for neonatal heart regeneration in mice (128, 133). However, no study thus far evaluated the potential ability of IL6 in inducing cardiomyocyte replication and heart regeneration in the adult stage.

Inflammatory cytokines of the tumor necrosis factor (TNF) ligand family, including Tnfsf11 (also known as **RANKL**), a cytokine secreted by Tregs already described in the "*Maternal factors*" section and Tnfsf12 (also known as **TWEAK**), were also found to positively regulate cardiomyocyte proliferative ability. TWEAK has been enlightened as a positive regulator of neonatal rat cardiomyocyte mitosis through fibroblast growth factor-inducible molecule 14 (FN14) receptor. However, early postnatal downregulation of **FN14** restrains TWEAK mitogenic potential in adult cardiomyocytes (134). Nevertheless, adenoviral expression of FN14 enables efficient induction of cell cycle re-entry in adult cardiomyocytes after TWEAK stimulation (134).

A few **anti-inflammatory cytokines**, such as Interleukin-4 (**IL4**, which is secreted by M2 macrophages described in the section entitled "*Cardiac cell populations*") and Interleukin-13 (**IL13,** a cytokine with anti-inflammatory activities but mediator of allergic inflammation), were suggested to exert a positive effect on cardiomyocyte proliferation. Interleukin-13 (IL13) stimulates neonatal cardiomyocyte replication *in vitro* by activation of IL13Ra1, and to a lesser extent IL4Ra, and downstream pathways, such as STAT6 and STAT3/Periostin (135), ERK and AKT (136). Furthermore, IL13 knock out mice display reduced cardiomyocyte cell cycle activity and impaired cardiac regeneration upon cardiac apex resection at the neonatal stage (136). However, the potential ability of IL13 in inducing cardiomyocyte proliferation and cardiac regeneration in the adult stage remains unexplored.

As described in the "*Maternal factors*" section, other cytokines secreted by Tregs, including Cystatin F (**Cst7**), and Fibrinogen-like 2 (**Fgl2**), were demonstrated to trigger cardiomyocyte proliferation and heart regeneration (50).

Administration of **Follistatin-like 1** (**FSTL1**), an epicardial-secreted cardiac mitogen, through an epicardial patch, improves survival, and sustains cardiac function in infarcted mouse and swine models, by promoting cell cycle re-entry and division of pre-existing cardiomyocytes (137). Some angiogenic factors were also demonstrated to induce myocyte proliferation. For example, cardiac overexpression of **VEGF (vascular endothelial growth factor)** paralog Vegfaa induces cardiac muscle hyperplasia in adult zebrafish, although inhibiting regeneration after injury, suggesting that spatio-temporal control of this factor is required (138). Overexpression of VEGF and **angiopoietin-1 (Ang1)** by adeno-associated viral vectors, in addition to improve angiogenesis, promotes cardiomyocyte cell cycle re-entry in infarcted swine models (139).

Finally, mutation of the **Ciliary Neurotrophic Factor (CNTF)** has been reported to impair cardiomyocyte proliferative response in injured zebrafish hearts, whereas CNTF injection facilitates cardiac regeneration (140).

Extracellular Matrix

The cardiac extracellular matrix (ECM) is a highly dynamic network of fibers comprised of matrix proteins in which cardiac cells, including cardiomyocytes, reside. It is continuously remodeled in response to environmental stimuli, aging, and pathological conditions, to support a wide variety of cellular responses. During the early postnatal development, the cardiac matrix changes its mechanical properties from a high hydrated structure, enriched with fibronectin, hyaluronic acid, and proteoglycans, to a stiffer structural network, enriched with collagen I, and laminin, thus supporting the strength of the cardiac muscle (141). Importantly, the increased rigidity of the heart after birth mechanically influences cardiomyocyte morphology and behavior, contributing to their cell cycle withdrawal. Indeed, rigid substrates interfere with rat and mouse cardiomyocyte cytokinesis, without affecting karyokinesis (nuclear division), thus leading to binucleation (142). On the other hand, softer substrates trigger cardiomyocyte rounding and cell division, coupled with a partial cardiomyocyte dedifferentiation process as documented by downregulation of sarcomere proteins (142).

A key pathway in matrix-stiffness mechano-transduction is the **Hippo pathway**, a well-known regulator of organ growth (143, 144). Importantly, the Hippo pathway has emerged as a key regulator of heart regeneration. If activated in the

cardiac tissue, it drives phosphorylation of **YAP (Yes-associated protein)**, thus preventing its translocation into the nucleus, in turn restraining cardiomyocyte proliferation in postnatal life [reviewed by Wang and colleagues (145)]. Inactivation of the Hippo signaling, by deletion of scaffold proteins, such as **Sav1 (Salvador homologue 1 protein)**, or downstream mediators, such as **Lats1** and **Lats2 (large tumor suppressor homologue 1 and 2)**, or alternatively constitutive expression of active YAP, in adult cardiomyocytes, have been shown to stimulate cardiomyocyte proliferation, reduce the scar size, and improve heart function in infarcted mouse models (146–149) [reviewed by Wang and colleagues (145)].

The stiffness is not the only way by which the extracellular matrix may impact on cardiomyocyte replicative and regenerative ability. Indeed, it has been demonstrated in the mouse model that the changes in the composition of the cardiac extracellular matrix during the early postnatal period influence cardiomyocyte growth and differentiation. For example, the proteoglycan **Agrin** is physiologically downregulated during the early postnatal cardiac development, contributing to the loss of cardiomyocyte proliferative potential (150). Agrin interacts with the dystrophin-glycoprotein complex (DGC), connecting the ECM to the F-actin cytoskeleton, through **Dag1 (dystroglycan 1)**. Administration of Agrin following myocardial infarction in juvenile and adult mice is sufficient to destabilize the cardiomyocyte cytoskeleton and facilitate cell cycle re-entry and cell division in the peri-infarcted region by activating downstream mediators such as the **extracellular signal-regulated kinase (ERK)** signaling and destabilizing YAP-Dag1 interaction, leading to **YAP** release and translocation into the nucleus (150). A single local delivery of recombinant human Agrin has been documented to enhance cardiomyocyte proliferation, improve cardiac function and reduce adverse remodeling, fibrosis, and infarct size in preclinical swine models (151).

Some members of the **fibronectin 1** family (fn1 and fn1b), the main components of the extracellular matrix, are produced and deposited after cardiac damage in the zebrafish model and are essential for the regenerative process (152). Fibronectin 1 (Fn1) and **collagen III**, produced by embryonic fibroblasts (described in the "*Cardiac cell populations*" section), have also been shown to induce mammalian cardiomyocyte proliferation (80). **β1-integrin** appears to be required for the proliferative response induced by embryonic fibroblast-secreted factors, and ventricular cardiomyocyte-specific deletion of β1-integrin in mice reduces myocardial proliferation and impairs ventricular compaction (80).

Periostin, a secreted extracellular matrix protein, promotes adult rat cardiomyocyte proliferation via activation of **αV**, **β1**, **β3**, and **β5 integrins** and downstream activation of **PI3K/AKT** (but not ERK) pathway (153). After myocardial infarction in adult rats, Periostin induces cardiomyocyte cell cycle re-entry and mitosis, improves ventricular remodeling and reduces infarct size (153). However, Periostin is also responsible for the recruitment of activated fibroblasts in the mouse model (154) and promotes extensive cardiac fibrosis in remote regions in infarcted swine models (155), thus its administration as a strategy for inducing cardiac regeneration is dampened.

Connective tissue growth factor (CTGF), also known as communication network factor 2a (Ccn2a), is a matricellular protein that is synthesized and secreted from endocardial cells after cardiac injuries (156). In zebrafish, CTGF has been reported necessary for heart regeneration by inducing cardiomyocyte proliferation and infiltration (156). CTGF triggers cardiomyocyte cell cycle activity also in neonatal mammals (135), however, its potential impact on adult cardiomyocyte proliferation and heart regeneration has not been explored thus far.

Finally, the extracellular matrix protein **Matrilin2 (Matn2)** is among the factors secreted by Tregs that trigger cardiac proliferation and heart regeneration (50) (described in the "*Maternal factors*" section). Thus, administration of extracellular matrix components, or modulation of the downstream signaling pathways, might be a promising approach for heart regeneration.

Cell-to-Cell Contact

Notch ligands are transmembrane proteins, therefore the signaling is activated when the cell expressing the ligand is adjacent to the cell expressing the notch receptor. Ligand binding leads to cleavage and release of the Notch intracellular domain (NICD), which then travels to the nucleus to regulate transcriptional complexes. The Notch signaling plays an essential role for trabeculation of the ventricular myocardium during mammalian cardiac development, as well as in heart health (157) [reviewed by MacGrogan and colleagues (158)]. The inhibition of Notch signaling has been shown to suppress the proliferation and to induce apoptosis of mammalian immature neonatal cardiomyocytes, highly expressing the notch receptor **Notch1**. However, Notch 1 expression levels decline during cardiac maturation (159). Enforced activation of the Notch signaling by constitutive expression of the active intracellular domain of Notch1 (N1 ICD), or stimulation with the ligand **Jagged1**, boosts the proliferation of immature cardiomyocytes (159).

Systemic Hormones

Hormones are signaling molecules that act distant from their site of production. Interestingly, some hormones belonging to steroid, eicosanoid, amino acid-derived, and protein subclasses have been investigated for their ability to modulate cardiomyocyte proliferation and heart regeneration.

Steroid hormones can be grouped into types according to the receptors to which they bind, namely glucocorticoids, mineralocorticoids, androgens, estrogens, progestogens and Vitamin D derivatives. Glucocorticoids and mineralocorticoids are typically synthesized in the adrenal cortex (hence they are also known as corticosteroids), whereas androgens, estrogens, and progestogens are sex steroids, typically synthesized in the gonads or placenta. All of them are released into the circulatory system.

Glucocorticoids (GCs) exert most of their actions through the **Glucocorticoid Receptor (GR)**, and in some tissues or conditions through Mineralocorticoid Receptor (MR). In zebrafish, stress-induced cortisol secretion blocks cardiomyocyte proliferation and cardiac regeneration after cryoinjury (160). In mammals, circulating active glucocorticoid levels physiologically

rise shortly before birth in preparation for postnatal life by promoting the maturation of the lungs and other organs. During late gestation, endogenous glucocorticoids were shown to induce the maturation of fetal cardiomyocytes via activation of GR receptor (161), whereas their impact on fetal cardiomyocyte proliferation is currently controversial (162, 163). A few studies reported the adverse side-effects of synthetic glucocorticoid therapy in preterm infants resulting from impaired cardiomyocyte proliferation and endowment (164–166). Importantly, a role for physiological glucocorticoids in postnatal cardiomyocyte growth and regenerative plasticity has been recently suggested in the mouse model. Indeed, a physiological increase in GR activation by endogenous glucocorticoids in the early postnatal development concurs to restrain the proliferative ability of neonatal cardiomyocytes [pre-publication by Pianca and colleagues (167)]. Cardiomyocyte-specific GR ablation (GR-cKO) appears sufficient to boost neonatal cardiomyocyte proliferation and to delay the early postnatal transition from hyperplastic to hypertrophic growth along with the maturation of myofibrils-mitochondria organization [pre-publication by Pianca and colleagues (167)]. Further analysis unveiled that GR ablation increases cardiomyocyte replication by regulating the energetic metabolism, favoring glucose catabolism over fatty acid oxidation [pre-publication by Pianca and colleagues (167)]. However, in later stages of postnatal life, no differences in cardiomyocyte proliferation rate were reported in GR ablated compared to control mice (168). Nevertheless, upon myocardial infarction, cardiomyocytes in GR ablated juvenile and adult mice are facilitated to re-enter into the cell cycle and divide, leading to regeneration of the lost cardiac tissue along with reduced scar formation [pre-publication by Pianca and colleagues (167)]. Altogether these results support a model where increased activation of GCs/GR axis restrains the regenerative plasticity of cardiomyocytes.

The lower incidence of cardiovascular disease and mortality rate in women compared to men of similar age, along with the increased occurrence in women after menopause, have suggested that gender-related differences in **sex steroid hormones** (in particular estradiol) play a key role in the development and evolution of cardiovascular disease [reviewed by Vitale and colleagues (169)]. Studies on lower vertebrates have demonstrated that sexual dimorphism reflects also a dimorphic cardiac regenerative response. Indeed, female zebrafish display higher rates of cycling cardiomyocytes in both cryoinjured and uninjured regenerating hearts compared to males (170). Furthermore, exposure to **estrogen** accelerates male zebrafish regeneration after damage, by enhancing cardiomyocyte dedifferentiation and proliferation. Instead, exposure to tamoxifen, an estrogen receptor antagonist, delays female heart regeneration (170). Nevertheless, the role of estrogens in cardiac regenerative plasticity in mammals remains so far unknown. Recently, **progesterone** has emerged as a mediator of sex-dependent transcriptional programs during cardiomyocyte maturation (171). Interestingly, progesterone supplementation has been suggested to increase cardiomyocyte proliferation and heart regeneration after myocardial infarction in a progesterone receptor-dependent manner, by increasing YAP expression and signaling (172).

Vitamin D has been reported to regulate cardiomyocyte proliferation both in zebrafish and mouse models. In zebrafish, Vitamin D promotes cardiomyocyte cycling and tissue regeneration, and this process requires intact Erbb2 signaling (173). In contrast, the administration of Vitamin D to cultured mouse cardiomyocytes has been reported to induce both anti-proliferative (168, 174–176) and pro-proliferative effects (173). Furthermore, the deletion of the Vitamin D receptor appears not sufficient to prolong the postnatal cardiomyocyte proliferative window in the mouse model (168). A potential explanation of these conflicting results could be that the effects of Vitamin D on cell proliferation may be context-dependent and/or concentration-dependent. Despite these discrepancies, Vitamin D supplementation was proved to reduce ventricular remodeling and improve cardiac function in heart failure patients [metanalysis of several clinical trials by Zhao and colleagues (177)].

Among eicosanoid hormones, **Prostaglandin E2 (PGE2)**, a principal mediator of inflammation, is upregulated in the injured zebrafish heart and the suppression of its production by administration of Cox2 inhibitors reduces cardiomyocyte proliferation in response to cardiac injuries (178).

Among amino acid-derived hormones, **thyroid hormones**, namely **triiodothyronine (T3)** and **thyroxine (T4)**, gained attention in the context of cardiomyocyte proliferative ability. The analysis of 41 different species unveiled an inverse correlation between cardiomyocyte diploid content (index of mitogenic potential) and plasma T4 levels. Interestingly, T4 levels raise soon after birth, coincident with cardiomyocyte withdrawal from the cell cycle and binucleation/polyploidization. Moreover, inactivation or cardiomyocyte-specific ablation of thyroid hormone receptor-α (TRα) counteracts mammalian cardiomyocyte polyploidization, increasing the number of diploid proliferating cells and therefore the regenerative potential (179). Furthermore, T3 administration to fetal cardiomyocytes promotes their maturation while suppressing their proliferation (180, 181) and reduces cardiomyocyte replication at the neonatal stage (122). In contrast, a surge in T3 levels has also been reported to initiate a brief but intense proliferative burst of predominantly binuclear cardiomyocytes during pre-adolescence (182), although the existence of this burst was disproved (183, 184).

Melatonin, an amino acid-derived hormone produced by the pineal gland, exerting a protective role against oxidative stress, apoptosis, and inflammation after cardiac injury, has also been documented to induce cardiomyocyte proliferation after myocardial infarction in the mouse model (185). The suggested mechanism involves the activation of the melatonin receptor and regulation of the miR-143-YAP axis (185) (further described in "*miRNAs*" section).

Among protein hormones, insulin-like growth factor signaling has been demonstrated to play a role in cardiomyocyte regenerative ability. During embryonic heart development, **Insulin-like growth factor 2 (IGF2)** appears to be the most prominent mitogen made by epicardial cells (186).

The expression of the zebrafish homolog Igf2b was found upregulated during zebrafish heart regeneration, and inhibition of its receptor IGF1R blocks cardiomyocyte proliferation during heart development and regeneration (187). Administration of IGF signaling agonist (NBI-31772) or antagonist (NVP) respectively boosts or reduces cardiomyocyte proliferation in zebrafish embryos (120). Furthermore, IGF signaling is required for cardiomyocyte replication during myocardial regeneration in zebrafish (120). As described in the "*Maternal factors*" section, IGF2 is among the factors secreted by maternal Treg cells during gestation, inducing cardiomyocyte proliferation and heart regeneration in adult mice (50).

The administration of low-dose IGF1 induces beneficial effects on remodeling in post-infarct patients, despite not improving heart function (188). Intramyocardial delivery of **Insulin-like growth factor 1 (IGF1)** together with **Hepatocyte growth factor (HGF)**, through hydrogel or saline injection, enables endogenous cardiac repair on infarcted swine hearts, leading to the generation of new immature cardiomyocytes (189). In this regard, intracoronary administration of adenovirus carrying the HGF gene modestly reduces heart dilation and improves heart function in heart failure patients (190).

Signaling Cascades

A large number of growth factors and cytokines transduce their effects via the RAS-mitogen activated protein (MAP) kinase signaling (also known as **Ras-Raf-MEK-ERK pathway**). The key role of ERK signaling in triggering cardiomyocyte dedifferentiation and proliferation has emerged in multiple studies, for example, downstream to NRG1/ERBB2 axis (102), Agrin (150), OSM (131), IL13 (136), and IGF signaling (186). Intriguingly, the suppression of **Dual specificity phosphatase 6 (DUSP6)**, which antagonizes the activation of the MAPK cascade, results in increased myocyte proliferation during embryonic and early postnatal development, as well as enhanced cardiac regeneration in zebrafish (191) and mice (192).

Proinflammatory cytokines (such as IL-1 and TNF-α), some mitogens, cellular stress (including UV irradiation, heat shock, and high osmotic stress), lipopolysaccharide, and protein synthesis inhibitors, may activate **P38 mitogen-activated protein (MAP) kinase signaling**, which has been enlightened as a negative regulator of cardiomyocyte division. P38 inversely correlates with cardiac growth during mammalian embryonic development (89). Its *in vivo* activation inhibits fetal cardiomyocyte DNA synthesis, whereas cardiac-specific ablation of p38α enables neonatal cardiomyocyte proliferation (89). Furthermore, pharmacological inhibition of p38 is sufficient to stimulate replication of adult ventricular cardiomyocytes (from 12-weeks-old rats), upregulating genes involved in cell cycle progression, mitosis and cytokinesis (including cyclin A2, cyclin B and aurora B) (89). P38 inhibition also boosts the mitogenic effect of growth factors, such as FGF1, NRG1 and IL1β (89, 90). After myocardial infarction in adult mice, combinatorial therapy with p38 inhibitor and FGF1 has been shown to induce cardiomyocyte proliferation and cardiac tissue regeneration, reduce scar formation and improve cardiac function (90). Interestingly, p38 MAP kinase inhibition alone is not able to boost heart function despite increased cardiomyocyte mitosis (90). A clinical trial to assess the safety and efficacy of losmapimod, a p38 inhibitor, has been initiated, however, it was stopped when non-encouraging trials of the Tumor Necrosis Factor-α (TNF-α)-targeting [whose cardio-depressant action is induced by activation of p38 (193)] in heart failure patients were reported (194) [reviewed by Javed and Murtaza (195)].

In the mouse model, ablation of **cardiac troponin I-interacting protein kinase (TNNI3K)**, a cardiomyocyte-specific MAPKKK, results in an increase of mononuclear diploid cardiomyocytes, facilitating heart regeneration after injury (196, 197). On the other hand, TNNI3K overexpression in zebrafish induces cardiomyocyte polyploidization and impairs heart regeneration (196).

Several cytokines activate the **Jak-STAT signaling**, which plays an important role in the maintenance of cardiac homeostasis and takes part in the acute inflammation occurring after heart injuries [reviewed by Barry and colleagues (198)]. In zebrafish, Jak1/STAT3 pathway is activated after cardiac injury (199). Furthermore, cardiomyocyte-specific deletion of **STAT3**, a downstream effector of inflammatory cytokines, such as IL6 and OSM, reduces cardiomyocyte proliferation during the injury-induced cardiac regenerative response in zebrafish (199) and neonatal mice (128). Thus, STAT3 is essential for heart regeneration. In addition, therapeutic activation of STAT3 by IL11 administration was shown to reduce fibrosis and attenuate cardiac dysfunction after myocardial infarction (200).

The administration of a **Glycogen synthase kinase 3 beta (GSK3β)** inhibitor, which leads to **β-catenin** nuclear accumulation, stimulates neonatal and adult cardiomyocyte dedifferentiation and proliferation (201). Moreover, germline deletion of GSK3β results in hyperproliferation of cardiomyocytes (202). However, in the latter model, no difference in β-catenin localization could be observed, suggesting that GSK3β may modulate cardiomyocyte replication in a β-catenin independent manner. Furthermore, inducible cardiomyocyte-specific deletion of GSK3-β stimulates cardiomyocyte mitogenesis and exhibits a protective role against cardiac remodeling after myocardial infarction (203). The administration of **N-cadherin** antibodies, which induce the release of sequestered β-catenin from adherent junctions, promotes cardiomyocyte cell cycle re-entry (204). Similarly, adenoviral induced overexpression of β-catenin in the cardiac tissue results in increased cardiomyocyte cell cycle activity and reduced myocardial infarct size, even if cardiomyocyte binucleation and hypertrophy, without an evident increase in cardiomyocyte number, have been documented (205). Intriguingly, the accumulation of β-catenin has been observed upon constitutive activation of ERBB2 signaling, specifically mediating cardiomyocyte dedifferentiation (102). Ablation of **lipoprotein-related receptor protein LRP6** (a coreceptor interacting with Frizzled receptor in Wnt/β-catenin signaling) in infarcted mouse hearts stimulates robust regenerative processes through the proliferation of pre-existing cardiomyocytes via a β-catenin independent mechanism, involving **ING5 (inhibitor of growth family member 5)**/p21 (206).

Finally, knockdown of the E3 ubiquitin ligases **Cbl** and **Itch** induces neonatal rat cardiomyocyte proliferation *in vitro* (207).

Transcription Factors

The decline of the proliferative and regenerative ability of cardiomyocytes in early postnatal development has been reported to be regulated by several transcription factors.

GATA4 (GATA binding protein 4) expression increases in cycling cardiomyocytes during heart regeneration in zebrafish (13). In neonatal mice ablation of GATA4 impairs cardiomyocyte proliferation and cardiac regeneration (92, 208). Furthermore, GATA abundance in the murine cardiac tissue decreases in the early postnatal period, and its overexpression by adenoviral gene transfer improves cardiac regeneration in 7-day-old mice (208). A suggested mechanism by which GATA4 exerts this regenerative effect is the increased expression of regenerative growth factors and cytokines, such as IL13 or FGF16, although the latter one is controversial (92, 208).

Meis1 (myeloid ecotropic viral integration site 1), whose abundance in the cardiac tissue modestly raises in the early postnatal period, is a crucial mediator of cardiomyocyte cell cycle arrest (209). Indeed, cardiomyocyte-specific deletion of Meis1 extends their proliferation, whereas its overexpression limits neonatal heart regeneration following myocardial infarction by upregulating cyclin-dependent kinase (CDK) inhibitors p15, p16 and p21 (209). Double knockout of Meis1 and **Hoxb13 (Homeobox B13)**, a cofactor of Meis1, reactivates cell cycle activity in adult cardiomyocytes, induces sarcomere disassembly and improves cardiac function following myocardial infarction (210).

Recently, combinatorial knockdown of **Meis2** (a member of the same family) and **Retinoblastoma (Rb1)**, through hydrogel-based delivery of small interfering RNAs in adult rats, was reported to significantly increase cardiomyocyte proliferation, to reduce infarct size and to improve cardiac function post-myocardial infarction (211).

Pitx2 (Paired-like homeodomain 2) has been reported to exert a key role in myocardial regeneration of neonatal and adult mice. Indeed, it is required for neonatal cardiac regeneration and sufficient to trigger adult myocardial regeneration in the mouse model (212). Mechanistically, it has been shown that Pitx2 promotes the expression of ROS scavengers, protecting cells from oxidative damage (212). Interestingly, Pitx2 is induced during heart regeneration triggered by Hippo deficiency (212) and its expression is stimulated by the transcription factor **Nrf2 (nuclear factor erythroid 2–related factor 2)**, whose ablation also impairs neonatal cardiac regeneration (212).

Multiple studies over time have pointed out the role in mammalian cardiac regeneration of **E2F family** members, transcription factors known to regulate cell cycle progression. Adenoviral delivery of **E2F1** triggers S-phase entry of adult rat cardiomyocytes *in vitro,* however, stimulating cell death (213, 214). Interestingly, **p53** ablation boosts E2F1-induced cardiomyocyte proliferation, despite not preventing apoptosis (214). Overexpression of **E2F2, E2F3**, and **E2F4** is sufficient to enhance the proliferation of neonatal cardiomyocytes *in vitro* (215) and, in the case of E2F2, also in terminally differentiated cardiomyocytes *in vivo* (216). Similar to E2F1, E2F3 overexpression was also associated with cell death (215). Instead, contrasting data have been obtained for the apoptotic response induced by **E2F2** and **E2F4**, with initial studies demonstrating a reduction in cell death upon E2F2 and E2F4 overexpression in cultured neonatal cardiomyocytes (215), and more recent studies describing an increase in apoptosis of cultured adult mammalian cardiomyocytes (217). Interestingly, co-expression of E2F2 and BEX1 [Brain Expressed X-Linked (Bex)] was demonstrated as a strategy to induce cardiomyocyte cell cycle activity, without cell death (217).

The transcriptional repressor **REST (transcriptional repressor element-1 silencing transcription factor)** can also trigger the proliferation of cultured cardiomyocytes (218). REST is required for normal embryonic cardiac development and neonatal regeneration upon injury, sustaining cardiomyocyte cell cycle activity by repressing the cell cycle inhibitor gene p21 (218).

Recently **Klf1 (Krüppel-like factor 1)** has been reported to be required for heart regeneration in zebrafish stimulating epigenetic and metabolic remodeling (219).

Other transcription factors that emerged as regulators of cardiomyocyte regenerative potential, such as **YAP**, **GR, HIF1α**, **SMADs**, **Tbx20**, **p53**, and **Jarid2** were described in other sections of this review.

Epigenetic Regulations

The remodeling of the epigenetic landscape has also been linked to cardiomyocyte regenerative ability. RNA sequencing analysis of cardiomyocytes unveiled a differential transcriptomic framework at various stages of postnatal life in healthy and infarcted mammals (220). The epigenetic modulation of specific genes, in particular those involved in chromatin compaction and cell cycle, has been suggested to contribute to the proliferative inability of terminally differentiated cardiomyocytes (220). Furthermore, chromatin-remodeling proteins contributing to mantain a fetal-like status are switched off in adult cardiomyocytes. It is the case of **Brg1 (Brahma-related gene-1)**, which promotes proliferation of embryonic mammalian cardiomyocytes by maintaining Bmp10 expression and repressing p57 (221). In addition, by interaction with HDAC (histone deacetylase) and PARP (poly ADP-ribose polymerase), Brg1 represses Myh6 (α-myosin heavy chain, mainly expressed in adult cardiomyocytes) and activates Myh7 (β-myosin heavy chain, mainly expressed in embryonic cardiomyocytes), thus controlling myosin heavy-chain switching during embryonic/neonatal development, as well in adulthood under cardiac stress-induced hypertrophy (221). Interestingly, transgenic inhibition of Brg1 in the zebrafish model impairs cardiomyocyte proliferation and myocardial regeneration by repressing CDK inhibitors, such as cdkn1a and cdkn1c (222).

Recent studies have unveiled a role in cardiomyocyte regeneration for **ALKBH5 (α-ketoglutarate-dependent dioxygenase alkB homolog 5)**, a N^6-methyladenosine eraser of messenger RNAs. Indeed, ALKBH5 expression levels decline in postnatal development and its overexpression

promotes cardiomyocyte replication and cardiac regeneration following myocardial infarction in juvenile and adult mice, by increasing YAP translation (223). In contrast, knock out of **methyltransferase-like 3 (METTL3)**, a N^6-methyladenosine writer, whose expression levels raise postnatally, induces cardiomyocyte cell cycle re-entry, reduces scar size and boosts cardiac function after myocardial infarction, by regulating the miR-143-YAP axis (224).

Finally, ablation of the DNA methylase **Dnmt3a**, which is significantly downregulated after injury-induced cardiac regeneration, triggers neonatal rat cardiomyocyte proliferation *in vitro* (207).

miRNAs

Several miRNAs were found to regulate cardiomyocyte proliferative and regenerative ability. High-throughput screening in neonatal rat cardiomyocytes led to the identification of 40 miRNAs able to stimulate karyokinesis and cytokinesis in neonatal cardiomyocytes (225). *In vitro* administration of two of them, **miR-590** and **miR-199a,** is sufficient to enhance the proliferation of adult rat cardiomyocytes (225). Importantly, cardiomyocyte proliferation was also observed after their delivery *in vivo* by intracardiac injection of lipid transfection complexes or by adenoviral vectors, leading to cardiac regeneration after myocardial infarction in the mouse model (225). The pro-regenerative efficacy of miR-199a has been also validated in larger animal models upon cardiac injury (infarcted swine), however, arrhythmic death after the persistent expression was reported (226).

miR-1825 has been reported to induce a pro-mitotic effect on adult cultured rat cardiomyocytes, along with alterations in the electron transport chain, and a decrease in mitochondrial numbers, oxygen species and DNA damage (227). Importantly, intra-cardiac delivery of miR-1825 enhances the proliferation of adult cardiomyocytes in the peri-infarcted region. Multiple pathways seem to mediate the pro-regenerative effects of miR-1825, including the upregulation of miR-199a, which in turn repress its targets p16, Rb1, and Meis2 (227).

A few miRNAs, including **miR-548c**, **miR-509**, and **miR-23b,** have been documented to stimulate the proliferation of adult cardiomyocytes through inhibition of Meis1 (228).

Overexpression of members of miR-17-92 cluster, such as **miR-17-92** (by employing α-MHC-Cre transgenic mice) (229) and **miR-19a/19b** (delivered through adeno-associated viruses) (230), is sufficient to enhance the proliferation of embryonic, postnatal, and adult cardiomyocytes (229). Intra-cardiac injection of miR-19a/19b promotes a robust regeneration process of the infarcted cardiac tissue and boosts heart function (230). Among the suggested mechanisms for the pro-proliferative/regenerative effects of miR-17-92 members, it has been suggested the targeting of the oncosuppressor **PTEN** and the repression of the immune and inflammatory injury-induced response (229).

miR302-367 has been shown to regulate the cell cycle of adult cardiomyocytes by repressing Mst and Lats kinases of Hippo signaling, as well as by altering differentiation and by down-regulating genes involved in fatty acid metabolism (231). Transient expression of miR302-367 promotes mouse cardiac regeneration, avoiding the adverse dedifferentiation and reduced function observed in long-term induction (231).

Interestingly, the pro-proliferative effects induced by several miRNAs, including miR-590, miR-302d, miR-302c, miR-373, miR-1825, miR-1248, miR-18a, miR-33b, miR-30e and miR-199a, were suggested to rely on YAP nuclear translocation, as well as actin polymerization by downregulation of **Cofilin2** (67).

Transgenic mice with cardiac overexpression of **miRNA-204** exhibit cardiomyocyte replication in the embryonic and adult stages. Mechanistically, miRNA-204 induces the degradation of **Jarid2** (jumonji), in turn promoting the expression of several cyclins. Overexpression of Jarid2 impairs cardiomyocyte pro-mitotic effect of miRNA-204 overexpression (232) and reduces embryonic cardiomyocyte proliferation by repressing cyclin D1 expression (233, 234).

Physical exercise has been demonstrated to induce cardiogenesis. Indeed, 2-months-old mice undergoing voluntary wheel running exhibited a greater ability to generate new cardiomyocytes at a projected annual rate of 7.5% compared to 1.63% in sedentary conditions (235). Interestingly, a higher frequency of diploid/mononucleated and proliferating cardiomyocytes was observed in the border zone of post-infarcted mice in exercise (235). The exercise-induced cardiogenesis has been suggested to be mediated by **miR-222**, which increases in response to exercise, and whose inhibition suppresses the mitogenic response (235).

Other miRNAs, including **miR-31a-5p** and **miR-708**, have been found crucial for neonatal cardiomyocyte replication *in vitro* (236, 237). Furthermore, *in vivo* delivery of **miR-708** in lipid nanoparticles was documented to confer cardiomyocyte protection against stress-induced apoptosis, although its role on cardiac regeneration remains unexplored (236).

Some miRNAs may negatively affect cardiomyocyte replication, thus their inhibition may be used to boost cardiac regeneration ability. For example, it has been demonstrated that **miR-99/100** and **let-7** are downregulated upon cardiac injury to facilitate cardiac regeneration in zebrafish (238). In contrast, their expression remains stable in mammals following cardiac damage. Anti-miR-99/100 and anti-let-7 *in vivo* delivery in infarcted mice results in cardiomyocyte dedifferentiation and proliferation, leading to reduced scar size and improved cardiac function (238).

miR-195, a member of the miR-15 family, has been identified as a positive mediator of cardiomyocyte cell cycle arrest by blocking the progress through the G2 checkpoint (239). Inhibition of miR-195, via administration of locked nucleic acid (LNA)-modified anti-miRs, from the early postnatal period until adulthood, extends the proliferative window of endogenous cardiomyocytes, regenerating the infarcted heart (239, 240).

Delivery of **miR-34a**, whose expression rises to adult levels within the first postnatal week, suppresses cardiomyocyte proliferation and regeneration in neonatal infarcted hearts (241). Conversely, anti-miR-34a treatment improves cardiomyocyte cell cycle activity and cardiac remodeling post-injury in adult mice (241).

Cardiac-specific overexpression of **miR-128** impairs neonatal and adult cardiomyocyte replication, whereas its ablation allows post-mitotic cardiomyocytes to re-enter the cell cycle driving epigenetic remodeling of pro-mitotic genes, including the chromatin modifier SUZ12, which ultimately triggers repression of CKI p27 (242).

Recently melatonin administration or METTL3 ablation have been shown to downregulate **miR-143**, in turn enhancing the expression of YAP, thus leading to neonatal cardiomyocyte proliferation (185) and heart regeneration (224).

Finally, anti-**miR-29a**, anti-**miR-30a**, and anti-**miR-141** increase neonatal cardiomyocyte cell cycle activity (243).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

In recent years, multiple approaches were suggested to reactivate the cell cycle machinery and regeneration of endogenous cardiomyocytes in the perspective of repairing the damaged myocardium and boosting cardiac function after severe cardiac injuries. In some cases, pre-clinical studies in larger animal models (swine) and clinical trials on post-infarct and/or heart failure patients were also performed, and promising results were documented. In this regard, combinatorial strategies deserve further investigation since they may be more effective. However, several of the suggested approaches still exhibit some limitations and require some precautions. To begin with, some treatments may have a differential response and/or side effects among mammals (for example miR-199a induced arrhythmia in large animals, but not in mice). Furthermore, the tissue specificity of the therapy is quite important to avoid potential side effects. More selective delivery systems are being developed, such as those based on biomaterials (cardiac patches, sponges, hydrogels, etc.), although further improvement in tolerance to immunogenic host responses is required [reviewed by Bar and Cohen (244) and Mei and colleagues (245)].

The delivery system may also have safety issues that should be carefully evaluated (for example, long-term effects induced by adenoviral vectors). Another important issue is the functionality and tissue integration of newly generated myocytes, especially in the strategies resulting in consistent dedifferentiation processes that may lead to altered cardiac function, if persisted. To this end, the duration of the stimulus that promotes cardiomyocyte proliferation is likely a key factor, which requires careful calibration. Additionally, strategies that facilitate re-differentiation of newly generated cardiomyocytes have also been suggested, for example by enhancing cell-cell coupling via overexpression of connexin 43 (246). Other safety issues include a careful evaluation of potential cancerogenic effects, especially for strategies employing genes that are known to play a major role in cancer development. Increased specificity to the cardiac tissue and the transient nature of the stimulus may likely reduce or avoid this problem.

Thus, induction of endogenous cardiomyocyte proliferation represents a promising and flourishing research approach to induce cardiac regeneration after major injuries, although further investigations are required to increase its efficacy and safety.

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CB and GD'U wrote the manuscript, with inputs and intellectual contribution from the other authors. All authors listed approved the manuscript for publication.

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Bioengineering Methods in MicroRNA-Mediated Direct Reprogramming of Fibroblasts Into Cardiomyocytes

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Ischemic heart disease is the major cause of mortality worldwide. Despite the most recent pharmacological progresses, cardiac regeneration is yet not possible, and heart transplantation is the only therapeutic option for end-stage heart failure. Traditional cardiac regenerative medicine approaches, such as cell therapies and tissue engineering, have failed in the obtainment of human functional cardiac tissue, mainly due to unavailability of high quantities of autologous functional cardiomyocytes (CMs), low grafting efficiency, and/or arrhythmic events. Direct reprogramming (DR) of fibroblasts into induced CMs (iCMs) has emerged as a new promising approach for myocardial regeneration by *in situ* transdifferentiation or providing additional CM source for cell therapy. Among available DR methods, non-viral transfection with microRNAs (miRcombo: miR-1, miR-133, miR-208, and miR-499) appears promising for future clinical translation. MiRcombo transfection of fibroblasts could be significantly improved by the development of safe nanocarriers, efficiently delivering their cargo to target cells at the required stoichiometric ratio and overall dose in due times. Newly designed *in vitro* 3D culture microenvironments, providing biomimetic biophysical and biochemical stimuli to miRcombo-transfected cells, significantly increase the yield of fibroblast transdifferentiation into iCMs, enhancing CM gene expression. Epigenetic regulation of gene expression programs, critical to cell lineage commitment, can also be promoted by the administration of specific anti-inflammatory and anti-fibrotic soluble factors, helping in suppressing fibroblast signature. The aim of this mini-review is to introduce the readers to a relatively unknown field of cardiac research integrating bioengineering tools as relevant for the progress of miRNA-mediated cardiac DR.

Keywords: myocardial infarction, microRNAs, fibroblasts, cardiomyocytes, cell reprogramming, tissue engineering, nanoparticles

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INTRODUCTION

Ischemic heart disease is a major cause of mortality with more than 23 million cases worldwide (1, 2). During myocardial infarction (MI), billions of cardiomyocytes (CMs) are irreversibly lost and replaced by cardiac fibroblasts (CFs) forming a non-contractile scar tissue, which undergoes continuous remodeling, causing left ventricle dilation and progressive heart failure (3, 4). Given the poor endogenous regenerative potential of the adult heart, recovery of cardiac functionality could be accomplished by the replenishment of lost CMs. However, cell therapies and cardiac

tissue engineering strategies have achieved limited success due to poor engrafting, survival, and integration of implanted cells, alone or in combination with biomaterials, into the host tissue and the unmet need for a source of mature and functional CMs (1–4).

Direct reprogramming (DR) of fibroblasts into induced CMs (iCMs) has emerged as a new source for CMs (5–7). Previous literature has reported several different cardiac DR strategies, including the upregulation of cardiac transcription factors (TFs) (8), the administration of complex combinations of small molecules (9), and the modulation of microRNAs (miRNAs) (6). MiRNAs are short non-coding RNAs (of ∼21 nucleotides) that regulate gene expression post-transcriptionally (10).

Despite excitement on DR potentialities for cardiac regeneration, the approach is in need of optimization. Main limitations include the low yield of fibroblast DR into iCMs, the wide use of unsafe viral vectors, and the generation of predominantly immature, partially reprogrammed iCMs (11, 12).

The present mini-review focuses on miRNA-mediated DR of fibroblasts into iCMs as a promising approach for future translation of cardiac DR into clinical settings. Herein, we discuss the key role of bioengineering research in improving cardiac DR efficiency and iCM maturation, through the design of efficient and safe miRNA-releasing nanocarriers and biomimetic *in vitro* culture microenvironments (**Figure 1**).

NON-VIRAL MicroRNA DELIVERY SYSTEMS FOR DIRECT REPROGRAMMING

MicroRNA-Mediated Reprogramming of Fibroblasts Into Cardiomyocytes

The use of miRNAs for cardiac DR was first studied in 2012 by Jayawardena et al. (6). An accurate screening of CM-specific miRNAs allowed the selection of six miRNA candidates involved in CM differentiation and development. A minimal combination of four miRNAs called miRcombo (miR-1, miR-133, miR-208, and miR-499) was then identified as able to promote DR of mouse fibroblasts into iCMs (6, 7). The role of such miRNAs in cardiac development was subsequently reported by several studies (13). MiR-1 and miR-133 are co-transcribed in the cardiac and skeletal muscle tissues during embryonic development, and their expression increases until adulthood (13). MiR-1 is involved in regulating CM proliferation and ventricular organization. MiR-133 shares common functions with miR-1. Conversely, miR-208 and miR-499 mostly regulate the expression of α and β isoforms of myosin heavy chain (MHC), which are involved in CM contraction (13). Transient transfection with miRcombo, using a commercial transfection agent (DharmaFECT™), was sufficient to induce *in vitro* DR of mouse neonatal and adult fibroblasts into iCMs (6, 14). Transfected cells expressed CM genes and proteins, showing sarcomeric organization and spontaneous calcium oscillations. Moreover, miRcombo delivery using a lentivirus induced *in vivo* DR of fibroblasts into iCMs (14).

Initially, microRNA delivery and upregulation of cardiac TFs were combined to induce DR of human fibroblasts into iCMs in mixed viral/non-viral approaches (15, 16). A reprogramming cocktail consisting of four TFs (Gata4, Tbx5, Hand2, and Myocardin) and two miRNAs (miR-1 and miR-133) was the most efficient in inducing DR of human foreskin fibroblasts (HFFs), adult human dermal fibroblasts (AHDFs), and adult human CFs (AHCFs) into iCMs (15).

However, in 2020, Paoletti et al. demonstrated that non-viral transient transfection with miRcombo (using DharmaFECT) is enough to trigger the *in vitro* transdifferentiation of AHCFs into iCMs (7). MiRcombo-transfected AHCFs showed enhanced expression of cardiac TFs (Gata4, Mef2c, Tbx5, and Hand2) at 7 days post-transfection, while at 15 days, 11% of cells expressed cardiac troponin T (cTnT), and at 30 days, 38% of cells showed spontaneous calcium oscillations (7).

Recently, miRcombo-mediated cardiac DR efficiency was improved by transfecting mouse fibroblasts with a polycistronic vector, inducing equivalent expression levels of the four microRNAs of miRcombo (17).

Recently, the administration of a polycistronic vector inducing equivalent expression levels of miRcombo was found to improve efficiency of mouse fibroblasts DR into iCMs (17), suggesting the need for delivery vectors, ensuring miRcombo delivery at a stoichiometric ratio.

Alternative Strategies for MicroRNA Delivery in Direct Reprogramming

In the last years, alternative non-viral strategies to lipid nano-formulations have been proposed for miRNA delivery. Biomaterial-based nanoparticles (NPs) should efficiently encapsulate miRNAs, protecting them from rapid degradation and ensuring their efficient release to target cells, through specific ligands on NP surface for receptor-mediated endocytosis (18). Synthetic polymers [e.g., poly(lactic-*co*-glycolic acid) (PLGA); poly(ethylene glycol) (PEG); and poly(ethylenimine) (PEI)], natural polymers (e.g., chitosan), and inorganic materials (e.g., gold and calcium phosphate) have been investigated as biomaterials for miRNA-loaded nanocarriers (18). Muniyandi et al. have proposed PLGA/PEI NPs encapsulating miR-1 and miR-133 for DR of AHCFs into iCMs (19). PLGA/PEI NPs showed cytocompatibility and pH-dependent payload release and induced the expression of structural (α-sarcomeric actinin) and functional (cTnT) CM proteins at 7 days post-transfection (19). Recently, Yang et al. have reported that branched-PEI coated nitrogen-enriched carbon dots (BP-NCDs) can encapsulate miRcombo for DR of neonatal mouse CFs into iCMs *in vitro* and *in vivo* (20). BP-NCDs efficiently delivered miRNAs to target cells, inducing the expression of specific CM genes (Nppa, Nkx2.5, and Myh7) and proteins (Gata4, Mef2c, Hand2, and Tbx5) *in vitro*. Furthermore, *in situ* delivery of miRcombo by BP-NCDs in an MI mouse model significantly reduced the infarcted area after 4 weeks compared with control groups. On the other hand, limitations of this study derive from the testing of neonatal mouse CFs rather than adult human

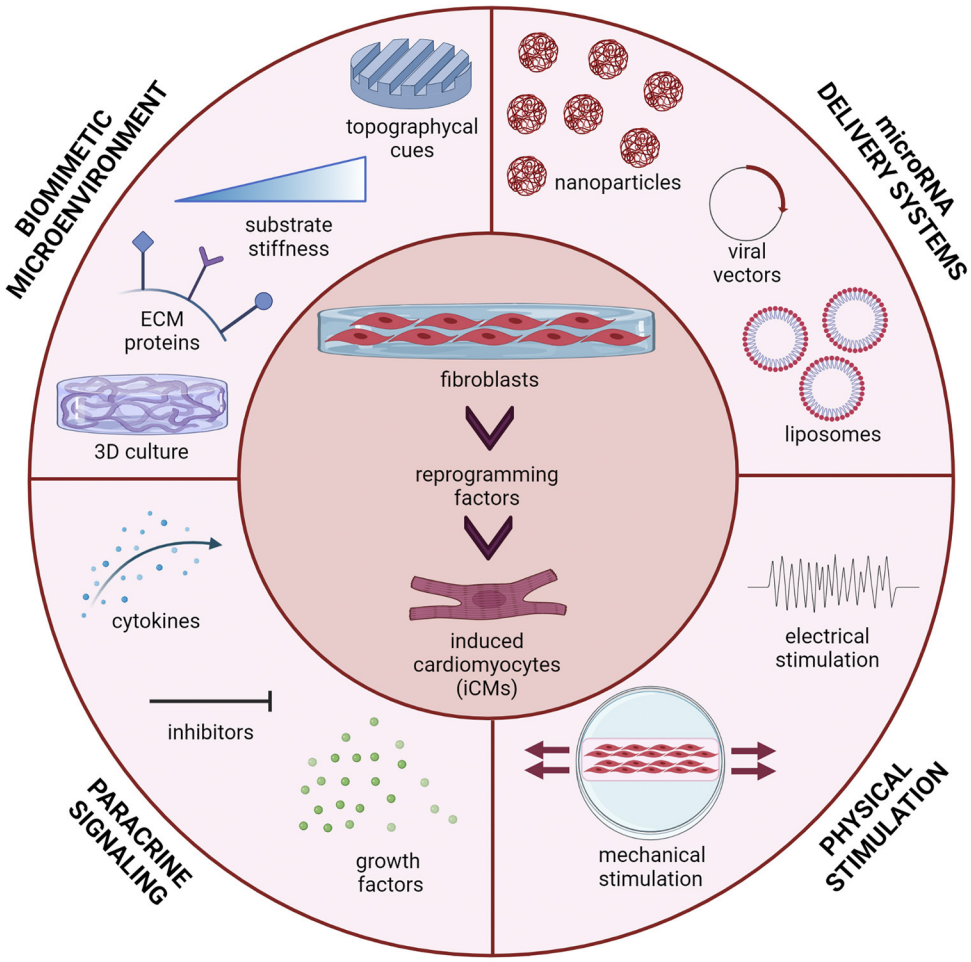

FIGURE 1 | Multiple stimuli can affect direct reprogramming (DR) of fibroblasts into induced cardiomyocytes (iCMs): microRNA delivery strategy (viral vectors, liposomes, polymeric nanoparticles); the microenvironment in which cells are cultured (three-dimensional culture, topographical cues, substrate stiffness and extracellular matrix proteins); paracrine signals (cytokines, inhibitors and growth factors) and physical stimuli (mechanical stretching, electrical stimulation). Figure was created using Biorender.com.

fibroblasts, the lack of extensive characterization of BP-NCDs in terms of encapsulation ability and release kinetics, and the non-degradability of the nanocarriers, which is associated with long-term safety concerns.

Hence, optimal nanocarriers for miRcombo delivery to human adult fibroblasts are currently missing. Furthermore, fibroblast specific targeting with functionalized NPs (e.g., using peptides, antibodies, or aptamers) could increase NP specificity of cargo release, thus maximizing DR effects and reducing off-target effects (21). However, specific ligands that recognize only fibroblasts populating the fibrotic scar are still under study.

BIOMIMETIC CULTURE MICROENVIRONMENT

Previous literature has reported higher DR yield achieved during *in vivo* experiments in mouse model compared with *in vitro* 2D cultures. This finding suggests that a three-dimensional (3D) culture microenvironment mimicking the biophysical and biochemical properties of cardiac tissue (**Table 1**) has the potential to significantly improve cardiac DR outcomes (13). *In vitro* culture of miRNA-transfected fibroblasts in cardiac tissue mimetic microenvironments may enhance DR efficiency and iCM maturation, as discussed in the next paragraphs (**Table 2**).

Cell-Substrate Interactions: Biochemical and Biophysical Properties of the Culture Substrate

In 2016, Sia et al. studied DR of neonatal tail tip fibroblasts (TTFs) transfected with retroviruses expressing Gata4, Mef2c, and Tbx5 (GMT). After transfection, the cells were seeded on Matrigel-coated polyacrylamide hydrogels with different stiffness (from 1 to 62 kPa). Reprogramming yield after 10 days of culture (∼17%) did not vary with substrate stiffness (27). On the contrary, microgroove culture substrates increased the yield (∼30%) of fibroblasts DR into iCMs. Cells showed sarcomere structures and

TABLE 1 | Reference cardiac tissue-like properties for biomimetic culture microenvironment.

Properties	Reference values in cardiac tissue	References
Human cardiac ECM composition	70% fibrillar collagen (collagen I and V), 20% basement membrane (collagen IV, laminin, agrin, perlecan, nidogen), 4% structural ECM (proteoglycans and fibrous glycoproteins), 3% matricellular components (collagen VI, fibronectin)	(22)
Stiffness	1–6 kPa (fetal); 10–15 kPa (adult); > 50 kPa (fibrotic)	(23)
Anisotropic ratio of stiffness	1.9–3.9	(24)
Cyclic mechanical deformation	1 Hz (in humans), 10% at early stage of diastole (stiffness: 10–20 kPa) up to 15–22 % at the end of diastole (stiffness: 50 kPa)	(24)
Electrical conductivity	0.57 S/m	(24)
Topographical cues	In native heart, myocardial fibers are arranged into distinct laminae (4–6 myocytes thick) separated by collagen-based ECM: helical-laminar assembly of hierarchically organized fibrillar structures.	(25)

spontaneous contractile activity, attributed to higher expression of Mkl1, a mechanosensitive TFs, and histone H3 acetylation for chromatin remodeling (27).

More recently, embryonic mouse fibroblasts, transfected with Gata4, Mef2c, Tbx5, and Hand2 (GMTH), were cultured on Matrigel-conjugated polyacrylamide hydrogels with different stiffnesses (1–126 kPa). Higher DR efficiency was obtained on substrates with similar stiffness (8 kPa) to healthy myocardium, compared with rigid polystyrene dishes (∼GPa). This result was attributed to the suppression of YAP/TAZ (Yes-associated protein/transcriptional coactivator with PDZ-binding domain) signaling and silencing of fibroblast gene programs, induced by a culture microenvironment with biomimetic stiffness (28). With respect the previous work by Sia et al. (27), the more efficient protocol for cardiac DR by GMTH transfection and the use of embryonic fibroblasts could account for the superior DR efficiency, despite the use of similar culture substrates. Although this result suggests variability of DR outcomes depending on fibroblasts types and reprogramming protocol, the role of mechanosensing was outlined. However, both studies were limited by the investigation of 2D cell cultures on the surface of hydrogels or microgroove substrates.

DR of fibroblasts embedded in 3D biomimetic matrices was only studied by Li et al. (26). In their work, miRcombo-transfected mouse fibroblasts cultured into 3D fibrin/Matrigel hydrogels showed higher DR efficiency compared with 2D cultures, as suggested by the higher expression of CM genes (α-MHC, cardiac troponin I, α-sarcomeric actinin, and Kcnj2) and proteins (cardiac troponin I and α-sarcomeric actinin). Such result was attributed to the upregulation of specific matrix metalloproteinases when cells were embedded in 3D hydrogels (26). Notably, 3D cell culture alone was sufficient to enhance the expression of CM TFs in non-transfected mouse fibroblasts compared with 2D cell cultures, suggesting that 3D culture microenvironment itself can promote the expression of CM genes (26).

Beyond biophysical characteristics of culture substrates, biochemical cues, such as proteins of the cardiac extracellular matrix (cECM), can help in recreating similar *in vitro* culture conditions to *in vivo* microenvironment (36). Indeed, in a different application, a 3D microenvironment containing brain ECM (bECM) was found to boost fibroblast DR into induced neuronal cells (iNs) (37). In this regard, gene set enrichment analysis (GSEA) of mouse embryonic fibroblasts (MEFs) transduced with MGT (Mef2c–Gata4–Tbx5) plasmids have shown that cECM proteins, such as collagens and laminins, are already expressed after 48 and 72 h post-transduction (38). Such findings suggest that in the early stages of fibroblast reprogramming, cells naturally create a suitable microenvironment, which enhances transdifferentiation. Indeed, Smith et al. have designed culture substrates based on PEG hydrogels functionalized with a high concentration of laminin and RGD peptide, achieving more efficient DR of mouse fibroblasts into iCMs, compared with hydrogels with low concentrations of RGD adhesion motifs or tissue culture polystyrene surfaces (30).

Paracrine Signaling and Small Molecules

Fibroblast DR *in vivo* is influenced by innumerable extrinsic factors of the cardiac microenvironment, encompassing not only mechanical forces or topographical cues but also the presence of cytokines, growth factors, and paracrine signals in the heart. After MI, pro-inflammatory cytokines are released in the wounded area, supporting cardiac remodeling through immune cells and fibroblast recruitment, inducing the deposition of stiffer ECM (39). Given the key roles that cytokines play during MI, it is worth studying how cytokines may influence cell reprogramming. Enrichment analysis of pathways that regulate cardiac reprogramming showed that anti-inflammatory cytokines (IFNA2, IFNA16, and IL10) are positively associated with DR and are called "activators," while pro-inflammatory molecules (IL1A, IL2, and IL26 cytokines and TF CEBPβ) were mostly identified as "inhibitors" (31). Indeed, TF ZNF281 was found to enhance cardiac DR via downregulation of genes involved in inflammatory response. Similarly, Testa et al. showed that treatment of mouse CFs with PTC-209, a Bmi1 inhibitor, before DR, negatively affected STAT3 and ERK1/2 phosphorylation, improving DR of fibroblasts into iCMs via inhibition of inflammatory pathways (32). Additionally, Jayawardena et al. found that JAK/STAT pathway suppression using Jak Inhibitor I, combined with miRcombo, enhanced DR of mouse fibroblasts into iCMs *in vitro* (6). Small molecule diclofenac, an inhibitor of cyclooxygenase-2 (COX-2) signaling, was also found to significantly enhance DR via PGE2/EP4 suppression, inducing sarcomere organization and increased number of beating cells as compared with GHMT alone in TTFs (33).

Moreover, a potential approach for improving DR relies also on inhibiting fibroblast endogenous signaling pathways

TABLE 2 | Selected bioengineering studies on DR of fibroblasts into iCMs.

Cells	DR agents	Transfection agent	Culture substrate signaling		Paracrine and/or small molecules	Physical stimulation	DR efficiency	References
			Biochemical	Biophysical				
Mouse neonatal and adult CFs	miRcombo	Dharmafect (*in vitro*) Lentivirus (*in vivo*)	–	TCP	Jak Inhibitor I	–	CM genes after 7 days, ∼28% reprogrammed cells, calcium transients, *in vivo* cardiac recovery after MI	(6)
Mouse neonatal CFs, TTFs	miRcombo	Dharmafect™	fibrin/Matrigel™	3D hydrogel	–	–	Increased cTnT and α-sarcomeric actinin, CM gene after 15 days compared to TCP	(26)
AHCFs	miRcombo	Dharmafect™	–	TCP	–	–	CM genes, ∼11% cTnT$^+$ cells at 15 days, calcium transients at day 30	(7)
AHCFs	miR-1 and miR-133	PLGA/PEI NPs	–	TCP	–	–	cTnT and α-sarcomeric actinin at 7 days	(19)
Mouse neonatal CFs	miRcombo	BP-NCDs	–	TCP	–	–	CM genes and proteins, *in vivo* cardiac recovery after MI	(20)
TTFs, mouse CFs	GMT	Retrovirus	–	Surface of Matrigel-conjugated polyacrylamide hydrogels; microgroove	–	Mechanical	CM genes, striated cTnT$^+$ cells at day 10, beating cells at 4 weeks (microgroove only)	(27)
MEFs	GHMT	Retrovirus	–	Surface of Matrigel-conjugated polyacrylamide hydrogels	–	–	CM genes, ∼13.8% cTnT$^+$ cells at 1 week, ∼33% αMHC-GFP$^+$ cells and beating cells at 4 weeks (8 kPa)	(28)
HNDFs	GMTHN	Plasmid transfection	Mouse CMs co-culture	Spin-coated nano-thin and nano-porous PLGA membrane	–	Electrical	CM genes, ∼6.4% cTnT$^+$ cells at 28 days	(29)
MEFs	OSKM (Oct4, Sox2, Klf4, and c-Myc)	Homozygous doxycycline-inducible OSKM mice	High laminin or RGD concentration	Functionalised poly(ethylene glycol) hydrogels	Jak Inhibitor I	–	∼6.21% α-sarcomeric actinin$^+$ cells, CM genes, beating cells at 18 days	(30)
Adult TTFs	AGHMT	Retrovirus	–	TCP	ZNF281	–	CM genes, ∼33% αMHC-GFP$^+$, ∼45% cTnT$^+$, and ∼28% αMHC$^+$/cTnT+ TTFs at 7 days, calcium transients, beating cells at 4 weeks	(31)
MEFs, adult CFs	Chemical cocktail (9)	–	–	TCP	PTC-209	–	∼40% of MEFs and ∼10% CFs αMHC$^+$, cTnT and Mlc-2v, CM genes, calcium transients	(32)
MEFs, post-natal and adult TTFs	GMT, GHMT	Retrovirus	–	TCP	Diclofenac	–	CM genes, αMHC- and α-sarcomeric actinin- positive cells, calcium transients, beating cells	(33)
MEFs, TFFs	GMT	Retrovirus	–	TCP	FGF2, FGF10, VEGF	–	CM genes, calcium transient, beating cells, αMHC- and α-sarcomeric actinin-positive cells	(34)
MEFs, adult CFs	HNGMT	Plasmid	–	TCP	SB431542	–	CM genes, ∼5 fold increase reprogrammed cells	(35)
MEFs, adult CFs	GHMT, miRs-1 and 133	Retrovirus	–	TCP	Y-27632, A83-01	–	CM genes, cTnT α-sarcomeric actinin- positive cells, reprogramming efficiency over 60%, beating iCMs	(16)

This table shows differential DR strategies for iCM generation combining cell source, reprogramming factors and delivery methods, biomimetic microenvironment, paracrine signaling and/or inhibitors and physical stimuli. DR efficiency reports the expression of cardiomyocyte genes and protein, electrophysiological characters and beating property. CFs cardiac fibroblasts, MEFs mouse embryonic fibroblasts, TTFs tail-tip fibroblasts, AHCFs adult human cardiac fibroblasts, HNDFs human neonatal dermal fibroblasts, α-MHC α-myosin heavy chain, cTnT cardiac troponin T, CM cardiomyocyte, TCP tissue culture polystyrene.

that maintain fibroblast identity. Silencing of transforming growth factor-beta (TGF-β) and rho-associated kinase (ROCK) signaling combined with different reprogramming cocktails was reported to improve DR. The use of TGF-β inhibitor SB431542 in combination with GHMT in mouse embryonic and adult fibroblasts was reported to induce ∼5-fold increase in cell reprogramming after 10 days of culture (35). Moreover, Zhao et al. have reported that DR is enhanced in GHMT-transfected mouse fibroblasts by overexpressing miR-1 and miR-133 combined with ROCK or TGF-β inhibitors, suggesting a synergistic effect in overcoming reprogramming barriers (16).

Yamakawa et al. have studied MEF reprogramming into iCMs in defined serum-free medium containing fibroblast growth factor (FGF) 2, FGF10, and vascular endothelial growth factor (VEGF) (34). The addition of these growth factors after cell transduction with Mef2c and Tbx5 successfully generated iCMs, exhibiting calcium oscillation and spontaneous contraction, by activating cardiac transcriptional regulators, including Gata4. Defined culture conditions influenced cardiac DR only in the later stage of transdifferentiation (34).

Further Physical Stimulations: Cyclic Mechanical Stretching and Electrical Stimulations

Mechanical stimulation of cultured induced pluripotent stem cell (iPSC)-derived CMs was found to enhance cellular alignment and sarcomere organization, calcium handling, and contractile properties, causing alterations in gene and protein expression toward a mature phenotype (40). As described above, Sia et al. have investigated different biophysical stimuli to induce fibroblast DR. Mechanical cyclic stimulation (10% strain, 1-Hz frequency) applied for 10 days reduced the percentage of reprogrammed cells compared with static cultures (27). Under mechanical stimulation, the expression of hallmarks of fibrotic scar tissue (collagen I and fibronectin) and reinforcement of fibroblast signature could account for the detected decrease in DR yield (41). On the other hand, mechanical cyclic stretching applied at a later stage of cardiac DR could potentially improve iCM maturation, as suggested by the wide literature on iPSC differentiation into CMs (42).

Electrical stimulation was also tested in cardiac DR, considering its beneficial effect on maturation of stem cell-derived CMs (43). Heart-like electric stimulation (1 V/cm, biphasic square pulse for 5 ms at 5 Hz) of GMTHN (Gata4, Mef2c, Tbx5, Hand2, and Nkx2.5) transfected human neonatal dermal fibroblasts (HNDFs), cocultured with murine CMs on nano-thin and nano-porous PLGA membranes, significantly increased DR yield, inducing the expression of CM genes and increasing the percentage of cTnT-positive cells (29). Additionally, cardiac cell sheets formed by reprogrammed cells were implanted in infarcted hearts, leading to cardiac function improvements and decreased adverse cardiac remodeling post MI (44). Although wider investigation is needed, such early studies suggested the positive role of electrical stimulation on DR efficiency.

DISCUSSION

Nowadays, MI still remains one of the leading causes of death worldwide. Hence, strategies for the replacement of CM loss are of primary interest in regenerative medicine.

DR of human fibroblasts into iCMs might represent a new therapeutic option for myocardial regeneration in addition to cell therapies with iPSC-CMs. Indeed, iPSC-CMs can now be obtained with high efficiency, although their maturation level is generally low, resembling fetal stage CMs (45). Finally, therapies using cells differentiated from pluripotent stem cells, such as iPSCs or embryonic stem cells (ESCs), suffer from the risk of teratoma formation (46).

DR could be exploited as a new source for autologous CMs derived from trans-differentiation of patients' fibroblasts with the advantage of low-to-null tumorigenicity risk if obtained by non-viral methods (9, 20). Current research efforts are addressed to increase DR yield and to approach a more adult iCM phenotype. However, one disadvantage of iCMs use in cell therapy is the need for high amounts of patients' fibroblasts (in which potential for *in vitro* expansion is reduced with respect to stem cells) to generate the required quantities of CMs (from tens to hundreds of millions).

With respect to iPSC-derived technologies, miRcombo-mediated DR also paves the way to new cell-free *in situ* strategies for cardiac regeneration, based on the local injection of reprogramming agents able to induce DR of CFs of fibrotic areas or their boundaries into iCMs. Non-viral approaches for *in situ* DR are safer than viral vectors, in which use is limited by possible off-target effects, mutagenesis risk for integrative virus (retrovirus and lentivirus), and strong immune response (47). Among non-viral strategies for cardiac DR, *in vivo* administration of small molecule combinations is complicated by the need to locally treat CFs with many drugs (up to nine small molecules) at specific relative concentrations (48, 49). On the contrary, the approach based on transient transfection of fibroblasts with miRcombo requires efficient simultaneous release of four miRNAs (miR-1, miR-133, miR-208, and miR-499) to CF cytoplasm. For efficient *in vivo* DR, nanocarrier surface could be functionalized with selected ligands for CF recognition, coupled to anti-fouling molecules (e.g., ethylene glycol oligomers) to ensure drug delivery specificity *in vivo*. Additionally, studies on optimal miRcombo dose and delivery kinetics into fibroblast cytoplasm are missing, while it would be fundamental to design efficient miRcombo delivery systems.

Although arrhythmic events have not been reported by reports on *in situ* cardiac DR in mouse models, this risk could potentially arise from the initial immaturity of early iCMs and be minimized by reducing DR time needed for effective CFs DR into mature iCMs (50). Additionally, based on very recent findings on iPSC-CMs therapies, administration of antiarrhythmic drugs could be considered and studied to assist early DR phases, enhancing patients' safety (51).

Another important feature of cardiac DR is its decreasing efficiency as a function of fibroblast aging (52). Scientific literature reported that embryonic vs. adult fibroblasts have

higher chances for conversion due to an open chromatin conformation (52). However, most DR studies employed embryonic, fetal, or post-natal mouse fibroblasts with superior transdifferentiation ability (52). Importantly, *in vitro* studies with mouse cells also provide specie-specific outcomes with limited relevance and predictivity for humans. As an exception, pig CFs might be employed considering their close features to human CFs (53). However, investigation on AHCFs is preferred in the perspective of future clinical translation of the approach. In this regard, DR is affected by "patient specificity," as its efficiency may vary significantly, depending on patients' age, sex, and genetic background. Overall safe and efficient standardized protocols taking into account patients' specificity should be defined, allowing more efficient cardiac DR based on clinical cases (52).

Currently, DR research is still at its basic steps. Hence, thorough *in vitro* studies are demanded, elucidating the role of biochemical and biophysical factors on DR efficiency of AHCFs into iCMs. Based on early findings (26), understanding and controlling the biochemical and biophysical cues of 3D culture substrates are the key for the design of instructive microenvironments improving DR efficiency and fostering the generation of mature iCMs (**Table 2**). Optimal 3D substrates should mimic cardiac tissue-like stiffness, composition, and architecture (**Table 1**). However, cell remodeling progressively alters the composition, permeability, and stiffness of 3D culture matrices, providing dynamic spatiotemporal cues affecting cell fate (54). New advanced techniques able to monitor 3D cell cultures could unravel the effects of dynamic microenvironmental changes on DR outcomes (55, 56). Such interdisciplinary research could be beneficial for efficient DR of human adult fibroblasts into iCMs, given their high epigenetic resistance to phenotype switch. Properly selected types and doses of anti-inflammatory and anti-fibrotic soluble factors could also help in suppressing fibroblast signature, to address the intrinsic epigenetic resistance of adult fibroblasts. Furthermore, more in-depth investigations of the effects of mechanical and electrical stimulations on DR yield and iCM maturation are still rather limited and deserve future attention.

CONCLUSIONS

The discovery of key biochemical and biophysical factors enhancing cardiac DR and the design of effective and safe nanocarriers for targeted miRcombo delivery will result in significant progresses of both *in vitro* and *in situ* cardiac DR approaches, fostering technological advances toward the future clinical application of cardiac DR strategies. However, full exploitation of DR potentialities requires an intense interdisciplinary research, in which bioengineering studies play a key role for the full exploitation of the potentialities of this new emerging approach.

AUTHOR CONTRIBUTIONS

The manuscript was conceived and written by CP and VC. VC supervised the project and acquired the funding. Both authors have given approval to the final version of the manuscript.

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REVIEW
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Cardiomyocytes Cellular Phenotypes After Myocardial Infarction

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Despite the increasing success of interventional coronary reperfusion strategies, mortality related to acute myocardial infarction (MI) is still substantial. MI is defined as sudden death of myocardial tissue caused by an ischemic episode. Ischaemia leads to adverse remodelling in the affected myocardium, inducing metabolic and ionic perturbations at a single cell level, ultimately leading to cell death. The adult mammalian heart has limited regenerative capacity to replace lost cells. Identifying and enhancing physiological cardioprotective processes may be a promising therapy for patients with MI. Studies report an increasing amount of evidence stating the intricacy of the pathophysiology of the infarcted heart. Besides apoptosis, other cellular phenotypes have emerged as key players in the ischemic myocardium, in particular senescence, inflammation, and dedifferentiation. Furthermore, some cardiomyocytes in the infarct border zone uncouple from the surviving myocardium and dedifferentiate, while other cells become senescent in response to injury and start to produce a pro-inflammatory secretome. Enhancing electric coupling between cardiomyocytes in the border zone, eliminating senescent cells with senolytic compounds, and upregulating cardioprotective cellular processes like autophagy, may increase the number of functional cardiomyocytes and therefore enhance cardiac contractility. This review describes the different cellular phenotypes and pathways implicated in injury, remodelling, and regeneration of the myocardium after MI. Moreover, we discuss implications of the complex pathophysiological attributes of the infarcted heart in designing new therapeutic strategies.

Keywords: myocardial infarction, apoptosis, autophagy, inflammation, senescence, dedifferentiation, cardioprotection

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INTRODUCTION

Myocardial infarction (MI) is defined as the myocardial injury that involves cell loss due to prolonged ischaemia (1). Onset of myocardial ischaemia results from an imbalance between oxygen supply and demand of the myocardial wall, mostly due to insufficient blood flow to the tissue. The causes for the reduced blood flow to the myocardium can be many e.g., atherothrombotic coronary artery disease (CAD), coronary embolism, but also coronary spasms, sustained tachyarrhythmia, blood loss, severe anaemia or respiratory failure (2).

As early as 10 min after the onset of ischemia, ultrastructural changes like glycogen depletion, relaxed myofibrils, disruption of the sarcolemma, and mitochondrial abnormalities can be seen in the human ischemic heart and in the next few hours necrosis and apoptosis will follow (1). A strategy to reduce damage and limit infarct size is ischemic post-conditioning, brief episodes of coronary re-occlusion and reperfusion after sustained ischemia, to activate the heart's self-defence

molecular programme against ischemia-reperfusion injury. The signalling pathways triggered by ischaemic conditioning are intricate and include activation of sarcolemmal receptors and cytosolic kinases, reduced mitochondrial permeability transition pore opening, calcium overload and proteolysis (3). The self-defence mechanism involves nitric oxide (NO), interleukin (IL)-10, and microRNA-144 as local triggers, while some know mediators are PKC, the RISK system, endothelial nitric oxide synthase (eNOS), and p38 (4). Despite the increasingly improved logistics of ischaemic pre- or post-conditioning, reperfusion still exacerbates the adverse changes induced by myocardial ischaemia and leading to infarction, such as rupture of mitochondria, even higher ROS levels than during ischaemia, disruption of sarcolemmal organization, and increased inflammation (3). Therefore, new approaches to confer cardioprotection, aiming to both reduce the infarct size and repair the injured myocardium, are still needed.

In this review we revisit the pathophysiology of MI and highlight the latest discoveries regarding cardiomyocyte cellular phenotypes and pathways implicated in cardiac injury and remodelling (**Figure 1**). Moreover, we discuss the complex attributes of the infarcted heart and its endogenous regenerative capacity as starting point for the design of new therapeutic strategies.

CELL DEATH

MI is characterized by loss of cardiomyocytes (CMs) through different mechanisms of cell death. To develop successful treatments to protect CMs from dying, it is important to understand the different ways cells can lose viability. In general, injured cells are removed from tissue in either a programmed manner, involving a defined series of molecular events, or in an uncontrolled manner, which usually results in rupture of the cell membrane and spilling of the cellular content into the surrounding environment. The main form of controlled cell death is apoptosis, whilst the main uncontrolled form of cell death is called necrosis (5).

Apoptosis is an orderly process by which a cell dies and has its contents phagocytosed by macrophages without spilling it into the surrounding tissue. Apoptosis can be initiated *via* two pathways, the intrinsic or the extrinsic pathway. Using the intrinsic pathway, the damaged cell itself activates the apoptosis-related signaling cascades after detecting damage *via* a number of intracellular sensors, such as Puma, Noxa, and Bax. The extrinsic pathway is activated when cells of the immune system interact with specific receptors on the surface of the damaged cell known as "death receptors" (6, 7). Once one of this two pathways is initiated, the cellular autodestruction is dependent on the intracellular actions of caspases, cysteine-aspartic proteases which are key features of apoptotic cell death. Activation of the caspase-dependent signalling cascade ultimately results in DNA fragmentation by endonucleases, degradation of nuclear proteins and cytoskeleton, crosslinking of proteins, expression of ligands for phagocytic cells, and formation of apoptotic bodies. The apoptotic bodies are then phagocytosed by macrophages or the surrounding cells before they fragment (8). This results in a containment of the injury and reduces the risk of inflammation and collateral damage to the surrounding tissue.

Apoptosis is a well-described phenomenon after MI, and proposed to occur in response to oxidative stress and proinflammatory cytokines (9, 10). One week after experimentally induced MI in mice there is an increase in the number of TUNEL and cleaved caspase-3-positive nuclei (two specific markers of apoptosis). This late onset of apoptosis is then sustained during the chronic phase of ischemia and reaches maximal levels 2 weeks after left coronary artery (LCA) ligation. Of note, apoptosis after MI is mostly present in the infarct border zone and the infarct area itself, or after reperfusion in globally hypoxic zones (5, 10).

Necrosis is an uncontrolled form of cell death where the cell is damaged so severely by a sudden shock, such as an hypoxic event, that it is unable to function. The damaged cell responds by swelling, as it fails to maintain homoeostasis with its environment, and it's characterized by metabolic failure, coincident with rapid depletion of ATP, failure of ion pumps and calcium overload. Ultimately, the necrotic cell undergoes plasma membrane rupture with spillage of intracellular contents into the surrounding areas, resulting in activation of inflammation and increased tissue damage (8). Necrotic CMs and matrix fragments are highly immunogenic and cause activation of innate immune receptors and pathways, including membrane-bound toll-like receptors (TLRs), HMGB1, and RAGE. Signal transduction from these pathways converges on activation of NF-κB which promotes the production of proinflammatory cytokines and chemokines (11–13).

More recently a caspase-independent programmed form of cell death which drives myocardial remodelling has been discovered. This process is called necroptosis and it involves processes typical of both the apoptotic and necrotic pathways. For example, necroptosis is triggered by death receptors such as tumor necrosis factor receptor-1 (TNFR1) followed by the induction of the receptor-interacting protein (RIP) necroptotic complex, but it also involves production of mitochondrial ROS and depletion of cellular ATP. Activation of RIP1-RIP3 signaling results in disrupted calcium homeostasis, cell membrane rupture, and consequent cell death (14). CMs necroptosis has been described to be involved in myocardial ischemia-reperfusion injury, potentially leading to heart failure. RIP1-RIP3 expression is enhanced in mouse hearts after permanent ligation of the left anterior descending artery (LAD), while knock-out of RIP3 results in reduced inflammation and oxidative stress comparable to the levels in wild type mice (15, 16).

How and to what extent these different forms of cell death interact during ischemic injury remains unclear. Yet, targeting of either form of cell death can have an impact on the infarct size and improve cardiac function. For example cyclosporine, which inhibits apoptosis by blocking mitochondrial permeability-transition pores, can decrease the infarct size in patients with acute MI (17). Similarly, downregulation of necroptosis can protect cells against reperfusion injury. Indeed, inhibition of RIP kinase-1 *in vitro* with the compound 6E11 rescues human aortic endothelial cells from necroptosis and

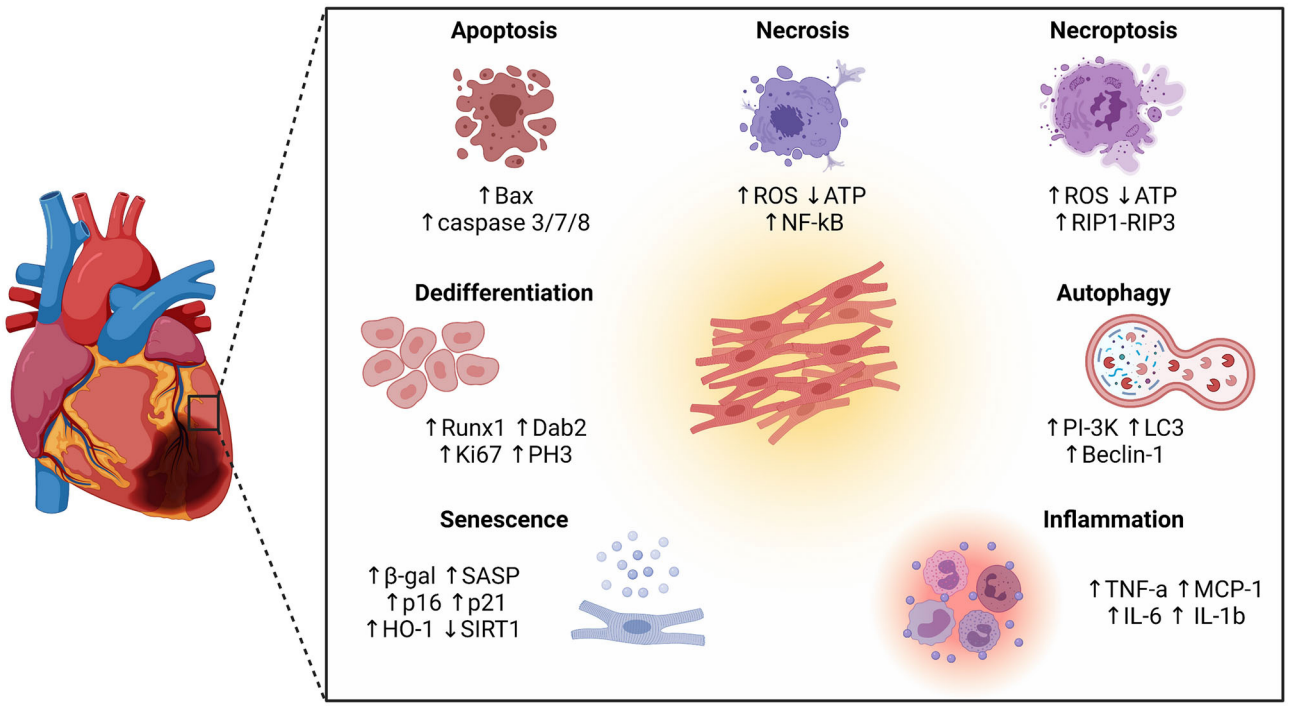

FIGURE 1 | Schematic representation of different cellular phenotypes and intracellular pathways involved in the pathophysiology of the infarcted heart. Besides different forms of cell death (apoptosis, necrosis, and necroptosis), other cellular phenotypes have emerged as key players in the ischemic myocardium, including autophagy, inflammation, senescence, and dedifferentiation.

protects them from cold hypoxia-reoxygenation (18). Also, inhibition of necroptosis through Necrostatin-1 administration after temporary ligation of the circum?ex artery in pigs reduces infarct size, oxidative stress, and inflammatory response (19). Interestingly, animal studies have demonstrated that combined inhibition of different forms of cell death reduces the infarct size more markedly than inhibition of either type of cell death alone. In particular, simultaneous inhibition of necroptosis with Necrostatin-1 and apoptosis with Z-VAD in ischemic guinea pig hearts enhances the cardioprotective effect, resulting in improved cardiac function (20).

AUTOPHAGY

Autophagy is a recycling and degradation mechanism essential for the quality control of intracellular proteins and organelles. In this process, cytoplasmic components are targeted and isolated from the rest of the cell within a double-membraned vesicle called autophagosome. The autophagosome then fuses with a lysosome, leading to the formation of the autolysosome. The contents of the autolysosome are eventually degraded (21). Autophagosome formation is initiated by the Class III phosphatidylinositol-3-kinase (PI-3K), Beclin-1 (also known as atg-6), and Microtubule-associated protein 1A/1B-light chain 3 (LC3) (22, 23). Control of the autophagic processes is exercised by the mammalian target of rapamycin (mToR), a serine/threonine protein kinase that can integrate information from multiple stimuli to inhibit autophagosome formation e.g., cellular nutrients, growth factors, and cellular redox state (5). Although this is a crucial process to maintain cellular homeostasis, excessive autophagy can result in overloading of cells with polyubiquitinated proteins, resulting in autophagic cell death. Indeed, autophagy is associated with neurodegenerative disorders, cancer, myopathies, and cardiomyopathies (24).

In cardiac cells, autophagy is upregulated when cells undergo stress. In experimental studies, the induction of autophagy has been observed to promote longevity, probably due to its clearance of damaged proteins and organelles (25). Conversely, the downregulation of autophagy has been observed to promote the development of hypertension, atherosclerosis, cardiac hypertrophy, ischemic heart disease and heart failure (26, 27). Autophagy has been demonstrated to have a protective role in the heart in response to ischemia by eliminating damaged mitochondria (28). Moreover, several investigators have demonstrated a dramatic increase in autophagy during the reperfusion phase of cardiac ischemia (29–33). Experiments using mouse models of MI report that autophagy occurs early after infarction and declines soon afterwards. In particular, autophagy signals increase immediately after LCA ligation in mice and are maximally expressed 1 week post ligation, in contrary to apoptosis which has a late onset. Autophagy is more prominent in the border zone of the infarct compared to remote areas and inhibition of the autophagic process by injection of

a TNF-α inhibitor (CAS1049741-03-8) leads to adverse cardiac remodelling after MI, suggesting an active role of autophagy in the determination of infarct size and cardiac function (10).

Data collectively point to a mainly protective role of autophagy in the heart early after MI. Enhancing this mechanism may be a cardioprotective strategy useful to limit remodeling after ischemia.

INFLAMMATION

The degree of systemic and local inflammation is considered a major determinant of the extent of myocardial damage post MI and subsequent cardiac remodelling and deterioration of function. The expression of pro-inflammatory markers is elevated in the post-ischemic heart and inflammatory cells are recruited in a time and site-specific manner (34–37). Inflammation, demonstrated by an accumulation of inflammatory cells and elevated levels of TNF-α, MCP-1, IL-6, and IL-1β, reaches a peak 1 week after LCA ligation in experimental animal models, and subsides thereafter. Interestingly, 4 weeks after MI in mice the levels of inflammatory cytokines are still substantially higher compared to control, suggesting an elevated state of inflammation in the chronically ischemic heart (10). These changes in inflammatory patterns can have both protective or detrimental effects on cardiac function during chronic ischemia.

Inflammation is a key regulator for autophagy. Yuan and colleagues reported an increase in autophagy in CMs *in vitro* and *in vivo* in response to LPS or TNF-α and enhanced autophagy by rapamycin protect against LPS-mediated myocyte apoptosis (38). A dramatic increase in autophagy signals (LC3 and beclin-1) was observed soon after exposure of CMs to hypoxia or Angiotensin II. The increase in autophagy and a decline afterwards coincided with the appearance of proinflammatory signals, suggesting a close link between the two phenomena (10, 39).

Inhibition of inflammation in patients with MI and heart failure (HF) through targeting of pro-inflammatory cytokines has yielded some promising data over the last few years. In the CANTOS (Canakinumab Anti-Inflammatory Thrombosis Outcome Study) trial, more than 10,000 patients with previous MI and evidence of systemic inflammation were randomized to receive variable doses of canakinumab, a human monoclonal antibody targeting IL-1β (40). In this trial, canakinumab was found to reduce the levels of IL-1β, IL-6 and high-sensitivity C-reactive protein, while reducing the risk of secondary cardiovascular events (41). Notably, canakinumab treatment also reduces HF hospitalization and HF mortality (42).

More recently, Li and colleagues developed microparticles containing anti-IL-1β antibodies. They reported that these particles prevented cardiac remodeling and promoted cardiac repair by neutralizing IL-1β when injected in a mouse LAD ligation model (43). Similarly, Xue and colleagues investigated a potential protective effect of nanoparticles containing miR199a-3p and constructed with the membrane of macrophages which overexpress receptors for TNF-α, IL-1β and IL-6. This nanoparticle had significant anti-inflammatory activity and promoted cardiac cell survival in mice after MI. It remains unclear if the anti-inflammatory effects in this study were due to the action of miR199a-3p, the absorption of pro-inflammatory cytokines by the particle, or both (44).

Even though inflammation is recognized as critically involved in the progression of MI, the therapeutic potential of modulating this process is still under investigation. The efficacy of different anti-inflammatory treatments may vary with the type and stage of the cardiovascular disease and other patient-specific variables, indicating that further research is needed.

SENESCENCE

Cellular senescence refers to a state of stable cell cycle arrest in which cells become resistant to growth-promoting stimuli. Physiologically, senescence prevents the expansion of damaged cells, serving an important anti-tumorigenic function. Senescence can occur in response to a wide range of damaging stimuli, including telomere shortening, oxidative stress, and DNA damage (45, 46).

Senescent cells are characterized by typical morphological and metabolic changes, although not all biomarkers of senescence (i.e., enlarged and flattened shape, elevated senescence-associated β-galactosidase (SA-β-gal) activity, chromatin remodeling and secretion of factors that promote inflammation and tissue deterioration) are present in all senescent cells (47). Senescence is characterized by a complex phenotype and its biomarkers are not unique to senescent cells, as some markers are also observed in apoptotic cells or quiescent cells, for example. Only cells with stable cell cycle arrest which don't respond to growth factors are considered senescent (48).

Cell cycle arrest is mediated by the p53/$p21^{CIP1}$ and $p16^{INK4A/pRb}$ tumour suppressor pathways (49, 50). Moreover, senescent cells typically have an enlarged size and flattened shape in comparison to dividing cells and they accumulate dysfunctional mitochondria and ROS. Senescent cells show an altered lysosomal content and activity, demonstrated by increased levels of β-gal activity, which is this characteristic widely excepted as a biomarker of cellular senescence (51). Senescence can also involve a persistent DNA damage response (DDR) and accumulation of DDR-related proteins (such as γ-H2AX) in nuclear foci called DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS) (52). Furthermore, many senescent cells acquire a senescence-associated secretory phenotype (SASP) that mediates non-cell autonomous effects of senescence, contributing to inflammation, and promoting tissue remodelling and repair or apoptosis (53).

Cellular senescence plays an important role in tissue remodelling both during development and in organ damage. After experimentally induced MI, ischemic injury initiates cell autophagy, apoptosis and immune-inflammatory reactions for clearance of damaged organelles and cells due to DNA damage, oxidative stress and mitochondrial dysfunction, ultimately leading to senescence in CMs. These CMs that become senescent after MI activate p16 and p53, upregulate enzyme activity of SA-β-gal and secretion of SASP, including the proinflammatory

factors IL-1, IL-6 and TNF-α (54), suggesting that accumulation of senescent cells is not only a reason for organ aging, but plays also a role in the progress of myocardial damage after ischemic injury and contributes to the decrease of heart function.

Eliminating senescent cells using senolytic drugs or downregulating senescence-associated processes using cardioprotective factors could facilitate or even stimulate myocardial regeneration in the infarcted heart. Senolytic drugs are drugs targeting pro-survival pathways and proteins that are upregulated during senescence, like the BCL-2 superfamily, p53 or PI3K/AKT (55). Navitoclax, a known inhibitor of BCL-2 and BCL-xL, was the first drug in a long series of compounds able to induce selective apoptosis in a variety of cells undergoing senescence both *in vitro* and *in vivo* (56). Other more selective BCL-2 family inhibitors are currently under development as a promising strategy against senescence and aging in the heart and other tissues.

Activation of Heme oxygenase (HO) was recently reported to inhibit the activation of β-gal and to prevent CMs from developing H_2O_2-induced senescence (54). HO is a rapidly inducible cytoprotective factor that catalyses the oxidative cleavage of Heme into equimolar amounts of carbon monoxide (CO), iron, and biliverdin, which is then converted to bilirubin by biliverdin reductase. In the past it was already reported that the inducible form of HO (HO-1) is upregulated after ischemia/reperfusion and that HO-1 mitigates cellular injury by exerting antioxidative, anti-apoptotic and anti-inflammatory effects (57–59). Additionally, knockout of HO-1 exacerbates ischemia/reperfusion-induced myocardial injury (60, 61). These studies suggest that HO-1 is involved in cardioprotection post-MI through inhibition of CMs senescence and that enhancing HO-1 expression could improve heart function after injury.

Another factor modulated by senescence is Sirtuin-1 (SIRT1), a NAD^+-dependent deacetylase which acts as a stress-response and survival protein (62). In mammals, SIRT1 is well-characterized to regulate several cardioprotective processes, including enhancing cell proliferation and cell survival by suppression of p53 (63, 64). Recently SIRT1 was found to be an active substrate of autophagy, which contributes to SIRT1 degradation during cellular senescence and aging (65). In ischemic conditions, SIRT1 can upregulate angiogenesis through hypoxia induced factor-1 (HIF-1), downregulate TGF-β and fibrosis, and inhibit apoptosis (64, 66). Additionally, it has been reported that Sirt1-induced p21 deacetylation can promote CMs cell cycle progression and proliferation in neonatal and adult mice, while depletion of Sirt1 reduces CMs proliferation both *in vitro* and *in vivo* (64). Of note, caloric restriction, a dietary restriction which has been described to attenuate tissue aging, promotes the activity of Sirtuins (67, 68). Furthermore, resveratrol, a polyphenol with anti-aging properties found in grapes, mimics caloric restriction by promoting FoxO and SIRT1 expression and activity (69, 70). These findings suggest that SIRT1 may be another suitable target to positively regulate cardiac protection and regeneration post-MI.

DEDIFFERENTIATION

After MI, injured CMs within the (Nppb–positive) infarct border zone can undergo ischaemia-induced gap junctional uncoupling from their neighbours. Uncoupling can be a mechanism enacted to protect the surviving regions of the heart by reducing the spread of proarrhythmic membrane depolarization and Ca^{2+} signals from dying myocytes into the surviving myocardium. Uncoupled CMs usually undergo apoptosis over the next few days or weeks, thereby expanding the infarct zone. Recently, it has been proposed that the border zone of the infarcted myocardium is involved in postinjury cardiac regeneration. Indeed, Nppb knockout mice are unable to recover from an ischemic injury, illustrating the importance of processes that occur in the border zone for myocardial repair after MI (71).

After uncoupling from the parent myocardium, myocytes in the border zone dedifferentiate and have the ability to proliferate and redifferentiate into functional CMs, thereby becoming a potential source of newly formed contractile cells in the post-MI heart. Nppb-positive border zone CMs express dedifferentiation (e.g., Runx1 and Dab2) and proliferation (e.g., Ki67 and PH3) markers and downregulate genes implicated in cardiac muscle contraction, oxidative phosphorylation, mitochondrial activity, and fatty acid β-oxidation, all of which are highly expressed in mature CMs. Additionally, these border zone CMs switch from a MEF2 to an AP-1 (activator protein 1)–responsive gene program (71, 72). MEF2a plays a role in maintaining CM differentiation and mitochondrial activity (73), therefore the reduced accessibility of MEF2-enriched elements in border zone CMs is in line with the reduction of the mature state and mitochondrial activity of these CMs.

It has been hypothesized that enhancing the dedifferentiation process itself by activating the mitotic signalling pathways involved in embryonic heart growth could be a complementary approach for cardiac regeneration. An inhibitor of glycogen synthase kinase-3 (GSK3β), 6-Bromoindirubin-3-oxime (BIO) isolated from mollusc Tyrian purple indirubins, has been shown able to induce dedifferentiation and induce cell cycle re-entry of CMs and endothelial cells by modulating Wnt signalling (74–77). The Wnt/β-catenin pathway is involved in cardiac specification during development and there is evidence supporting a role of Wnt in response to cardiac injury (78). However, although proliferative, dedifferentiated CMs fail to efficiently induce neonatal programs for proliferation and metabolic switching to glycolysis. Additionally, in transgenic mice in which the CM cell cycle is stimulated through cardiac overexpression or inhibition of factors like cyclin D2, Tbx20, PI3K-Akt, Wnt/β-catenin, Notch, Hippo or YAP to facilitate the transition at cell-cycle checkpoints, CMs proliferate preferentially in the border zone (79–85), suggesting that the dedifferentiated state of the border zone CMs acquire after MI is necessary but not sufficient for CM renewal after injury.

Efficient redifferentiation of the dedifferentiated CMs into contractile units is necessary for the new CMs to contribute to the pump function of the heart. For successful initiation of the redifferentiation process, uncoupled CMs need to regain contact with the surviving myocardium, allowing intercellular

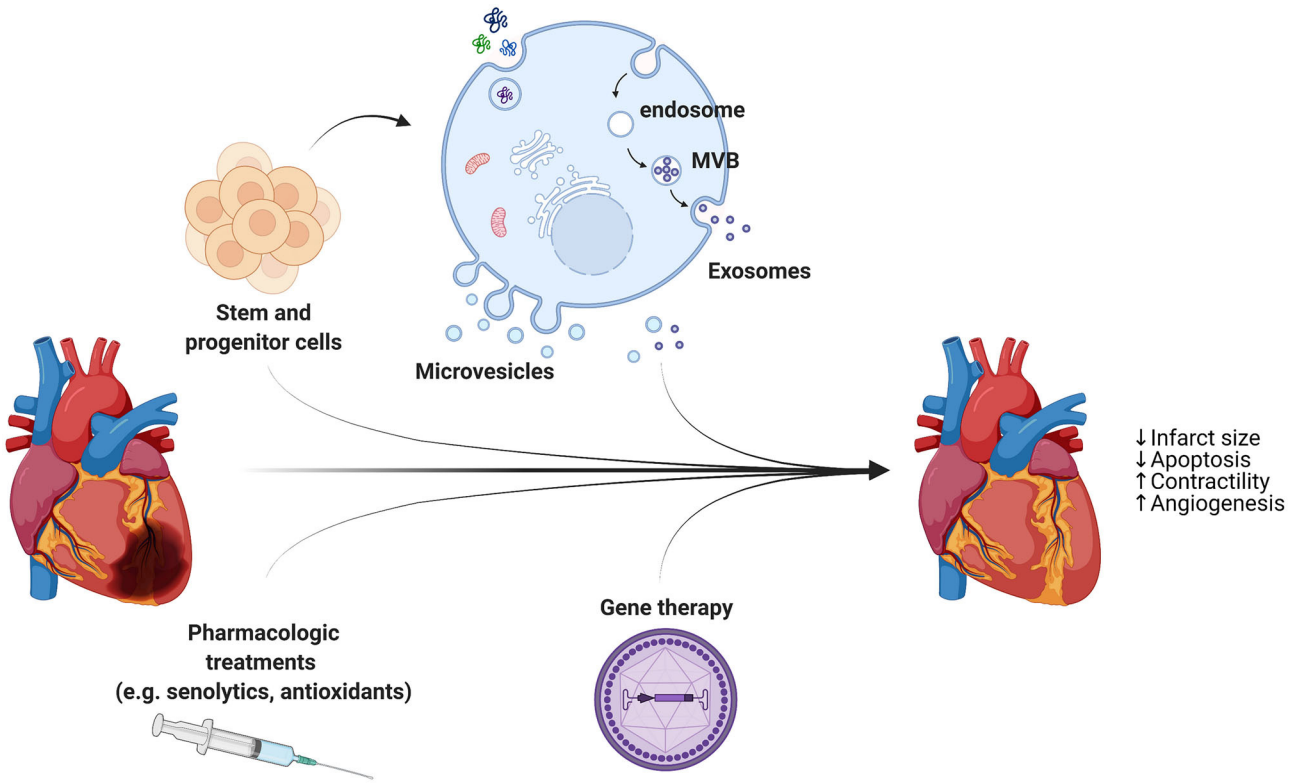

FIGURE 2 | In addition to reperfusion therapy, cardioprotective strategies are necessary to reduce the infarct size and repair the injured myocardium. A combination of different approaches, including stem cell- and progenitor cell-derived extracellular vesicles, pharmacologic treatments, and AAVs for gene therapy, holds great promise for heart regeneration through limiting cell death and stimulating cardiomyocytes proliferation and blood vessel growth.

transfer of cytoplasmic contents through gap junctions. The major isoform of gap junction proteins expressed in mammalian ventricular myocytes is Cx43. Wang and colleagues reported that CMs redifferentiation was not observed unless dedifferentiated CMs made gap junction (Cx43)–mediated cell-cell connections with neonatal ventricular myocytes in a coculture system. Additionally, they demonstrated through the use of a Cx43–small interfering RNA (siRNA), designed to inhibit Cx43 expression in CMs, reduced redifferentiation of uncoupled CMs (72). Considering these studies, targeting the dedifferentiated CMs in the infarct border zone specifically and promoting the endogenous redifferentiation process by facilitating gap junction formation may be a promising strategy for heart regeneration after MI.

CARDIOPROTECTIVE STRATEGIES AGAINST MI

Patients suffering from cardiovascular diseases undergo treatments, surgeries and medications which alleviate symptoms and decelerate disease progression, but fail to repair damaged tissue. In animal studies, the ischemic conditioning protocols have consistently been shown to reduce infarct area and increase myocardial salvage after prolonged ischemia. However, both animal and human studies have demonstrated mixed effects of pre- and post-conditioning on ischemia or reperfusion-induced arrhythmias. Therefore, it has been proposed that cardioprotective and regenerative strategies are necessary in addition to reperfusion to reduce the infarct size and repair the injured myocardium (**Figure 2**).

Since the proliferating and self-healing capacity of CMs in adults is limited, the first approaches in the cardiac regenerative medicine field focused on exploiting the potential of autologous or allogeneic transplants of stem cells for heart repair. Stem cells are specified as undifferentiated cells possessing the ability to generate, sustain, and replace terminally differentiated cells *via* unlimited replication. However, the therapeutic use of pluripotent stem cells (ESCs and iPSCs), potentially able to differentiate into mesodermal-derived CMs, is still limited mainly due to the risk of immune rejection, genetic instability, teratoma risk, low induction efficiency, and ethical issues (86, 87). Additionally, several independent studies have demonstrated that, while providing significant improvement in heart function, injection of stem and progenitor cells into the damaged myocardium results only in limited differentiation, mainly into vascular lineages (88, 89).

Alternative mechanisms and explanations for the beneficial effects of stem cell transplants despite low levels of differentiation have been thoroughly investigated. Stem cell-derived paracrine

effects have emerged as a very promising strategy for the reactivation of endogenous mechanisms of repair and regeneration in several disease models (90–94). In these studies, cell transplantation has been demonstrated to indirectly contribute to tissue regeneration by modulating cellular processes rather than direct differentiation into new functional tissue. Interestingly, fibroblasts engineered to resemble cardiosphere-derived cells (CDCs) by overexpression of β-catenin and Gata4, are able to improve cardiac function and mouse survival when transplanted into a model of acute MI through activation of cardioprotective signals and reduction of fibrosis in the surrounding tissue (95). These discoveries have led to a significant paradigm shift, from exploring the stem cell genome to analyzing the stem cell "secretome" as the whole of growth factors and chemo-attractant molecules produced by paracrine secretion. In the analysis of the stem cell secretome, there is growing interest on the characterization of extracellular vesicles (EVs). These EVs are membrane-bound cellular components enriched with soluble bioactive factors (e.g., proteins, lipids) and RNA (regulatory miRNAs and mRNA) eliciting wide-ranging effects while mediating horizontal intercellular transfer of genetic information to recipient cells and modulating their function (96). EVs are secreted as micro-sized (microvesicles, diameter 0.2–1 μm) and nanosized (exosomes, diameter 40–150 nm) particles. Microvesicles are released as shedding vesicles by direct budding of the plasma membrane, while exosomes are produced in endosomal multivesicular bodies (MVB) and secreted as the MVB fuses with the plasma membrane (97). The isolation and characterization of exosomes (Exo) is still difficult, and distinct techniques such as chromatography, centrifugation, precipitation, and affinity-isolation are used, often in combination (98–101).

Some studies suggest that the beneficial effects observed in preclinical models of ischemic heart disease following stem cell transplantation are mediated by progenitor cell-derived exosomes. These beneficial effects include the activation of pro-survival, angiogenic, anti-inflammatory and anti-fibrotic pathways, and the stimulation of resident endogenous progenitors, overall enhancing organ function (102). EVs from adult mesenchymal stem cells (cardiac progenitor cells and bone marrow) have been demonstrated to provide cardioprotection during acute myocardial infarction (90, 103–108), enhance wound healing (109), counteract graft-vs.-host-disease (GVHD) (110), reduce renal injury (111), mediate liver regeneration (112), stimulate neural plasticity following stroke (113), and counteract Doxorubicin-induced cardiotoxicity (114). Moreover, evidence suggests that EVs produced by epicardial cells are able to enhance proliferation in primary neonatal murine CMs and H9C2 cells *in vitro* and promote cell cycle re-entry when injected into the injured area of infarcted neonatal hearts (115). Since cell-free delivery of bioactive cargos by EVs recapitulates similar beneficial responses to stem cell transplantation, EVs offer more benefits over conventional cell therapy, being an immunologically unresponsive agents (116). Therefore, exosomes from stem and progenitor cells are promising candidates to promote autophagy and CMs survival, while working against senescence and SASP, but they may be engineered to guarantee substantial therapeutic effects.

Other cell-free therapies that are being investigated for their potential in mammalian heart regeneration include adeno-associated viruses (AAVs). These are non-integrating viruses and have been shown to achieve high levels of transduction in quiescent cells and CMs, with AAV1, AAV6, AAV8 and AAV9 identified as the most cardiotropic ones (117, 118). Gene therapies employing AAVs could potentially be used to jumpstart the cell cycle and promote CMs proliferation. However, it must be taken into consideration that this strategy may lead to increased cancer risk in non-cardiomyocytes. Indeed, multiple candidate genes that have been considered for this approach, including Hippo and YAP, are involved in certain types of cancer (119, 120), therefore requiring precise targeting to CMs to minimize oncogenic risks while using this approach.

CONCLUSION

A combination of different approaches, including stem cell- and progenitor cell-derived Exo, AAVs for gene therapy, and specific medication (e.g., senolytic drugs), to target the border zone of the infarct holds great promise for heart regeneration through stimulating CM proliferation and redifferentiation and blood vessel growth in the damaged hearts. Yet multiple issues, like specific induction of CMs, potential cancer risk in non-cardiomyocytes, and incomplete electrical coupling between newly generated cells and host cardiac tissue still need to be fully addressed before clinically applying these strategies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Tailoring Cardiac Synthetic Transcriptional Modulation Towards Precision Medicine

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Molecular and genetic differences between individual cells within tissues underlie cellular heterogeneities defining organ physiology and function in homeostasis as well as in disease states. Transcriptional control of endogenous gene expression has been intensively studied for decades. Thanks to a fast-developing field of single cell genomics, we are facing an unprecedented leap in information available pertaining organ biology offering a comprehensive overview. The single-cell technologies that arose aided in resolving the precise cellular composition of many organ systems in the past years. Importantly, when applied to diseased tissues, the novel approaches have been immensely improving our understanding of the underlying pathophysiology of common human diseases. With this information, precise prediction of regulatory elements controlling gene expression upon perturbations in a given cell type or a specific context will be realistic. Simultaneously, the technological advances in CRISPR-mediated regulation of gene transcription as well as their application in the context of epigenome modulation, have opened up novel avenues for targeted therapy and personalized medicine. Here, we discuss the fast-paced advancements during the recent years and the applications thereof in the context of cardiac biology and common cardiac disease. The combination of single cell technologies and the deep knowledge of fundamental biology of the diseased heart together with the CRISPR-mediated modulation of gene regulatory networks will be instrumental in tailoring the right strategies for personalized and precision medicine in the near future. In this review, we provide a brief overview of how single cell transcriptomics has advanced our knowledge and paved the way for emerging CRISPR/Cas9-technologies in clinical applications in cardiac biomedicine.

Keywords: single cell sequencing, CRIPSR/Cas9 system, endogenous gene activation, cardiomyocytes, gene regulation

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INTRODUCTION

Cardiovascular disease, a leading global cause of death, is accounting for 17.3 million deaths per year; a number that is expected to grow to more than 23.6 million per year by 2030 (1). Common cardiovascular disease conditions leading to heart failure include myocardial ischemia or cardiomyopathies such as ventricular hypertrophy that can be inherited or stress-induced. These

conditions result among others in deficient oxygen supply, eventually affecting cardiomyocytes' transcriptional and differentiation states that precede substantial cardiomyocyte loss due to the lack of regenerative capacity. Simultaneously, non-cardiomyocytes also undergo significant phenotypic and transcriptional changes leading to imbalanced intercellular communication. Comorbidities, including hypertension, dyslipidemia, and glucose imbalance, produce a hostile environment that modifies disease progression increasing heart failure risk (2). Collectively, these factors determine the extent of myocardial damage, which has a direct impact on patient specific disease progression while also depending on time of injury (3). Hence, a heterogeneous tissue remodeling characterizes heart failure progression. Current therapeutic guidelines for cardiovascular diseases involve reperfusion-based treatments and drug therapies mostly alleviating the symptoms generated by cardiac functional deterioration (1, 4–6). Therapeutic concepts targeting favorable reparative myocardial remodeling remain challenging. A big problem lies in the inefficient regenerative capacity of the heart. Based on the studies of various animal models as well as human data on cardiac regeneration, the attention has recently shifted toward the possibility of repairing diseased hearts by reawakening the intrinsic regenerative potential (7). The analysis of the integration of 14C generated by nuclear bomb tests during the Cold War allowed to estimate that fewer than 50% of cardiomyocytes are physiologically exchanged during the course of life in the human heart (8), indicating the intrinsic potential of cardiomyocytes renewal in the human myocardium. Indeed, in response to heart injury, the rate of cardiomyocyte cell cycling increases in the peri-infarct region; however, this is far too limited to effectively replace the lost cardiomyocytes (9). Thus, efforts have been made toward stimulating cardiomyocytes proliferation based on factors responsible for the transient neonatal heart regeneration in animal models. To promote endogenous cardiomyocyte proliferation, initial approaches targeted universal cell cycle regulators such as cyclins, cyclin-dependent kinases (CDKs), tumor suppressor genes, and cell-intrinsic signaling pathways that regulate cardiomyocytes proliferation during development (7, 10). These include mainly developmental transcription factors comprising the Hippo, Hedgehog (HH), Wnt pathway, HIF1α, SMADs, TBX20, p53, Jarid2, GATA4, MEIS1/2, Retinoblastoma, PITX2, E2F family members, KLF1, REST (11–25) as well as chromatin remodeling proteins (26), and microRNAs (miR-590, miR-199a, miR-548c, miR-509, miR-23b, miR-17-92 cluster, miR302-367, miR-143) (27–30). Recently, induced expression of the pluripotency factors OCT4, SOX2, KLF4, AND C-MYC (OSKM) was shown to trigger cardiomyocyte dedifferentiation by reprogramming cardiomyocytes to a fetal-like regenerative state. Short-term OSKM expression ameliorated myocardial damage and improved cardiac function upon myocardial infarction (31). These findings serve as a proof-of-concept to unleash the intrinsic regenerative potential of cardiomyocytes.

Another challenge is the application of exogenous factors to the adult heart, while preventing aberrant cell proliferation (26). Secreted factors such as Neuregulin 1 (NRG1), an agonist for the ERBB2 and ERBB4 receptor tyrosine kinases and a key mitogen during heart development, were shown to promote the reactivation of cell cycle (32–34). NRG1 is reactivated in both, zebrafish and mouse heart regeneration, stimulating cardiomyocyte proliferation and metabolic reprogramming; a potentially less risky approach than direct overexpression of cell cycle modulators or kinases (33, 35–37). Although stimulating cardiomyocyte proliferation is a promising strategy to boost myocardial regeneration in adult hearts, several obstacles must be overcome before reaching clinical applications. These include, for instance, inefficient and uncontrolled cell proliferation with an increased risk of cancer (9). Furthermore, not only proliferation, but also cardiomyocytes maturation needs to be coordinately re-activated; which implies both structural remodeling and dramatic metabolic alterations driven by different mechanisms (38). In order to tackle this problem, the fundamentals of complex regulatory networks that govern cardiomyocyte regeneration and repair embedded in their pathophysiological environment, need to be understood in the disease context (39, 40). This will help to tailor therapeutic strategies correcting specific cellular defects.

A powerful tool capable of deciphering individual cellular responses within tissues is single cell sequencing (SCS). Using the SC transcriptomic data, the spatiotemporal interplay of different cell types within tissues enables to delineate dynamics during disease progression (41, 42). Further bioinformatic assessment of ligand–receptor interactions allows us not only to measure the expression of ligands and receptors in multiple cell types, but also to systematically decode intercellular communication networks that function in homeostasis and are altered in disease states (43). Having a better understanding of how exactly transcriptomic changes in both, healthy and pathological conditions mediate phenotypic effects at the SC resolution, will allow the use of synthetic transcription for correcting disease conditions. This can be achieved by using programmable nucleases such as DNA targeting class II clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 systems (44). These rapidly advancing technologies have expanded the applications of genetic research across the world by mediating transcriptional control of endogenous genes to model and, moreover, to treat common and multifactorial diseases in one-in-a-lifetime approaches in the near future. This review aims to summarize the current status toward deciphering the cardiac disease transcriptome and to describe the novel approaches in which molecular tools including CRISPR/Cas9 can be used to modulate and revert disease conditions.

REAL-TIME SINGLE CELL TRANSCRIPTOME PROFILE OF THE HEART, THE BASIS OF PRECISE THERAPEUTIC TARGET IDENTIFICATION

The heart is composed of four morphologically as well as functionally distinct chambers, which requires an exact orchestration of all heterogeneous cell populations to guarantee its proper function (45). This is governed by a spatiotemporal pattern of gene regulation and cell-cell communication which, when altered upon disease condition, results in

significant phenotypic changes and imbalanced intercellular communication. This leads to organ tissue remodeling triggering a vicious circle driving disease progression. Knowing crucial factors, which perturbation results in alteration of gene regulatory networks, maladapted cellular behaviors, and ultimately to a disease condition, will help to develop more efficient and tailored therapies. Despite decades of research, the medical interventions for treating cardiac abnormalities have not dramatically changed from the classical symptomatic treatment, remaining the clinical need unmet worldwide.

One of the critical advancements to understand disease states in the past years was the adaptation of several SCS platforms for profiling all heart cells' transcriptome. Even though application of SCS for many tissues was rapidly and successfully adapted, the use of this method in the heart remained quite challenging for some time. While isolation and sequencing of several non-cardiomyocyte cell populations, including immune and endothelial cells, were relatively straightforward, singularization and dispensing of large, elongated cardiomyocytes for sequencing were challenging. To circumvent this issue, single nuclei sequencing (SNS) has been adapted for cardiomyocytes as well as recent advances have been made toward whole cell sequencing (46–48). These technological innovations have allowed accurate use of SCS for all heart cells embedded in a tissue including cardiomyocytes. The different platforms used for SCS of the mammalian heart including human are well-described elsewhere (46, 47), and are not the focus of this review; only few examples of their application for heart tissue and their potential therapeutical implications will be discussed. Several studies provided comprehensive transcriptomic data that can be used to extract valuable information of diseased vs. healthy heart. Litvinuková et al. (41) provided comprehensive transcriptomic data on six distinct cardiac regions of the healthy adult heart deploying SNS of cardiomyocytes and SCS of enriched fibroblast, stromal, vascular and immune cell populations. An important observation in this study was the identification of cardiomyocyte population heterogeneity among the atrial and ventricular compartments (41). This study highlighted not only chamber-specific and lineage-specific profiles, but also sex differences of the healthy heart.

Other heart-disease oriented studies have focused on the cellular composition upon ischemic injury in the murine heart (42, 49, 50). SCS of the interstitial cell population showed comprehensive dynamics of cardiac stromal, vascular and immune cells of healthy and ischemic hearts. A novel activated fibroblast population characterized by an anti-WNT signaling transcriptome signature was identified (49). This particular observation is advancing our understanding of the role of transcriptional activation of WNT effectors and inhibitors, previously observed in the whole heart tissue upon stress (51). WNT signaling plays a complex role in cardiac biology and disease, affecting different cell types including cardiomyocytes, fibroblasts, and endothelial cells (51). Many drugs inhibiting WNT signaling are currently under investigation for their potential impact in heart repair (49, 51, 52). It is therefore pivotal to identify the cell-specific transcriptional profiles in order to target the correct cell population. A transient phenotypic change has been identified upon ischemic injury in a murine model. In this model, endothelial cells undergo a transient mesenchymal activation within the first days after myocardial damage, but do not acquire a long-term mesenchymal fate (42). The authors concluded that the transient mesenchymal fate of endothelial cells may facilitate cell migration and clonal expansion to promote regeneration of vascular networks (42). These data indicate the intrinsic regenerative potential of the heart, which is rendered inefficient in long-term remodeling and can be used as regenerative therapeutic targets. Using a different protocol, SCS was also performed using infarct and border zone regions and was compared to control hearts (50). Similar to other studies, they could detect cell type-specific upregulation of various genes between healthy and diseased subpopulations of various cell types. In this study, *Ckap4* was reported as a novel marker specifically upregulated in activated fibroblasts. The authors further identified a subset of epicardially located cardiomyocytes expressing *Myoz2*, a protein that tethers α-actinin to the hypertrophy inducer calcineurin, thereby inhibiting hypertrophic response (53). This indicates that subpopulations of cardiomyocytes respond differently to known hypertrophic factors, and would limit therapies that assume homogenous hypertrophic response among cardiomyocytes populations.

Yekelchyk et al. specifically investigated the transcriptional profile of mono and multi-nucleated adult cardiomyocytes under baseline conditions and in pressure-induced cardiac hypertrophy in the murine heart (48). Using an image-based quality control system and strict exclusion criteria, they concentrated on rod-shaped adult cardiomyocytes. This differs from other studies using the same system (3, 41). A noteworthy observation of this study is the elucidation of cardiomyocytes clusters correlating with the expression of basic helix-loop-helix transcription factor HIF1α, a master regulator of hypoxic stress response which was the main driver of heterogeneity in this pathological condition. This is in line with CreERT2-based lineage-tracing studies revealing a population of hypoxic cycling cardiomyocytes resembling neonatal proliferative cardiomyocytes that contribute to the slow cardiomyocyte turnover occurring in the adult mammalian heart (54). Interestingly, overexpression of a downstream target of HIF1α, the Zinc finger E-box-binding homeobox 2 (ZEB2), improves cardiomyocyte survival and cardiac function as well as angiogenesis following cardiac damage (55). Activation of HIF1α expression is well described in pathological conditions (56) now the prospective analyses of its activation in distinct cell types will offer a new perspective to interfere with pathological phenotypes in a cell-dependent manner, which may be applicable to other transcription factors. Indeed, HIF1α regulates cardiac fibroblasts activation upon ischemic injury by limiting their proliferative capacity (57), highlighting even more the necessity for cell-targeted therapies.

Furthermore, Wang et al. (3) have analyzed heart cells and their interconnection on normal healthy and patients with heart failure as well as those with functional recovery after treatment with a left ventricular assist device (LVAD) at single-cell resolution. Applying bioinformatic tools, the authors studied transcription-factor-centered regulatory networks and evaluated

regulon activities in cardiomyocytes. They demonstrated regulation of transcription factor-depending regulons such as JUN, CEBPD and TCF7L2, which were previously described in disease conditions (58–60). However, the data exhibited distinct profiles of regulation among the different conditions, strongly indicating the presence of specific target disease-induced pathways and networks, warranting more disease-specific treatment of cardiovascular diseases. Wang et al. also provided evidence supporting a model, in which non-cardiomyocytes undergo substantial changes during the loss of normal heart function that may direct disease progression and prognosis. The comparison of cell types of diseased and healthy hearts led them to identify non-cardiomyocyte cell types necessary for maintaining myocardial homeostasis and for protecting the heart tissue from failing. As an example, based on the data they obtained, they performed transplantation of ACKR1+ endothelial cells into the ischemic heart, which significantly enhanced cardiac function (3). More importantly, they showed that transcriptome profiles of all cell populations from the patients with improved heart function shifted considerably toward normal physiological state. Thus, this observation implies the plasticity and substantial recovery potential of cardiac cells in the adult human heart, even in end-stage heart failure (3). This data holds promise for the development of strategies that can reverse disease cell-states by exploiting endogenous recovery programs of the heart. Using SCS analysis in cardiac biopsy samples from patients with heart failure before treatment, the presence of failing cardiomyocytes characterized by the activation of DNA damage response genes only in patients showing poor prognosis was validated (61, 62). Hence, these methods present a realistic approach to determine clinical prognosis and treatment response.

Another study analyzed human left ventricular samples including control non-failing, hypertrophic and end-stage cardiomyopathy as well as heart failure samples along with mouse hearts at different stages after experimentally-induced pressure overload to investigate the pathological progression of cardiac hypertrophy (63). Specifically, their findings suggested a pivotal role of macrophage subtype switching toward an inflammatory state upon reduction of cardiac function during pathological cardiac hypertrophy. Therefore, they tested the effectiveness of the anti-inflammatory treatment on the stage-specific macrophages between 2 and 5 weeks after induced pressure overload. This resulted in ameliorated cardiac hypertrophy. However, an earlier anti-inflammatory treatment before 2 weeks failed to avoid decline in cardiac function (63). This suggests that stage-specific targeting of macrophages may serve to suppress pathological cardiac hypertrophy and influence the course of disease progression. This study also revealed conserved cellular and molecular basis of cardiac hypertrophy between mouse and human, providing an excellent platform to investigate mechanisms that can be translated toward improved therapies. The extraction of (sub-)cell types and their respective transcriptome profiles allows for analyses of differential gene and gene cluster expression as well as gene regulatory networks in health and disease states. This will help to exactly specify their role for targeted functional phenotyping (64). For instance, ischemic and non-ischemic human heart samples subjected to SNS yielded a catalog of cardiomyocyte and non-cardiomyocyte (vascular endothelial cells, endocardial endothelial cells, fibroblasts, mesothelial cell, smooth muscle cells, adipocytes, immune cells, and neurons) and their individual, disease-specific gene expression profile (65). The authors identified gene regulatory networks and disease driver candidates by intersection of the SNS data sets with the disease risk GWAS data (65). This data extended descriptive cellular characterization of the human heart (3, 41) toward understanding the precise underlying disease progression mechanisms.

Altogether, these recent discoveries serve as a blueprint for how knowledge about gene expression dynamics, collected from single cell responses under physiological and pathological conditions, provides unprecedented information. Invaluable insight that supports context-specific therapeutic approaches based on transcriptional modulation is the observation that altered SC transcriptomic profiles in the human diseased hearts seem to reverse toward normal state with improved organ function (3). This indicates that transcriptional profile and function are coupled. Furthermore, as abovementioned, the information collected from current studies, such as HIF1α and ZEB2 expression promoting cardiomyocytes proliferation endogenously, can be used to transiently boost clusters of cardiomyocytes toward a more regenerative state in ischemic conditions. Based on the data that ACKR1+ endothelial cell transplantation preserves cardiac function upon ischemia, an approach using suitable adeno-associated virus (AAV) serotype or other non-viral vector delivery approach can be designed for enhancing *Ackr1* expression in this cell population. This is particularly motivating toward developing more specific strategies that can restore homeostatic cell states for a wide number of human cardiovascular pathologies ranging from adaptive cardiac remodeling to heart failure. The next logical step is to actively interfere with the identified aberrant gene expression and to rewire gene programs for the prevention of heart failure progression. In this context, synthetic control of transcription to restore endogenous homeostatic transcriptional programs of the heart specifically in the desired cell-types offers a suitable platform. In terms of budget, current advances of protocols as well as standardization of bioinformatics pipelines warrants the SCS approach as a realistic option for clinical applications in the near future. Importantly, conventional single-cell RNA-seq analysis may not be sufficient for obtaining the information necessary for a deeper understanding of molecular behavior and therefore combined bulk sequencing analysis will be mandatory for a more precise analysis (66).

ADAPTING SYNTHETIC CONTROL OF TRANSCRIPTION TO THE HEART

Regulation of gene expression relies on transcription factors availability and their activity, as well as on chromatin state and nucleosome positioning that determines RNA polymerases recruitment to a specific gene locus (67). This complex

process has turned transcriptional control "undruggable" for many years (67). Recent approaches tackling this issue deploy epigenetic modifiers and synthetic transcription factors driven by DNA binding element systems such as engineered zinc finger, transcriptional activator-like elements (TALEs) or aim at repurposing programmable CRISPR/Cas9 systems (68–70). Beyond genome editing activity of the CRISPR system, epigenomic modifications can now be achieved at a specific genomic locus by using mutated catalytically inactive dead (d) Cas9 protein fused to effector domains (68). This works by carefully choosing where guide (g) RNA molecules bind relative to transcriptional start sites (TSS) in the genome. This allows for recruitment of dCas9 with activator or inhibitor domains to a specific locus of interest. Consequently, chromatin landscape modification or further recruitment of factors that lead to tailored transcriptional modulation is possible. This is extensively reviewed elsewhere (67, 71, 72). Hence, the limitation of strict control over endogenous gene expression *in vivo* that has long been a tedious work for researchers is now alleviated by the use of the RNA-guided programmable endonuclease systems associated with transcriptional modifiers (67). This offers the ability to precisely modify endogenous gene expression to program cell and tissue behavior (67). Thus, all the efforts that have been conducted for decades in order to understand how exactly transcriptomic changes cause disease conditions can now be exploited to develop therapeutic concepts by applying rapidly evolving CRISPR/Cas9 technologies.

CRISPR/Cas9 technology is rapidly advancing in the medical world with the development of therapies for blood disorders, Duchenne muscular dystrophy, cystic fibrosis, and cancer (73–75). Yet, the CRISPR-mediated transient transcriptional activation or repression of genes, desirable when considering changing the course of complex diseases such as metabolic diseases or tissue regeneration (76), is still in its infancy. Endogenous regulatory mechanisms of genome function as well as issues concerning CRISPR-mediated transcriptional engineering need to be addressed before this technology reaches use in the clinic, and is discussed elsewhere (67). Initial generation of dCas9-based transcriptional modulation platforms consisted of transcriptional activators derived from herpes simplex virus, VP16. Second-generation systems resulted from combination of bi/or tripartite activators such as VP64, VPR, SAM, the peptide scaffold-based activator SunTag-VP64 and RNA containing aptamers with increased activation efficiency (67, 72). The same is true for synthetic repression, where the initial KRAB repressor domain has been improved by several bipartite repressors consisting of KRAB and a secondary repressor domain (ZIM3, KOX1, MeCP2, DNMT3A, DNMT3L) (72, 77, 78). All these systems allow to fine-tune the intensity of gene modulation according to the biological needs and represent promising tools to modulate the cellular epigenome. Importantly, titration of gene activity is possible with the development of advanced gene activator platforms by expanding homomeric (79) or by using heteromeric transactivation domains (80). Additionally, the selection of gRNA target sites upstream of the TSS was sufficient to modulate drug resistance phenotypes according to expression levels indicating fine-tuning of gene activity to biologically relevant levels (79). Furthermore, tiling of gRNAs in the TSS upstream region was consistently reported as an option to adjust gene activation strength (81–83). Altogether, Cas9-transcription factor characteristics and careful gRNA selection are therefore suitable for unprecedented control of endogenous gene activity, an advantage over classical cDNA delivery via AAV which will be further discussed below.

CRISPR-MEDIATED CONTROL OF TRANSCRIPTION IN PRECLINICAL MODELS

CRISPR-based synthetic transcriptional control may lay the basis for personalized and precision medicine. Efficient transcription regulation mediated by CRISPR-mediated gene activation (CRISPRa) systems was demonstrated *in vivo* in the brain, liver, kidney and skeletal muscle as well as mouse model of human diseases including muscle dystrophy, diabetes, kidney and brain diseases using different delivery methods (84–87). Preclinical models using dCas9-targeted transcription factor regulation have shown great promise for treating disorders such as Duchenne's muscular dystrophy, type 1 diabetes, acute kidney disease and retinitis pigmentosa (84, 85, 88). Liao at al. developed a mouse model, in which transcriptional activators were separated from constitutively expressed Cas9 (active or inactive, Cas9a and Cas9i, respectively) (85). This consisted of a combination including gRNAs engineered to contain two MS2 domains for recruiting the MS2:P65:HSF1 (MPH) transcriptional activation complex to the target locus, which was introduced with an AAV serotype 9. MS2 binds a specific stem-loop structure allowing assembly of RNA-protein complexes and it is used as tagging technique (89). Using this system, they showed amelioration of acute kidney injury by induced expression of the protective protein *Klotho* or the anti-inflammatory IL-10. Next, they triggered trans-differentiation of liver cells into insulin-producing cells by induction of pancreatic and duodenal homeobox gene 1 (*Pdx1*) in liver cells; which improved hyperglycemia in streptozotocin (STZ)-induced diabetes model via tail vein injection of AAV-gRNAs. Moreover, they showed that transcriptional induction of *utrophin*, a protein product which is very similar to dystrophin, improved muscle strength in a mouse model of Duchenne muscular dystrophy (DMD) by local application of the AAV-gRNAs (85). Additionally, a kidney-specific epigenetic modifier with dCas9TET3 fusion proteins to induce gene activity was shown to be efficient by Xu et al. (90). A double transgenic mouse was generated in a *Sim1* heterozygous background, which normally develops obesity. The second transgene consists of a dCas9 fused to a transcriptional activator VP64 as well as a sgRNA targeting the *Sim1* promoter or enhancer (88). By using this system to activate *Sim1* expression from the healthy intact allele, the obesity phenotype was rescued upon targeting of both, *Sim1* promoter or enhancer. The same rescue was observed upon direct delivery of three different AAV particles carrying the dCas9VP16, the gRNA-promoter *Sim1* and the gRNA-enhancer *Sim1* into the hypothalamus. These examples elegantly demonstrate that

CRISPR-based transcriptional modulation can also be applied for epigenetically-mediated correction of a genetic disease without the need of modifying the mutated gene-coding DNA sequence. Meng et al. engineered bone marrow derived mesenchymal stem cell (MSC) to overexpress *Il-10* using CRISPRa based on dCas9-VP64-MS2 system (91).

IL-10 improved myocardial infarction; which is hampered in patients with diabetes due to MSC dysfunction. Engineered MSCs overexpressing *Il-10* were transplanted in a diabetic mice model with myocardial infarction, which substantially suppressed inflammation, improved cardiac functional recovery, alleviated cardiac injury, decreased apoptosis of cardiac cells, and increased angiogenesis (91). Other studies showed that CRISPRa approaches can decrease seizures and rescue cognitive deficits in a rodent model of epilepsy as well as demonstrated the utility of CRISPRa system for *in vivo* screening, e.g., in liver or brain (86, 87). Common efforts from E. Olson's and L. Zelarayán's Labs resulted in the establishment of a mouse model for cardiomyocyte-specific, CRISPR-mediated transcriptional modulation. The system is based on the constitutive expression of dCas9VPR combined with systemic administration of gRNA driving dCas9 to specific loci via AAV serotype 9, which showed robust, safe and specific single or multiplex activation of targeted genes (92). This model represents a rapid and powerful technical platform for gene activation in postnatal cardiomyocytes in preclinical proof-of-concepts. All of these studies demonstrate the feasibility of CRISPR-based methods for transcriptome modulation and set the stage for future optimization in both, basic and clinical research.

CRISPR-MEDIATED CONTROL OF TRANSCRIPTION TO ENHANCE CARDIAC REGENERATION

Although modulating individual factors in adult cardiomyocytes did enable some proliferative activity (93, 94), overexpression of a combination of cell cycle regulators increased the effect on cardiomyocyte proliferation and improved cardiac function after ischemic injury (95). Moreover, as aforementioned, effective cardiomyocytes' regeneration requires coordinated structural and metabolic alteration, which demands the targeting of multiple mechanisms. A highly attractive feature of the CRISPR-based technology is the possibility of simultaneous manipulation of multiple genes that can be exploited to efficiently induce cardiomyocytes renewal. The notion that a disease phenotype is triggered by dysregulation of several factors affecting one or more networks supports the use of multiple genes manipulation targeting dynamic gene networks, which perturbation results in a disease phenotype. In this context, CRISPR-associated RNA scaffolds were shown to provide a powerful way to construct not only multiple, but also orthogonal synthetic gene expression programs (96, 97). Such system was applied to modulate a branched metabolic pathway in yeast, in which multiplexed transcription activation and repression is carried out using distinct single gRNAs modified with RNA aptamers, termed scaffold RNAs. These aptamers can recruit either binding protein fused to a Krüppel-associated box (KRAB) domain for transcription repression or the MS2 coat protein (MCP) fused to VP64 for transcription activation (97). Another strategy made use of different dCas9 orthologs in a dual inducible and repressible systems for precise and dynamic control of CRISPR-dCas9- and 12a-mediated epigenetic editing tested in HEK293T cells (98). These studies provide promising evidence of the ability to use CRISPR-mediated gene modulation for modeling complex gene networks and reversing a disease condition using orthogonal systems for parallel activation and repression in the same cell. The combination of single cell transcriptomics and the bioinformatic assessment of network activities will provide the information for tailored CRISPR-based synthetic control of transcription. This will allow steering gene expression profiles in order to detour a cell toward a physiological state and prevent organ deterioration in a multifactorial and disease-specific manner (**Figure 1**).

Besides targeting cardiomyocytes, which depending on the disease condition and extent of the damage may not be efficient, a promising approach to promote regeneration of heart tissue is to convert resident non-cardiomyocytes cells directly into *de novo* cardiomyocytes (10). Direct reprogramming *in vivo* has been reported in mice by using GATA4, MEF2C, and TBX5 (GMT) or GMT factors plus HAND2 (GHMT) reprogramming cocktails with retroviral delivery in order to infect proliferating cells such as activated cardiac fibroblasts after myocardial injury (99, 100). This approach generated new cardiomyocyte-like cells from activated cardiac fibroblasts. However, direct reprogramming showed relatively low reprogramming efficiency (10). Adding of ZNF281 to the reprogramming cocktail repressed genes associated with the inflammatory response as well as regulated cardiac gene expression by interacting with the transcription factor GATA4 (101). Additional factors improving cardiac reprogramming efficiency include the modification of endogenous signaling pathways such as RAC-α serine/threonine-protein kinase (AKT1), transforming growth factor-β (TGFβ), WNT, and Notch signaling (10, 102, 103). Furthermore, enhanced cardiac reprogramming was observed by suppressing the expression of the Polycomb complex protein BMI1 and the splicing factor polypyrimidine tract-binding protein 1 (PTB), while indicating the repressive role of these factors for cardiac reprogramming (104, 105). Trans-differentiation induced by activating endogenous gene expression with the use of the CRISPR-dCas9 system has been reported in different cell lines including neonatal mouse fibroblasts (10, 85, 106–108). However, it was shown that endogenous cardiac transcription factor activation is necessary for expression of maturation genes but not sufficient to induce efficient cardiac fibroblast trans-differentiation (108). It will be interesting to evaluate whether combinatorial activation and/or repression, allowing for more precise control of multiple pathways in orthogonal directions, could enhance cardiomyocytes reprogramming efficiencies by harnessing knowledge about the epigenetic landscape and modulating factors of cardiomyogenic cells (109).

A further application of CRISPR-dCas9 systems will include patient-specific induced pluripotent stem cells (iPSC) for derivation of specific cell types for transcriptomic approaches.

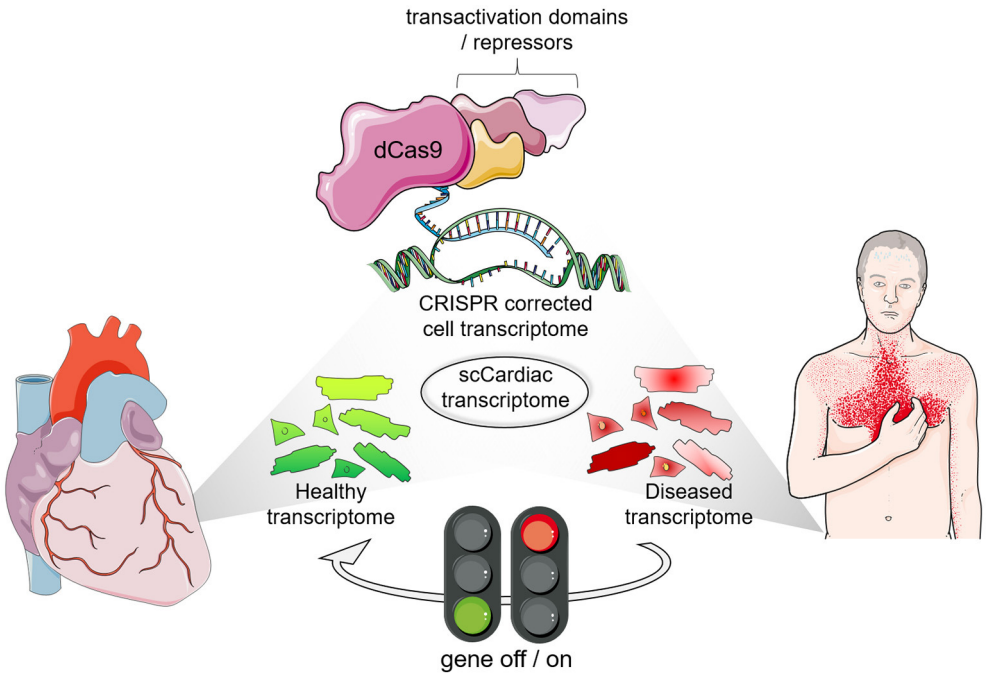

FIGURE 1 | The era of transcription profile analyses and transcriptional engineering at single cell resolution. Single cell (SC) transcriptomics identified cell types in healthy and diseased cardiomyocytes along with their transcriptional profile. Interference with gene expression is possible with CRISPR-based synthetic transcription factors to steer gene expression profiles of specific subpopulations of target cells in the heart.

The iPSCs offer an attractive experimental platform, paving the way for the development of personalized medicine in cardiovascular diseases (110). CRISPR/dCas9 activation and interference systems were widely used for genome-scale screening (96, 111–113). Upon identification of transcriptional networks dysregulation in patient specific iPSC-derived cells, the amalgamation of CRISPR-mediated gene modulation with iPSC technology may allow reverting disease condition in a dish as a basis to translate the personalized approach to the patient without affecting the genomic DNA. This will include the delivery of a CRISPR-gene modulation molecular tool that will restore the altered transcriptome in specific cells for precise (patient and disease specific) therapeutical applications. With these improvements, personalized medicine could be a reality for many patients, minimizing side effects (**Figure 2**).

The concept of using single cell (SC) transcriptome for personalized medicine is already an important research focus for the investigation and treatment of multifactorial diseases. Recently, a pan-European initiative ("Life Time": Revolutionizing Healthcare by Tracking and Understanding Human Cells during Disease) as well as national initiatives (e.g., Berlin Cell Hospital and Virchow 2.0, Germany) have been initiated. These and similar consortia aim at targeting human cells during the onset and progression of complex diseases as well as at analyzing their response to therapy at single-cell resolution (114). Integration of large molecular and clinical datasets will identify molecular mechanisms and create predictive computational models of disease progression allowing the implementation of gene or pathway-directed targeted therapy (114, 115). SCS technology has already contributed to the identification of novel disease biomarkers helping in the diagnosis and refinement of treatments. High-resolution SC transcriptomics will be vital in dissecting how these new treatments affect cell populations receiving the cell precision therapies (115). The CRISPR toolbox is an emerging opportunity to therapeutically modulate cellular states by the use of gene or base editing and synthetic transcription. CRISPR/Cas9-based gene editing approaches for prevention of cardiovascular disease have been demonstrated (71, 116). CRISPR base editors that are delivered *in vivo* using lipid nanoparticles were shown to efficiently and precisely modify disease-related genes in living cynomolgus monkeys (117). In this study, *PCSK9*, a well-established target in atherosclerosis, was mutated *in vivo* using CRISPR base editors leading to a variant that resulted in lower levels of low-density lipoprotein (LDL) cholesterol in the blood and reduced the risk of atherosclerotic cardiovascular disease (117). In the same manner, identification of specific targets by omics approaches will allow to combine with CRISPR-based modulation approaches in order to target a specific (genomic or epigenomic) perturbation in a disease and patient-specific manner. Logistically, primary patient tissue or iPSC cells, differentiated into a desired cell type, can be used for extracting omics information to be further applied in pathway analysis and network perturbation identification. This would lead to deciphering the set of factors that may need to be modulated for reverting a disease condition, which will be tested experimentally *in vitro* before ultimately reaching

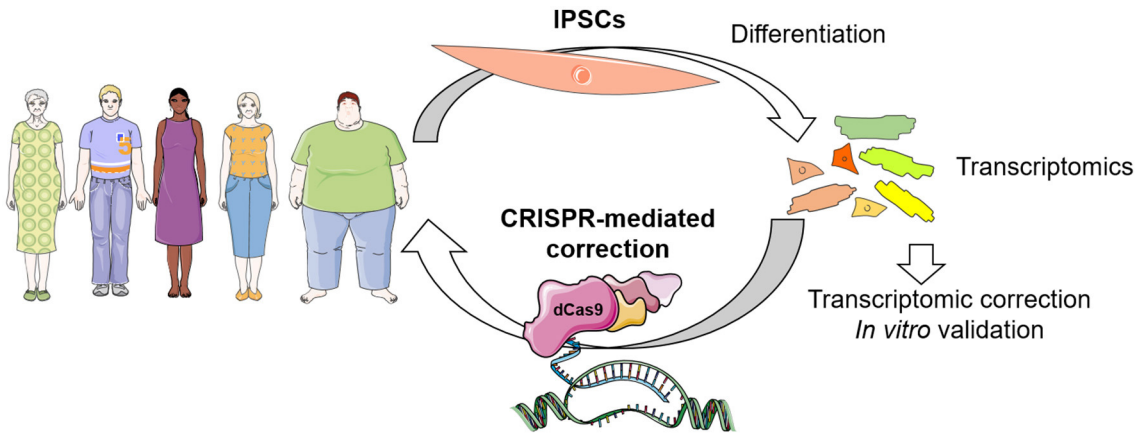

FIGURE 2 | Envisioned concept of CRISPR-dCas9 system in patient-specific therapeutics. Identification of transcriptional networks dysregulation can be achieved in patient specific iPSC-derived cells, which can be corrected by CRISPR-mediated gene modulation *in vitro* for validation, and finally *in vivo* for personalized therapeutics in the near future.

the patient. Technical challenges that need to be overcome include standardization of the methods, costs and user-friendly analysis tools.

ADVANTAGES AND CHALLENGES OF CRISPR-MEDIATED TRANSCRIPTIONAL CONTROL

While the CRISPR/Cas9 system has demonstrated a great promise for a variety of applications, there are several factors that influence its efficacy as well as its safety which must be addressed, especially when the goal is *in vivo* human gene therapy. Last but not least, there are ethical implications that need to be carefully considered, and are discussed extensively elsewhere (118–120). A more worrisome problem includes specific biosafety regulatory challenges and ethical issues concerning applications of CRISPR technology for irreversibly editing the human genome. Nevertheless, ethical concerns need to be addressed properly, which may not be unique when considering other interventions that influence human biology (119).

In respect to tool design, the necessary factors that require careful examination include target DNA site selection, sgRNA design, off-target effects and the method of delivery, the latter representing a major obstacle for use of CRISPR-based editors for *in vivo* applications (121). A powerful advantage of the CRISPR/Cas9 system is the ability to especifically target any 23-bp sequence that contains a PAM motif on either strand of DNA (121). However, single and multiple-base mismatches can be tolerated specially at greater distances from the PAM resulting in off-target effects (122–129). Importantly, the catalytically inactive Cas9 leaves the genome unaffected, significantly reducing the concerns over off-target effects (92, 130). Therefore, lower risk of side-effects are introduced by using a dCas9; however, this needs to be addressed on individual sgRNAs and in a context-specific manner. In order to reduce off-target events, rational design of the sgRNA has been the subject of a significant body of work resulting in many criteria and no simple rules (121). Comparing predictions from several sgRNA design tools with experimental results published in SpyCas9 off-target studies, showed evidence of algorithmic overfitting (124). They indicated the importance of using a model trained on data from the same gRNA expression system, which are currently few, especially for tissues when *in vivo* experiments are deployed.

From the clinical perspective, CRISPR-mediated control of gene expression offers several advantages compared to previous methods based on the expression of an open reading frame of the gene of interest, lacking physiologically relevant splice variants with exogenous, and thus uncontrolled expression. CRISPR-mediated regulation has overcome these obstacles and can now generate unprecedented levels of endogenous control while simultaneously offering a multiplexing possibility (131). The major challenge of CRISPR-based therapies is the delivery. Delivery systems for the CRISPR machinery can be classified into three general groups: physical delivery, viral vectors, and non-viral vectors. Viral delivery vectors include specifically engineered AAV, and full-sized adenovirus and lentivirus vehicles. These are the most common CRISPR/Cas9 delivery methods for *in vivo* approaches (121). AAV is considered a suitable vehicle for gene therapy since it is not known to cause any pathologies in humans, and there is a wide range of serotypes allowing for infection of a multitude of cells with different specificities. Moreover, the virus itself is able to efficiently transduce cells, while provoking little to no innate or adaptive immune response or associated toxicity, at least upon the first treatment with a certain serotype (121, 132). Thus, due to their well-proven safety profiles, AAVs are currently the best choice for nucleic acid-based therapy in clinical trials. AAVs, however, have the disadvantage of a small payload of ∼4.7 kb, which can become a limitation considering all the necessary components of the CRISPR activation or repression systems (76, 133). Several approaches are under development to circumvent these hurdles, including the profiling of non-viral nanoparticles for gene delivery and decreasing the size

of individual Cas9 components (134). Successful packaging of the SpyCas9 and sgRNA into two separate AAV particles and using them for co-transduction has been already reported (135). On one hand, this increases the overall size of the constructs that can be used. On the other hand, this naturally adds more complexities than those existing with a single vector (121). A further approach includes a split Cas9 system, in which the Cas9 C-terminal region is packaged into one AAV vector and the Cas9 N-terminus is packaged into a second AAV vector (136, 137). Reconstitution of the two Cas9 halves results in a functional Cas9 with editing efficiency comparable to the native Cas9, allowing for the use of larger overall Cas9 variants with AAV particles. This has also been proven to be effective in gene editing in pig and human models of Duchenne muscular dystrophy (116, 138). Moreover, the identification of a small Cas9 ortholog from Staphylococcus auricularis (SauriCas9) that can be packaged into an AAV for genome editing, has broadened the possibilities of efficient delivery and can be adapted for gene modulation, further expanding the CRISPR toolbox for epigenetic regulation (139). Non-viral delivery of Cas9 for genome editing have been demonstrated less efficient than viral methods, however, they could allow repeated dosing by using e.g., lipid-based nanoparticles (140). Other delivery strategies have been applied *in vivo*, including direct mRNA delivery and ribonucleoprotein (RNP) delivery with lipid nanoparticles (LNP), especially for genome editing (140). Lipofectamine has been used to deliver base editors to the murine ear, however, entailing toxicity, which promoted the development of more biocompatible lipid formulations that can be used to deliver the Cas9 RNP *in vivo*. These formulations include gold nanowires, gold nanoclusters, black phosphorus nanosheets and nanoscale zeolitic imidazole frameworks (ZIFs). *In vivo* efficacies of these delivery systems are yet to be determined. Thus, there is a growing need for the next-generation more efficient vectors to be developed (141–144).

Despite these hurdles, CRISPR/Cas9-based therapies have begun their path into the clinic. CRISPR-based gene editing clinical trials for sickle cell disease and beta-thalassemia (CTX001) have paved the way for CRISPR-mediated therapies and further optimizations (145). This has been followed by AAV-based clinical trials and planned non-viral nanoparticle-based delivery of CRISPR to the liver (NTLA-2001) (145). Future studies are necessary to determine pre-existing immunity against candidate Cas9 proteins in humans. Also, the combination of cell- and tissue-specific regulatory components with broad tropism AAV vectors will help to fine-tune the localization of the effector components, while providing increased specificity and safety (76). Many classical targets considered "undruggable" came into play with expression interference strategies such as siRNA (146) and proteolysis-tags technologies (147). With CRISPR/Cas9 gene activity modulation, a powerful approach to precisely target candidate expression mechanisms at the transcriptional level emerges, further expanding our targeting scope. While enzyme activation with classical pharmacological approaches such as small molecules is limited to a small fraction of candidate targets (148), CRISPR gene modulation harbors the potential for gain-of-function mode of actions including transcription factors [i.e., Pdx1 (85) and c-myc (86)], formerly deemed difficult-to-drug (149). Furthermore, CRISPR gene modulation was shown to be efficient for congenital diseases based on haploinsufficiency and diseases caused by loss of a gene product in animal models. An endogenous gene product can be normalized from the healthy allele [as shown for *Sim1* haploinsufficiency in obesity (88)] or replaced by a similar transcript [as shown for *utrophin*, replacing the lack of dystrophin for Duchenne muscular dystrophy (85)]. These therapies present challenges when using pharmacological applications.

Simultaneous activation and repression of multiple genes leading to network modulation rather than unidirectional regulation may be of more therapeutic relevance. With the intensified investigation of endogenous gene regulatory networks in the SC-specific context, the gene network engineering via CRISPR systems is highly attractive for the reestablishment of homoeostatic gene regulatory networks upon disease conditions. Such a high precision tuning of the defined sets of synergistic genes will result in the extraordinary control over cell behavior (131), allowing the induction of tailored reparative responses using the own cell machinery in the mature organ. While they can be exploited to enhance regenerative processes of cells, tissues, and organs, these advances need further technological development along with a better understanding of how exactly epigenomic and transcriptomic changes mediate phenotypic effects at the single cell resolution (67). This will allow for more precisely targeted approaches adjusted to the physiological needs.

CONTROLLING Cas9 FUNCTION AND SIDE-EFFECTS

To restrict Cas9 activity, and thus reduce the off-target effects, attempts for temporally restricted (d)Cas9 expression were developed including chemical and light controlled gene activity modulation. Detailed reviews regarding inducible Cas9 systems discussed background (leaky) activity, editing effectivity, and reversibility for gene editing approaches (150, 151). We therefore summarize inducible systems specifically adapted for endogenous gene activity modulation here. Temporal control was harnessed by decoupling Cas9 from transcriptional modulators with conditional chemical or light induced assembly of the synthetic transcription factor. Tested chemical and light inducible elements included: (1) Absicic acid (ABI-PYL1), Giberrellin (GIB1-GAI), and Rapamycin (FKBP-FRB) systems as well as (2) red-light (PHYB-PIF), and blue-light (CRY2PHR-CIBN) inducible systems (98, 152, 153).

Small molecule based Cas9-DNA interference was demonstrated and applicable for CRISPRa approaches reducing gene expression of up to 89% (154). While effectively limiting (d)Cas9 activity, these systems rely on constitutive expression and presence of (d)Cas9 and effector domain proteins harboring potential for unwanted cellular and organismal effects. To overcome this, Trimetoprim or Doxycycline responsive promoter elements driving dCas9 transcription were successfully tested for induced dCas9 expression with concomitant

(multiplexed) gene activation (155–157). Additionally, degron motif-based "suicide-tags" for protease inhibition-dependent Cas9 expression was presented as a promising option for Cas9 activity restriction (158). Taming Cas9 expression or activity using pharmacological approaches might therefore be a prospective route for temporally restricted or pulsed endogenous gene activation, possibly reducing expected immune responses upon constitutive Cas9 expression (159) and deserves a future validation in *in vivo* models. Bioengineering of Cas9 proteins as well as elaborate cell-type specific and temporally resolved Cas9 expression systems (151) are therefore essential ventures for safe and limited CRISPR/Cas9 based applications (71). Protein-based anti-CRISPRs, which are accessory proteins with fewer than 200 amino acids called "anti-CRISPRs," can function as antagonists of CRISPR systems and achieve context-specific inhibition of Cas9. This will offer a solution for mitigating the problem of off-target cleavage as well as for limiting Cas9 activity on the genome (160). To what extent these approaches can be transferred to the clinic is still uncertain, nevertheless they warrant further studies.

CONCLUSION

Research efforts during the past decades have broadened our insights into the molecular determinants of cardiac disease. With the recent emergence of the SCS multi-omics profiling, more detailed and comprehensive understanding of the basic molecular profiles of disease-associated perturbations within each cell in the human heart could be achieved, fine-tuning our previous knowledge. Combining this information with the revolutionizing CRISPR technology will enormously advance medical research and open a new chapter of precision and personalized medicine. Within the last few years of research, CRISPR-mediated gene editing has already entered the clinic, making the application of further CRISPR approaches for synthetic transcription a realistic option for more specific treatments of different cardiac disease entities. Furthermore, integration of patient-specific data and human-based *in vitro* models, will help to identify more personalized therapies for rare diseases. Such an approach will necessarily include collection of biological information from a patient, integration of a suitable iPSC-model to test patient- and/or disease-specific synergistic network modulation and their safety, and ultimately fine-tuning of a precise therapeutic approach. All these tools will broaden our understanding of human- and disease-specific effects as well as provide us with information about safety of proposed modulations. With these advances, a reshaped personalized management of complex human diseases will become a realistic approach. Reduction of costs for development and production of such approaches is mandatory to allow disease-specific and individualized therapies in the future. Joined efforts of clinicians, basic researchers and industry partners is already now facilitating rapid advancements of the discussed technologies that will dramatically impact biomedical research and disease treatments in the future.

AUTHOR CONTRIBUTIONS

ES, SL, DP, and LZ conceptualized, wrote, and revised the manuscript and figures. All authors contributed to the article and approved the submitted version.

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Acute myocardial infarction induces remodeling of the murine superior cervical ganglia and the carotid body

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A role for cardiac sympathetic hyperinnervation in arrhythmogenesis after myocardial infarction (MI) has increasingly been recognized. In humans and mice, the heart receives cervical as well as thoracic sympathetic contributions. In mice, superior cervical ganglia (SCG) have been shown to contribute significantly to myocardial sympathetic innervation of the left ventricular anterior wall. Of interest, the SCG is situated adjacent to the carotid body (CB), a small organ involved in oxygen and metabolic sensing. We investigated the remodeling of murine SCG and CB over time after MI. Murine SCG were isolated from control mice, as well as 24 h, 3 days, 7 days and 6 weeks after MI. SCG and CBs were stained for the autonomic nervous system markers β3-tubulin, tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT), as well as for the neurotrophic factors brain derived neurotropic factor (BDNF), nerve growth factor (NGF) and their tyrosine receptor kinase (pan TRK). Results show that after MI a significant increase in neuron size occurs, especially in the region bordering the CB. Co-expression of TH and ChAT is observed in SCG neuronal cells, but not in the CB. After MI, a significant decrease in ChAT intensity occurs, which negatively correlated with the increased cell size. In addition, an increase of BDNF and NGF at protein and mRNA levels was observed in both the CB and SCG. This upregulation of neurotropic factors coincides with the upregulation of their receptor within the SCG. These findings were concomitant with an increase in GAP43 expression in the

SCG, which is known to contribute to axonal outgrowth and elongation. In conclusion, neuronal remodeling toward an increased adrenergic phenotype occurs in the SCG, which is possibly mediated by the CB and might contribute to pathological hyperinnervation after MI.

KEYWORDS

myocardial infarction, superior cervical ganglion, carotid body, neurotrophic factors, GAP43, neuronal remodeling, BDNF (brain derived neurotrophic factor), NGF (nerve growth factor)

Introduction

About one third of all global deaths are attributed to cardiovascular diseases (1). In western countries, the incidence of sudden cardiac death (SCD) is 50–100 per 100,000 which is attributed to coronary artery disease (CAD) in 70–80% of cases, despite the development of reperfusion strategies and medical therapies (2). SCD after myocardial infarction (MI) has been classically linked to heterogeneous conduction in the infarct border zone caused by surviving cardiomyocytes surrounding the scar area, resulting in polymorphic ventricular tachycardia (VT) based on micro-re-entry (3, 4). Interestingly, in the past decades a role for the cardiac autonomic nervous system in arrhythmogenesis after MI has increasingly been recognized (5, 6). The heart is innervated by the autonomic nervous system, divided in sympathetic and parasympathetic branches, regulating cardiac function. In order to maintain a regular heartbeat, a balance is needed between sympathetic and parasympathetic tone. Parasympathetic input toward the heart is provided by (branches of) the vagal nerve that synapse in parasympathetic ganglia at the epicardial layer of the heart. Preganglionic cardiac sympathetic axons synapse with postganglionic sympathetic neurons in the sympathetic chain (7). In humans, cardiac input from the sympathetic chain is provided by both cervical as well as thoracic contributions (7, 8).

A myriad of studies have reported a potential association of cardiac sympathetic hyperinnervation, usually defined as an increased density of sympathetic nerve fibers in the area of damage, with SCD after MI. To date the exact underlying mechanism of the relation between sympathetic hyperinnervation and VT after MI is still uncertain. Likely, factors secreted by the ischemic myocardium retrogradely stimulate axonal outgrowth and remodeling of sympathetic ganglia, altering electrophysiological properties, thereby increasing the risk of VT and SCD (9–11). Recent data shows an upregulation of nerve growth factor (NGF) in the ischemic zone after MI, that supports this concept (12).

Although several studies have shown sympathetic hyperinnervation as well as neuronal remodeling after MI, the exact timeline of this phenomenon is less clear. In several species, neuronal remodeling has been described to occur 1–8 weeks after MI, characterized by increased expression of growth associated protein (GAP43) and synaptophysin – both markers for neuronal outgrowth – and increased amounts of tyrosine hydroxylase (TH), suggesting an increase in innervation and a switch toward a more adrenergic phenotype (13–16). Most studies, however, focus on the stellate ganglion, whereas limited information is available on the relevance of the other ganglia providing sympathetic input to the heart.

The superior cervical ganglion (SCG) gives input to the carotid plexus whose fibers run along the carotid arteries and provide sympathetic input toward the head where it stimulates parts of the eye, mouth and small blood vessels. The SCG also participates in innervation of the heart, providing the superior cardiac nerve that joins with postganglionic sympathetic fibers originating from other sympathetic ganglia at the cardiac plexus (7, 8). Remarkably, in mice it has been shown that ganglionectomy of the SCG before MI leads to an almost entire loss of myocardial sympathetic innervation of the left ventricular anterior wall, in addition to a significantly reduction in chronic consequences of MI, such as myocardial inflammation, myocyte hypertrophy, and overall cardiac dysfunction (17). In human, it has been established that the SCG is involved in cardiac innervation (8), although the impact of a potential remodeling of this ganglion after MI, is unclear. In this respect, it may be relevant that the SCG is situated adjacent to the carotid body (CB), a small organ involved in oxygen, carbon and pH sensing, that has been shown to produce many neurotrophic factors (18). Of interest, Rocha et al. report that in rabbits the response of the chemo sensitive cardiac reflex of the CB was enhanced in the acute phase of MI (19). Hypertonicity of the CB has been linked with cardiac disease such as hypertension and chronic heart failure. In rats with induced chronic heart failure, denervation of the CB performed early after MI, resulted in improved survival due to reduction of ventricular remodeling, less fibrosis and reduction of arrhythmias (20). Whether this is a transient phenomenon is unclear, as is the time-course of remodeling of the superior cervical ganglion and CB after MI.

Given the relevance of the SCG in cardiac innervation of the murine heart, as well as the still enigmatic role of this ganglion and the CB in the innervation of the human heart in health and

disease, in the current study we investigated the remodeling of the murine SCG as well as the bordering CB over time after MI.

Materials and methods

Animals

C57BL/6J (Jackson Laboratory) male mice of 13 weeks old (n = 20) were used. All animal experiments were approved by the Animal Ethics Committee of the Leiden University (License number AVD1160020185325), Leiden, The Netherlands. All mice were maintained in a specific pathogen-free facility on a 12 h day and night cycle and regularly monitored.

Induction of myocardial infarction and superior cervical ganglia isolation

Myocardial infarction was induced as previously described (12). Briefly, mice were anesthetized with 2% isoflurane, intubated and ventilated. The left anterior descending coronary artery (LAD) was permanently ligated and ischemia was confirmed by discoloration of the anterior wall of the left ventricle. The mice were given the analgesic drug Temgesic, 24 h before and after the operation to relieve pain. As control, untreated mice (n = 4, 8 SCGs) were included. All mice were euthanized by CO_2 asphyxiation; untreated control mice (n = 4; 8 SCGs) and 24 h (n = 5; 10 SCGs), 3 days (n = 3; 6 SCGs), 7 days (n = 5; 10 SCGs), or 6 weeks (n = 3; 6 SCGs) after MI (**Figure 1A**). The SCG and hearts were removed as previously described (21). Briefly, to dissect the left- and right SCG, an incision was made in the skin of the neck area, the submandibular glands were moved aside, whereafter the carotid artery bifurcation with the SCG could be captured bilaterally. After excision, both SCG were fixed with 4% paraformaldehyde (104005; Merck Millipore), and embedded in paraffin. The ganglion sections, 5 μm thick, were adhered in a series of 1:3 on silane adhesive slides (Klinipath KLINKP-SIL-3057) to allow different stainings to be assessed in the same ganglion.

Immunofluorescence detection and quantification of neuronal- and neurotrophic markers

The illustration in **Figure 1B** shows the schematic overview of; (i) studied markers; (ii) used microscope and scanners; (iii) location of the sections; (iv) quantification method; and (v) output parameters. All slides were deparaffinized prior to the antigen retrieval, by heating slides in Tris-EDTA buffer (pH 9, 98°C) for 12 min, and incubated with primary antibodies overnight at 4°C.

To study the cell size and autonomic nerve markers (**Figures 1B1,B2**), every third slide was incubated with primary antibodies: anti-tyrosine hydroxylase (TH, a marker for sympathetic nerves) (Fisher Scientific PA14679; 1:1,000), anti-choline acetyltransferase (ChAT, considered a marker for parasympathetic nerves) (Abcam ab181023; 1:1,000) and anti-β-tubulin III (β3-tubulin, a general nerve marker) (Santa Cruz; SC-80005; 1:1,000). To study neurotropic markers and receptors (**Figure 1B2**), one slide per ganglion with paraffin sections that contained the CB were used. The slide was incubated with the primary antibodies: anti-brain derived neurotrophic factor (BDNF) (Abcam ab108319; 1:250), anti-nerve growth factor (NGF) (Abcam ab6199; 1:100) or anti-pan tyrosine receptor kinase (pan TrK) (Abcam ab181560; 1: 500) combined with anti-TH (Fisher Scientific PA14679; 1:1,000).

On the second day, the slides were incubated with their corresponding secondary antibodies: donkey anti-rabbit Alexa Fluor 488 (Invitrogen A-21206; 1:250), donkey anti-sheep Alexa Fluor 568 (Invitrogen A21099; 1:250) or donkey anti-mouse Alexa Fluor 647 (Invitrogen A31571; 1:250) for 1 h followed by a 10 min nuclear staining with DAPI (Invitrogen D3571: 1:1000). The slides were mounted with ProLong Gold Antifade Mountant (Invitrogen P36930) and the images were captured with the Zeiss AxioscanZ1 slide scanner or Leica WLL confocal microscope under the same exposure time and gain settings.

For quantification of the neuronal size (derived from the neuronal area) three sections per region were selected (**Figure 1B1**) and for fluorescent intensity, 3 sections per slide were selected (**Figure 1B2**). The individual sections were opened with ImageJ (version 1.52p) and a 500 × 500 pixels (equals 162.5 × 162.5 μm) square was placed within each section. Within each square 20 cells were drawn in as ROI and the area or intensity were measured. The intensity was corrected by subtraction of the background intensity.

Immunohistochemical staining and quantification of growth-associated protein 43

To study neuronal outgrowth, one slide per ganglion with 5 μm-thick paraffin sections was stained with growth associated protein 43 (GAP43), a marker for nerve sprouting (22, 23) (**Figure 1B3**). As previously described, the sections were first deparaffinized and antigen retrieval was performed. Hereafter, the sections were incubated with the primary anti-GAP43 antibody (Abcam ab75810, 1:2,000) overnight. The next day, the primary antibody was washed away and thereafter the slides were incubated with the secondary biotinylated anti-rabbit IgG (H + L) antibody (Vector Laboratories BA-1000, 1:200) for 1 h, followed by an incubation with ABC-AP (Vector Laboratories AK-5000) for 30 min. To visualize GAP43, the slides were incubated with alkaline phosphatase (AP) substrate (Vector

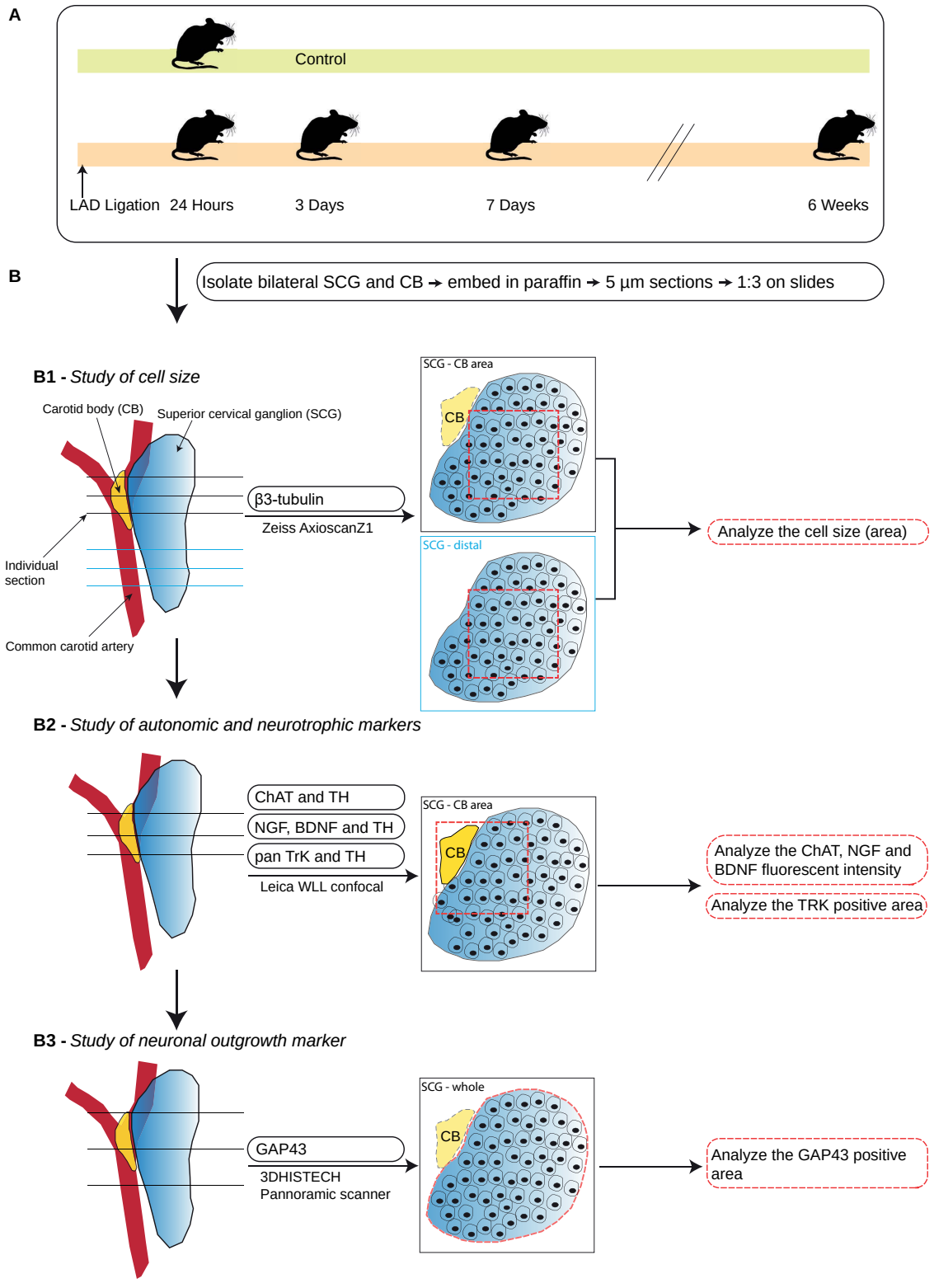

FIGURE 1
Schematic workflow of the time-course study. **(A)** Schematic overview of the studied timepoints after induction of MI. **(B)** A step-by-step illustration of the performed immunofluorescence stainings in this timepoint study of the SCG and CB after MI. Left panels display an overview of the selection of sections for each analysis. The midline panels explain the regions within the sections that were selected for each quantification method. **(B1)** Analysis of neuronal size in the SCG-CB area and SCG-distal area. **(B2)** Analysis of the fluorescent intensity of autonomic and neurotrophic markers in the SCG-CB area. **(B3)** Analysis of the area of growth associated protein 43 (GAP43) in the SCG.

Laboratories SK-5105) in the dark for 5 min. The substrate was then washed away and the slides were counterstained with haematoxylin (Klinipath VWRK4085-9002) in order to visualize the nuclei. After dehydration the sections were mounted with Entellan mounting medium (Merck 107961) and all images were captured with the 3DHISTECH Pannoramic scanner.

To quantify GAP43 expression, 3 sections of each ganglion were quantified. Individual sections were imported into ImageJ and the ganglion region was selected by hand with the ImageJ selection function and set as ROI. To calculate the GAP43 + area (fractional area,%) within the SCG the measurements were performed as follows: within the green channel the total area of the ganglion was measured by setting the threshold on the maximum and this was divided by the GAP43 + area which was measured using the default threshold in ImageJ (version 1.52p).

Hybridization chain reaction RNA fluorescent *in situ* hybridization

Hybridization chain reaction RNA fluorescent *in situ* hybridization (HCR-RNA FISH) was carried out in control and 7 days after MI SCG sections that contained the CB. The manufacturers protocol was followed and the DNA probes, DNA HCR amplifiers, hybridization buffer and wash buffer were purchased from Molecular Instruments[1] (24). Briefly, slides were first heated for 1 h at 60°C to improve adhesion. For RNA retrieval, the slides were deparaffinized and heated in TRIS buffer for 15 min at 95°C, followed by a 10 min Proteinase K (10 μg/ml) (Promega, V3021) digestion. A humidified chamber was used in all following incubation steps. Probe hybridization with BDNF-B1 (NM_007540.4, LOT PRM659), NGF-B2 (NM_013609.3, LOT PRM660), and GAP43-B3 (NM_008083.2, LOT PRF293) was performed with 4 pmol/ml probes for 16 h at 37°C. Prior to the hairpin amplification, 6 pmol/ml of the hairpins B1-h1 + h2 (fluorophore 546), B2-h1 + h2 (fluorophore 647), and B3-h1 + h2 (fluorophore 488) were snap-cooled by heating to 95°C for 90 s and incubated for 30 min in the dark at room temperature. Sections were incubated with the hairpin amplifiers for 90 min at room temperature. To stain the nuclei, slides were incubated for 10 min with DAPI (Invitrogen, D3571; 1:1,000) and washed in PBS. Sections were mounted with Prolong Gold Antifade (Invitrogen, P36930) and imaged with a Zeiss Airyscan LSM 900 confocal microscope under the same exposure time and gain settings. mRNA expression was quantified in 3 sections per ganglion, the ganglia and CB were separately selected as ROI in ImageJ and threshold settings to measure the area were kept the same throughout the quantification. Artifacts that were highly fluorescent were manually deleted from the ROI.

1 https://www.molecularinstruments.com/

Statistics

Data are presented as mean ± standard error of the mean (SEM). One-way ANOVA and multiple comparisons followed by a Tukey's *post hoc* analysis were used to determine statistically significant differences among groups. An unpaired Student's was used to statistically analyze the HCR-RNA FISH data. All quantifications were performed in a blinded fashion and results were considered significantly different when the p-value was <0.05. GraphPad Prism (GraphPad Software, San Diego, CA, USA; version 9) was used for statistical analysis. Pearson correlation coefficients were used to test the linear relationship between two variants. R (version 4.0.2) was used for Pearson correlation coefficients and linear regression.

Results

Regional differences in neuronal enlargement in the superior cervical ganglia after acute myocardial infarction

To investigate the remodeling of the murine SCG over time after MI by permanent LAD ligation, SCG were collected and analyzed after 24 h, 3 days, 7 days, and 6 weeks. Although small interindividual variations in MI infarction sizes were observed, no significant differences were detected between hearts at 1 week and 6 weeks after MI (**Supplementary Figures 1A,B**). The presence of hyperinnervation was verified by β3-tubulin staining of the infarction region and border zone (**Supplementary Figure 1A**).

In order to study potential effects of sidedness, both left and right-sided ganglia were examined. **Supplementary Figure 2** shows that there was no significant difference in neuronal cell size (A and B), the area of ChAT positive nuclei/total nuclei area (%) (C and D), intensity of NGF expression (E and H), intensity of BDNF expression (I and L) and GAP43 positive area (M and N) between the left and right SCG at 24 h and 7 days after MI. Since no differences could be detected between the left and right ganglia for the parameters tested in our study, ganglia were treated as independent samples onward (**Supplementary Figures 2A–N**).

Staining of serial sections of the murine SCG with the general nerve marker β3-tubulin provided an overview of the distribution of neurons and nerves for each timepoint

(Figure 2A). Sections were studied that either contained (SCG – CB area) or lacked (SCG – distal) the CB. Quantification of the neurons in these sections revealed a significant difference in the cell size (area) at 24 h and 7 days after MI in the SCG – CB area when compared to SCG – distal area (Figure 2B). In the SCG – CB area a significant increase in cell size was observed 24 h, 3 days and 7 days after MI, when compared to control. Since we established a regional difference in neuronal enlargement, only the SCG – CB area was evaluated in this study onward.

The area bordering the carotid body displays a decrease in choline acetyltransferase intensity

Immunostainings of sections that contained the CB, were performed for the parasympathetic marker ChAT as well as the sympathetic marker TH at several timepoints after MI. The CB type I glomus cells could be identified as clusters of bright TH positive cells, that lack ChAT expression, bordering the SCG. The murine SCG neuronal cells co-expressed TH and ChAT in the SCG throughout the MI timeline (Figure 3A). Overall, the peripheral part of the ganglion displayed a higher TH intensity as compared to the center of the ganglion. While the ChAT expression showed the opposite pattern. However, after MI diminished expression of ChAT was found most markedly in the SCG-CB area and no clear difference in TH expression was observed (Figure 3A, arrows).

We carried out multiple control experiments to confirm the reliability of the detected co-expression of TH and ChAT in murine SCG neuronal cells. It has previously been demonstrated that a cell bridge, which is present in about 30% of the murine ganglia, connecting the cranial pole of the sympathetic SCG with the parasympathetic nodose ganglion (NG) exists and that the NG is mostly ChAT positive and TH negative (25). To validate our antibody specificity, a double immunofluorescence staining for ChAT and TH was performed on the SCG, the cell bridge and the nodose ganglion (NG). Almost all neuronal cells of the NG were solely positive for ChAT, whereas co-expression of TH and ChAT were observed in the SCG. The cell bridge showed more double positive cells more toward the SCG, and less TH positive cells were observed toward the NG (Supplementary Figure 3A). No obvious differences in ChAT intensity between the SCG and NG could be detected. To validate the presence of ChAT protein in SCG neurons, immunoblot analysis of protein lysates from either whole SCG tissue or isolated nuclei was carried out and showed the presence of a 69KDa protein, in accordance with the presence of ChAT (Supplementary Figures 3B,C). The cell lysate of human epicardial cells, that lack ChAT expression, was used as a negative control (Supplementary Figure 3C). To validate the presence of ChAT mRNA in SCG neurons, HCR-RNA FISH was carried out and shows immunofluorescence representing ChAT mRNA (Supplementary Figure 3D). Lastly, a co-staining of TH and ChAT in a human sympathetic stellate ganglion showed a similar ChAT staining pattern as observed in the murine control ganglion (Supplementary Figure 3E). These findings provide evidence for the presence of ChAT mRNA as well as a 69 kDa ChAT protein, validates the specificity of the ChAT antibody and rules out a cross reaction between ChAT and TH antibodies.

In sympathetic stellate ganglia a phenotypic switch has previously been described, where ChAT expression was downregulated providing a more arrhythmogenic environment (14). Timepoint comparison showed a significant loss or decrease in ChAT intensity of neurons with a significant difference at 24 h after MI, and a similar trend was observed at day 3 and 7 after MI, when compared to control (Figure 3B). Of interest, at day 7 after MI, in 2 out of 5 samples a diminished expression of ChAT in the nuclei was seen, but as this was not visualized in the other 3 out of 5 ganglia, quantification of the overall ChAT positive area within the nucleus throughout the timepoints showed no significant difference (Supplementary Figure 4). As the enlarged neuronal cells displayed a low expression of ChAT, a correlation analysis was performed to examine whether an increase in cell size in the SCG correlated with a decrease of ChAT intensity. This revealed a negative correlation between neuronal cell size and relative ChAT expression, which was significant at 24 h, 3 and 7 days after MI (Figures 3C–F). This correlation disappeared 6 weeks after MI (Figure 3G).

Brain derived neurotropic factor and nerve growth factor expression is increased in the carotid body and superior cervical ganglia after myocardial infarction

The CB type I glomus cells secrete neurotrophic factors during development as well as in response to environmental stimuli (26, 27), therefore we assessed brain derived neurotropic factor (BDNF) and nerve growth factor (NGF) expression in the CB and SCG neurons. Expression of BDNF was present in the CB and SCG neurons at all examined timepoints (Figure 4A). When quantifying the BDNF fluorescent intensity over time, in the CB a significant increase was detected 7 days after MI (Figure 4B). No significant difference could be detected in the SCG neurons in the CB area, although a similar trend was observed (Figure 4C).

When examining NGF expression at different timepoints, an increase in NGF fluorescent intensity was observed 7 days after MI in the CB and SCG neurons (Figure 5A). These data corresponded with quantification data that showed a significant upregulation of NGF fluorescent intensity in the SCG neurons and the CB at 7 days after MI (Figures 5B,C). In addition,

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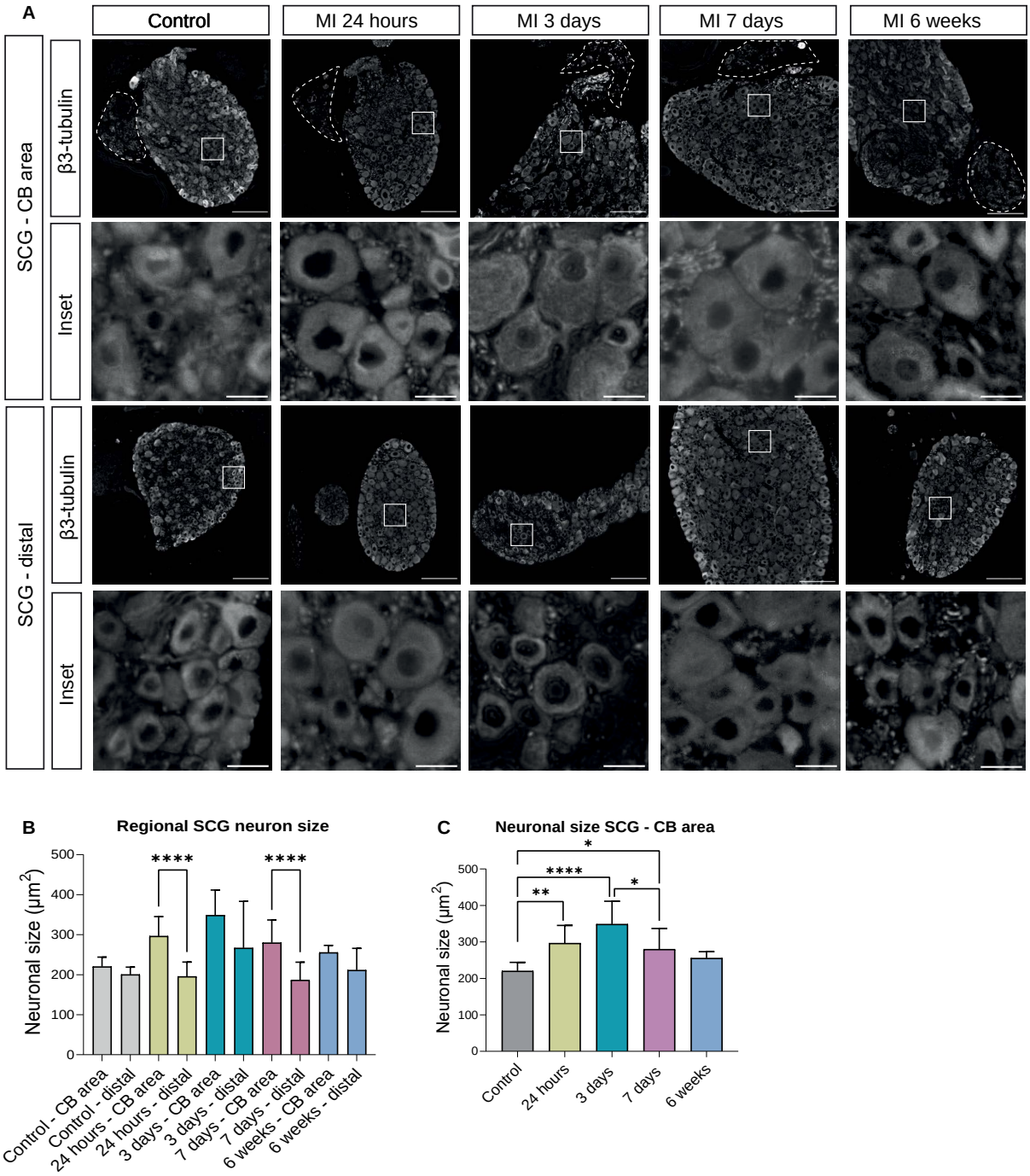

FIGURE 2

Regional differences of neuronal enlargement in the SCG after MI. **(A)** β3-tubulin immunofluorescence staining in distinct SCG regions of control mice (n = 4, 8 SCG) and mice 24 h (n = 5, 10 SCG), 3 days (n = 3, 6 SCG), 7 days (n = 6, 12 SCG), and 6 weeks (n = 3, 6 SCG) after MI. Scale bar indicates 100 µm in the upper panels, and 15 µm in the insets. **(B)** Bar graphs that display the alterations of the neuronal size (µm^2) in the SCG – CB and SCG – distal region. **(C)** Bar graphs that display the alterations of neuronal size (µm^2) in the SCG – CB area. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$.

the NGF fluorescent intensity in the SCG neurons showed a significant upregulation 24 h after MI when compared to control, as well as a significant decrease 3 days after MI when compared to 24 h and 7 days after MI (Figure 5C). At 6 weeks after MI, no significant difference as compared to control could be observed anymore.

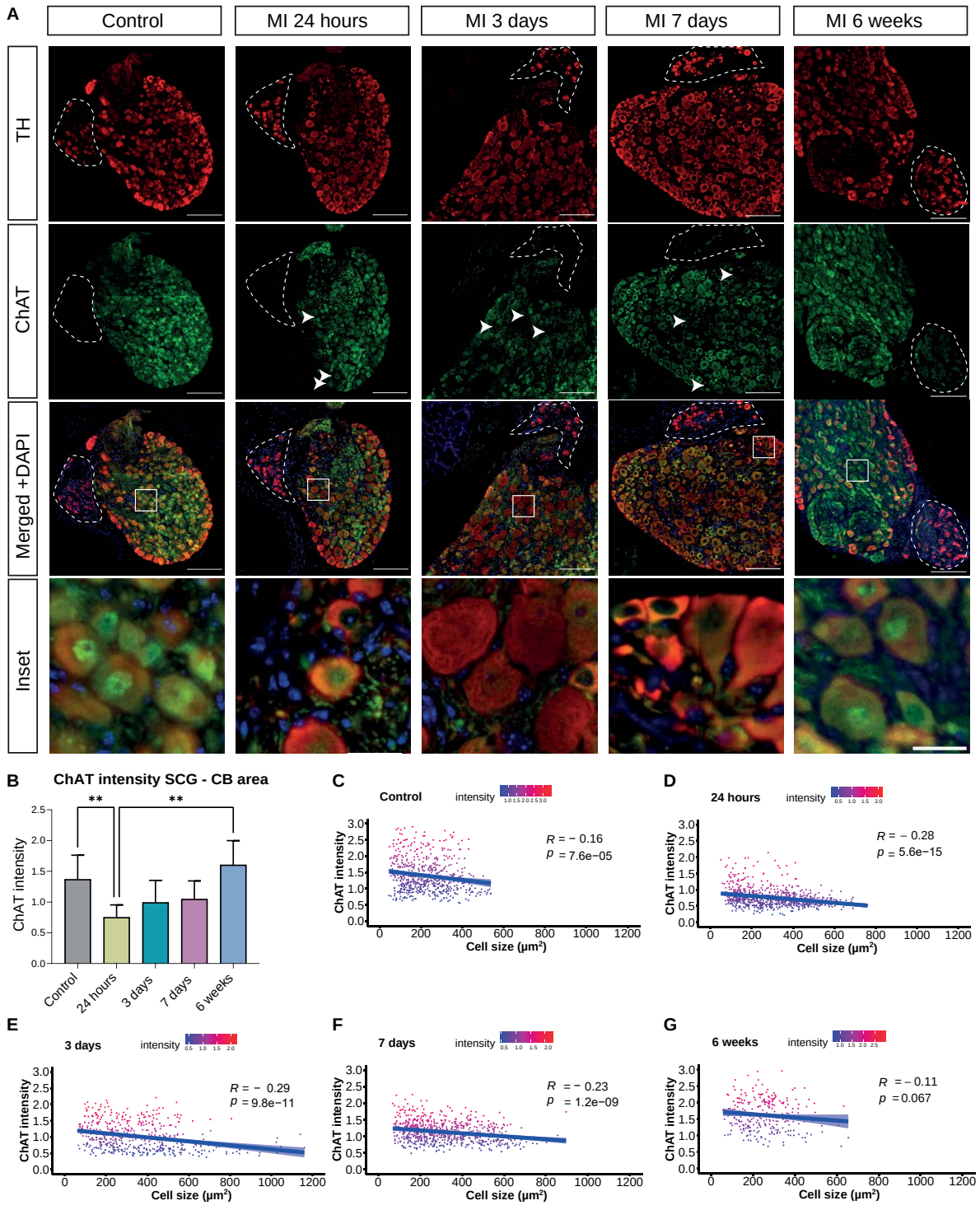

FIGURE 3

Decreased ChAT intensity in the SCG after MI. **(A)** Immunofluorescence staining of TH (red), ChAT (green) and DAPI (nuclei, blue) in the SCG-CB region of control mice (n = 4, 8 SCG) and mice 24 h (n = 5, 10 SCG), 3 days (n = 3, 6 SCG), 7 days (n = 6, 12 SCG) and 6 weeks (n = 2, 4 SCG) after MI. The CB is positive for TH and is indicated with dashed lines. The scalebar indicates 100 and 20 μm in the insets. **(B)** Bar graph displays the ChAT intensity in the SCG-CB region over time. **(C–G)** Linear regression analysis of the neuronal ChAT intensity plotted against the cell size of control mice and mice 24 h, 3 days, 7 days, and 6 weeks after MI. Each dot represents a single cell and the light blue regions indicate the standard error of the mean (SEM) of the ChAT intensity. The R value demonstrates the correlation coefficients at the indicated p values.
$^{**}P < 0.01$.

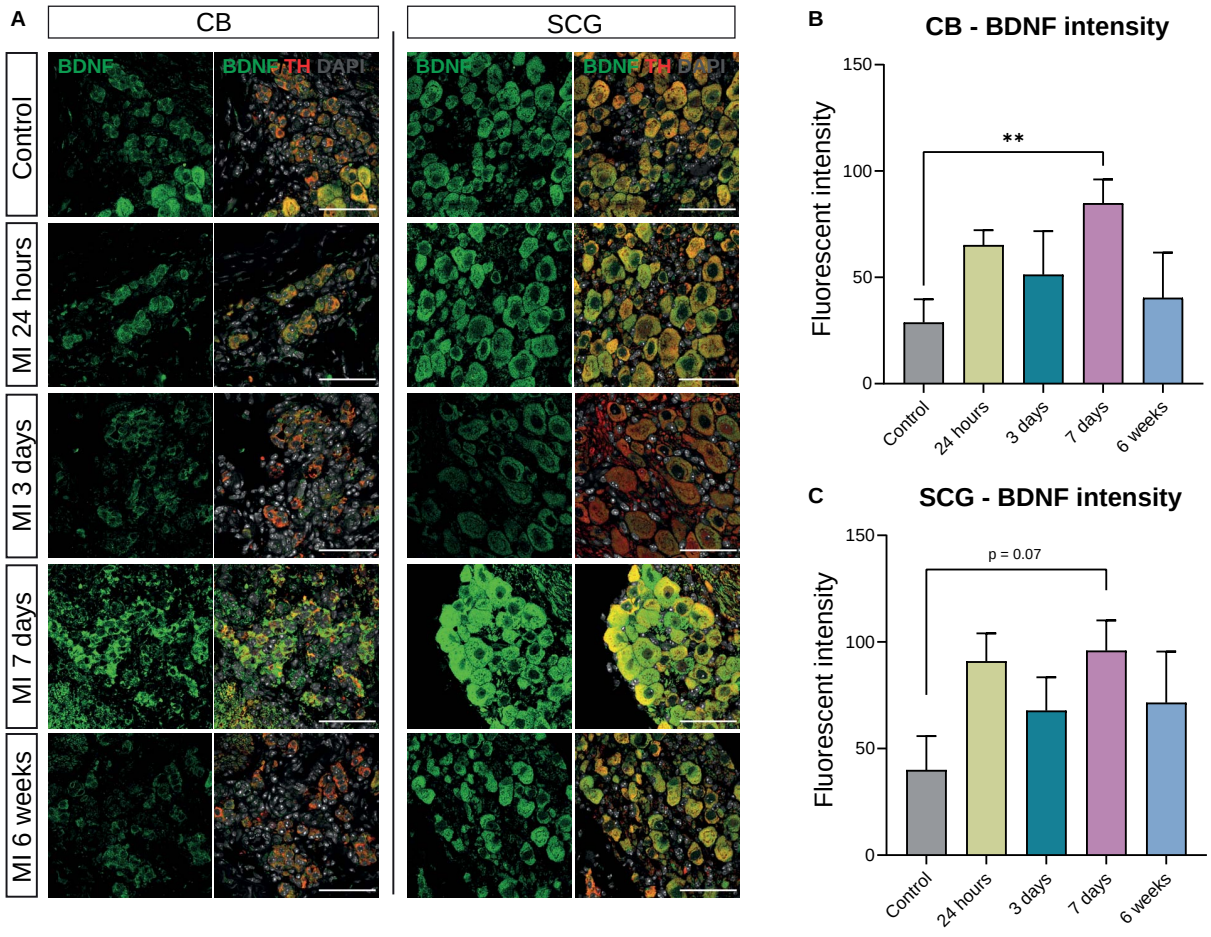

FIGURE 4
BDNF is increased in CB and SCG neurons after MI. **(A)** Immunofluorescence staining of BDNF (green), TH (red), and DAPI (nuclei, gray) in the CB and SCG of control mice (n = 4, 8 SCG) and mice 24 h (n = 4, 8 SCG), 3 days (n = 2, 4 SCG), 7 days (n = 4, 8 SCG), and 6 weeks (n = 2, 4 SCG) after MI. Scale bar indicates 50 μm. **(B,C)** Bar graphs display the BDNF intensity of the CB and SCG neurons at different timepoints after MI. Both the CB glomus type I cells and SCG neurons are TH positive cells. $^{**}P < 0.01$.

High affinity receptors of brain derived neurotropic factor and nerve growth factor in superior cervical ganglia neurons are increased after myocardial infarction

The neurotrophic tyrosine receptor Kinase (TrK; e.g., TrKA and TrKB) expression in neurons facilitates the binding of NGF and BDNF and mediates their subsequent impact on neuronal survival and axonal growth (28–30). To study the presence of these receptors in the SCG, immunostaining of the SCG with a pan TrK antibody was carried out and showed a low expression of pan TrK in control SCG neurons (**Figure 6A**). After MI, pan TrK expression in SCG neurons gradually increased and was significantly upregulated at day 7 after MI. Interestingly, this upregulation persisted at 6 weeks after MI (**Figures 6A,B**).

Growth associated protein 43 is upregulated in the superior cervical ganglia after myocardial infarction

As we observed neuronal remodeling concomitant with an increased expression of the neurotrophic factors BDNF, NGF and their receptors, we postulated that this contributes to new axon formation and axonal elongation in the SCG. SCG sections of control mice and mice post-MI were therefore stained for growth associated protein 43 (GAP43), a growth- and plasticity-related protein that is involved in axon elongation and nerve regeneration during early development (31). As shown in **Figure 7A**, after MI a strong increase in GAP43 expression was observed in neurites and, to a lesser extent, also inside the neuronal cell bodies, while in the control SCG a very low number of neurites expressed GAP43. Timepoint comparison showed a significant upregulation of GAP43

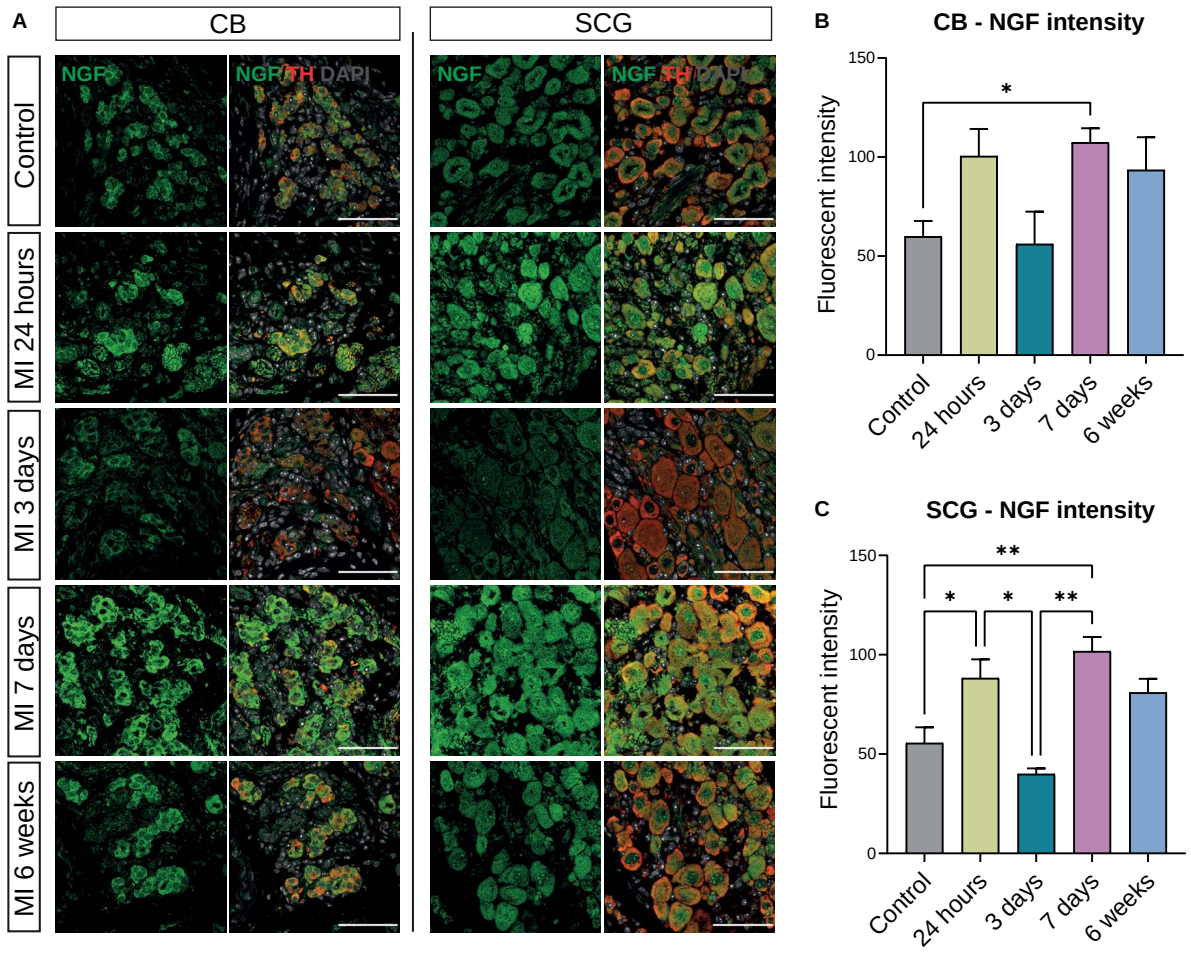

FIGURE 5
NGF is increased in the CB and neurons in SCG after MI. **(A)** Immunofluorescence staining of NGF (green), TH (red), and DAPI (nuclei, gray) in the CB and SCG neurons of control mice (n = 4, 8 SCG) and mice 24 h (n = 4, 8 SCG), 3 days (n = 2, 4 SCG), 7 days (n = 4, 8 SCG), and 6 weeks (n = 2, 4 SCG) after MI. Scale bar indicates 50 μm. **(B,C)** Bar graphs display the NGF intensity of the CB and SCG neurons at different timepoints after MI. $^{*}P < 0.05$, $^{**}P < 0.01$.

expression at all examined timepoints after MI (Figure 7B). Interestingly, GAP43 was found to be present in the CB as well, Supplementary Figure 5 shows GAP43 staining within the CB at 24 h, 3 days and 6 weeks after MI.

mRNA levels of brain derived neurotropic factor, nerve growth factor and growth associated protein are elevated 7 days post- myocardial infarction

To further substantiate our findings, HCR-RNA FISH was performed to evaluate whether the observed changes on a protein level, could also be established at mRNA level. Figure 8A shows representative images of the mRNA content in the SCG neurons (left panel) and CB (right panel). NGF, BDNF and GAP43 are present in both the SCG neurons and CB, where each dot represents a single mRNA molecule. Quantification data in Figures 8B–G show that NGF, BDNF and GAP43 mRNA content are significantly upregulated in both the SCG and CB at 7 days post MI when compared to control.

Discussion

In the current study, we assessed the neuronal remodeling of murine SCG neurons and the CB at several time points after MI. Key findings are: (i) After MI, neuronal enlargement takes place and the enlargement is amplified in the neurons within the area bordering the CB (referred to as SCG-CB area); (ii) ChAT and TH are co-expressed in SCG neuronal cells, but not in the CB that expresses only TH and not ChAT; (iii) ChAT intensity is

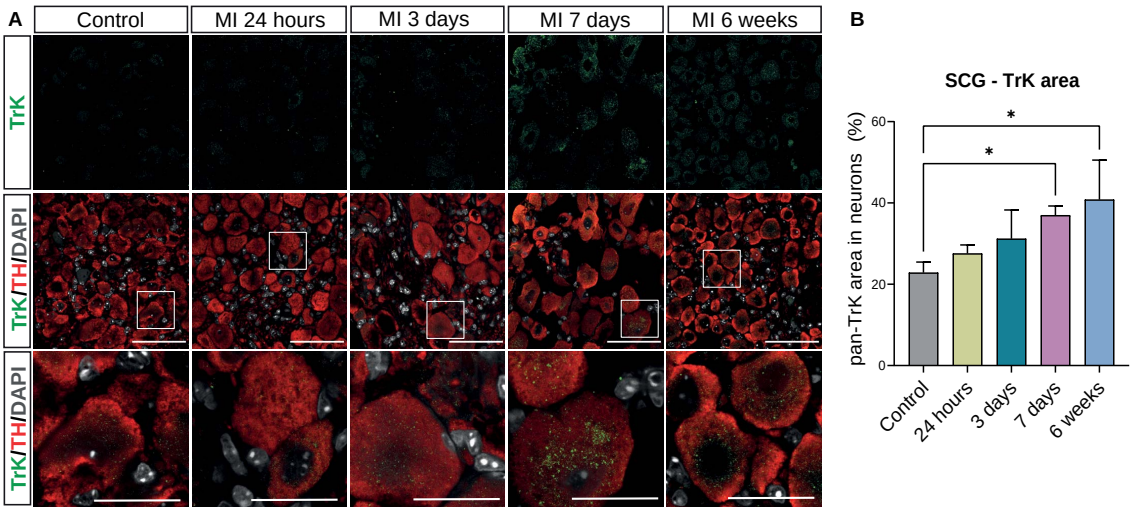

FIGURE 6
Increase in high affinity receptors for BDNF and NGF in SCG neurons after MI. **(A)** Immunofluorescence staining of pan Trk (green), TH (red), and DAPI (nuclei, gray) in SCG of control (n = 4, 8 SCG) mice and mice 24 h (n = 4, 8 SCG), 3 days(n = 2, 4 SCG), 7 days (n = 4, 8 SCG), and 6 weeks (n = 2, 4 SCG) after MI. Scale bar indicated 50 μm and 20 μm in the insets. **(B)** Bar graph displays the timepoint comparison of the percentage, the positively stained area out of the total neuronal area, of pan TrK expression in the SCG after MI. $^{*}P < 0.05$.

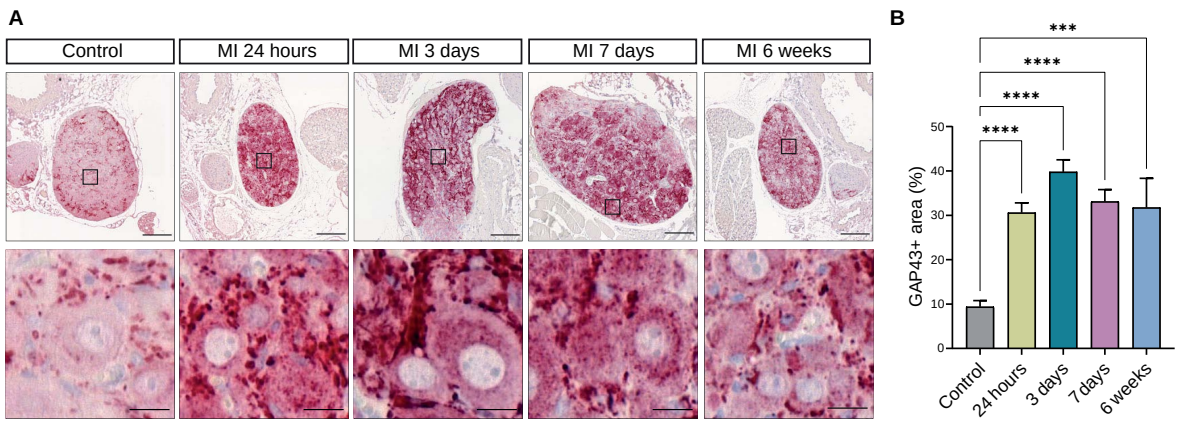

FIGURE 7
Growth Associated Protein 43 reveals neuronal outgrowth after MI. **(A)** Representative images of a GAP43 immunohistochemistry staining in SCG from control mice (n = 4, 8 SCG) and mice 24 h (n = 5, 10 SCG), 3 days (n = 2, 4 SCG), 7 days (n = 5, 10 SCG), and 6 weeks (n = 2, 4 SCG) after MI. Scale bar indicates 100 μm and 20 μm in the insets. **(B)** Bar graph displays the timepoint comparison of the percentage, the positively stained area out of the total area, of GAP43 expression in the SCG after MI. $^{***}P < 0.001$, $^{****}P < 0.0001$.

significantly downregulated 24 h after MI in SCG neurons; (iv) A significantly negative correlation between neuronal cell size and relative ChAT expression was established in the SCG; (v) Expression of neurotrophic factors BDNF and NGF protein and mRNA was increased in the CB and SCG after MI, concomitant with an increase in their TrK-receptor in the SCG; and (vi) An increased expression of GAP43 protein as well as mRNA, indicative of neuronal remodeling resulting in hyperinnervation after MI.

The role of the autonomic nervous system in post-MI arrhythmogenicity has gained increased attention over the past decades. Whereas vagal innervation is considered cardioprotective, sympathetic overdrive is associated with arrhythmias and sudden cardiac death (32, 33). Remarkably, although nerve tissue is generally notorious for its lack of regeneration capacity in adults, after cardiac damage the intriguing phenomenon of cardiac sympathetic hyperinnervation has been reported in multiple animal species, suggesting a renewed capacity of neuronal outgrowth of sympathetic neurons (34, 35). In line with this, several studies in human, rat, rabbit and pig indicated neuronal and electrical remodeling in the stellate ganglia after MI (9, 13, 14,

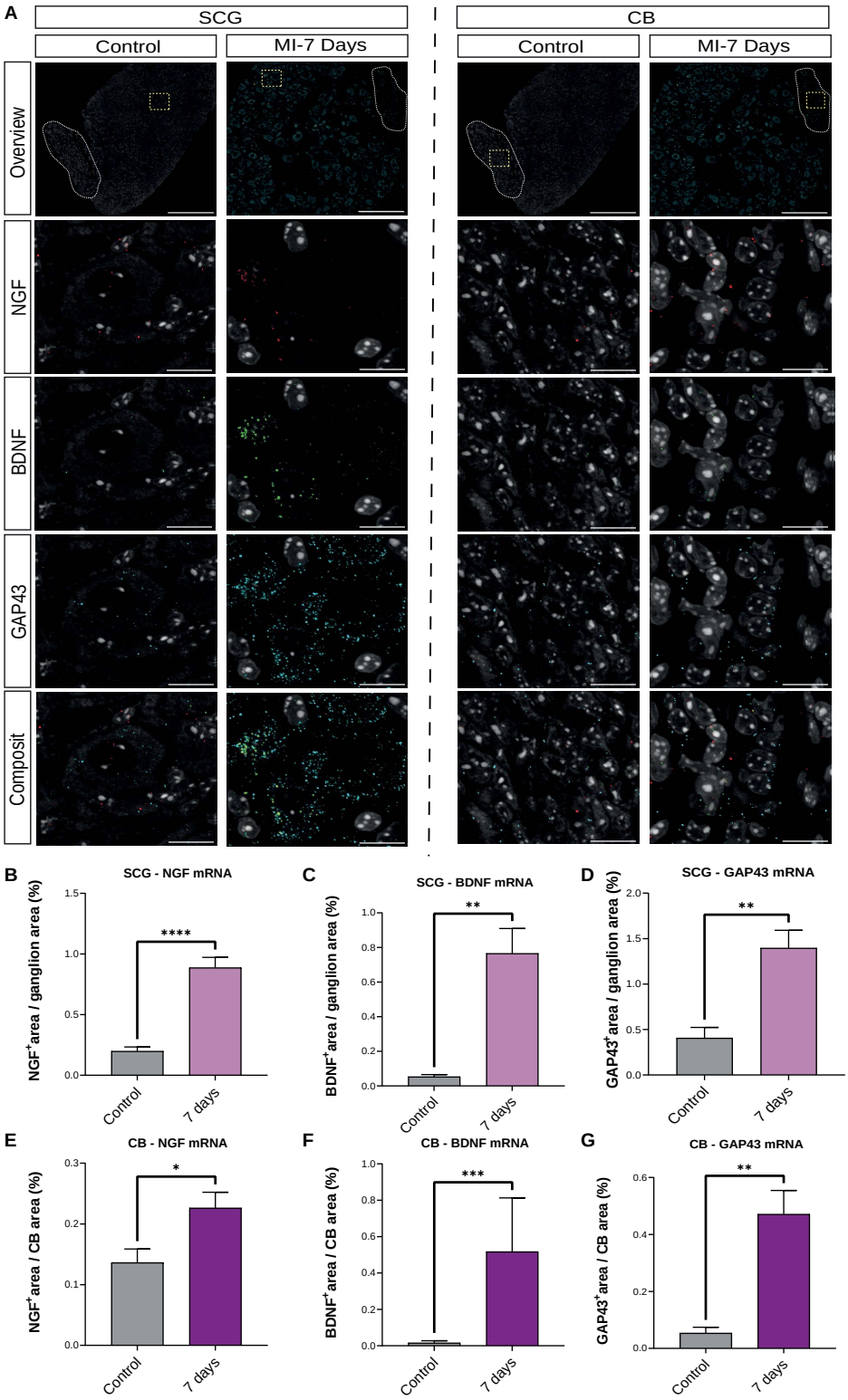

FIGURE 8

mRNA levels of BDNF, NGF and GAP43 are elevated 7 days post-MI. **(A)** Representative HCR-RNA FISH images of NGF, BDNF and GAP43 in the SCG (left panel) and CB (right panel) in control (n = 3, 6 SCG) and 7 days (n = 4, 8 SCG) after MI mice. Scale bar indicates 100 μm in the overview panels and 20 μm in the enlarged panels. **(B–D)** Bar graphs display the percentage, the positively stained area out of the total area, of NGF, BDNF and GAP43 in control SCG compared to 7 days after MI. **(E–G)** Bar graphs display the percentage, the positively stained area out of the total area, of NGF, BDNF and GAP43 in control CB compared to 7 days after MI. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

36). In addition to the stellate ganglion and upper thoracic ganglia, the cardiac plexus also receives contributions from sympathetic nerves derived from the SCG that participate in cardiac ventricular innervation in both human and mouse (8, 17, 37). However, in contrast to the stellate ganglion, data on the time course of neural remodeling in the SCG – that is bordering the oxygen- and PH- sensing CB – after MI is still limited.

Expression of choline acetyltransferase in the sympathetic superior cervical ganglia

Sympathetic neuronal cells are classically considered as adrenergic cells, expressing the rate-limiting enzyme TH, that plays a pivotal controlling role in the synthetic pathway of catecholamines (adrenergic neurotransmitters) (38). In contrast, ChAT is the enzyme that catalyzes the synthesis of acetylcholine (cholinergic neurotransmitter in the peripheral nervous system) (39) and is generally considered as a marker for parasympathetic neurons. Remarkably, we observed that almost all neuronal cells co-express TH and ChAT in the murine SCG. The antibodies that were used stained the CB glomus type I cells (TH positive and ChAT negative) and the parasympathetic nodose ganglion (TH negative and ChAT positive), validating the specificity of the TH and ChAT antibodies. This was further supported by the detection of ChAT mRNA and protein, in both the cytoplasm and the nucleus, in the SCG at different timepoints after MI. In addition, neurons co-expressing TH and ChAT were also observed in human sympathetic ganglia in the current study.

These findings contradict with what has been reported in rat and pig sympathetic ganglia, where only few neurons are either bi-phenotypic or ChAT positive (14, 40), and no ChAT expressing neurons were observed in rabbit sympathetic ganglia (13). Of interest, in rat, the presence of an alternative splice variant of common ChAT (cChAT), that lacks exons 6–9, has been shown. This splice variant favors the nerves and neurons within the peripheral nervous system and is therefore called peripheral ChAT (pChat) (41). In the dorsal root ganglia, pChAT has been shown to possess sufficient enzyme activity to supply the neurons with acetylcholine (42). Whether this pChAT has similar functions in the SCG is yet to be determined. These data indicate that phenotype differences in sympathetic neurons among species may exist. Alternatively we hypothesize that, taking into account the different ChAT splice variants, the target of the ChAT antibody may be a determining factor at play. Nevertheless, with regard to the ChAT and TH expression profile, the mouse model seems to resemble human ganglia, thus holding potential as an adequate model to study processes of transdifferentiation (i.e., switch in neuronal phenotype) after cardiac damage, as has been described in animal species as well as in human (43–45).

The SCG does not solely give input to the anterior part of the heart, but has many connections to different glands, vessels and muscles, indicating that it needs to be able to exert multifunctional signals. This is underlined by studies of Matsumoto et al. who detected up to quadruple function, in which these neurons would exert cholinergic, adrenergic, purinergic and non-adrenergic excitatory effects (46). The connection of the SCG to the parasympathetic nodose ganglion has been previously shown and although its function is still unclear, it is considered as a potentially relevant gateway for interaction between sympathetic and parasympathetic neurons (25).

Neuronal remodeling after acute myocardial infarction

MI timepoint analysis demonstrated neuronal enlargement in the SCG, especially in neurons in the region bordering the CB. Within these neurons a significant decrease in ChAT intensity at 24 h after MI was observed. Remarkably, the level of ChAT showed a negative correlation with neuronal size. We speculate that, as previously shown in heart failure patients, the neuronal enlargement in the SCG are -due to the swelling- harder to excite and thereby contribute to the withdrawal of parasympathetic effects leading to a more pro-arrhythmic environment (47). In addition, we observed a loss of nuclear ChAT in a subpopulation of mice after MI. It has previously been reported that the 69 kDa isoform of ChAT can shift between the cytoplasmic and nuclear compartments. Once located in the nucleus, ChAT can act as a transcriptional activator of high affinity choline transporter 1 (CHT1) which is also involved in the regulation of acetylcholine synthesis in neurons (48). The nuclear localization has also been shown to sustain epigenetic regulations of neuronal structures (49), which might also further mediate the neuronal remodeling observed in the SCG after MI. We speculate that the stress response upon MI induced a loss of nuclear ChAT, which has been observed in 2 out of 5 mice (**Supplementary Figure 4**), thereby disrupting the balance between autonomic sympathetic and parasympathetic regulation.

Neurotrophic factors and role of the carotid body

The CB is a neural crest derived structure located at the carotid bifurcation and is the main peripheral chemoreceptor in mammals (26). It can sense and respond to changes in blood flow, O_2- and CO_2 levels, PH as well as changes in metabolites such as glucose and lactate (18, 50). Neuron-like glomus cells in the CB express a wide range of growth factors and neurotrophic factors during development (27). In our timepoint analysis, after MI an increase in both protein and mRNA expression of neurotrophic factors (BDNF and NGF) in the CB and SCG was observed. Moreover, an upregulation of the pan TrK (including the high affinity receptors of BDNF and NGF) receptors in the SCG neurons was observed. Surprisingly, the TrK expression in SCG neurons was maintained at high levels at 6 weeks post-MI

with a significant difference compared to control (**Figure 6B**). Results indicate that neuronal remodeling can be influenced by neurotrophic factors via paracrine and/or autocrine effects.

Although neurotropic factors and receptors were upregulated, the question arose whether this could actually contribute to the development of hyperinnervation after MI. GAP43, a growth-associated protein, participates in the developmental regulation of axonal growth and the formation of new synapses, neurite outgrowth, and synaptogenesis after injury (51–53). This might be related to its function in growth cones by stabilizing F-actin, preventing actin polymerization and promoting microtubule-based neurite outgrowth (52, 54, 55). Its transcriptional expression is lowly expressed in mature neurons, but up-regulated in differentiating and regenerating neurons (56). It is thereby a suitable marker to examine the neo-outgrowth during development or after damage. In addition to previous findings of GAP43 expression in sprouting axons in the infarcted heart (57), in the present study we showed a striking upregulation of the GAP43 expression in SCG neurons post-MI starting from as early as 24 h after MI compared to control.

Strengths and limitations

The strength of this study is that for the first time an elaborate timeline is presented of SCG and CB remodeling post-MI. Key findings have been generated by precise and blinded quantifications with validated antibodies and probes. A limitation is the rather small n-numbers for the different stages, although we strived for a number of at least 8 ganglia for the most relevant stages. As our aim was to study ganglion remodeling after MI at the RNA and protein level, no functional studies were included, and we did not study the paracrine function of neurons or the effects of interventions such as anti-adrenergic therapy or anti-growth factor therapy. Future studies especially aimed at the potential function of ChAT in influencing sympathetic neural activity in the same neurons are warranted.

Conclusion

In conclusion, neuronal remodeling toward an increased adrenergic phenotype occurs in the SCG and is potentially mediated by the CB. This is substantiated by the marked increase in neuronal cell size of the SCG after MI, especially in the region bordering the CB. A significant decrease in ChAT intensity at 24 h after MI was observed and this coincided with a significantly negative correlation with neuronal size. In addition, upregulation of neurotrophic factors and their high affinity receptors, indicate a paracrine/autocrine neurotrophic effect that is accompanied with an increased upregulation of GAP43 in the SCG. These results suggest an interplay of the SCG and CB after MI, that is likely to contribute to pathological cardiac sympathetic hyperinnervation.

Future perspectives

In this time-course study, we show that the cholinergic marker ChAT is expressed in sympathetic neurons of the SCG and that expression of ChAT displays a transition in expression after MI. Further studies are required to study the functional implication of ChAT in adrenergic neurons and the mechanisms behind the changes of ChAT caused by MI. Taking into consideration that the CB could influence SCG neuronal remodeling, as was indicated in the present study, the further exploit of the potential interaction between CB and SCG could empower our integrative understanding of cardiac (hyper)innervation after damage.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by the Animal Ethics Committee of the Leiden University, Leiden, Netherlands.

Author contributions

YG, LR, and MJ designed the experiments. YG, LR, and TG performed the experiments. YG and LR performed data collection and analysis. JG and MJ supervised by data collection and analysis. YG, LR, JG, and MJ wrote the first draft of the manuscript. All authors contributed to the study conception and design and commented on previous versions of the manuscript, and read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.758265/full#supplementary-material

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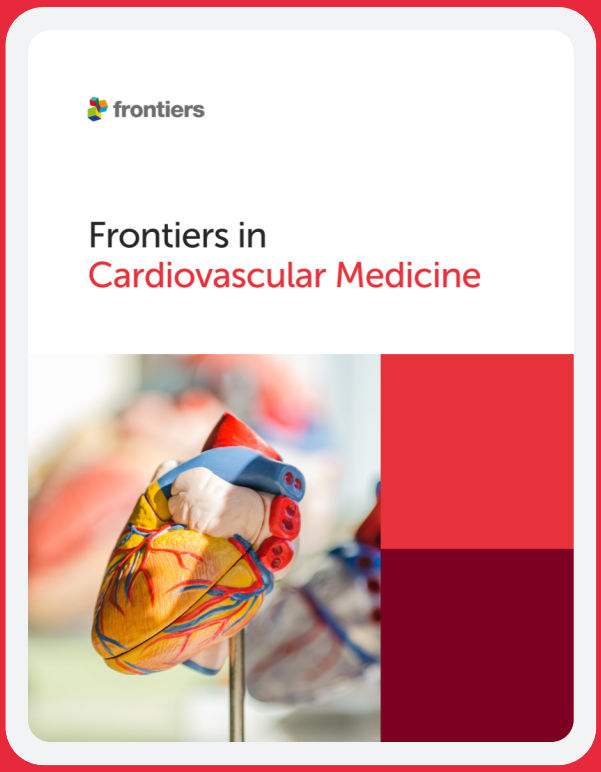

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