NON-CODING RNA AS THERAPEUTIC TARGET: A GAME CHANGER IN CARDIAC REGENERATIVE STRATEGIES?

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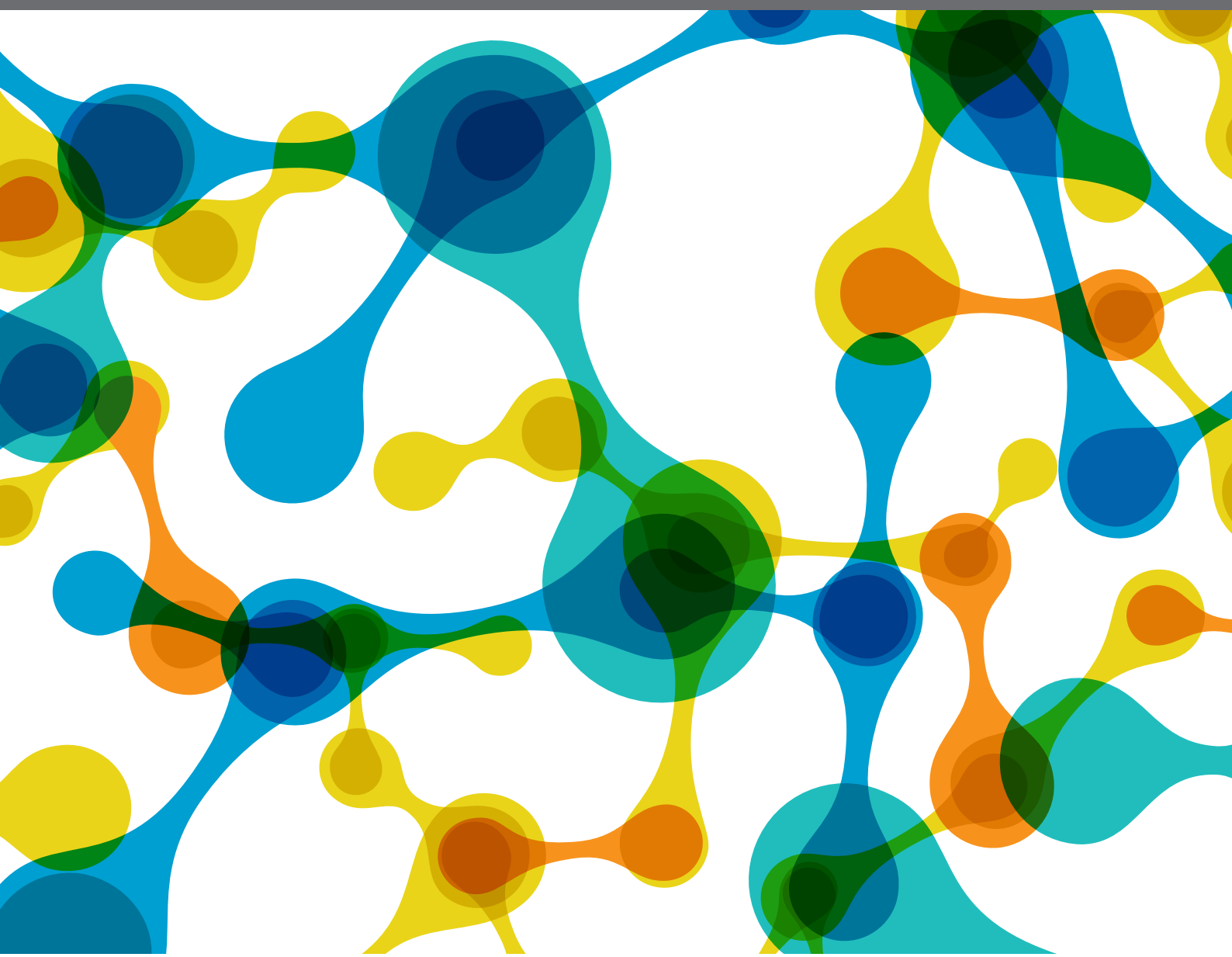

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NON-CODING RNA AS THERAPEUTIC TARGET: A GAME CHANGER IN CARDIAC REGENERATIVE STRATEGIES?

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Editorial Research Topic: Non-coding RNA as Therapeutic Target: A Game Changer in Cardiac Regenerative Strategies?

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Keywords: non-coding RNA, microRNAs, lncRNAs, cardiac regeneration and development, cardiovascular disease, circRNA

Editorial on the Research Topic

Non-coding RNA as Therapeutic Target: A Game Changer in Cardiac Regenerative Strategies?

INTRODUCTION

Cardiovascular diseases are the most common cause of death worldwide (Roth et al., 2017). Though recent advances in therapeutic drug treatments made it possible to better manage the disease and increase the life span of heart failure patients, there is still no option to cure the patient's heart after a myocardial infarction (MI). This results mainly from a lack of cardiac regeneration after massive cardiomyocyte loss due to MI. Adult cardiomyocytes are mostly inable to divide. Their proliferation capacity gets lost after birth when maturation takes place and the cardiomyocyte become quiescent (Tzahor and Poss, 2017). Recent research is focused on deciphering of the molecular switches from neonatal to adult cardiomyocytes, i.e., regenerative vs. post-mitotic states of cardiomyocytes.

Non-coding RNAs (ncRNAs) account for the vast majority of mammalian transcripts and it has been shown in the last two decades that this class of molecules is involved in the regulation of many, if not all, physiological and pathological settings, including cardiac disease and regeneration (Bär et al., 2016; Beermann et al., 2016; Hunkler et al., 2021).

Due to their involvement in many different pathological settings, ncRNAs serve as attractive therapeutic targets. Synthetic ncRNA mimics can be used to restore decreased function, whereas anti-sense oligonucleotides can be used to inhibit disease-promoting functions. Several ncRNA-based drugs are already in clinical trials, including those to target pathological heart conditions (Huang et al., 2020).

This Frontiers Research Topic entitled "Non-coding RNA as Therapeutic Target: A Game Changer in Cardiac Regenerative Strategies?" has collected nine contributions from experts who showcase recent findings of different aspects of cardiac regeneration and provide novel insights into the major subtypes of ncRNAs, particularly, long non-coding RNA (lncRNA), microRNA (miRNA), and circular RNA (circRNA) biology and their regulation in cardiovascular disease.

FEATURED PUBLICATIONS

In the last decade, the miRNA-family 212/132 was shown to be crucial for the development of cardiac remodeling after pressure overload. Transgenic overexpression in cardiomyocytes led to the

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development of cardiac hypertrophy, whereas miR-212/132-knockout mice were protected (Ucar et al., 2012). In this Research Topic, Lei et al. investigated the effect of miR-212/132 knockout in mice after myocardial infarction (MI). Under baseline conditions cardiac contractile function in miR-212/312 knockout mice was improved, whereas 1 day and 4 weeks after MI no significant difference was found. These findings are partially surprising as Ucar et al. (2012) observed no differences in cardiac functions between knockout (KO) and wildtype (WT) mice under basal conditions and several other reports highlight cardiac improvement after miR-212/132 knockout in MI (Foinquinos et al., 2020; Batkai et al., 2021). These contradictory findings could be due to timing issues. Moreover, in a recent publication Lei et al. (2021) show that in a mouse model of pressure overload miR-212/132 knockout leads to improved cardiac function in comparison to WT animals. Importantly, the underlying mechanisms seem to be conserved in mammals, as therapeutic targeting of the miR-212/132 family in mice and pigs showed promising results for the treatment of heart failure (Foinquinos et al., 2020; Batkai et al., 2021). Based on these data, the worldwide first miRNA-antisense therapy targeting miR-132 in patients with chronic heart failure was launched and showed encouraging results (Täubel et al., 2021), underlining the outstanding role of miR-132 in the cardiac remodeling process.

Atherosclerosis is a leading cause of death and disability. Recent research showed that abnormal proliferation of vascular smooth muscle cells (VSMC) has a critical role in the formation of atherosclerotic lesions. One therapeutic option is to target mitogenic-induced proliferation of VSMCs. Tian et al. addressed this question by generating an expression profile of circRNAs in VSCMs treated with the mitogen PDGF-BB. They not only found 112 differentially expressed circRNAs, but also investigated the circRNA parental genes. Furthermore, Tian et al. provided bioinformatics-based analysis of a regulatory relationship between differentially regulated circRNA and VSCM-related miRNA paving the way for future therapeutic options.

Desjarlais et al. describe a RNA-sequencing approach to identify deregulated miRNAs in bone marrow-derived proangiogenic cell (PAC) after exposure to cardiovascular risk factors. PACs are important for postnatal neovascularization and thus play a crucial role in the handling of peripheral artery disease (Asahara et al., 1999). Interestingly, it became evident that several miRNAs were affected in all exposed conditions, indicating a general pattern to target for therapy.

A detailed and in-depth review and meta-analysis from Zhai et al. focused on studies from the last decade of miRNAs circulating in the blood after acute myocardial infarction (AMI). First, the authors summarize the findings from the selected publications. Next, the authors re-assessed the reported results. Even though they found that cardiomyocyte-specific miR-499 had better diagnostic accuracy than other single miRNAs, they still suggest to test a panel of several miRNAs rather than a single one in the future to serve as diagnostic markers for AMI.

Restricted blood flow in the heart due to blockage of the artery can lead to myocardial infarction and cardiac cell death. To overcome this problem, promoting cardiac neovascularization has been in the focus of recent research. In the review article of Kesidou et al. the process of neovascularization and the possibilities to target this process after MI is discussed. Recently, it has been shown that extracellular vesicles (EV) are important for cell-cell communication. Kesidou et al. describe current developments in targeting neovascularization using miRNAs and EV-bound miRNAs.

The review of Yuan and Krishnan summarizes the current knowledge on ncRNAs in cardiac regeneration and its potential to treat heart failure through the induction of cardiac regeneration. The role of miRNAs in cardiac differentiation, cardiomyocyte proliferation, cardiac reprogramming, and cardiomyocyte survival is described in detail, since this class of ncRNAs was the first discovered and many studies have been conducted since then. In addition, lncRNAs and circRNAs are described as important players in the field of cardiac regeneration, which becomes evident by the raising numbers of published manuscripts. Furthermore, the authors summarize recent attempts and successes of novel therapeutic strategies to treat heart failure through ncRNA-driven regeneration.

Santos et al. focus specifically on the role of lncRNA in cardiomyocyte proliferation and cardiac regeneration. The authors state, that molecular and physiological alterations during the aging of the heart facilitates the development of heart failure. This provides a potential role for lncRNAs as targets for therapeutic options. Furthermore, the possibility of direct reprogramming of endogenous cardiac fibroblasts to cardiomyocytes is discussed in order to replace damaged cells in the heart.

Another important aspect of lncRNAs and cardiac regeneration is the regulation of metabolic signaling. The switch from neonatal to adult cardiomyocytes is accompanied by a change of energetic source. The review of Correia et al. highlights findings from a possible molecular interplay of lncRNAs and metabolic signaling in regard to cardiac regeneration.

Complementing the other reviews in this Research Topic, the article of Mester-Tonczar et al. summarizes the function of circRNAs in cardiac regeneration. The authors give a comprehensive overview about circRNA biogenesis and their function in general. Later, they focus on their role in the setting of cardiomyocyte biology, cardiovascular diseases, and a possible therapeutic potential in this field. Mester-Tonczar et al. address in detail the implication of cirRNAs in MSC-derived and iPSC-derived cardiomyocytes and show the obstacles to cope with when studying circRNA biology.

SUMMARY

Non-coding RNAs have been shown to be involved in several pathological and physiological settings, including cardiovascular diseases and cardiac regeneration. In this Research Topic, the reports highlight the current knowledge and provide state-of-the-art data. In addition, obstacles and future

challenges in ncRNA research and regenerative medicine are discussed.

AUTHOR CONTRIBUTIONS

JL and CB wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Differential Expression and Bioinformatics Analysis of CircRNA in PDGF-BB-Induced Vascular Smooth Muscle Cells

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Atherosclerosis is mediated by various factors and plays an important pathological foundation for cardiovascular and cerebrovascular diseases. Abnormal vascular smooth muscle cells (VSMCs) proliferation and migration have an essential role in atherosclerotic lesion formation. Circular RNAs (circRNA) have been widely detected in different species and are closely related to various diseases. However, the expression profiles and molecular regulatory mechanisms of circRNAs in VSMCs are still unknown. We used high-throughput RNA-seq as well as bioinformatics tools to systematically analyze circRNA expression profiles in samples from different VSMC phenotypes. Polymerase chain reaction (PCR), Sanger sequencing, and qRT-PCR were performed for circRNA validation. A total of 22191 circRNAs corresponding to 6273 genes (host genes) in the platelet-derived growth factor (PDGF-BB) treated group, the blank control group or both groups, were detected, and 112 differentially expressed circRNAs were identified between the PDGF-BB treated and control groups, of which 59 were upregulated, and 53 were downregulated. We selected 9 circRNAs for evaluation of specific head-to-tail splicing, and 10 differentially expressed circRNAs between the two groups for qRT-PCR validation. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analyses enrichment analyses revealed that the parental genes of the circRNAs mainly participated in cardiac myofibril assembly and positive regulation of DNA-templated transcription, indicating that they might be involved in cardiovascular diseases. Finally, we constructed a circRNA-miRNA network based on the dysregulated circRNAs and VSMC-related microRNAs. Our study is the first to show the differential expression of circRNAs in PDGF-BB-induced VSMCs and may provide new ideas and targets for the prevention and therapy of vascular diseases.

Keywords: cardiovascular disease, RNA-seq, circRNA, VSMC, ceRNA

Abbreviations: α-SMA, smooth muscle actin-α; AS, Atherosclerosis; ceRNAs, competitive endogenous RNAs; circRNA, circular RNA; DE circRNAs, differentially expressed circRNAs; ECM, extracellular matrix; miRNA, microRNA; NC group, the blank control group did not receive PDGF-BB; ncRNAs, noncoding non-coding RNAs; PS group, Cells were treated with PDGF-BB; SM22α, smooth muscle 22α; VSMCs, Vascular smooth muscle cells.

INTRODUCTION

Atherosclerosis (AS) is the main pathological basis of cardiovascular and cerebrovascular diseases and is mediated by various factors. With socioeconomic development, the morbidity and mortality of cardiovascular diseases are increasing worldwide; consequently, cardiovascular disease has become one of the important diseases threatening public health, and its causes and pathomechanism are not yet clear (Zhao et al., 2019). Vascular smooth muscle cells (VSMCs) are the major cellular components of the blood vessel wall, where they exist in a differentiated contractile phenotype to respond to arterial contraction and to produce extracellular matrix (ECM; Basatemur et al., 2019). Accumulating evidence shows that abnormal VSMC proliferation and migration have an essential role in atherosclerotic lesion formation. Genetic lineage tracing studies have illustrated that in atherosclerotic plaques, especially progressing plaques, extensive lipids are released by damaged or dying macrophages, and VSMCs. Then, accumulating lipid infiltration appeared in the center of the plaque, forming the necrotic core. VSMCs migrate and proliferate to the surrounding of the necrotic core and play an important role in creating a fibrous cap that stabilizes the atherosclerotic plaque (Feil et al., 2014; Misra et al., 2018). Moreover, VSMCs can differentiate into many other cell types found in the plaque core, suggesting that these cells might participate in multiple processes underlying atherosclerotic plaque stability (Tang et al., 2012; Ackers-Johnson et al., 2015; Durham et al., 2018; Wang et al., 2019).

An increasing body of evidence shows that the expression level of contractile SMC markers decreased is related with injury and inflammation, and which is associated with the reduced expression of MYOCD (a key factor regulating the contractile VSMC state in the development of plaques; Ackers-Johnson et al., 2015). *In vitro*, studies have demonstrated that SMCs, stimulated by growth factors, oxidative stress, and inflammatory cytokines, can phenotypically switch into proliferating, and/or migrating cells. Among them, platelet-derived growth factor (PDGF-BB) is considered to be one of the most effective mitogens in the proliferation and migration of VSMC, which can initiate various biological effects by activating intracellular signal transduction pathways and play a significant role in regulating the proliferation and migration of VSMC (Heldin and Westermark, 1999; Dzau et al., 2002; Shawky and Segar, 2017). Consequently, it will be necessary to find a new target to inhibit PDGF-mediated VSMC proliferation and migration which will exert an important therapeutic intervention in AS development.

Non-coding RNAs (ncRNAs) are a group of biomolecules acting as pivotal regulators that play powerful and diverse roles in pathological and physiological processes (de Almeida et al., 2016). Their gene expression patterns can also reveal changes in biological pathways that correlate with disease progression or even the risk of disease progression (Bayoumi et al., 2016; Zhang et al., 2019). Circular RNAs (circRNAs) are an emerging group of ncRNAs that are ubiquitous, stable, and evolutionarily conserved in eukaryotes (Memczak et al., 2013). Though the phenomenon of RNA cyclization was first reported in the 1970s (Sanger et al., 1976), circRNAs were considered as byproducts of aberrant splicing during transcription and remained underappreciated. As RNA sequencing technologies evolve, accompanied by the development of computational algorithms, numerous circRNAs have been discovered (Salzman et al., 2012). Notably, circRNAs have been found extensively in different species and are closely related to various diseases, including cardiopathy, which has a great impact on human health (Burd et al., 2010; Holdt et al., 2016; Huang et al., 2019). ncRNAs, especially microRNAs (miRNAs), and circRNAs, can function as competitive endogenous RNAs (ceRNAs) which can construt gene regulatory networks to regulate the expression of multiple genes with spatiotemporal specificity. Given the characteristics of ncRNAs, they could have great potential application in the treatment of diseases. However, the circRNA expression profiles and whether circRNAs participate in the regulatory of VSMCs still remain not clear. In the present study, we aimed to perform high-throughput RNA sequencing in paired PDGF-BB-treated VSMCs (PS) and a normal control group (NC) to investigate VSMC-specific circRNA profiles, as well as potential functional characterization of the representative candidate circRNAs. Our study is the first to show the differential expression of circRNAs in PDGF-BB-induced VSMCs and may provide new ideas and targets for the prevention and therapy of vascular diseases.

MATERIALS AND METHODS

Cell Cultures

Primary human aortic smooth muscle cells (HASMCs; ScienCell, United States) were cultured with Smooth Muscle Cell Medium (SMCM; ScienCell, United States) in a humidified incubator with 5% CO_2 at 37°C. HASMCs were inoculated at a density of 3×10^5 cells/well in 6-well culture plates overnight. Before the subsequent experiments, cells were made quiescent by starvation for 24 h and then treated with 10 ng/ml platelet-derived growth factor (PDGF-BB; Sigma-Aldrich) for 48 h. The blank control group did not receive PDGF-BB. Each group had three samples.

Immunofluorescent Staining

Human aortic smooth muscle cells treated with PDGF-BB and the contol were stained for a-SMA (abcam, United States). Brifly, cells of two groups were fixed with 4% paraformaldehyde for 20 min or overnight at 4°C, then, washed for 20 min in 0.5% Triton X-100 (Solarbio, China). Afterward, cells were blocked in 2% BSA for 30 min, then, washed in phosphate-buffered saline (PBS) for twice/10 min each time. Primary antibodies (a-SMA, 1:400 dilution) were added over night at 4°C, rinsed 3 times/10 min each time in PBS the following day, and subsequently the secondary antibody (1:1000 dilution) incubate at 37°C for 1 h. After incubation, the cells were washed 3 times with PBS, for 10 min each. Cell nucleus were labeled using DAPI for 5 min, then washed 3 times (5 min each) in PBS. Finally, cells were imaged with a fluorescent microscope.

RNA Library Construction and Sequencing

According to manufacturer's instructions, total RNA was isolated from each sample using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). The amount and purity of the total RNA were quantified using NanoDrop ND-1000 (NanoDrop, Wilmington, DE, United States), and the integrity of total RNA was assessed by Agilent 2100 with RIN number >7.0. Then circRNA library was constructed. Firstly, approximately 5 μg RNA was subjected to ribosomal RNA depletion with the Ribo-Zero^{TM} rRNA Removal Kit (Illumina, San Diego, CA, United States). Secondly, linear RNAs were removed with RNase R (Epicentre Inc., Madison, WI, United States) to enrich circRNAs (3U RNase R for per μg RNA). Finally, the RNA fragmentation was obtained using divalent cations under high temperature for reverse-transcribed to generate first-stranded cDNA, then, the second-stranded DNAs were next synthesized with *Escherichia coli* DNA polymerase I, RNase H, and dUTP. To constrcut strand-specific cDNA, we added specificity terminal amino modification of the DNA fragment ends to prepare them for ligation to the adapters. After amplified by Polymerase chain reaction (PCR), the library was purified and the average insert size was 300 bp (± 50 bp). Finally, paired-end were sequenced on an Illumina HiSeq 4000 (LC Bio, China) according to the recommended protocol. The sequencing data used and/or analyzed during the current study are available in NCBI databases. (BioProject PRJNA607375).[1]

Bioinformatics Analysis

Low-quality and undetermined bases was removed and sequence quality was verified using FastQC[2]. Then, we used Bowtie2 and Tophat2 to map reads to the reference genome. CIRCExplorer and TopHat-fusion were utilized for *de novo* assembly of the mapped reads to circRNA and recognizing back splicing reads in unmapped reads. All samples generated unique circRNA. The differentially expressed circRNAs with log2 (fold change) >1 or log2 (fold change) <−1 and with statistical significance (p value <0.05) by R package–edgeR were selected for further studies.

CircRNA Validation by PCR

Polymerase chain reaction was used to validate the reliability of the high-throughput RNA sequencing data. A Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) was used for reverse transcription of circRNAs. According to manufacturer's instructions, appropriate volume of master mix as well as RNA sample were prepared, then the reaction for reverse transcription was initiated at 25°C for 10 min, 55°C for 30 min, and 85°C for 5 min. Then, cDNA and gDNA templates were PCR amplified for 35 cycles using Taq PCR MasterMix (Tiangen, China) following the manufacturer's protocol, and PCR products were visualized using 2% GelRed-stained agarose gel. To confirm the PCR results, we further performed Sanger sequencing to directly examine the PCR product. To verify the accuracy of the differential expression of circRNAs, qRT-PCR was conducted using a FastStart Universal SYBR Green Master Kit (Roche, Germany). Briefly, the first strand cDNA was synthesized using random hexamer primer and then amplified by SYBR Green Kit following the standard procedure that is denaturation 95°C (10 min) followed by amplification by a total of 40 cycles of 95°C (15 s) and 60°C (1 min) on an ABI7500 system (Applied Biosystems, Foster City, CA, United States). GAPDH was used as an internal control, and PCR primers are listed in **Supplementary Table S1**.

GO and KEGG Pathway Analyses

The differentially expressed circRNA-host gene data were analyzed by the DAVID tool (V6.8; Huang da et al., 2009) with its GO function enrichment and KEGG pathway analyses. An enrichment gene count ≥2 and hypergeometric test significance threshold P value <0.05 were considered to indicate significant enrichment.

Interaction Between CircRNA and miRNA

Vascular smooth muscle cell-associated miRNAs were selected from disease-miRNA interactions validated in previous studies (Leeper and Maegdefessel, 2018; Wang and Atanasov, 2019). For the obtained VSMC-related miRNAs, we predicted whether there was a regulatory relationship between them and the selected differentially expressed circRNAs. We used miRanda and TargetScan to predict the relationships between the VSMC-related miRNAs and the differentially expressed circRNAs, and the Cytoscape tool was used to construct a network map of target miRNAs and circRNAs.

Statistical Analysis

Data were analyzed and visualized with SPSS 22.0 (IBM Corporation, Somers, NY, United States) and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, United States). Data are presented as the mean ± standard deviation. Wilcoxon rank-sum test, Student's t-test and fold change were used to analyze the significant differences between the sequencing data of samples. A t test was applied to compare qRT-PCR results. Differences with p <0.05 were considered statistically significant.

RESULTS

CircRNA Expression Profiles in PDGF-BB-Treated VSMCs

Cells were treated with 10 ng/ml PDGF-BB (PS), and the blank control group did not receive PDGF-BB (NC). After stimulation for 48 h, morphological changes and the expression levels of SM22α and α-SMA in the two groups were detected and are shown in **Supplementary Figures S1A–C**. As expected, VSMCs tended to phenotypically switch to function as proliferative and/or migratory cells in response to stimulation by PDGF-BB (Heldin and Westermark, 1999; Allahverdian et al., 2018). Compared to the NC, the cell morphology became spindle-shaped and elongated, and the expression of differentiation-associated genes and proteins was decreased in the PS, suggesting

[1] https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA607375

[2] http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

that the cells had a stronger capacity for proliferation. In the meantime, 7 known SMC markers were detected in NC and the PS group and we added a heatmap of the differential mRNA expression between the two groups (**Supplementary Figure S1D**). The result showed that the synthesis markers of KRT8 and TLR4 were significantly increased and contractile markers including MYH11, SMTN, CNN1, SMM22α, and α-SMA were reduced in PDGF-BB treated HASMC, which corresponded to the privious study. These studies fully confirmed that the cells gain a proliferative phenotype after treatment with PDGF-BB.

To investigate the effects of PDGF-BB on circRNA expression in VSMCs, two groups of HASMCs were prepared for high-throughput sequencing using an Illumina HiSeq 4000 (LC Bio, China). We used CIRCExplorer to *de novo* assembly of the mapped reads and to identify back splicing reads. The following criteria were restricted for circRNA identification: (1) mismatch ≤ 2; (2) back-spliced junction reads ≥ 1; and (3) two splice sites <100 kb apart on the genome. Accordingly, a total of 22191 circRNAs, corresponding to 6273 genes (host genes) in the PS, NC, or both groups, were detected, of which 7322 and 7870 circRNAs were specifically expressed in the NC and PS groups, respectively. A total of 6999 circRNAs were identified in both the PS and NC groups (**Figure 1A**). Further analysis revealed that three categories of circRNAs were represented: exons (94.06%), introns (5.43%), and intergenic regions (0.50%; **Figure 1B**). The results showed that the majority of the circRNAs originated from protein-coding exons. By analyzing the sequencing data, we identified 5794 circRNAs recorded in circBase[3] and 16397 novel circRNAs that were discovered in this study. The chromosomal distribution of all circRNAs showed that these circRNAs were distributed on almost all human chromosomes (**Figure 1C**).

By the criteria of log2 (fold change) >1 or log2 (fold change) <-1 and p value <0.05, we identified 112 differentially expressed circRNAs between the PS and NC groups, of which 59 were upregulated, and 53 were downregulated. The DE circRNAs in the samples of the two groups are displayed with a Volcano plot, bar graph, and heatmap. Additionally, the chromosome distributions of the DE circRNAs are shown in the bar graph (**Figures 2A–D**). The top ten upregulated and downregulated circRNAs are listed in **Table 1**.

Validation of VSMC-Enriched CircRNAs

To verify the accuracy of the RNA-seq data, 9 circRNAs (circRNA2637, circRNA4624, circRNA4487, circRNA3875, circRNA4209, circRNA5591, circRNA5550, circRNA5497,

[3]http://circrna.org/

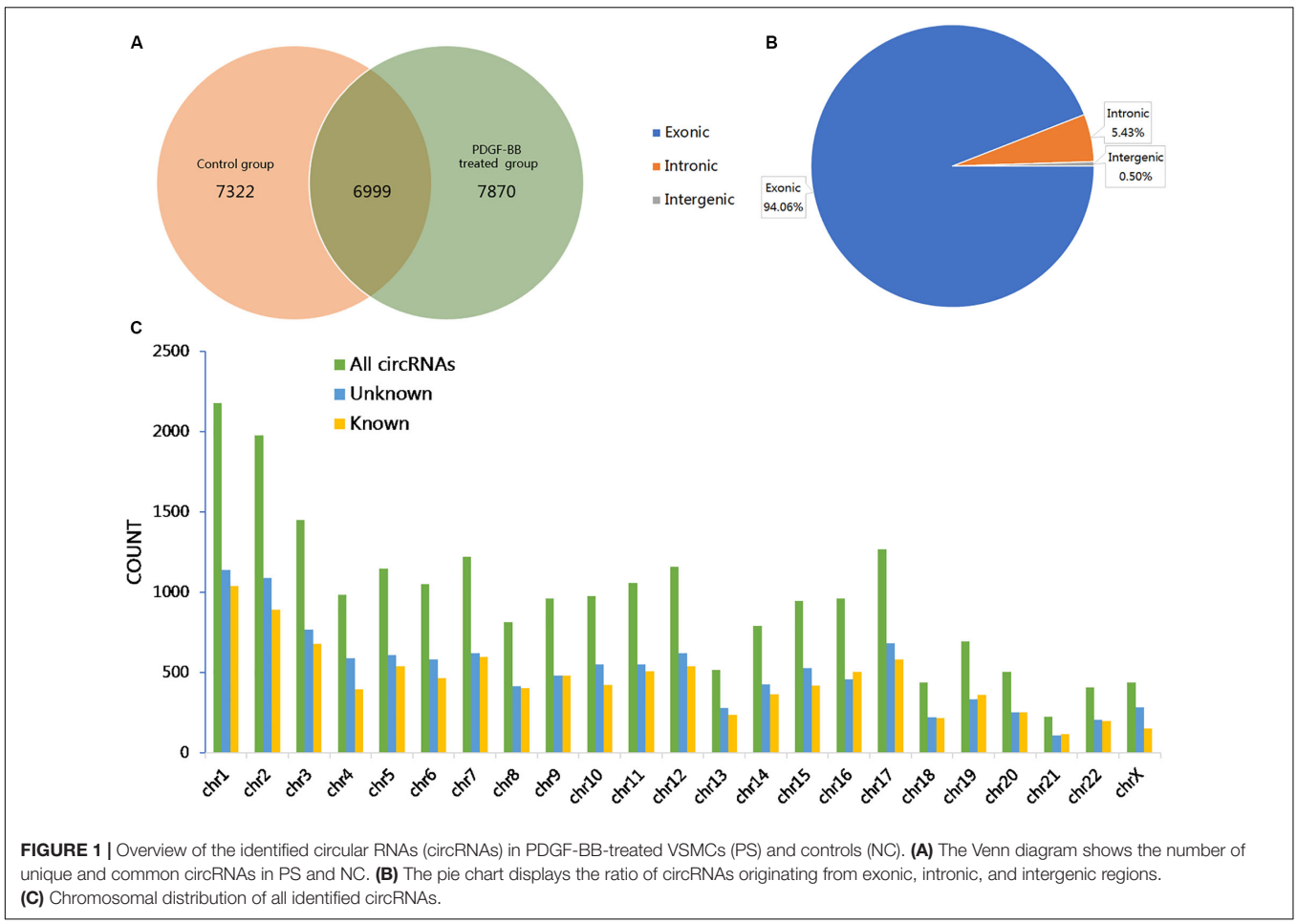

FIGURE 1 | Overview of the identified circular RNAs (circRNAs) in PDGF-BB-treated VSMCs (PS) and controls (NC). **(A)** The Venn diagram shows the number of unique and common circRNAs in PS and NC. **(B)** The pie chart displays the ratio of circRNAs originating from exonic, intronic, and intergenic regions. **(C)** Chromosomal distribution of all identified circRNAs.

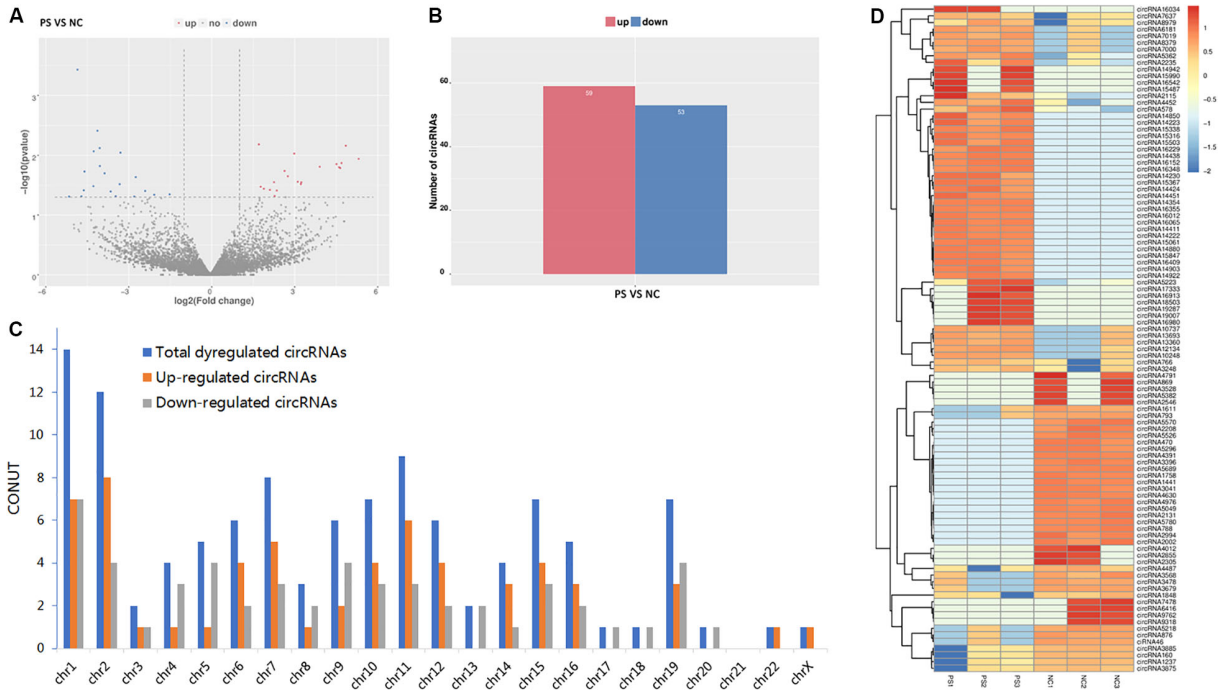

FIGURE 2 | Differentially expressed (DE) circRNAs in PS and NC. **(A)** and **(B)** display the volcano plot and bar graph for the DE circRNAs in the samples of the two groups. **(C)** The chromosome distributions of differentially expressed circRNAs. **(D)** The DE circRNAs and samples are coclustered by hierarchical clustering analysis. The upper maps are based on DE circRNAs with log2 (fold change) >1 or log2 (fold change) <-1 and *p* value <0.05 for the comparisons of PS vs NC.

TABLE 1 | Top ten upregulated and downregulated circRNAs.

CircRNA Name	Gene symbol	Regulation	Annotation	Chromosome	Fold change (log2)	*P* value
CircRNA12134	AC004922.1	Up	Exon	Chr7	5.312	0.011
CircRNA6181	SSH1	Up	Exon	Chr12	4.847	0.007
CircRNA7019	AP1G1	Up	Exon	Chr16	4.691	0.013
CircRNA7000	SNTB2	Up	Exon	Chr16	4.646	0.016
CircRNA8379	HMGCR	Up	Exon	Chr5	4.605	0.016
CiRNA10248	CDR1	Up	Exon	ChrX	4.509	0.014
CircRNA10737	HINFP	Up	Exon	Chr11	3.905	0.016
CircRNA13693	SOX13	Up	Exon	Chr1	3.238	0.028
CircRNA13360	GIGYF2	Up	Exon	Chr2	3.222	0.030
CircRNA8979	HPCAL1	Up	Exon	Chr2	3.111	0.028
CircRNA5297	TBCE	Down	Exon	Chr1	−5.159	0.049
CircRNA876	CCNY	Down	Exon	Chr10	−4.855	0.000
CircRNA198	PCCA	Down	Exon	Chr13	−4.714	0.049
CircRNA5218	NEK7	Down	Exon	Chr1	−4.629	0.039
CircRNA3568	PRELID2	Down	Exon	Chr5	−4.602	0.019
CircRNA3679	AFAP1	Down	Exon	Chr4	−4.283	0.033
CircRNA1611	IST1	Down	Exon	Chr16	−4.270	0.009
CiRNA46	SRF	Down	Intron	Chr6	−4.130	0.004
CircRNA4487	MITD1	Down	Exon	Chr2	−4.052	0.008
CircRNA793	ZMYND11	Down	Exon	Chr10	−4.046	0.015

and circRNA5223) were randomly selected for validation experiments. We used PCR to evaluate specific head-to-tail splicing. First, divergent (circular) and convergent (linear) circRNA-specific primers were designed for RT-PCR to verify that the selected candidate RNAs are indeed circRNAs. The results showed that the divergent primers produced amplicons from RNA-derived samples and not from genomic DNA. The PCR products were visualized using 2% GelRed-stained agarose

gel (**Figure 3A**). Then, Sanger sequencing was performed to validate the PCR product, and the head-to-tail splice junctions were identified, unambiguously demonstrating that the selected candidates were circRNAs (**Figure 3B**). Finally, ten circRNAs (threshold: a fold change greater than 1 and a p value less than 0.05 in two comparisons that were differentially expressed in the two groups) were selected for qRT-PCR validation, and three biological replicates were performed. The results showed that the expression levels of circRNA-4452, circRNA-13360, circRNA-1698, circRNA-8979, and circRNA-14411 were significantly upregulated, and the expression levels of circRNA-3041, circRNA-5780, circRNA-1848, and circRNA-3875 were significantly downregulated. Accordingly, Three of the candidate circRNAs were identified the sequence of head-to-tail splice junctions directly by Sanger sequencing of PCR amplicons (**Supplementary Figure S2**). The qRT-PCR assay results were consistent with our RNA-seq assay results, confirming the accuracy of sequencing. However, circRNA-536 was not consistently and/or significantly differentially expressed between the two groups (**Figures 3C,D**).

GO and KEGG Analyses of the Dysregulated CircRNA Parental Genes

Previous studies have demonstrated that circRNAs are closely related to their parental genes and have the ability to regulate their parental genes (Zhang et al., 2016; Wei et al., 2017). Thus, to further investigate the panorama of circRNA functions and interactions in VSMCs, we performed GO function enrichment analysis and KEGG pathway enrichment analysis based on the significantly differentially expressed circRNA host genes. The results of GO enrichment analysis showed that 782 GO BP (biological process), 198 GO CC (cellular component), and 241 GO MF (molecular function) terms were enriched. The top 25 (GO BP), top 15 (GO CC), and top 10 (GO MF) are displayed in **Figure 4A**. GO enrichment showed that 66 genes were enriched in protein binding, 53 genes were expressed in the cytoplasm, and 16 genes were mainly associated with regulation of transcription, DNA-templated. In the GO category "cellular component," the most significant terms were actin cytoskeleton, clathrin-coated vesicle and flotillin complex, while in the GO category "molecular function," the main molecular functions were actin binding, clathrin heavy chain binding, coenzyme binding, transcription factor activity, and RNA polymerase II transcription factor binding. In the GO category "biological process," neural crest cell migration, positive regulation of transcription via serum response element binding, cardiac myofibril assembly, and positive regulation of DNA-templated transcription were the most representative significant terms. The top 20 GO terms are displayed in a scatter plot (**Figure 4B**). KEGG pathway enrichment analysis suggested that there were 81 signaling pathways related to the differentially expressed genes, among which the "RNA degradation," and the "phototransduction" signaling pathways were the most significant pathways. The top 20 pathways are shown in **Figure 4C**. The two most significant pathways are displayed in **Figure 5**.

Construction of a CircRNA and Disease-Related miRNA Network

To further explore the regulatory mechanism of the DE circRNAs and investigate the relationships between DE circRNAs and the function of VSMCs, we compared VSMC-associated miRNAs in validated disease-associated miRNA. A detailed list of the miRNAs is provided in **Table 2**. Based on the ceRNA regulatory mechanism, TargetScan, and miRanda software were used to analyze the miRNAs binding sites for DE circRNA. Finally, we constructed a circRNA-miRNA interaction network, 73 nodes (23 DE circRNAs and 50 miRNAs) and 83 edges were identified in the circRNA–miRNA network (**Figure 6**). According to the network, we predicted that 12 down- and 11 upregulated circRNAs have miRNA binding sites that could act as ceRNAs participating in the regulation of posttranscriptional gene expression. Notably, we found that circRNA160 (hsa_circ_0008776) showed the highest degree of connectivity in the network, with up to 11 miRNA binding sites, followed by circRNA13360 (hsa_circ_0003341), and circRNA7637 (hsa_circ_0001222), indicating their potential important function in VSMCs and cardiovascular disease.

DISCUSSION

AS is a complex pathological process characterized by endothelial dysfunction, lipid infiltration, oxidative stress, inflammation, cell proliferation, and apoptosis (Weber and Noels, 2011; Lu and Daugherty, 2015). During the development of AS, the arterial wall is stimulated by multiple harmful conditions, such as hyperlipidemia, hypertension, diabetes, smoking, homocysteinemia, and other agents that may respond to multiple signaling molecules that interact with the lining of the endothelium, altering the homeostatic condition of the arterial wall and resulting in the migration and proliferation of VSMCs within the lesions (Ross, 1995). It has been considered that phenotypic transformation of VSMCs is an important contributor to vascular disease development, including the pathologic process of atherosclerotic plaque development (Bennett et al., 2016). In recent years, through the combination of high-throughput sequencing and bioinformatics analysis, an increasing number of RNA categories and important potential targets for gene therapy have been discovered (Dixon et al., 2005; Guo et al., 2014). A variety of studies confirmed a strongly association between circRNAs and cardiovascular disease (Tan et al., 2017). Holdt et al. (2016) demonstrated that circANRIL could induce cell apoptosis and inhibit cell proliferation through inducing nucleolar stress and p53 activation. Huang et al. (2019) found that circRNA Nfix regulated by superenhancers (SEs) acts as a pivotal element in regulating cardiac regeneration. However, the expression profiles of circRNAs in different VSMC phenotypes are not yet known.

In this study, it is the first time to show the differential expression of circRNAs in two groups of VSMCs. We screened circRNA profiles to identify the dynamically changed circRNAs in order to discover pivotal biomarkers for vascular biology. A total of 22191 circRNAs were identified in both the PS and NC groups. Currently, five classes of circRNAs have

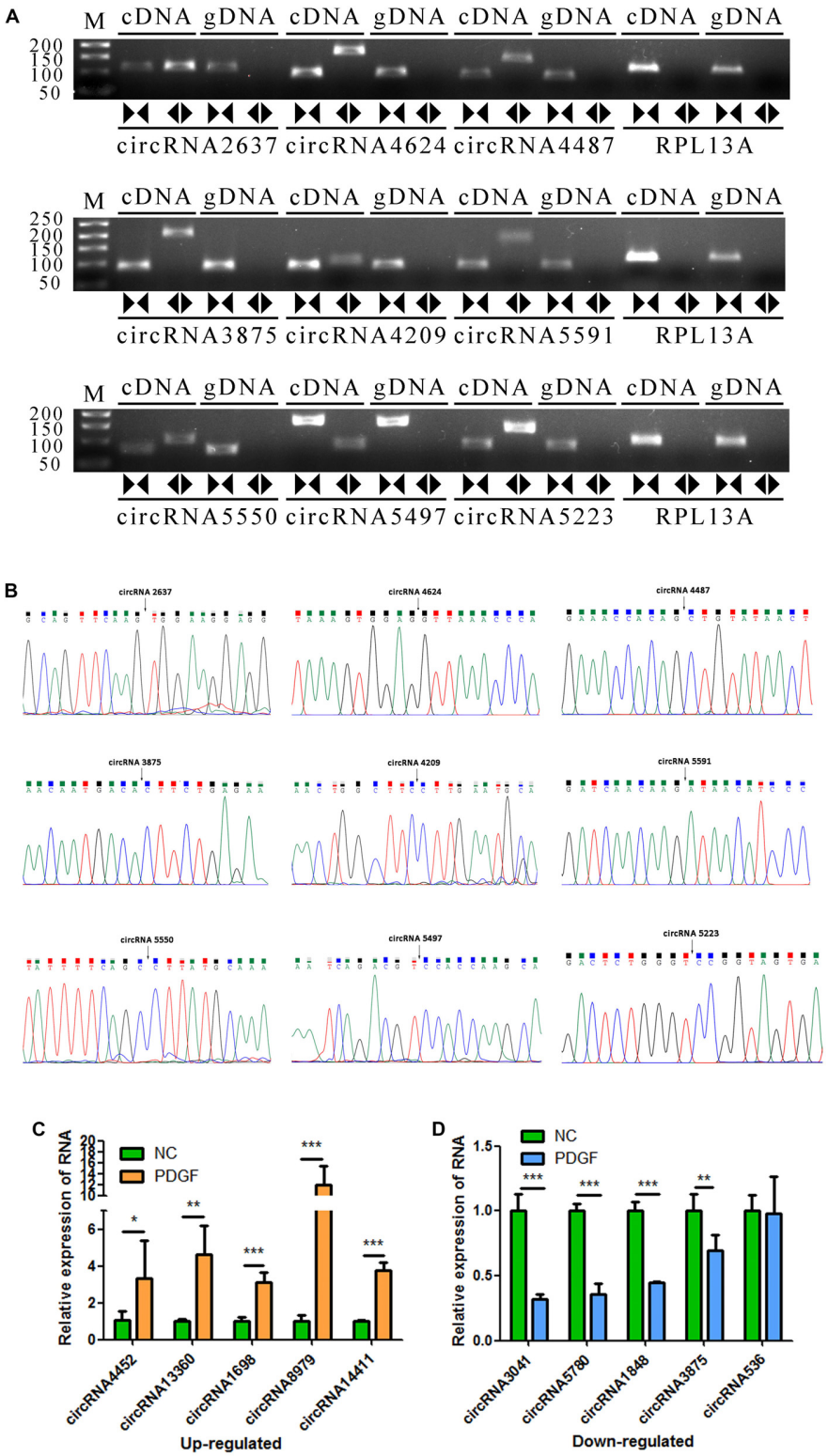

FIGURE 3 | Validation of VSMC-enriched circRNAs. **(A)** Nine circRNAs were selected for validation experiments. RT-PCR with divergent (circular) and convergent (linear) primers was used to confirm the candidate circRNAs. Divergent (circular) primers (◀▶) successfully amplified a single fragment at the expected sizes from cDNA but not from genomic DNA (gDNA). Convergent (linear) primers (▶◀) could amplify from both cDNA and gDNA. **(B)** Sanger sequencing of the selected candidate circRNAs shows the back-splice junctions. **(C,D)** The relative expression levels of 10 DE circRNAs were determine by qRT-PCR. The data are presented as the mean ± SD, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

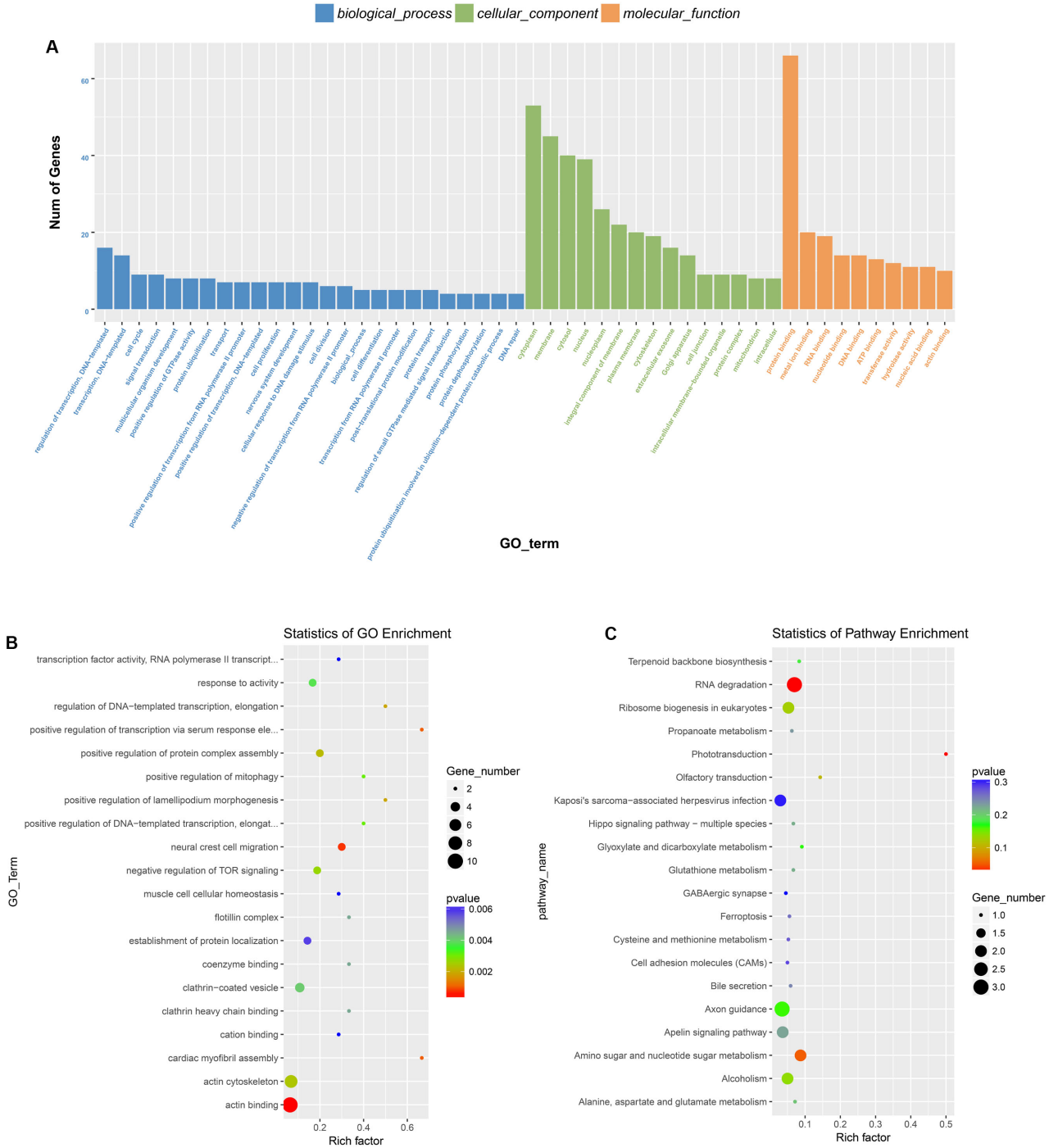

FIGURE 4 | GO and KEGG pathway analysis of the parental genes of the identified circRNAs. **(A)** The top 25 GO BP, top 15 GO CC, and top 10 GO MF are displayed. **(B)** The top 20 most represented significant GO terms are displayed in a scatter plot. **(C)** The top 20 enriched KEGG pathways of the circRNA parental genes.

been detected, circular RNA genomes (viroid and hepatitis delta virus circles), circular RNA from introns, circular RNA intermediates in RNA processing reactions, circRNA from exons, and circRNA in archaea with snRNP functions (Lasda and Parker, 2014). Further analysis of the present study revealed that three categories of circRNAs were represented, among which exon-based circRNAs were the overwhelming majority (94.06%). Using a calculation of FDR to analyze the difference molecules of 22191 circRNAs, we found that the positive results were few. Based on the identification of two groups of cell

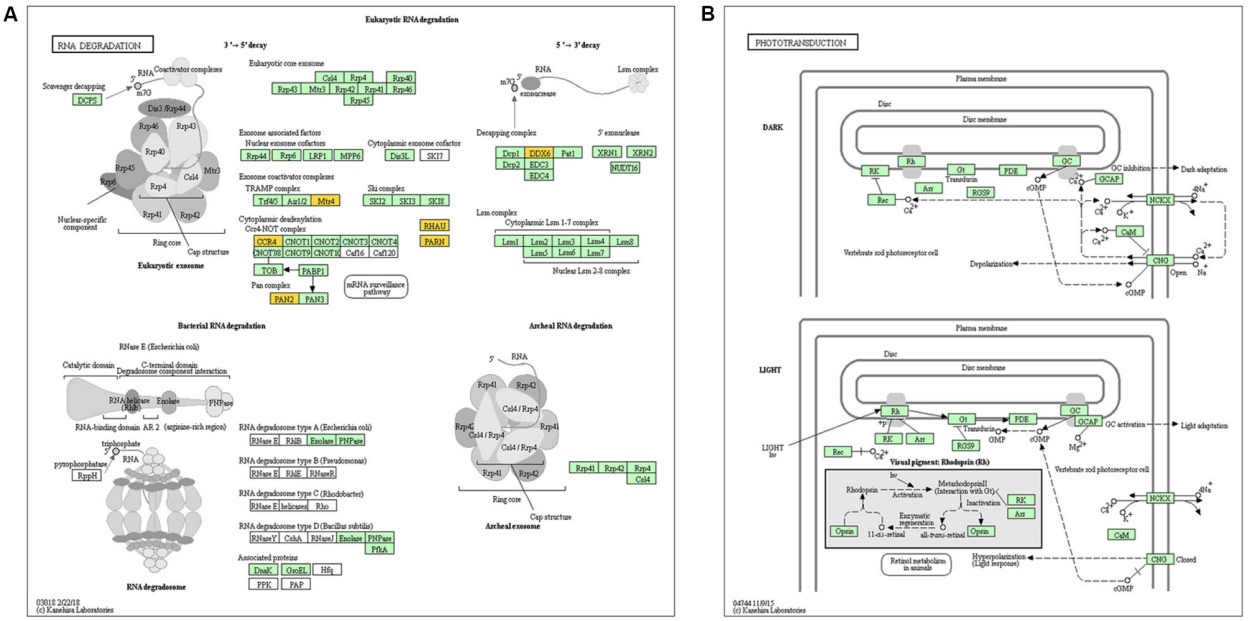

FIGURE 5 | Genes mapped to KEGG pathways by pathway analysis. **(A)** and **(B)** show the signaling pathways "RNA degradation" and "phototransduction," which were the most significant pathways.

TABLE 2 | Detailed list of the VSMC-associated miRNAs.

miRNA	Target(s)	Role and function in SMC dynamics	References
miRNA-214	NCK associated protein 1 (NCKAP1)	Migration, Proliferation, and Neointima Hyperplasia	Afzal et al., 2016
miRNA-130a	MEOX1	Proliferation, migration	Wu et al., 2011
miRNA-675	PTEN	Proliferation	Lv et al., 2018
miR-221/-222	CDKN1B, CDKN1C	Proliferation, migration, and anti-apoptotic effects	Davis et al., 2009; Liu et al., 2009
miRNA-22-3p	High mobility group box-1 (HMGB1)	Proliferation and Migration and Neointimal Hyperplasia	Huang et al., 2017
miRNA-23b	The transcription factor forkhead box O4 (FoxO4)	Phenotypic switching	Iaconetti et al., 2015
miRNA-195	The Cdc42, cyclin D1, and fibroblast growth factor 1 (FGF1) genes	Regulate cell phenotype and prevents neointimal formation.	Wang et al., 2012
miR-206	ARF6, SLC8A1	Differentiation	Lin et al., 2016
miRNA-34a	Neurogenic locus notch homolog protein-1 (Notch1)	Proliferation and migration	Chen et al., 2015
miR-146a	KLF4/5	Differentiation, proliferation	Sun et al., 2011

phenotypes, we think that the screening algorithm we used may be too strict, and the results may lose a large number of true positive results. Therefore, we changed the screening criteria, using the criteria of log2 (fold change) >1 or log2 (fold change) <-1 and P value <0.05 as previous studies (Dang et al., 2017; Xu et al., 2018; Hu et al., 2019) to screen out 112 different expression circRNAs between PS and NC, and then randomly selected 10 circRNAs for verification experiment. On the basis of verification, we calculated the false positive rate (FPR) to generate the Q value. The false-positive rate is only 10%, which shows that the range of difference we selected is of great reference value. And the PCR results were quite consistent with the sequencing results, confirming the high reliability of the high-throughput sequencing data. Cluster analysis was used to display the differences of circRNA expression between the PDGF-treated and control groups. From the analysis, we found that the expression of circRNAs showed a significant difference between the treatment group compared to the control group. The results showed that different circRNAs are turned off and on in different cell states at different times and are involved in numerous metabolic processes. Such circRNAs may become novel prognostic markers for diseases.

GO and KEGG pathway enrichment analyses of the host genes we detected showed that the parental genes of the circRNAs mainly participate in cardiac myofibril assembly and positive regulation of DNA-templated transcription, indicating that they might be involved in cardiovascular diseases. Moreover, according to the BP, CC, and MF terms with substantial enrichment, the genes were mainly associated with gene

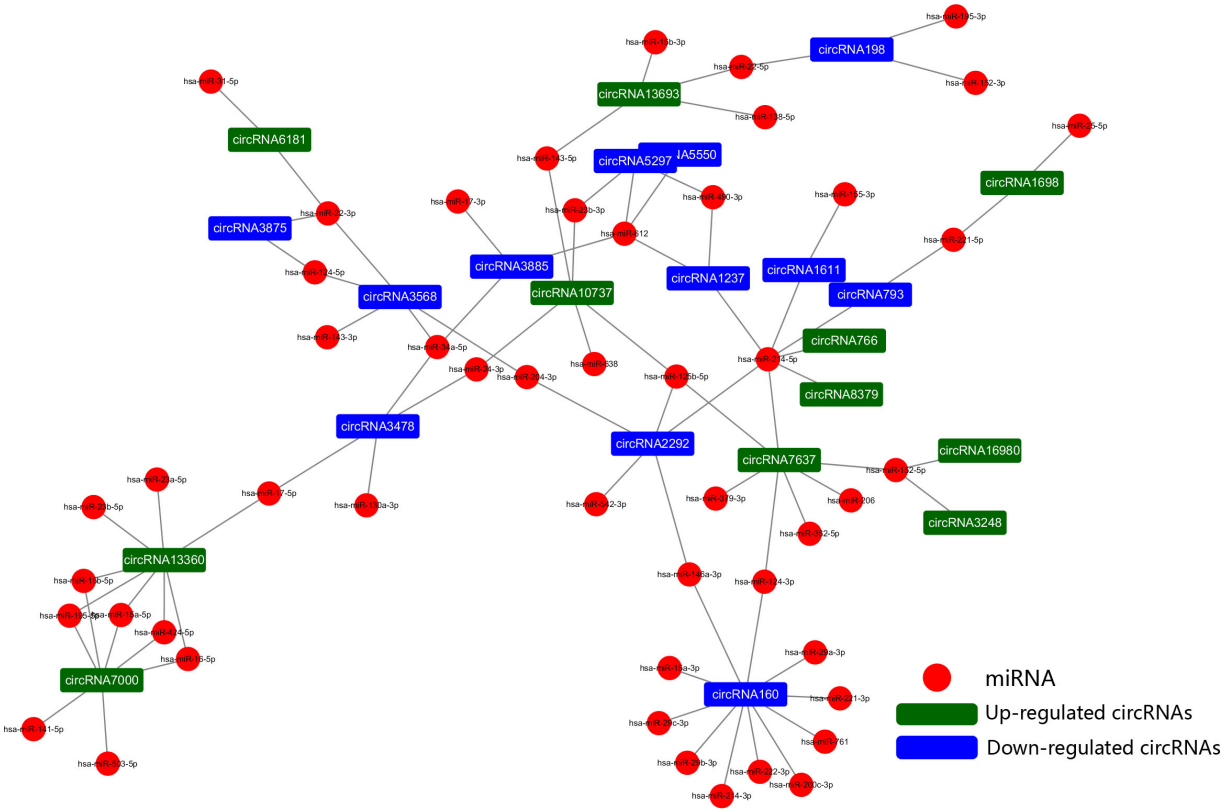

FIGURE 6 | The circRNA-miRNA network. The generated network consists of 73 nodes (23 dysregulated circRNAs and 50 miRNAs) and 83 edges. According to the network, we predicted that 12 down- and 11 upregulated circRNAs have miRNA binding sites and could act as ceRNAs to regulate posttranscriptional gene expression.

expression at the transcriptional or posttranscription level in the cytoplasm. The "RNA degradation" pathway is an important signaling pathway associated with many biological processes in eukaryotes.

A variety of studies have revealed that circRNAs can function as sponges for related miRNAs, thus playing a vital regulatory role in influencing physiological processes as well as various diseases, including cardiovascular disease, of which miRNAs act as indispensable bridges joining various RNAs (Salmena et al., 2011; Tay et al., 2014). The circRNA-miRNA network has been proven to be a widely accepted mechanism of gene expression regulation. Hence, a circRNA-miRNA network was constructed based on the dysregulated circRNAs and VSMC-related miRNAs. Based on ceRNA theory, circRNAs containing MRE binding site could act as a miRNA sponge to regulate gene expression, in other words, there was a negative correlation in the expression of circRNA and miRNA. Among the altered circRNAs in the network, we found that circRNA160 (hsa_circ_0008776) which is significantly downregulated after PDGF-BB treated VSMC, showed the highest degree of connectivity throughout the network, with up to 11 miRNA binding sites. Among them, it is demonstrated that the expression of miR-221-3p, miR-222-3p, and miR-146a-3p were increased by growth stimulators and overexpression these miRNAs could dramatically enhance VSMC proliferation, growth, and migration (Liu et al., 2009; Sun et al., 2011; Li et al., 2019). In addition, circRNA13360 (hsa_circ_0003341) was upregulated in PS group and has been predicted to have an MRE binding site and may act as a miRNA sponge for miR-23b-5p and miR-424-5p et al., studies suggested that overexpression of miR-23b and miR-424 inhibited VSMC proliferation and migration (Merlet et al., 2013; Iaconetti et al., 2015). Since the function of these RNAs in the network has been partially demonstrated and our results are in line with previous studies, suggesting a potential vital function of circRNAs in the pathology of AS. Additional analyses revealed that circRNA160, encoded by the parental gene THSD1 thrombospondin type I domain (1), was significantly downregulated in PDGF-BB-treated VSMCs. Although the regulatory roles of circRNA160 in the proliferation or migration of VSMCs remain largely unknown, THSD1 is a novel regulator during vascular development and functions to protect the intraplaque microvasculature and prevent hemorrhaging in advanced atherosclerotic lesions (Haasdijk et al., 2016). The expression of THSD1 could be regulated, depending on activation by multiple microenvironmental factors. However, studies that elucidate the regulation of THSD1 are still lacking, and whether circRNA160 can regulate the transcription of its parental genes remains to be further studied.

In this study, we aimed to discover pivotal biomarkers for vascular biology to provide evidence supporting molecular therapy for the diseases. But objectively, circRNAs have diverse functions, apart from miRNA sponging, they can directly influence or control mRNA transcripts or effect protein translation and function. In addition, some of them have the ability to translate peptides (Lasda and Parker, 2014; Du et al., 2017; Pamudurti et al., 2017). However, the data information in our study could provide clues for further research. We could conduct in-depth studies to explore the regulatory role in disease development, providing important targets for disease treatment or diagnosis.

CONCLUSION

In summary, the proliferation and migration of VSMCs are important contributing factors to vascular disease development, including the pathologic process of atherosclerotic plaque progression. In this study, we identified the differential expression of circRNA in PDGF-BB-induced VSMCs. A circRNA-miRNA network was constructed, and bioinformatics analysis suggested that circRNAs may play vital roles in the pathology of AS, especially at the posttranscriptional level. This is only the beginning toward a better understanding of the roles of circRNAs in VSMCs, and more functional experiments are still needed to confirm the precise molecular regulatory mechanisms of circRNA functions.

DATA AVAILABILITY STATEMENT

The sequencing data used during the current study are available in NCBI databases (BioProject PRJNA607375).

AUTHOR CONTRIBUTIONS

JiaT and YF performed the bioinformatics analyses and wrote the manuscript. QL and YX designed the primers and conducted qRT-PCR assays. XX and YZ performed the cell experiments. LY and ZW analyzed the data. JinT and BY conceived, designed, and supervised the study. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.00530/full#supplementary-material

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Loss of miR-132/212 Has No Long-Term Beneficial Effect on Cardiac Function After Permanent Coronary Occlusion in Mice

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Background: Myocardial infarction (MI) is caused by occlusion of the coronary artery and induces ischemia in the myocardium and eventually a massive loss in cardiomyocytes. Studies have shown many factors or treatments that can affect the healing and remodeling of the heart upon infarction, leading to better cardiac performance and clinical outcome. Previously, miR-132/212 has been shown to play an important role in arteriogenesis in a mouse model of hindlimb ischemia and in the regulation of cardiac contractility in hypertrophic cardiomyopathy in mice. In this study, we explored the role of miR-132/212 during ischemia in a murine MI model.

Methods and Results: miR-132/212 knockout mice show enhanced cardiac contractile function at baseline compared to wild-type controls, as assessed by echocardiography. One day after induction of MI by permanent occlusion, miR-132/212 knockout mice display similar levels of cardiac damage as wild-type controls, as demonstrated by infarction size quantification and LDH release, although a trend toward more cardiomyocyte cell death was observed in the knockout mice as shown by TUNEL staining. Four weeks after MI, miR-132/212 knockout mice show no differences in terms of cardiac function, expression of cardiac stress markers, and fibrotic remodeling, although vascularization was reduced. In line with these *in vivo* observation, overexpression of miR-132 or miR-212 in neonatal rat cardiomyocyte suppress hypoxia induced cardiomyocyte cell death.

Conclusion: Although we previously observed a role in collateral formation and myocardial contractility, the absence of miR-132/212 did not affect the overall myocardial performance upon a permanent occlusion of the coronary artery. This suggests an interplay of different roles of this miR-132/212 before and during MI,

including an inhibitory effect on cell death and angiogenesis, and a positive effect on cardiac contractility and autophagic response. Thus, spatial or tissue specific manipulation of this microRNA family may be essential to fully understand the roles and to develop interventions to reduce infarct size.

Keywords: miR-132/212, myocardial infarction, cardiac function, adverse cardiac remodeling, permanent coronary occlusion

INTRODUCTION

Although the mortality rate of myocardial infarction (MI) in patients is going down due to recently developed post-infarction treatments and secondary prevention, MI is still one of the leading causes of mortality (Mozaffarian et al., 2015). 12.5% of patients that suffered a MI with ST-segment elevation may die within 6 months (Steg et al., 2012), suggesting that novel effective treatments are still required.

microRNAs are small non-coding RNAs that play essential roles in cardiac development, and dysregulation of microRNAs promote the pathological progression of many cardiac diseases. Therapeutic interventions are beneficial to slow down the myocardial pathological progression (Bonauer et al., 2009; Montgomery et al., 2011; Grueter et al., 2012; Hullinger et al., 2012; Wahlquist et al., 2014), and therefore microRNAs have been considered as promising therapeutic targets for cardiovascular diseases (van Rooij, 2014; van Rooij and Kauppinen, 2014).

The miR-132/212 family plays essential roles in maintaining physiological function and pathological disease progression of the cardiovascular system. The expression level of this family goes upon angiogenic stimulation, including hypoxia (Burek et al., 2019) or loss-of-VHL (Lei et al., 2020) and during hypertrophic growth upon Angiotensin II treatment (Eskildsen et al., 2013). Loss of miR132/212 shows impaired angiogenesis response in hindlimb ischemia model (Lei et al., 2015) and overexpression of miR132/212 enhance neovascularization. They are also reported to be upregulated in the failing human heart, where they play a detrimental role in the regulation of cardiomyocyte contractility and the cardiac hypertrophy in hypertension-induced heart failure models (Ucar et al., 2012). However, the regulation and biological function of this family in the response to a MI has never been investigated. We, therefore, used the miR-132/212 genetic knockout (KO) mice and induced MI by permanent occlusion of the coronary artery to explore the functional effects on cardiac function compared to wild-type controls.

MATERIALS AND METHODS

Generation and Genotyping of miR-132/212 KO Mice

The miR-132/212 KO mice have been generated as described previously (Kayo et al., 2014). In brief, the miR-132/212$^{flox/flox}$ mice were generated by gene targeting in mouse ES cell from the C57BL/6N background and then crossed with a Cre deleter line to remove the miR-132/212 genome region (see **Figure 1A**). The resulted miR-212-132$^{-/-}$ line is subsequently maintained in C57BL/6J mice background. To reduce the ES C57BL/6N background, it was back crossed with C57BL/6J for 6 times. All the animal experiment in this study was carried out using age and sex-matched C57BL/6J mice as wildtype (WT) control from the same bread. miR132/212 localizes between the exon1 and exon2 od HIC1 gene. After the removal of the miR-132/212 region, HIC1 and neighboring gene expression is not altered. For genotyping, DNA samples were obtained by ear clipping and used in a GC-Rich PCR kit (Roche, cat. 12140306001) with miR-132/212 primers as shown in **Table 1**. PCR products were revealed on a 1% agarose gel: WT genotype display a predicted band at 1076 bp and the KO genotype at 392 bp.

LAD Ligation and Echocardiography

This study was approved by the Animal Ethical Experimentation Committee (DEC. 2013.II.02.019, Utrecht University) and was carried out in accordance with the Guide for the care and use of Laboratory Animals.

Myocardial infarction (MI) was induced by ligation of the Left anterior descending artery (LAD) and applied on 10–12 weeks old WT (C57B6) and miR-132/212 KO mice, as described previously (Grundmann et al., 2011). In brief, mice were anesthetized with fentanyl (0.05 mg/kg), midazolam (5 mg/kg) and medetomidine (0.5 mg/kg) by intraperitoneal injection and surgical procedures were performed under sterile conditions. LAD was ligated just below the left atrial appendage with an 8–0 Ethilon monifil suture. The chest was then closed and animals received atipamezole (2.5 mg/kg) and flumazenil (0.5 mg/kg) to recover quickly. Temgesic (0.1 mg/kg) was given every 8 h after surgery for 6 times to reduce discomfort. Cardiac function was assessed with echocardiography (Vevo® 2100 System, Visualsonics) and analyzed with Vevo2100-1.6.0 (Visualsonics) before and after the surgical procedure (days 0, 7, 14, and 28). During the procedure, the animals were kept under 2% isoflurane anesthesia and the body temperature was strictly maintained between 36.5–37.5°C. To terminate the mice, mice were anesthetized by an overdose anesthesia with fentanyl (0.1 mg/kg), midazolam (10 mg/kg), and medetomidine (1 mg/kg) by intraperitoneal injection.

Neonatal Rat Cardiomyocytes Isolation and Hypoxia Treatment

Neonatal rat cardiomyocytes isolation was performed with Pierce Primary Cardiomyocyte Isolation Kit (Life Technologies, Cat. 88281) following manufacture's instruction. In brief, neonatal rat hearts were collected within 3 days after birth. After washing with ice cold Hank's Balanced Salt Solution (HBSS) (Gibco),

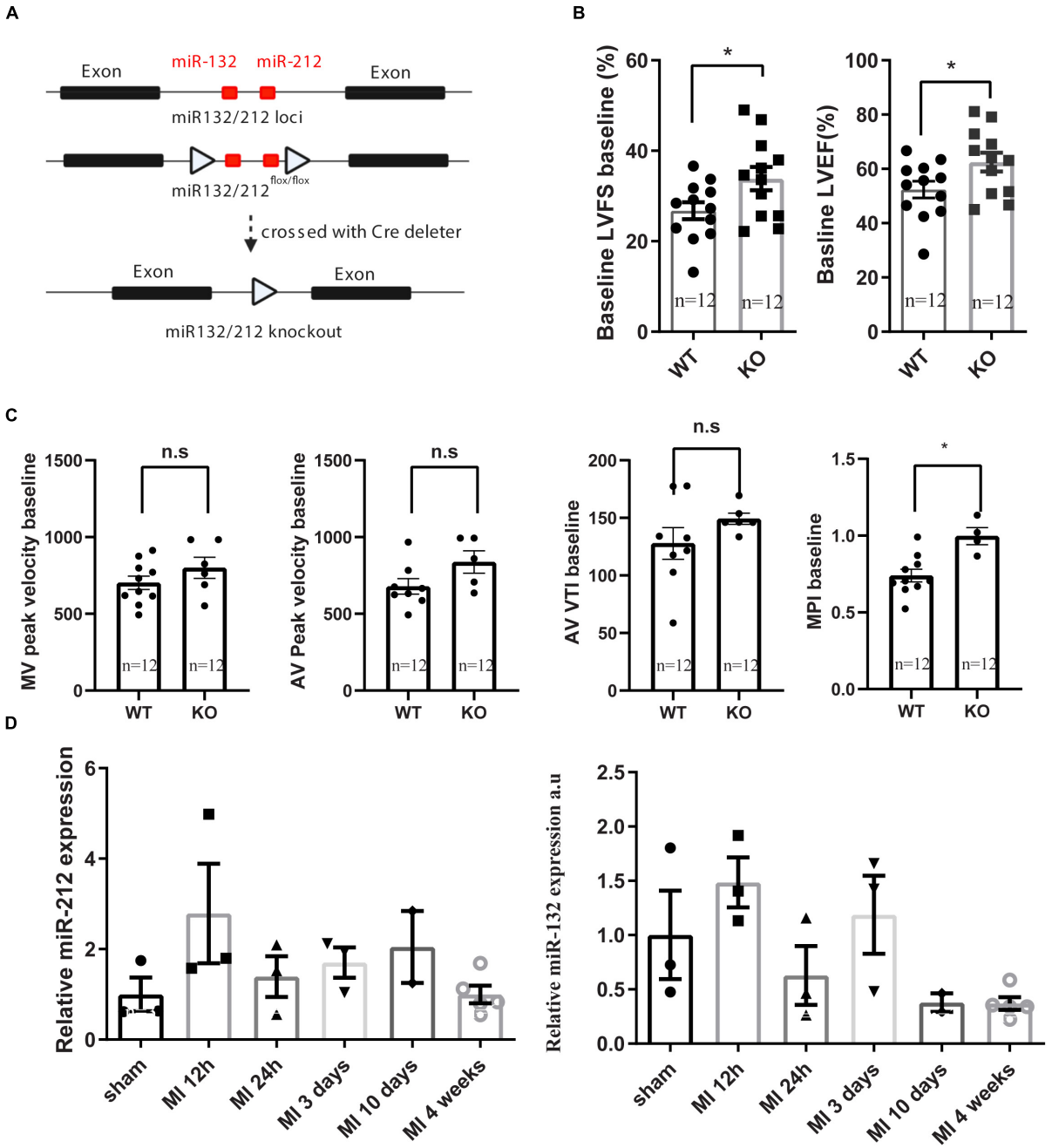

FIGURE 1 | Generation of miR132/212 KO mice and base line characterization the cardiac function of the knockout mice. **(A)** Gene targeting strategy of miR132/212. **(B,C)**. base line characterization the cardiac function of the knockout mice. **(D)** expression of miR132 and mir212 after myocardial infarction. LVFS: left ventricular fraction shorting; LVEF: left ventricular enjection fraction; MPI: myocardial performance index; MV: Mitral valve; AV: Aortic valve; AV VTI: Aorta velocity time integral.

hearts are cut into small pieces before enzymatic digestion for 35 min. After digestion, pieces are washed with cold HBSS once again and disassociated with cardiomyocyte culture medium with 10% FBS and single cells generated by filtrating over a 40 μm filter to remove undigested tissue. After centrifuge, cells were re-suspended in culture medium with 10% FBS and seeded at 2.5×10^5cells/cm^2. The next day, cells were transfected with microRNA mimics mirVana miRNA mimic scramble control (4464085), hsa-miR-132-3p mimics (MC10166), hsa-miR-212-3p mimics (MC10340) with RNAiMAX (Life Technologies) at 50nM following manufacture's instruction. Six hours after transfection, medium was replaced with fresh DMEM medium with 10% FBS containing 1× Cardiomyocyte Growth Supplement. Forty-eight hours after transfection, cells were transferred to a hypoxia

TABLE 1 | Cardiac function analysis of miR-132/212 knockout and WT mice by echocardiography at baseline: LVFS; LVEF; myocardial performance index (MPI), peak velocity of MV and AV.

	Base line		1 week after MI		2 weeks after MI		4 weeks after MI	
	WT (*n* = 12)	KO (*n* = 12)	WT (*n* = 6)	KO (*n* = 5)	WT (*n* = 6)	KO (*n* = 5)	WT (*n* = 5)	KO (*n* = 5)
Heart_Rate	460.9 ± 10.4	485.4 ± 8.0	643.49 ± 69.1	501.92 ± 11.23	456.2 ± 21.1	398.7 ± 12.9	614.8 ± 96.3	506.6 ± 16.2
Diameter_systolic	2.8 ± 0.1	2.4 ± 0.1	4.18 ± 0.28	3.86 ± 0.25	4.4 ± 0.4	5 ± 0.4	5.1 ± 0.2	5.1 ± 0.5
Diameter_diastolic	3.8 ± 0.1	3.7 ± 0.1	4.83 ± 0.24	4.51 ± 0.14	5.1 ± 0.3	5.5 ± 0.4	5.7 ± 0.2	5.6 ± 0.5
Volume_systolic	30.2 ± 2.6	22.6 ± 3.1	80.59 ± 12.49	66.18 ± 9.48	94.5 ± 15.5	123.9 ± 21.9	126.9 ± 8.7	131.2 ± 24.3
Volume_diastolic	63.0 ± 3.1	57.8 ± 4.2	110.96 ± 12.31	93.7 ± 6.65	124.3 ± 15.7	151.6 ± 24.1	159.1 ± 11.4	160.1 ± 29.4
Stroke_volume	32.8 ± 2.6	35.2 ± 2.4	30.38 ± 3.08	27.52 ± 5.31	29.8 ± 2.6	27.6 ± 6.7	32.3 ± 4.8	28.9 ± 9.5
Ejection_fraction	52.4 ± 3.1	62.5 ± 3.5*	29.06 ± 3.67	30.61 ± 6.72	26.8 ± 5.6	19 ± 5.1	20.2 ± 2	18.8 ± 4.6
Fraction_shortening	26.7 ± 1.9	33.8 ± 2.6*	13.69 ± 1.81	14.68 ± 3.54	12.8 ± 3	8.9 ± 2.5	9.3 ± 1	8.7 ± 2.2
Cardiac_output	15.3 ± 1.6	17.1 ± 1.3	20.13 ± 3.73	14.04 ± 3.03	13.5 ± 1.1	10.9 ± 2.5	19.2 ± 2.8	15 ± 5.4

chamber with 5% CO_2 and 1% O_2 for 24 h. Then cells were fixed with 4% PFA for 15 min before TUNEL staining.

TUNEL Staining

Twenty-four hours after MI surgery (n = 4/group), mice were terminated. Hearts were explanted, rinsed and fixed with 0.2 PFA in 15% sucrose at 4°C overnight before cryopreservation with Tissue Tek for sectioning. Ten μm cryosections were prepared. For TUNEL staining, sections were first dried for 10 min at room temperature, then digested with 5 μg/ml proteinase K (cat. Roche) for 20 min at 37°C. Sections were subsequently used for TUNEL staining (*In situ* Death Detection Kit, Cat. 1684795, Roche) following manufacturer's instructions. After TUNEL, sections were counterstained with Hoechst for nuclei and Troponin for cardiomyocytes. Images were taken and analyzed by a blinded investigator with Cellsens imaging system at 20× magnification. TUNEL staining for hypoxia treated Rat neonatal cardiomyocytes (RNCM) was performed in a similar approach without Proteinase K treatment.

Infarct Size Quantification

Twenty-four hours after MI surgery (n = 6/group), infarct size (IS) was determined as a percentage of the area at risk (AAR). Four% Evans Blue solution was injected via the thoracic aorta and hearts were explanted, rinsed and filled with paper before placement in −20°C freezer for 1 h. Hearts were subsequently sliced into 1mm cross sections and incubated with 1% triphenyltetrazolium chloride (TTC, Sigma) for 1 h at 37°C, then fixed with formaldehyde 4% for 15 min. Images from both sides of the cardiac sections were taken sequentially from apex to atrium. IS, AAR and left the ventricular area were measured with Photoshop and reconstructed as previously described in ImageJ (Koudstaal et al., 2015).

Lactate Dehydrogenase and Troponin Measurement

After termination at 24 h post-MI, blood samples (n = 4/group) were collected by cardiac puncture. Samples were centrifuged at 12,000×g for 10 min and cleared plasma then transferred to another tube. For total lactate dehydrogenase assay (LDH), 10 μl of plasma was used to determine total LDH concentration using the Toxicology Assay Kit (Cat. TOX7-1KT, Sigma) according to manufacturer's instructions with an arrayscan at 492 nM (Thermo Fisher). cTnI levels were measured by ELISA (Synchron Lxi 725 integrated clinical chemistry, Beckman Coulter) in the Laboratorium Klinische Chemie en Hematology (LKCH) of UMC Utrecht as previously described (Oerlemans et al., 2012).

Histological Analysis and Immunohistochemical Staining

28 days after MI (long-term group, n = 6/group), mice were terminated. Hearts were explanted, rinsed and fixed with 0.2 PFA in 15% sucrose at 4°C overnight before cryopreservation with Tissue Tek for sectioning. HE staining and Picrosirius red staining were performed for morphological and fibrotic remodeling assessment, respectively, as described before (Timmers et al., 2008). For evaluation of large vessels, sections were first blocked with 2% BSA for 30 min, FITC-labeled anti-αSMA antibody was applied for 1 h at room temperature. After incubating the slides with 1 mg/ml Hoechst to visualize the nuclei, sections were mounted in fluoromount G (Southern Biotech). The complete sections were then scanned for both αSMA and Hoechst channel. Images were analyzed with ImageJ. All the αSMA positive signal larger than 500 arbitrary unit (a.u.) (proximately two nuclei) were considered as a vessel. The vessel coverage was calculated by total vessel area divided by total number of cells and vessel density was calculated by the total number of vessels divided by the total number of the cells.

RNA Isolation and RT-PCR Analysis

DNA-free RNA was extracted with Tripure (Roche applied science). To perform qPCR for gene expression, RNA is transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions, and quantitative real-time PCR was performed on a MyIQ single-color qRT-PCR system (Bio-Rad), as described previously (van Mil et al., 2012). All primers used for qPCR analysis are listed in the **Table 1**. Mature miR-132 and miR-212 expression levels were measured by TaqMan® MicroRNA Assay following manufactory's instruction, using U6 as control.

Statistical Analysis

Data was analyzed using Graphpad Prism 8 and comparisons were performed with *t*-test between two groups. For multiple groups, and time-course measurement, two-way ANOVA were used with *post hoc* Bonferroni correction. Data are presented as mean ± SEM. $p < 0.05$ is considered as significant, labeled with*.

RESULTS

miR-132/212 Knockout Mice Show More Damage in the Heart After MI

Consistent with our previous observations, miR-132/212 knockout mice display enhanced cardiac contractile function as shown by higher left ventricular fraction shortening (LVFS), left ventricular ejection fraction (LVEF) and myocardial performance index (MPI) at baseline, as shown in **Figures 1B,C**.

For assessing the role of miR-132/212 in the setting of an acute MI, LAD ligations were performed in miR-132/212 knockout and WT control mice **Figure 2A**. 24 h post-MI, cardiac damage was assessed by TTC staining on cardiac slices from operated WT and KO mice (**Figure 2B**). No significant differences were observed in infarct size (IS), as measured both in the percentage of LV (IS/LV) and in the percentage of area at risk (IS/AAR). Although KO mice display a trend toward higher IS/AAR (**Figure 2C**), circulating Lactate Dehydrogenase (LDH) levels and Troponin levels, markers for cardiac damage, did not differ between WT and KO mice (**Figure 2D**).

To further explore the effect of miR-132/212 in cell death, we overexpressed miR-132 and miR-212 in hypoxic RNCM for 24 h. Cardiomyocytes with overexpression of miR-132 or miR-212 are more resistant to ischemia-induced cell death, as shown by TUNEL staining (**Figures 2E,G**) indicating that miR-132 and 212 are indeed protective for ischemia in cardiomyocytes.

TUNEL staining was then performed on cross-sections of infarcted hearts 24 h post-MI to determine differences in cell death. A trend to an increased percentage of TUNEL positive cells in the KO mice is observed but did not reach significance in both border zone and infarcted area compared to WT control hearts (**Figures 2F,G**).

Loss of miR-132/212 Shows No Benefit in Cardiac Function Preservation or Adverse Cardiac Remodeling

To see the long term consequence of miR-132/212 loss post-MI, we exposed another set of mice to MI and followed their cardiac function by echocardiography for 4 weeks **Figure 3A**. Consistent with the previously observed effect in cell death, we observed that KO mice demonstrated a stronger reduction in cardiac function than WT mice within the first 2 weeks (**Figure 3B**). However, eventually both WT and KO animals display similar cardiac function at 4 weeks, exemplified by a similar reduction in ejection fraction and fractional shortening (**Figure 3B**).

After the termination of these mice at 4 weeks post-MI, we further characterized their hearts at the histological and molecular levels. Both WT and KO displayed extensive cardiac remodeling and expansion of the IS (**Figure 3C**). No differences in fibrotic remodeling, both in the infarct and remote areas could be observed between WT and KO mice (**Figure 3C**). To assess the stress status of the hearts, we checked the expression of several cardiac stress markers, but no significant difference was detected in Anp, Bnp, nor in the βMHC/αMHC ratios (**Figure 3E**).

Neovascularization has been shown to play a role in cardiac healing and remodeling after MI (Takeda et al., 2009; Zarrinpashneh et al., 2013) and we have previously observed that miR132/212 did affect the arteriogenic response after hind-limb ischemia. To see if the loss of miR-132/212 could also affect the neovascularization after MI, we stained for αSMA to visualize larger vessels which are mainly responsive for actual blood supply. We observed that KO mice display a lower number of vessels upon MI. There was a trend toward a lower vessel density and vessel coverage rate in the KO mice, but these were not statistically significant (**Figure 3D**).

DISCUSSION

In this study, we tested the role of miR-132/212 during myocardial infarction using genetic knockout mouse both on the short and long term post-MI. Four weeks after MI loss of miR-132/212 did not show any differences in the cardiac function or adverse cardiac remodeling. These results indicated that general inhibition of miR-132/212 in the setting of MI has no beneficial effect in the preservation of cardiac function.

Loss of miR-132/212 did not show any beneficial effects on cardiac function at 4 weeks, both on cardiac function as well as histological levels, although ths miRNA family is described to play a role in neovascularization and myocardial contractility regulation. Several distinct mechanisms might explain this observation. First, we and others found that miR-132/212 modulate the Ras-MAPK pathway by synergistically suppressing multiple intrinsic inhibitors of the Ras-MAPK [Rasa1 (Katare et al., 2011), Spred1 and Spry1] and PI3K-AKT pathway (PTEN) in Human Umbilical Vein Endothelial Cells (HUVECs). It also has been shown that miR-132/212 has an anti-apoptotic role by activating the PI3K-AKT pathway in a mouse cardiomyocyte line (Ucar et al., 2012). Thus, miR-132/212 may have a positive effect on cardiomyocyte cell death by directly regulating survival signaling during ischemia. Secondly, miR-132/212 regulates the contractility of the heart. Reducing wall stress after MI, either by mechanically unloading the heart (Kapur et al., 2013) or pharmaceutically by using ACE inhibitors or beta-blockers (Gajarsa and Kloner, 2011), is beneficial to cardiac healing after MI. Therefore we believe that the loss of miR-132/212 enhances both cardiac contractility and increases stress levels post-MI. In this sense, miR-132/212 inhibition may induce more damage following MI, a detrimental effect undesirable in the clinical setting.

Although miR-132/212 plays a protective role in the immediate-early phase post-MI, associated with an early increase

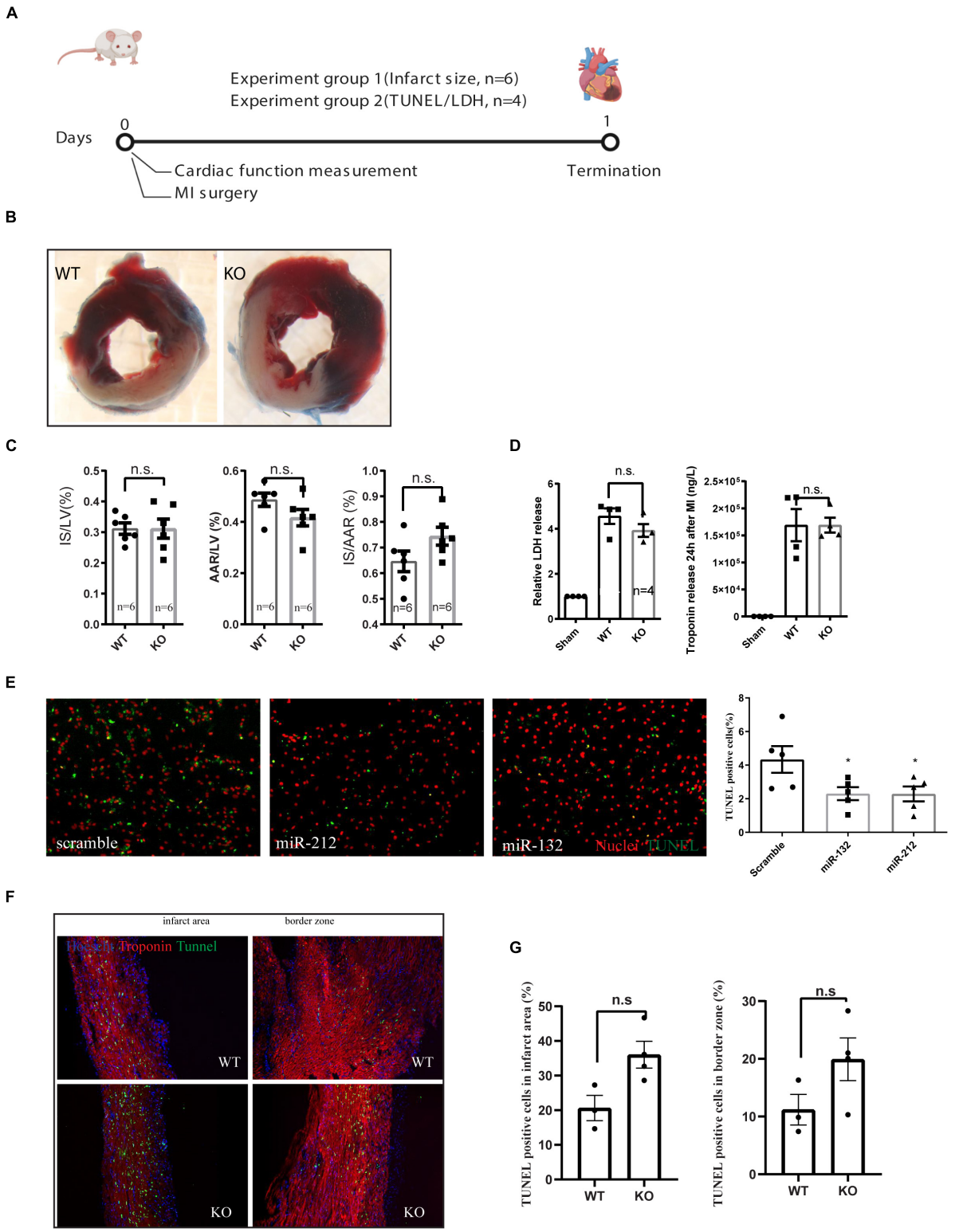

FIGURE 2 | Characterization of cardiac damage after MI by TTC and TUNEL staining. **(A)** Experiment design for short term cardiac damage assessment. **(B)** representative images of WT and KO hearts stained with TTC 24 h after MI. **(C)** Quantification of infarct size (IS), area at risk (AAR) of left ventricle (LV), **(D)** LDH and Troponin release in the serum at 24 h after MI as measured by ELISA. **(E)** representative images of TUNEL staining on rat neonatal cardiomyocytes, transfected with indicated microRNA mimics or scramble controls and treated for 24 h of ischemia and the quantification of TUNEL positive cells. **(F,G)** Representative images of TUNEL staining on the left ventricle 24 h after MI and their quantifications.

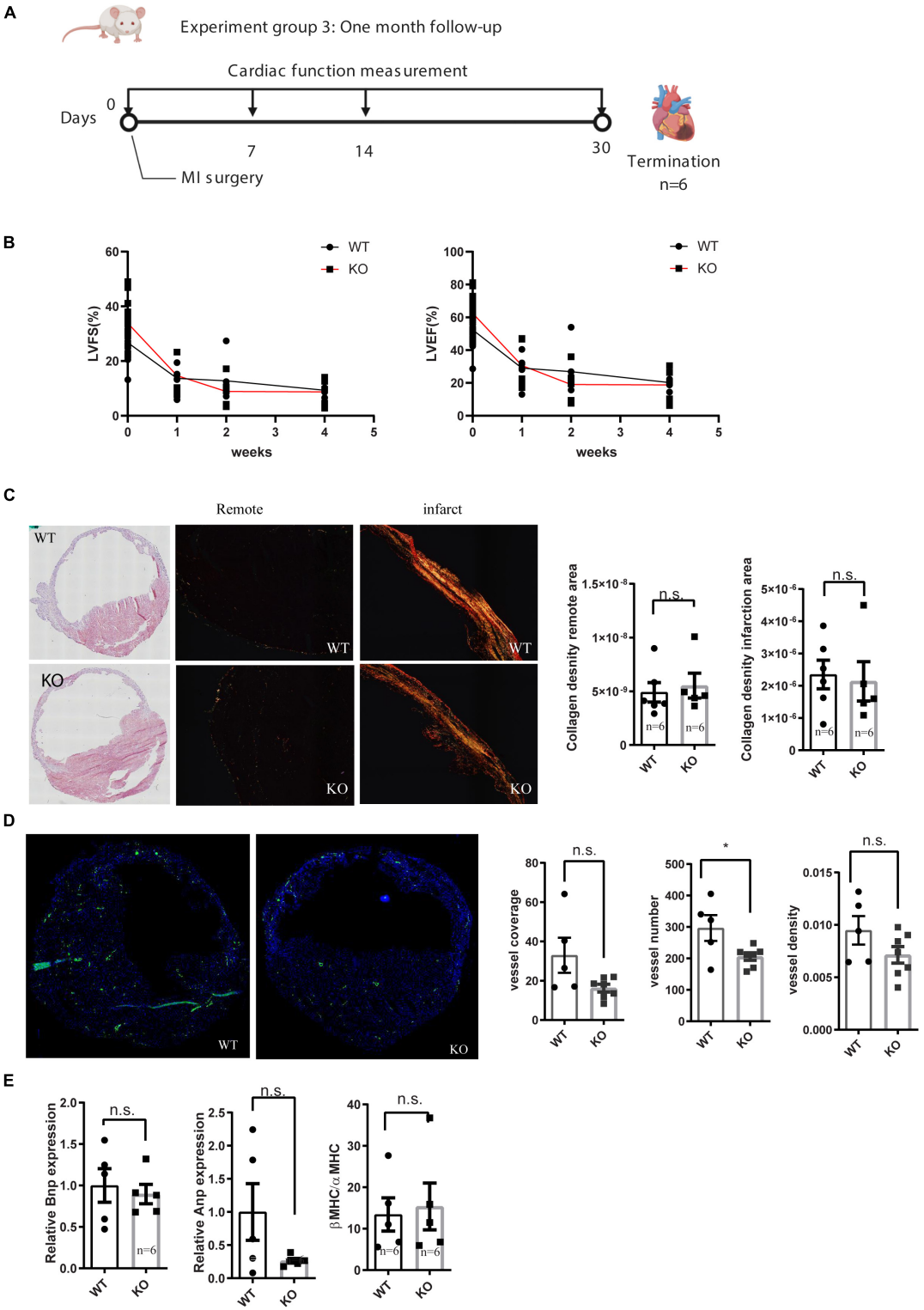

FIGURE 3 | Characterization of cardiac function of miR-132/212 knockout mice by echocardiography 4 weeks after MI. **(A)** experiment design for month cardiac function follow-up after myocardial infarction. **(B)** LVEF as a percentage in WT and KO mice during 4 weeks post- MI. **(B)** LVFS as a percentage in WT and KO mice during 4 weeks post- MI. **(C)** representative images of HE staining of the WT and KO cardiac section 4 weeks post-MI, representative images of Picrosirius red staining for collagen content of the WT and KO cardiac section and quantification of collagen density. **(D)** Representative images of αSMA staining in WT and KO cardiac section and quantification of number of the αSMA positive vessel, normalized vessel coverage and vessel density. **(E)** Molecular characterization of the hearts 4 weeks post-MI by qPCR for cardiac stress markers: Anp, Bnp, and βMHC/αMHC ratio.

in expression 12h post-MI, its expression is decreased at 24 h but increased again in a second wave in later phases but didn't reach statistical significance **Figure 1D**. The upregulation of this miR-132/212 in the later phase is potentially impairing cardiac contractility and the associated autophagic response (Ucar et al., 2012). It remains to be tested if inhibition of miR-132/212 at later stages after MI may still help to maintain cardiac contractile function while keeping or even increasing the expression of miR-132/212 in the early phase. For that purpose, conditional knockout mice, antagomiRs or better targeting of therapeutics should be used (Kwekkeboom et al., 2014), and at least a substantial amount of viable myocardium has to be preserved before any effect of miR-132/212 inhibition can be observed. Nevertheless, our results demonstrate yet another example for multifunction properties of a single microRNA, emphasizing that a spatial and/or tissue-specific intervention may be critical to achieving desired therapeutic effects (Kwekkeboom et al., 2014).

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethical Experimentation Committee (Utrecht University). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

ZL and JS conceived the study. ZL, JF, JD, and CM performed the experiment, collected and analyzed the data. ZL, JF, HA, PD, and JS wrote the manuscript. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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frontiers in Physiology

SYSTEMATIC REVIEW
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Value of Blood-Based microRNAs in the Diagnosis of Acute Myocardial Infarction: A Systematic Review and Meta-Analysis

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Background: Recent studies have shown that blood-based miRNAs are dysregulated in patients with acute myocardial infarction (AMI) and are therefore a potential tool for the diagnosis of AMI. Therefore, this study summarized and evaluated studies focused on microRNAs as novel biomarkers for the diagnosis of AMI from the last ten years.

Methods: MEDLINE, the Cochrane Central database, and EMBASE were searched between January 2010 and December 2019. Studies that assessed the diagnostic accuracy of circulating microRNAs in AMI were chosen. The pooled sensitivity, specificity, positive likelihood ratio, negative likelihood ratio, diagnostic odds ratio, and area under the curve (AUC) were used to assess the test performance of miRNAs.

Results: A total of 58 studies that included 8,206 participants assessed the diagnostic accuracy of circulating miRNAs in AMI. The main results of the meta-analyses are as follows: (1) Total miRNAs: the overall pooled sensitivity and specificity were 0.82 (95% CI: 0.79–0.85) and 0.87 (95% CI: 0.84–0.90), respectively. The AUC value was 0.91 (95% CI: 0.88–0.93) in the overall summary receiver operator characteristic (SROC) curve. (2) The panel of two miRNAs: sensitivity: 0.88 (95% CI: 0.77–0.94), specificity: 0.84 (95% CI: 0.72–0.91), AUC: 0.92 (95% CI: 0.90–0.94). (3) The panel of three miRNAs: sensitivity: 0.91 (95% CI: 0.85–0.94), specificity: 0.87 (95% CI: 0.77–0.92), AUC: 0.92 (95% CI: 0.89–0.94). (4) Results by types of miRNAs: miRNA-1: sensitivity: 0.78 (95% CI: 0.71–0.84), specificity: 0.86 (95% CI: 0.77–0.91), AUC: 0.88 (95% CI: 0.85–0.90); miRNA-133a: sensitivity: 0.85 (95% CI: 0.69–0.94), specificity: 0.92 (95% CI: 0.61–0.99), AUC: 0.93 (95% CI: 0.91–0.95); miRNA-208b: sensitivity: 0.80 (95% CI: 0.69–0.88), specificity: 0.96 (95% CI: 0.77–0.99), AUC: 0.91 (95% CI: 0.88–0.93); miRNA-499: sensitivity: 0.85 (95% CI: 0.77–0.91), specificity: 0.95 (95% CI: 0.89–0.98), AUC: 0.96 (95% CI: 0.94–0.97).

Conclusion: miRNAs may be used as potential biomarkers for the detection of AMI. For single, stand-alone miRNAs, miRNA-499 may have better diagnostic accuracy compared to other miRNAs. We propose that a panel of multiple miRNAs with high sensitivity and specificity should be tested.

Keywords: miRNAs, acute myocardial infarction, diagnosis, biomarker, meta-analysis

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INTRODUCTION

Although advanced clinical medications have recently been developed for the diagnosis and prevention of coronary heart disease (CAD), acute myocardial infarction (AMI), which includes ST-elevation myocardial infarction (STEMI) and non-STEMI (NSTEMI), is still considered a primary public health threat, with high morbidity and mortality worldwide (Moss et al., 1996; GBD 2013 Mortality and Causes of Death Collaborators, 2015). Acute-phase reaction during ischemic damage is a crucial pathogenesis of ischemia myocardial issue (Hoffmeister et al., 2003). Dependent on accurate recognition and diagnosis, early effective revascularization treatment is an important strategy to repair ischemic myocardium and can significantly reduce the mortality of AMI patients (Hung et al., 2013). Currently, the most widely used biomarkers of myocardial injury during clinical practice are cardiac troponin and creatine kinase-MB (CK-MB), which may provide effective benefits for patients with revascularization therapy (Dohi et al., 2015; Anand et al., 2019). However, the elevation of cardiac troponin may be involved in serious, non-cardiac disease such as neuromuscular disorders, severe sepsis, and chronic renal insufficiency (Lamb et al., 2006; Finsterer et al., 2007; Vallabhajosyula et al., 2017). High levels of cardiac troponin have also been detected in patients with heart failure (Myhre et al., 2018). Therefore, early diagnostic biomarkers and improvement of the accuracy of approaches for the early prediction of AMI are still warranted.

Potential novel genetic and molecular biomarkers are currently being explored (Lorenzano et al., 2019). MicroRNAs (miRNAs/miRs) are endogenous, non-coding RNAs ∼19–25nt that play crucial post-regulatory roles in animals and plants by targeting mRNAs for translational or cleavage repression (Bartel, 2004). MiRNAs can inhibit or reduce target gene expression, subsequently affecting protein expression (Saxena et al., 2018). Thus, miRNAs play important regulatory roles in cell growth, development, and differentiation (Gabisonia et al., 2019). Further, miRNAs have been identified in extracellular fluid and can be extremely stable despite the presence of endogenous RNase (Chevillet et al., 2014). In recent years, a number of studies have reported that miRNAs are dysregulated in CAD, and that specific circulating miRNA signatures might be useful as biomarkers for the diagnosis of AMI and as therapeutic targets (Jakob et al., 2017). However, the results of previous studies were significantly different, potentially due to sample size, specimen types, and different detection technologies. Therefore, the purpose of this systematic review and meta-analysis was to summarize the diagnostic values of blood-based miRNA levels from published articles from the last 10 years and to appraise the accuracy of results to determine whether miRNAs may be used as novel biomarkers for the diagnosis of AMI.

MATERIALS AND METHODS

This study was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-analysis guidelines (Hutton et al., 2015; Moher et al., 2015). Two reviewers (CN Zhai, R Li) were independently involved with study selection, data extraction, and quality assessment.

Study Selection

An electronic search of MEDLINE (including PubMed), the Cochrane Central database, and EMBASE was performed to identify relevant articles published between January 2010 and December 2019. The following medical subject heading terms were used: ("plasma" OR "serum" OR "circulating") AND ("microRNA" OR "miRNAs" OR "miR*") AND ("myocardial infarction" OR "AMI" OR "coronary heart disease" OR "coronary artery disease" OR "coronary syndrome" OR "ischemic heart disease"). No language restrictions were imposed. All relevant review articles were retrieved, and duplicates were removed by manually searching. Based on the title and abstract, manuscripts of interest were obtained for full-text review. Only full-text references were included.

Inclusion and Exclusion Criteria

Inclusion and exclusion criteria were developed by the investigative team. The inclusion criteria were: (1) human studies, (2) studies related to circulating miRNAs levels and AMI, and (3) studies that contained enough data to evaluate the diagnostic value of miRNAs in AMI. Exclusion criteria were based on the following: (1) studies evaluating tissue miRNA or miRNA in other body fluids; (2) case reports, conference abstracts, and reviews; and (3) non-human studies.

Data Extraction, Meta-Analysis, and Quality Assessment

Each manuscript was assessed independently by two researchers (CN Zhai and K Hou). Disagreements among reviewers were resolved by consensus. Data extracted included the following: authors, publication year, country, type of blood-based fluid (serum or plasma), characteristics of the study population (both case and control), study design (qRT-PCR detection method), whether miRNA screening was performed, number of miRNAs assessed, listing of the specific dysregulated miRNAs in AMI patients compared with controls, and outcome of statistical analyses including details of miRNA analysis, such as type of reference miRNA utilized. Studies reporting on single miRNA were included in the meta-analysis and were evaluated according to the Quality Assessment of Diagnostic Accuracy Studies 2 (QUADAS-2) checklist (Whiting et al., 2011), which is designed to assess the risk of bias and the applicability of studies of diagnostic accuracy. The following four key domains were included: patient selection, the index test, the reference standard, and flow and timing. Each was assessed with respect to the risk of bias, and the first three domains were assessed with respect to applicability.

Statistical Analysis

Analysis was based on the accuracy of the identified miRNAs for diagnosing the presence of AMI, as determined using Receiver Operator Characteristic (ROC) curves via the Area Under the Curve (AUC) value, and sensitivity and specificity where available (Carter et al., 2016). We calculated the pooled sensitivity,

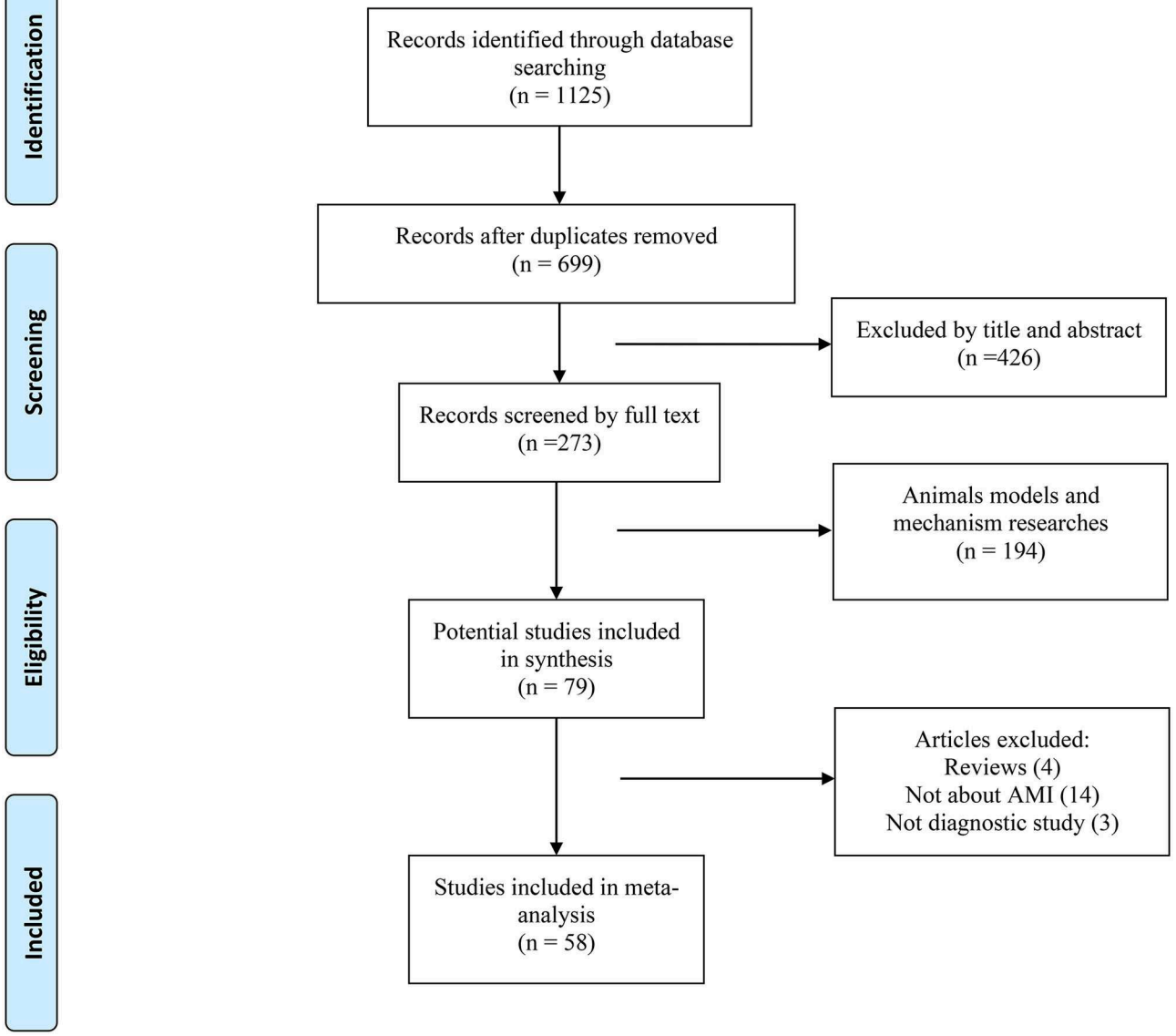

FIGURE 1 | Flow diagram of the literature search process and study inclusion.

specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), and diagnostic odds ratio (DOR), generated the bivariate summary receiver operator characteristic (SROC) curve, and calculated the area under the curve (AUC) to assess the overall diagnostic accuracy of miRNAs in distinguishing AMI patients from controls. Forest plots were constructed using STATA (15.0 StataCorp LP, College, Station, TX, USA). Due to the presumed heterogeneity of studies, a random-effects model (DerSimonian–Laird method) was used (Mahid et al., 2006). The heterogeneity of included studies was assessed using I^2, and the *P*-value was considered significant if I^2 was >50% or $P < 0.05$. Subgroup analyses were performed to explore the potential source of heterogeneity as follows: (1) based on the type of blood sample (plasma or serum), (2) the method of qRT-PCR detection (SYBR Green or TaqMan), (3) the type of reference control used for normalization (RNU or Cel-miRNA), (4) sample size (Sample size $\geq$ 100 or Sample size < 100), and (5) different populations (Caucasian or East Asian). To assess the publication bias of the included studies, we performed Deeks' test of funnel plot asymmetry (Deeks et al., 2005).

RESULTS

Literature Search Results and Characteristics of the Included Studies

The PRISMA flow diagram of the literature search and inclusion of relevant studies are shown in **Figure 1**. Overall, 79 full-text articles were deemed relevant for a more detailed evaluation. Of these articles, 21 were excluded for the following reasons: review

TABLE 1 | Characteristics of studies included in the systematic review.

References	Country	Specimen	Diseases	Case (*n*)	Control (*n*)	Age [mean ± s.d. /(range)]		Gender (male: female)	
						Case	Control	Case	Control
Corsten et al. (2010)	Netherlands	Plasma	STEMI vs. NC	32	36	62 ± 13	62 ± 13	24/12	23/13
Adachi et al. (2010)	Japan	Plasma	AMI vs. NC	9	10	66.8 ± 9.28	41.5 ± 8.0	6/3	5/5
Ai et al. (2010)	China	Plasma	AMI vs. NC	93	66	58.2 ± 10.2	55.1 ± 9.6	67/26	39/27
Wang et al. (2010)	China	Plasma	AMI vs. non-AMI	33	33	63.5 ± 10.1	64.3 ± 7.6	23/10	22/11
Cheng et al. (2010)	China	Serum	AMI vs. NC	31	20	NA (45-71)	NA	18/13	NA
D'Alessandra et al. (2010)	Italy	Plasma	STEMI vs. NC	33	17	57.9 ± 8.6	46.1 ± 13.9	31/2	13/4
Gidlöf et al. (2011)	Sweden	Plasma	STEMI vs. NC	25	11	64.56 ± 2.7	65.09 ± 3.51	20/5	7/4
Meder et al. (2011)	Germany	Blood	AMI vs. NC	20	20	59.3 ± 14	63.3 ± 14.8	16/4	14/6
Wang et al. (2011)	China	Plasma	AMI vs. NC	51	28	60.06 ± 11.53	57.86 ± 10.36	43/8	19/9
Zile et al. (2011)	USA	Plasma	AMI vs. NC	12	12	58 ± 3	61 ± 2	9/3	5/7
Devaux et al. (2012)	Netherlands	Plasma	STEMI & NSTEMI vs. NC	510	87	62 (0.32–91)	53 (40–60)	303/94	87/0
Long et al. (2012a)	China	Plasma	AMI vs. NC	17	25	53 ± 12.5	51 ± 12.3	13/4	18/7
Long et al. (2012b)	China	Plasma	AMI vs. NC	18	30	55 ± 11.4	50 ± 12.3	13/5	17/13
Li Y. Q. et al. (2013)	China	Plasma	AMI vs. NC	67	32	63.84 ± 11.17	61.75 ± 9.58	52/15	22/10
Li C. et al. (2013)	China	Serum	AMI vs. NC	117	100	62.7 ± 11.4	65.3 ± 9.98	20/97	19/81
Lu et al. (2013)	China	Plasma	AMI vs. non-AMI	40	15	66.8 ± 11.1	54.3 ± 17.5	30/10	6/9
Gidlöf et al. (2013)	Sweden	Plasma	AMI vs. non-AMI	319	88	NA	NA	NA	NA
Olivieri et al. (2013)	Italy	Plasma	NSTEMI vs. NC	92	99	82.6 ± 6.9	79.5 ± 5.4	39/53	40/59
Wang et al. (2013)	China	Plasma	AMI vs. NC	13	27	NA	NA	NA	NA
Li L. M. et al. (2014)	China	Plasma	AMI vs. NC	56	28	63.95 ± 11.34	60.50 ± 9.10	44/12	20/8
Li Z. et al. (2014)	China	Plasma	AMI vs. NC	27	31	54.15 ± 11.34	51.21 ± 12.25	20/7	16/15
Huang et al. (2014)	China	Plasma	AMI vs. NC	150	150	60.93 ± 12.86	60.66 ± 9.94	122/28	123/27
Hsu et al. (2014)	Tai Wan	Serum	STEMI vs. NC	31	31	59.0 ± 11.5	53.7 ± 14.8	29/2	29/2
He et al. (2014)	China	Plasma	AMI vs. non-AMI	359	30	58 ± 14	57 ± 10	301/58	21/9
Peng et al. (2014)	China	Plasma	AMI vs. non-AMI	76	110	64.6 (46–88)	60 (52–81)	43/33	61/49
Wang et al. (2014)	China	Plasma	AMI vs. NC	17	28	52 ± 11	58 ± 11	12/5	12/16
Xiao et al. (2014)	China	Serum	AMI vs. NC	NA	NA	NA	NA	NA	NA
Białek et al. (2015)	Poland	Plasma	STEMI vs. non-AMI	19	20	58 (55–65)	63 (58–74)	15/4	8/12
Chen et al. (2015)	China	blood	AMI vs. non-AMI	53	50	68.8 ± 7.3	68.3 ± 6.5	44/9	39/11
Devaux et al. (2015)	Luxembourg	Plasma	AMI vs. non-AMI	224	931	72 (61–80)	61 (49–74)	158/66	610/321
Gao et al. (2015)	China	Plasma	STEMI vs. non-AMI	35	160	NA	NA	NA	NA
Ji et al. (2015)	China	Serum	AMI vs. NC	98	23	64 ± 13.8	63.6 ± 12.2	82/16	15/8
Han et al. (2015)	China	Plasma	AMI vs. non-AMI	42	42	69.2 ± 7.4	67.4 ± 5.4	34/8	33/9
Li et al. (2015)	China	Plasma	STEMI & NSTEMI Vs. NC	87	87	56.93 ± 9.17	57.28 ± 10.82	64/23	62/25
Liu et al. (2015)	China	Plasma	AMI vs. NC	70	72	64.2 ± 11.2	62.3 ± 10.3	34/36	35/37
Yao et al. (2015)	China	Plasma	AMI vs. NC	50	39	63.2 ± 11.4	62.7 ± 10.5	33/17	27/12
Zhang L. et al. (2015)	China	Plasma	AMI vs. non-AMI	142	85	64.86 ± 12.84	66.45 ± 10.61	102/40	59/26
Zhang R. et al. (2015)	China	Plasma	STEMI & NSTEMI	110	110	57.74 ± 12.03	58.28 ± 11.32	87/23	83/27
Zhao et al. (2015)	China	Serum	AMI vs. NC	59	60	60.1 ± 11.3	61.9 ± 12.1	34/25	30/30
Ke-Gang et al. (2016)	China	Plasma	AMI vs. non-AMI	233	79	63 (20-91)	69 (35-70)	163/70	46/33
Wang et al. (2016)	China	Plasma	AMI vs. NC	32	36	55.62 ± 9.17	58.57 ± 11.54	18/14	24/12
Yang et al. (2016)	China	Plasma	AMI vs. NC	54	30	NA	NA	NA	NA
Zhang et al. (2016)	China	Plasma	AMI vs. NC	17	10	59.7 ± 8.4	56.2 ± 5.5	12/5	5/5
Zhu et al. (2016)	China	Plasma	STEMI vs. NC	60	60	64.1 ± 10.3	62.1 ± 11.2	46/14	45/15
Shalaby et al. (2016)	Egypt	Serum	NSTEMI vs. NC	48	25	54.3 ± 8.3	49.2 ± 10.2	32/16	17/8
Fawzy et al. (2018)	Egypt	Serum	STEMI vs. NC	110	121	NA	NA	61/49	63/58
Guo et al. (2017)	China	Plasma	AMI vs. NC	90	45	35.2 ± 5.6	36.2 ± 5.2	48/42	25/20
Yang et al. (2017)	China	Serum	AMI vs. NC	76	30	63.6 ± 11.7	62.3 ± 10.5	48/28	19/11
Agiannitopoulos et al. (2018)	Greece	Plasma	AMI vs. NC	50	50	62.12 ± 10.99	59.30 ± 9.82	38/12	33/17

(Continued)

TABLE 1 | Continued

References	Country	Specimen	Diseases	Case (*n*)	Control (*n*)	Age [mean ± s.d. /(range)]		Gender (male: female)	
						Case	Control	Case	Control
Liu et al. (2018)	China	Plasma	NSTEMI vs. NC	145	30	67	65	56:89	15/15
Wang et al. (2019)	China	Plasma	AMI vs. NC	66	70	61.84 ± 1.71	61.90 ± 3.69	30/36	26/44
Yi and An (2018)	China	Plasma	AMI vs. NC	30	30	61.35 ± 8.65	57.64 ± 5.91	NA	NA
Zhang et al. (2018)	China	Plasma	STEMI vs. NC	80	60	62.83 ± 7.52	63.15 ± 7.32	46/34	35/25
Bukauskas et al. (2019)	Lithuania	Serum	STEMI vs. NC	62	26	64 ± 12	42 ± 13	46/16	20/6
Li H. et al. (2019)	China	Plasma	AMI vs. NC	35	55	60.86 ± 11.25	56.36 ± 12.36	15/20	27/28
Li P. et al. (2019)	China	Serum	AMI vs. non-AMI	41	32	62.95 ± 11.04	63.16 ± 10.63	30/11	19/13
Xue et al. (2019a)	China	Plasma	STEMI & NSTEMI	29	21	68.0 ± 10.4	58.5 ± 14.3	23/6	16/5
Xue et al. (2019b)	China	Plasma	STEMI & NSTEMI	31	27	61.1 ± 10.0	60.1 ± 12.2	25/6	19/8

STEMI, ST-elevation myocardial infarction; AMI, acute myocardial infarction; NSTEMI, non ST-elevation myocardial infarction; NC, normal control.

article ($n = 4$), not about AMI ($n = 14$), and not a diagnostic study ($n = 3$). Finally, 58 studies investigating plasma, serum, or peripheral venous blood miRNAs in the diagnosis of AMI were identified as eligible for inclusion in this systematic review (Adachi et al., 2010; Ai et al., 2010; Cheng et al., 2010; Corsten et al., 2010; D'Alessandra et al., 2010; Wang et al., 2010, 2011, 2013, 2014, 2016, 2019; Gidlöf et al., 2011, 2013; Meder et al., 2011; Zile et al., 2011; Devaux et al., 2012, 2015; Long et al., 2012a,b; Li C. et al., 2013; Li Y. Q. et al., 2013; Lu et al., 2013; Olivieri et al., 2013; He et al., 2014; Hsu et al., 2014; Huang et al., 2014; Li L. M. et al., 2014; Li Z. et al., 2014; Peng et al., 2014; Xiao et al., 2014; Białek et al., 2015; Chen et al., 2015; Gao et al., 2015; Han et al., 2015; Ji et al., 2015; Li et al., 2015; Liu et al., 2015, 2018; Yao et al., 2015; Zhang L. et al., 2015; Zhang R. et al., 2015; Zhao et al., 2015; Ke-Gang et al., 2016; Shalaby et al., 2016; Yang et al., 2016, 2017; Zhang et al., 2016, 2018; Zhu et al., 2016; Guo et al., 2017; Agiannitopoulos et al., 2018; Fawzy et al., 2018; Yi and An, 2018; Bukauskas et al., 2019; Li H. et al., 2019; Li P. et al., 2019; Xue et al., 2019a,b). These studies were performed in 12 countries; most of the subjects involved were East Asian, with Caucasian as the second most common population investigated. The major clinical characteristics of the included studies are shown in **Table 1**. In total, 8,206 patients were included in the study: 4,526 AMI patients and 3,680 healthy/non-AMI subjects (1,975 health controls, 1,705 non-AMI patients). Ten studies were relevant to the evaluation of patients with STEMI, three studies only included NSTEMI patients, and the other 45 articles included both types of myocardial infarction. The population demographics of our study are shown in **Table 1**. In total, 2,692 men and 1,834 women were included among AMI groups, and 2,066 men and 1,614 women were included in the control groups.

Identification of Dysregulated miRNAs in the Included Studies

All included studies used Quantitative reverse transcription polymerase chain reaction (qRT-PCR) to detect the expression levels of miRNAs. A summary of all study methods is provided in **Table 2**. Seven studies performed miRNA screening to compare blood-based miRNAs between AMI patients and control groups (Adachi et al., 2010; D'Alessandra et al., 2010; Meder et al., 2011; Li C. et al., 2013; Hsu et al., 2014; Huang et al., 2014; Li et al., 2015). Fifty-one articles identified miRNAs based on their own previous studies or based on the literature (Ai et al., 2010; Cheng et al., 2010; Corsten et al., 2010; Wang et al., 2010, 2011, 2013, 2014, 2016, 2019; Gidlöf et al., 2011, 2013; Zile et al., 2011; Devaux et al., 2012, 2015; Long et al., 2012a,b; Li Y. Q. et al., 2013; Lu et al., 2013; Olivieri et al., 2013; He et al., 2014; Li L. M. et al., 2014; Li Z. et al., 2014; Peng et al., 2014; Xiao et al., 2014; Białek et al., 2015; Chen et al., 2015; Gao et al., 2015; Han et al., 2015; Ji et al., 2015; Liu et al., 2015, 2018; Yao et al., 2015; Zhang L. et al., 2015; Zhang R. et al., 2015; Ke-Gang et al., 2016; Shalaby et al., 2016; Yang et al., 2016, 2017; Zhang et al., 2016, 2018; Zhu et al., 2016; Guo et al., 2017; Agiannitopoulos et al., 2018; Fawzy et al., 2018; Yi and An, 2018; Bukauskas et al., 2019; Li H. et al., 2019; Li P. et al., 2019; Xue et al., 2019a,b). The expression of 50 miRNAs were identified as either significantly higher or lower expression in AMI cases; specific details were shown in **Table 3**. Thirty-three miRNAs were upregulated (miRNA-1, miRNA-17-5p, miRNA-19b-3p, miRNA-21, miRNA-23b, miRNA-26a-1, miRNA-30a, miRNA-122-5p, miRNA-124, miRNA-126, miRNA-133a/b, miRNA-134, miRNA-145-3p, miRNA-146a, miRNA-150, miRNA-181a, miRNA-186, miRNA-195, miRNA-199a-1, miRNA-208a/b, miRNA-210, miRNA-223, miRNA-302b, miRNA-328, miRNA-361-5p, miRNA-423-5p, miRNA-486, miRNA-494, miRNA-497, miRNA-499, miRNA-663b, miRNA-1291, and miRNA-1303) (Adachi et al., 2010; Ai et al., 2010; Cheng et al., 2010; Corsten et al., 2010; D'Alessandra et al., 2010; Wang et al., 2010, 2011, 2013, 2014, 2016, 2019; Gidlöf et al., 2011, 2013; Meder et al., 2011; Zile et al., 2011; Devaux et al., 2012, 2015; Long et al., 2012a,b; Li C. et al., 2013; Li Y. Q. et al., 2013; Olivieri et al., 2013; He et al., 2014; Hsu et al., 2014; Li L. M. et al., 2014; Li Z. et al., 2014; Xiao et al., 2014; Białek et al., 2015; Chen et al., 2015; Han et al., 2015; Ji et al., 2015; Li et al., 2015; Liu et al., 2015, 2018; Yao et al., 2015; Zhang L. et al., 2015; Zhang R. et al., 2015; Ke-Gang et al., 2016; Shalaby et al., 2016; Zhang et al., 2016, 2018; Guo et al., 2017; Yang et al., 2017; Agiannitopoulos et al., 2018; Fawzy et al., 2018; Li P. et al., 2019; Xue et al., 2019a,b), and 17 miRNAs were downregulated (miRNA-22-5p, miRNA-23a-3p, miRNA-26a, miRNA-30d-5p, miRNA-99a,

TABLE 2 | Study methods and corresponding dysregulated miRNAs identified.

References	qRT-PCR detection method	Reference control	miRNA screening performed	Dysregulated miRNAs[a] (n)	Significantly dysregulated miRNAs on validation (*n*)	AMI definition	Time of blood sampling
Corsten et al. (2010)	SYBR	n/a	N	6	2	ST segment elevation; increase CK and TnI	within 12 h of chest pain onset
Adachi et al. (2010)	TaqMan	n/a	Y	3	1	NA	Within 48 h after onset of chest pain
Ai et al. (2010)	SYBR	RNU6	N	2	1	Ischemic symptoms; increase cTnI and CK-MB; ST segment elevation or depression; pathological Q wave	NA
Wang et al. (2010)	TaqMan	Cel-miR-39	N	40	4	Ischemia symptom; increase cTnI; ST segment change; coronary angiography	Within 12 h after admission
Cheng et al. (2010)	n/a	n/a	N	1	1	Ischemic chest pain; increase CK-MB; ST segment elevation	Within 24 h after AMI
D'Alessandra et al. (2010)	TaqMan	n/a	Y	48	6	NA	Within 12 h after the onset of symptoms
Gidlöf et al. (2011)	SYBR	miR-16	N	5	4	ST-segment elevation; coronary angiography	Within 24 h of the onset of ischemic symptoms
Meder et al. (2011)	n/a	RNU6	Y	40	20	ESC/AHA guidelines	NA
Wang et al. (2011)	SYBR	RNU6	N	2	2	Chest pain lasting >20 min; increase CK-MB and cTnI; ST segment and T- wave changes; pathological Q wave	Within 24 h after the onset of syndromes
Zile et al. (2011)	TaqMan	RNU6	N	6	5	AHA/ACC guidelines	Within 24 h after admission
Devaux et al. (2012)	TaqMan	n/a	N	2	2	ST segment elevation or depression; increase CK and TnI; coronary angiography	With acute and ongoing chest pain for 12 h
Long et al. (2012a)	SYBR	RNU6	N	2	2	Ischemic symptom; increase cTnI and CK-MB; ST segment elevation or depression; pathological Q wave;	Within 4 h after onset of symptom
Long et al. (2012b)	SYBR	RNU6	N	3	3	Ischemic symptom; increase cTnI and CK-MB; ST segment elevation or depression; pathological Q wave;	Within 4 h after onset of symptom
Li Y. Q. et al. (2013)	SYBR	Cel-miR-39	N	4	4	Chest pain lasting >30 min; increase CK-MB and cTnI; ST segment elevation or depression; pathological Q wave	Within 12 h of the onset of symptoms
Li C. et al. (2013)	TaqMan	n/a	Y	21	6	Chest pain; ST segment elevation or depression; increase cTnI and CK-MB; pathological Q wave	Within 2h after hospitalization
Lu et al. (2013)	TaqMan	RNU6	N	1	1	Typical chest pain; increase cTnI; coronary angiography	The next morning after admission
Gidlöf et al. (2013)	SYBR	miR-17	N	3	2	STEMI: ECG criteria; NSTEMI: increase troponin and clinical symptoms	71% were taken within 24 h, 82% within 48 h and 93% within 72 h after onset of chest pain
Olivieri et al. (2013)	TaqMan	miR-17, cel-miR-39	N	6	5	Ischemic symptom; ST segment elevation or depression >1 mm/negative T wave/new onset LVBB; increase cTnT	Immediately after hospitalization
Wang et al. (2013)	SYBR	RNU6	N	1	1	Acute ischemic chest pain within 24 h; ECG changes; increase cTnI	Immediately after admission

(Continued)

TABLE 2 | Continued

References	qRT-PCR detection method	Reference control	miRNA screening performed	Dysregulated miRNAs[a] (n)	Significantly dysregulated miRNAs on validation (*n*)	AMI definition	Time of blood sampling
Li L. M. et al. (2014)	SYBR	Cel-miR-39	N	1	1	Increased cTnT or CK-MB; chest pain lasting for >30 min; pathological Q waves/ST-segment changes	Within 12 h after onset of chest pain
Li Z. et al. (2014)	SYBR	RNU6	N	1	1	Ischemic symptoms; increase cTnI and CK-MB; ST segment elevation or depression; pathological Q wave	Within 4 h after onset of symptom
Huang et al. (2014)	n/a	Cel-miR-39	Y	77	2	Chest paining lasting >20 min; ST segment changes; pathological Q wave; increase cardiac biomarkers	NA
Hsu et al. (2014)	SYBR	n/a	Y	25	5	ACC/AHA guideline	NA
He et al. (2014)	SYBR	n/a	N	2	2	Ischemic symptoms; increase cTn and CK; ST segment change; pathological Q wave	6 h after the onset of symptoms
Peng et al. (2014)	TaqMan	miR-16	N	3	3	STEMI: ST segment elevation; NSTEMI: ischemic symptom and increase cTnI	Within 3 h after admission
Wang et al. (2014)	SYBR	RNU6	N	3	3	Acute ischemic-type chest pain; ECG changes; increase cTnI	Immediately after admission
Xiao et al. (2014)	TaqMan	Cel-miR-39	N	2	2	NA	NA
Białek et al. (2015)	TaqMan	HY3	N	1	1	Chest pain; ST segment elevation; coronary angiography	NA
Chen et al. (2015)	TaqMan	RNU6	N	1	1	Biochemical markers; acute ischemic-type chest pain; ECG changes; coronary angiography	Immediately after admission
Devaux et al. (2015)	SYBR	n/a	N	6	3	ACC/AHA guideline	With acute chest pain for 12 h
Gao et al. (2015)	SYBR	Cel-miR-39	N	1	1	ACC/AHA guidelines	Immediately after hospitalization
Ji et al. (2015)	SYBR	miR-16	N	3	3	Ischemia symptoms; ST segment elevation; increase cTnI and CK-MB	Immediately after hospitalization
Han et al. (2015)	TaqMan	RNU6	N	1	1	ESC/AHA/ACC guidelines	Within 12 h after the symptom onset
Li et al. (2015)	TaqMan	RNU6	Y	28	3	Ischemia symptoms; ST segment abnormality; pathological Q wave; increase cTnI and CK-MB	Within 4 h after onset of symptoms
Liu et al. (2015)	TaqMan	Cel-miR-39	N	3	3	Increased cTnT or CK-MB; chest pain lasting for >30 min; pathological Q waves/ST-segment changes	Within 2 h after the onset of symptom
Yao et al. (2015)	SYBR	RNU6	N	1	1	Ischemic symptoms; increase cTnI and CK-MB; ST segment changes and pathological Q wave	Within 4 h after the onset of symptoms
Zhang L. et al. (2015)	TaqMan	RNU6	N	1	1	Ischemic-type chest pain; increase cTnI; ECG change; coronary angiography	Within 12 h after the onset of acute chest pain
Zhang R. et al. (2015)	TaqMan	RNU6	N	2	2	Acute ischemic chest pain; abnormal ECG; increase cTnI and CK	Within 24 h after admission
Zhao et al. (2015)	SYBR	Cel-miR-39	N	1	1	ESC/ACC guidelines	Within 24 h after hospitalization
Ke-Gang et al. (2016)	TaqMan	Cel-miR-39	N	1	1	ESC Guidelines	Immediately after hospitalization

(Continued)

TABLE 2 | Continued

References	qRT-PCR detection method	Reference control	miRNA screening performed	Dysregulated miRNAs[a] (n)	Significantly dysregulated miRNAs on validation (*n*)	AMI definition	Time of blood sampling
Wang et al. (2016)	SYBR	Cel-miR-39	N	3	3	ESC/AHA/ACC guidelines	Within 12 h after the onset of chest pain
Yang et al. (2016)	SYBR	RNU6	N	1	1	Coronary angiography	NA
Zhang et al. (2016)	TaqMan	Cel-miR-39	N	1	1	ESC/ACC guidelines	Immediately after admission
Zhu et al. (2016)	TaqMan	RNU6	N	1	1	Ischemic chest pain lasting>30 min; increase cTnI; new ST segment elevation	Immediately after admission
Shalaby et al. (2016)	SYBR	RNU6	N	2	2	Ischemic symptom; no ST segment elevation; increase cTnI; coronary angiography	Within 24 h of onset of chest pain
Fawzy et al. (2018)	TaqMan	RNU6	N	1	1	Ischemic symptoms; ECG changes; coronary angiography	Within the first 12 h of the chest pain
Guo et al. (2017)	n/a	GAPDH	N	1	1	Guideline for AMI by Chinese Medicine Academy	Within 12 h of chest pain onset
Yang et al. (2017)	SYBR	RNU6	N	1	1	Clinical symptoms; ECG changes; increase cTnI, CK and CK-MB	Within 24 h of symptoms onset
Agiannitopoulos et al. (2018)	TaqMan	RNU24	N	2	2	Ischemic chest pain; ST segment elevation or depression; pathological Q wave; rise of cardiac biomarkers	Within 24 h of onset of chest pain
Liu et al. (2018)	SYBR	n/a	N	4	4	ACC guideline	Within 2–4 h after admission
Wang et al. (2019)	n/a	Cel-miR-39	N	6	2	Ischemic symptoms; ST segment-T wave changes or new LBBB; pathological Q wave; coronary angiography	Within 4 h of onset of chest pain/dyspnea
Yi and An (2018)	SYBR	RNU6	N	1	1	Coronary angiography; increase cTns and CK-MB	NA
Zhang et al. (2018)	SYBR	n/a	N	1	1	ST segment elevation; coronary angiography	Within 6 h after the onset of symptoms
Bukauskas et al. (2019)	SYBR	Cel-miR-39	N	3	3	2015 ESC Guidelines for the management of ACS	the first 24 h of admission
Li H. et al. (2019)	SYBR	Cel-miR-39	N	3	3	ACC/AHA guideline	With chest pain for 10 h
Li P. et al. (2019)	SYBR	RNU6	N	4	4	Ischemic symptoms; ST segment elevation or pathological Q wave; increase cTnI	With chest pain for 3 h
Xue et al. (2019a)	SYBR	Cel-miR-39	N	3	3	2012 ESC/AHA/ACC guidelines	Within 4 h of onset of chest pain
Xue et al. (2019b)	SYBR	Cel-miR-39	N	6	3	2012 ESC/AHA/ACC guidelines	Chest pain onset < 4 h duration

SYBR, SYBR Green; RNU6, small nuclear RNA U6; STEMI, ST-elevation myocardial infarction; NSTEMI, non ST-elevation myocardial infarction; CK, creatine kinase; CK-MB, creatine kinase-MB. Cardiac troponin I/T, cTnI/T. ECG, electrocardiogram.

(n): the number of dysregulated miRNAs.

[a]*Based upon miRNA screening, or based upon literature review or prior work (see text).*

Y or N, Yes or No; NA: not available.

miRNA-125b, miRNA-126-3p, miRNA-132-5p, miRNA-145, miRNA-146a-5p, miRNA-191, miRNA-214, miRNA-320b, miRNA-375, miRNA-379, miRNA-519-5p, and miRNA-let-7d) (D'Alessandra et al., 2010; Meder et al., 2011; Long et al., 2012b; Lu et al., 2013; Hsu et al., 2014; Huang et al., 2014; Wang et al., 2014, 2019; Gao et al., 2015; Li et al., 2015; Yang et al., 2016; Yi and An, 2018; Bukauskas et al., 2019; Li H. et al., 2019). Thirteen upregulated miRNAs (miRNA-1, miRNA-19b-3p, miRNA-21, miRNA-122-5p, miRNA-126, miRNA-133a/b, miRNA-134, miRNA-150, miRNA-186, miRNA-208a/b, miRNA-486, miRNA-499, and miRNA-663b) (Adachi et al., 2010; Corsten et al., 2010; D'Alessandra et al., 2010; Wang et al., 2010, 2011, 2013, 2016,

TABLE 3 | Significantly dysregulated miRNAs of patients with AMI as compared with controls.

miRNAs	References	Expression	Area under the curve (AUC)	Sensitivity	Specificity
Single miRNA					
miRNA-1	Ai et al., 2010	Upregulated	0.774	70.0%	75.0%
	Corsten et al., 2010		—[a]	90.0%	95.0%
	Cheng et al., 2010		—	—	—
	D'Alessandra et al., 2010		—	—	—
	Wang et al., 2010		0.847	78.0%	80.0%
	Gidlöf et al., 2011		0.979	88.9%	100%
	Li Y. Q. et al., 2013		0.827	80.0%	90.0%
	Long et al., 2012a		0.92	93.0%	90.0%
	Olivieri et al., 2013		—	—	—
	Li C. et al., 2013		—	78.0%	86.0%
	Gidlöf et al., 2013		0.59	55.0%	65.0%
	Li L. M. et al., 2014		0.854	80.0%	90.0%
	Liu et al., 2015		0.81	70.0%	90.0%
	Liu et al., 2018		0.773	75.7%	53.5%
miRNA-17-5p	Xue et al., 2019a	Upregulated	0.857	85.2%	85.7%
miRNA-19b-3p	Wang et al., 2016	Upregulated	0.821	72.2%	66.7%
	Wang et al., 2019		0.667	66.1%	61.3%
miRNA-21	Zile et al., 2011	Upregulated	—	—	—
miRNA-21-5p	Olivieri et al., 2013		—	—	—
	Zhang et al., 2016		0.892	78.6%	100%
	Wang et al., 2014		0.949	—	—
miRNA-23b	Zhang et al., 2018	Upregulated	0.809	88.1%	60.3%
miRNA-26a-1	Xue et al., 2019b	Upregulated	0.965	100%	85.2%
miRNA-30a	Long et al., 2012b	Upregulated	0.88	88.3%	82.5%
miRNA-122-5p	Yao et al., 2015	Upregulated	0.855	100%	60.2%
	Wang et al., 2019		0.626	63.8%	73.9%
miRNA-124	Guo et al., 2017	Upregulated	0.86	52.0%	91.0%
miRNA-126	Long et al., 2012a	Upregulated	0.86	75.1%	83.1%
miRNA-126-5p	Xue et al., 2019a		0.802	100%	61.9%
miRNA-133	Wang et al., 2011	Upregulated	0.89	98.0%	73.0%
miRNA-133a	Peng et al., 2014		0.912	81.1%	91.2%
	Liu et al., 2018		0.928	71.0%	96.5%
miRNA-133b	Corsten et al., 2010		—	—	—
	D'Alessandra et al., 2010		—	—	—
	Wang et al., 2010		0.867	87.3%	84.9%
	Gidlöf et al., 2011		0.859	99.6%	63.6%
	Li Y. Q. et al., 2013		0.947	82.7%	100%
	Olivieri et al., 2013		—	—	—
	Wang et al., 2013		—	—	—
	Ji et al., 2015		0.787	85.4%	99.8%
	Ke-Gang et al., 2016		0.667	61%	68%
	D'Alessandra et al., 2010		—	—	—
	Ji et al., 2015		0.823	66.5%	100%
miRNA-134	He et al., 2014	Upregulated	0.818	79.4%	77.1%
	Li C. et al., 2013		0.657	53.6%	78.1%
miRNA-134-5p	Wang et al., 2016		0.827	77.8%	77.8%
	Wang et al., 2019		0.702	70.6%	79.1%
miRNA-145-3p	Xue et al., 2019a	Upregulated	0.720	81.8%	61.9%
miRNA-146a	Xue et al., 2019b	Upregulated	0.911	100%	66.7%
miRNA-150-3p	Hsu et al., 2014	Upregulated	0.715	71.3%	70.1%
	Li H. et al., 2019		0.904	88.2%	75.9%
miRNA-150	Zhang R. et al., 2015		0.678	80.6%	49.8%
miRNA-181a	Zhu et al., 2016	Upregulated	0.834	81.5%	81.8%
miRNA-186	Li C. et al., 2013	Upregulated	0.715	77.8%	60.9%
miRNA-186-5p	Wang et al., 2016		0.824	77.8%	77.8%
	Wang et al., 2019		0.692	56.9%	84.3%

(Continued)

TABLE 3 | Continued

miRNAs	References	Expression	Area under the curve (AUC)	Sensitivity	Specificity
miRNA-195	Long et al., 2012b	Upregulated	0.89	100%	44.7%
miRNA-199a-1	Xue et al., 2019b	Upregulated	0.855	96.8%	66.7%
miRNA-208b	Corsten et al., 2010	Upregulated	0.944	90.6%	94.1%
	Gidlöf et al., 2011		1.0	100%	100%
	Devaux et al., 2012		0.90	79.5%	99.8%
	Li Y. Q. et al., 2013		0.89	82.4%	99.8%
	Gidlöf et al., 2013		0.82	79.0%	70.0%
	Devaux et al., 2015		0.76	64.7%	80.2%
	Li et al., 2015		0.674	59.8%	73.6%
miRNA-208a	Agiannitopoulos et al., 2018		0.999	98%	100%
	Wang et al., 2010		0.965	90.9%	100%
	Xiao et al., 2014		—	—	—
miRNA-208	Białek et al. (2015)		—	—	—
	Li C. et al., 2013		0.778	75.8%	73.1%
	Han et al., 2015		—	—	—
	Liu et al., 2015		0.72	65.0%	90.0%
	Liu et al., 2018		0.9940	90.0%	100%
	Li P. et al., 2019		0.868	70.0%	97.5%
miRNA-210	Shalaby et al., 2016	Upregulated	0.90	83.3%	100%
miRNA-223	Li C. et al., 2013	Upregulated	0.741	77.8%	68.3%
miRNA-302b	Yang et al., 2017	Upregulated	0.95	88.2%	93.3%
miRNA-328	He et al., 2014	Upregulated	0.887	86.3%	74.6%
miRNA-361-5p	Wang et al., 2014	Upregulated	0.881	—	—
miRNA-423-5p	Olivieri et al., 2013	Upregulated	—	—	—
miRNA-486-3p	Hsu et al., 2014	Upregulated	0.629	38.8%	84.1%
miRNA-486	Zhang R. et al., 2015		0.731	56.5%	86.5%
miRNA-494	Li P. et al., 2019	Upregulated	0.839	79.6%	82.1%
miRNA-497	Li Z. et al., 2014	Upregulated	0.88	82%	94%
miRNA-499	Corsten et al., 2010	Upregulated	0.918	84.5%	93.9%
	Adachi et al., 2010		—	—	—
	Wang et al., 2010		0.822	60.0%	90.3%
	Devaux et al., 2012		0.98	95.0%	100%
	Li Y. Q. et al., 2013		0.884	80.0%	94.0%
	Li C. et al., 2013		0.755	80.0%	93.0%
	Xiao et al., 2014		—	—	—
	Chen et al., 2015		—	—	—
	Devaux et al., 2015		0.65	35.7%	90.3%
	Liu et al., 2015		0.88	82.0%	94.0%
	Zhang L. et al., 2015		0.86	80%	80.28%
	Zhao et al., 2015		0.915	86.37%	93.47%
	Shalaby et al., 2016		0.97	93.4%	100%
	Agiannitopoulos et al., 2018		0.999	98%	100%
	Liu et al., 2018		0.995	98.4%	100%
	Li P. et al., 2019		0.852	71.5%	89.3%
	D'Alessandra et al., 2010		—	—	—
miRNA-499-5p	Gidlöf et al., 2011		0.989	87.7%	100%
	Olivieri et al., 2013		0.88	88.0%	75.0%
	Gidlöf et al., 2013		0.79	64.0%	90.0%
miRNA-499a	Fawzy et al., 2018		0.953	97.2%	75%
miRNA-663b	Meder et al., 2011	Upregulated	0.94	90%	95%
	Peng et al., 2014		0.611	72.4%	76.5%
miRNA-1291	Peng et al., 2014	Upregulated	0.695	78.4%	89.5%
miRNA-1303	Li H. et al., 2019	Upregulated	0.884	81.2%	89.3%
miRNA-22-5p	Wang et al., 2019	Downregulated	0.975	96.7%	96.7%
miRNA-23a-3p	Bukauskas et al., 2019	Downregulated	0.806	73.3%	79.6%
miRNA-26a-5p	Hsu et al., 2014	Downregulated	0.675	57.8%	90.2%
miRNA-26a	Li et al., 2015		0.745	73.6%	72.4%

(Continued)

TABLE 3 | Continued

miRNAs	References	Expression	Area under the curve (AUC)	Sensitivity	Specificity
miRNA-30d-5p	Bukauskas et al., 2019	Downregulated	0.745	80.9%	64.9%
miRNA-99a	Yang et al., 2016	Downregulated	—	—	—
miRNA-125b	Huang et al., 2014	Downregulated	0.858	—	—
miRNA-126-3p	Hsu et al., 2014	Downregulated	0.694	64.1%	80.0%
miRNA-132-5p	Li H. et al., 2019	Downregulated	0.886	85.3%	74.1%
miRNA-145	Gao et al., 2015	Downregulated	—	—	—
miRNA-146a-5p	Bukauskas et al., 2019	Downregulated	0.800	84.5%	70.4%
miRNA-191-5p	Hsu et al., 2014	Downregulated	0.652	48.6%	93.8%
miRNA-191	Li et al., 2015		0.669	62.1%	69.0%
miRNA-214	Lu et al., 2013	Downregulated	—	—	—
miRNA-320b	Huang et al., 2014	Downregulated	0.866	—	—
miRNA-375	D'Alessandra et al., 2010	Downregulated	—	—	—
	Wang et al., 2019		0.510	92.6%	33.1%
miRNA-379	Yi and An, 2018	Downregulated	0.751	—	—
miRNA-519-5p	Wang et al., 2014	Downregulated	0.798	—	—
miRNA-let-7d	Long et al., 2012b	Downregulated	0.86	64.7%	100%
Two miRNA panels					
miRNA-191-5p,-486-3p	Hsu et al., 2014	Upregulated and downregulated[b]	0.867	83.87%	83.33%
miRNA-486-3p,-126-3p	Hsu et al., 2014	Upregulated	0.849	61.29%	93.55%
miRNA-126-3p,-150-3p	Hsu et al., 2014	Upregulated	0.843	93.55%	64.52%
miRNA-486-3p,-26a-5p	Hsu et al., 2014	Upregulated and downregulated	0.821	87.10%	64.52%
miRNA-26a-5p,-150-3p	Hsu et al., 2014	Upregulated and downregulated	0.821	83.87%	70.97%
miRNA-191-5p,-150-3p	Hsu et al., 2014	Upregulated and downregulated	0.789	77.42%	77.42%
miRNA-150,-486	Zhang R. et al., 2015	Upregulated	0.771	72.6%	72.1%
miRNA-499,-210	Shalaby et al., 2016	Upregulated	0.98	97.9%	100%
miRNA-22-5p,-122-5p	Wang et al., 2019	Upregulated and downregulated	0.976	98.4%	96.2%
Three miRNA panels					
miRNA-30a,-195, let-7d	Long et al., 2012b	Upregulated and downregulated	0.930	94%	90%
miRNA-21-5p,-361-5p,-519-5p	Wang et al., 2014	Upregulated and downregulated	0.989	93.9%	100%
miRNA-19b-3p,-134-5p,-186-5p	Wang et al., 2016	Upregulated	0.898	88.9%	77.8%
miRNA-22-5p,-150-3p,-132-5p	Li H. et al., 2019	Upregulated and downregulated	0.942	91.2%	87.0%
miRNA-17-5p,-126-5p,-145-3p	Xue et al., 2019a	Upregulated	0.857	84.0%	85.7%
miRNA-26a-1,-146a,-199a-1	Xue et al., 2019b	Upregulated	0.913	97.8%	71.6%
Panels with ≥ 4 miRNAs					
miRNA-1,-134,-186,-208,-223,-499	Li C. et al., 2013	Upregulated	0.811	55.3%	90.1%

AMI, acute myocardial infarction; miRNA, microRNA.

[a]*Not reported.* [b]*Upregulated and downregulated: where the individual miRNAs were either upregulated or downregulated in AMI compared with control groups.*

2019; Gidlöf et al., 2011, 2013; Devaux et al., 2012, 2015; Li Y. Q. et al., 2013; Olivieri et al., 2013; He et al., 2014; Hsu et al., 2014; Peng et al., 2014; Xiao et al., 2014; Białek et al., 2015; Chen et al., 2015; Han et al., 2015; Ji et al., 2015; Li et al., 2015; Liu et al., 2015, 2018; Zhang L. et al., 2015; Zhang R. et al., 2015; Ke-Gang et al., 2016; Shalaby et al., 2016; Agiannitopoulos et al., 2018; Fawzy et al., 2018; Li H. et al., 2019; Li P. et al., 2019) and three downregulated miRNAs (miRNA-26a, miRNA-191,

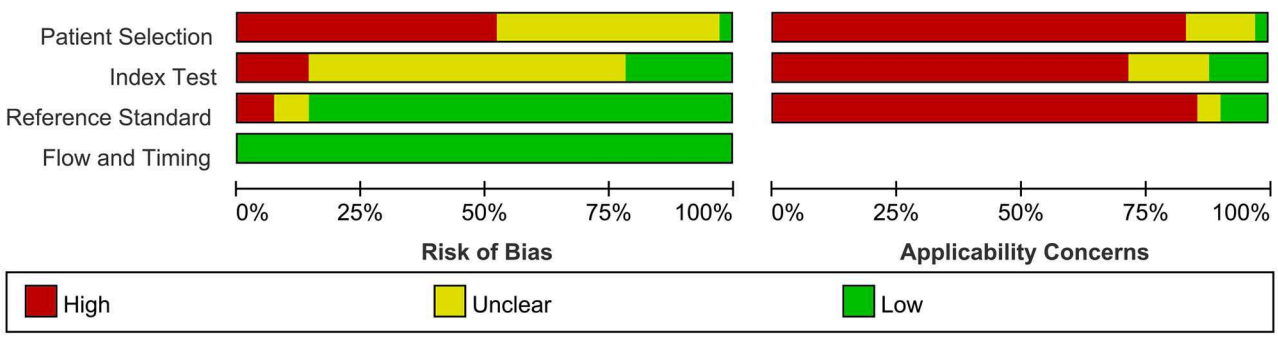

FIGURE 2 | Bar graphs of the methodological quality assessment. Each risk-of-bias and applicability item is presented as percentages across included studies, which indicates the proportion of different levels for each item.

and miRNA-375) (D'Alessandra et al., 2010; Hsu et al., 2014; Li et al., 2015; Wang et al., 2019) were identified by more than one study. Among these articles, which included 22 original studies, miRNA-499 was the most frequently identified dysregulated miRNA in AMI patients (Adachi et al., 2010; Corsten et al., 2010; D'Alessandra et al., 2010; Wang et al., 2010; Gidlöf et al., 2011, 2013; Devaux et al., 2012, 2015; Li C. et al., 2013; Li Y. Q. et al., 2013; Olivieri et al., 2013; Xiao et al., 2014; Chen et al., 2015; Liu et al., 2015, 2018; Zhang L. et al., 2015; Zhang R. et al., 2015; Shalaby et al., 2016; Agiannitopoulos et al., 2018; Fawzy et al., 2018; Li P. et al., 2019). 16 studies focused on the miRNA-208 family (miRNA-208a/b) (Corsten et al., 2010; Wang et al., 2010; Gidlöf et al., 2011, 2013; Devaux et al., 2012, 2015; Li C. et al., 2013; Li Y. Q. et al., 2013; Xiao et al., 2014; Białek et al., 2015; Han et al., 2015; Li et al., 2015; Liu et al., 2015, 2018; Agiannitopoulos et al., 2018; Li P. et al., 2019), 14 studies focused on miRNA-1 (Ai et al., 2010; Cheng et al., 2010; Corsten et al., 2010; D'Alessandra et al., 2010; Wang et al., 2010; Gidlöf et al., 2011, 2013; Long et al., 2012a; Li C. et al., 2013; Li Y. Q. et al., 2013; Olivieri et al., 2013; Li L. M. et al., 2014; Liu et al., 2015, 2018), and 14 studies focused on the miRNA-133 family (miRNA-133a/b) (Corsten et al., 2010; D'Alessandra et al., 2010; Wang et al., 2010, 2011, 2013; Gidlöf et al., 2011; Li Y. Q. et al., 2013; Olivieri et al., 2013; Peng et al., 2014; Ji et al., 2015; Ke-Gang et al., 2016; Liu et al., 2018). Additionally, four studies identified a panel of two miRNAs (Hsu et al., 2014; Zhang R. et al., 2015; Shalaby et al., 2016; Wang et al., 2019), six studies identified a panel of three miRNAs (Long et al., 2012b; Wang et al., 2014, 2016; Li H. et al., 2019; Xue et al., 2019a,b), and one study identified a panel of more than four miRNAs that were elevated in AMI (Li C. et al., 2013).

Sensitivity, Specificity, and Area Under the Curve

Among the included studies, the most common methods of assessing the diagnostic accuracy of dysregulated miRNAs were AUC and sensitivity and specificity, as determined from ROC curves. In the identified dysregulated miRNAs, AUC values ranged from 0.510 to 1.0, sensitivity ranged from 48.6% to 100%, and specificity from 33.1% to 100%. In the single identified miRNAs, the highest AUC sensitivity and specificity combination was reported for miRNA-208b (AUC 1.0, sensitivity 100%, specificity 100%) (Gidlöf et al., 2011). In the panel of two miRNAs, the highest value combination was reposted for miRNA-499 and miRNA-210 (AUC 0.98, sensitivity 97.9%, specificity 100%) (Shalaby et al., 2016). In the panel of three miRNAs, the highest value combination was reported for miRNA-21-5p, miRNA-361-5p, and miRNA-519-5p (AUC 0.989, sensitivity 93.9%, specificity 100%) (Wang et al., 2014). In the panel of $\geq$ 4 miRNAs, there was one study about AUC 0.811, which had sensitivity 55.3% and specificity 90.1% (Li C. et al., 2013).

Meta-Analysis Outcomes of Diagnostic Accuracy of miRNAs in AMI

The Quality Assessment of Included Studies

The pooled result of the meta-analysis for the diagnostic accuracy of blood-based miRNAs in AMI was pooled from 42 studies (Ai et al., 2010; Corsten et al., 2010; Wang et al., 2010, 2011, 2014, 2016, 2019; Gidlöf et al., 2011, 2013; Meder et al., 2011; Devaux et al., 2012, 2015; Long et al., 2012a,b; Li C. et al., 2013; Li Y. Q. et al., 2013; Olivieri et al., 2013; He et al., 2014; Hsu et al., 2014; Li L. M. et al., 2014; Li Z. et al., 2014; Peng et al., 2014; Ji et al., 2015; Li et al., 2015; Liu et al., 2015, 2018; Yao et al., 2015; Zhang L. et al., 2015; Zhang R. et al., 2015; Ke-Gang et al., 2016; Shalaby et al., 2016; Yang et al., 2016; Zhang et al., 2016, 2018; Zhu et al., 2016; Guo et al., 2017; Agiannitopoulos et al., 2018; Fawzy et al., 2018; Bukauskas et al., 2019; Li H. et al., 2019; Li P. et al., 2019; Xue et al., 2019a,b). Quality assessment results of the studies reporting on miRNAs included in the meta-analysis using the QUADAS-2 evaluation tool are shown in **Supplementary Figure 1**. Results are presented as percentages across the studies (**Figure 2**).

Total miRNAs

Dysregulated miRNAs in AMI patients compared with controls, the SROC curve with AUC, sensitivity, specificity, PLR, NLR, and DOR for miRNAs were included in our meta-analysis (**Table 4**). A random effect model was used for the meta-analysis due to significant heterogeneity (all $I^2 > 50\%$). Forty-four individual

TABLE 4 | The overall and subgroups meta-analysis results for comparison of diagnostic value of miRNAs.

Comparisons (*n*, study)	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PLR (95% CI)	NLR (95% CI)	DOR (95% CI)
POOLED SINGLE miRNAs (*n* = 102)						
Total miRNAs	0.91 (0.88–0.93)	0.82 (0.79–0.85)	0.87 (0.84–0.90)	6.27 (4.97–7.90)	0.21 (0.18–0.24)	30.40 (21.60–42.77)
Multiple combinations						
Two miRNAs panel (*n* = 9)	0.92 (0.90–0.94)	0.88 (0.77–0.94)	0.84 (0.72–0.91)	5.40 (2.84–10.26)	0.15 (0.07–0.30)	37.00 (10.52–130.16)
Three miRNAs panel (*n* = 6)	0.92 (0.89–0.94)	0.91 (0.85–0.94)	0.87 (0.77–0.92)	6.70 (3.83–11.72)	0.11 (0.07–0.18)	62.24 (27.40–141.38)
SUBGROUP ANALYSIS						
Type of blood sample						
Plasma (*n* = 75)	0.92 (0.89–0.94)	0.84 (0.80–0.87)	0.87 (0.82–0.90)	6.22 (4.68–8.27)	0.19 (0.15–0.23)	32.97 (21.48–50.61)
Serum (*n* = 26)	0.89 (0.86–0.92)	0.78 (0.72–0.82)	0.87 (0.82–0.91)	6.13 (4.23–8.89)	0.26 (0.20–0.33)	23.88 (14.15–40.28)
Type of miRNA detection method						
SYBR green (*n* = 62)	0.92 (0.89–0.94)	0.84 (0.80–0.87)	0.87 (0.83–0.90)	6.33 (4.77–8.40)	0.19 (0.15–0.24)	33.83 (22.46–50.94)
TaqMan (*n* = 30)	0.90 (0.87–0.92)	0.80 (0.74–0.85)	0.88 (0.82–0.93)	6.82 (4.27–10.88)	0.23 (0.17–0.30)	30.15 (15.03–60.45)
Type of miRNA reference						
RNU (*n* = 27)	0.93 (0.91–0.95)	0.86 (0.80–0.91)	0.89 (0.81–0.94)	8.08 (4.42–14.78)	0.16 (0.11–0.23)	51.49 (22.59–117.35)
Cel-miRNA (*n* = 50)	0.90 (0.87–0.92)	0.82 (0.78–0.85)	0.85 (0.81–0.89)	5.55 (4.22–7.29)	0.21 (0.17–0.26)	26.36 (17.70–39.25)
Included studies size						
Sample size ≥ 100 (*n* = 51)	0.89 (0.86–0.91)	0.79 (0.75–0.83)	0.86 (0.81–0.90)	5.82 (4.04–8.37)	0.24 (0.19–0.31)	24.00 (13.91–41.42)
Sample size < 100 (*n* = 51)	0.93 (0.90–0.95)	0.85 (0.81–0.89)	0.87 (0.83–0.90)	6.65 (5.03–8.78)	0.17 (0.13–0.21)	39.75 (27.00–58.53)
Different population						
Caucasian (*n* = 21)	0.95 (0.93–0.97)	0.86 (0.78–0.91)	0.94 (0.86–0.97)	14.15 (5.89–34.00)	0.15 (0.09–0.24)	95.63 (28.08–325.74)
East Asian (*n* = 78)	0.89 (0.86–0.91)	0.80 (0.77–0.83)	0.84 (0.81–0.87)	5.12 (4.13–6.35)	0.23 (0.20–0.28)	21.88 (15.97–29.99)
Type of different miRNAs						
miR-1 (*n* = 11)	0.88 (0.85–0.90)	0.78 (0.71–0.84)	0.86 (0.77–0.91)	5.41 (3.18–9.19)	0.26 (0.18–0.36)	21.07 (9.17–48.38)
miR-133 a/b (*n* = 9)	0.94 (0.92–0.96)	0.85 (0.72–0.92)	0.92 (0.78–0.98)	10.79 (3.63–32.09)	0.17 (0.09–0.31)	64.18 (19.03–216.49)
miR-133a (*n* = 5)	0.93 (0.91–0.95)	0.85 (0.69–0.94)	0.92 (0.61–0.99)	10.63 (1.69–66.96)	0.16 (0.07–0.36)	66.15 (7.41–590.92)
miR-208 a/b (*n* = 13)	0.94 (0.91–0.95)	0.83 (0.74–0.89)	0.98 (0.88–0.99)	35.45 (5.90–212.88)	0.18 (0.11–0.28)	201.13 (24.36–1660.71)
miR-208b (*n* = 7)	0.91 (0.88–0.93)	0.80 (0.69–0.88)	0.96 (0.77–0.99)	19.30 (2.78–134.22)	0.21 (0.12–0.35)	92.61 (9.07–945.66)
miR-499 (*n* = 17)	0.96 (0.94–0.97)	0.85 (0.77–0.91)	0.95 (0.89–0.98)	16.27 (7.31–36.22)	0.16 (0.10–0.26)	103.54 (31.08–345.01)

miR, microRNA. AUC, the area under cure in the overall summary receiver operator characteristic (SROC) curve. CI, confidence interval; PLR, positive likelihood ratio. NLR, negative likelihood ratio; DOR, diagnostic odds ratio; n, the number of included studies.

miRNAs were identified in 43 studies (Ai et al., 2010; Corsten et al., 2010; Wang et al., 2010, 2011, 2014, 2016, 2019; Gidlöf et al., 2011, 2013; Meder et al., 2011; Devaux et al., 2012, 2015; Long et al., 2012a,b; Li C. et al., 2013; Li Y. Q. et al., 2013; Olivieri et al., 2013; He et al., 2014; Hsu et al., 2014; Li L. M. et al., 2014; Li Z. et al., 2014; Peng et al., 2014; Ji et al., 2015; Li et al., 2015; Liu et al., 2015, 2018; Yao et al., 2015; Zhang L. et al., 2015; Zhang R. et al., 2015; Ke-Gang et al., 2016; Shalaby et al., 2016; Yang et al., 2016; Zhang et al., 2016, 2018; Zhu et al., 2016; Guo et al., 2017; Agiannitopoulos et al., 2018; Fawzy et al., 2018; Bukauskas et al., 2019; Li H. et al., 2019; Li P. et al., 2019; Xue et al., 2019a,b). The sensitivity, specificity, and the corresponding SROC value with 95% CIs (95% confidential intervals) of the total miRNAs in the diagnostic of AMI were 0.82 (95% CI: 0.79–0.85), 0.87 (95 %CI: 0.84–0.90), and 0.91 (95% CI: 0.88–0.93), respectively (**Supplementary Figures 2A,B**, **3A**). The Deeks' funnel plot asymmetry test suggested a potential for publication bias in the total miRNAs (p-value = 0.00, **Supplementary Figure 3B**).

Panels of Multiple miRNAs

As shown in **Table 3**, four studies (Hsu et al., 2014; Zhang R. et al., 2015; Shalaby et al., 2016; Wang et al., 2019) focused on the diagnostic value of a panel of two types of miRNAs, and the pooled sensitivity (**Figure 3A**) and specificity (**Figure 3B**) estimates were 0.88 (95% CI: 0.77–0.94) and 0.84 (95% CI: 0.72–0.91), respectively. The area under the SROC curve (**Figure 3C**) was 0.92 (95% CI: 0.90–0.94). The Deeks' test (**Figure 3D**) was performed to evaluate publication bias, and results suggested a low probability of publication bias.

From the analysis of a panel of three types of miRNAs in six studies (Long et al., 2012b; Wang et al., 2014, 2016; Ke-Gang et al., 2016; Li H. et al., 2019; Xue et al., 2019a,b) (**Table 3**), the pooled sensitivity (**Figure 4A**) and specificity (**Figure 4B**) estimates were 0.91 (95% CI: 0.85–0.94) and 0.87 (95% CI: 0.77–0.92), respectively. The area under the SROC curve (**Figure 4C**) was 0.92 (95% CI: 0.89–0.94). The Deeks' test was performed and suggested that publication bias likely had a low effect on the summary estimates (**Figure 4D**).

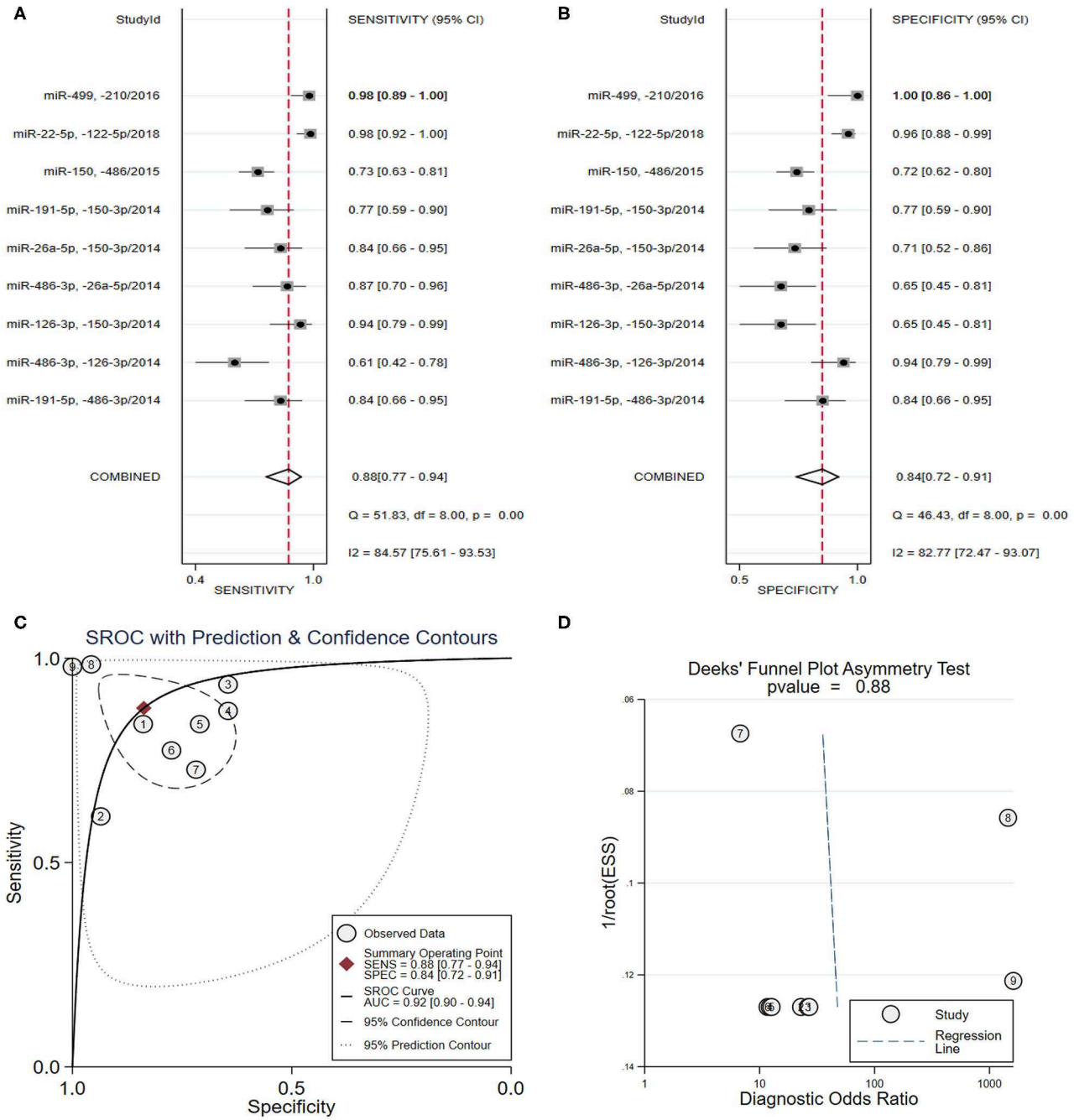

FIGURE 3 | The sensitivity, specificity, summary receiver operator characteristic (SROC) curve with area under curve (AUC), and funnel graph of the combination of two miRNAs in the diagnosis of acute myocardial infarction. **(A)** Sensitivity. **(B)** Specificity. **(C)** SROC curve with AUC. **(D)** Funnel graph.

Sensitivity Analyses

Sensitivity analyses were performed on the included studies according to the following factors: (1) Type of patient blood sample (plasma vs. serum): the SROC values were 0.92 vs. 0.89, the pooled sensitivity and specificity were 0.84 vs. 0.78 and 0.87 vs. 0.87, respectively; (2) Type of miRNA detection method (SYBR green vs TaqMan): the SROC values were 0.92 vs. 0.90, the pooled sensitivity and specificity were 0.84 vs. 0.80 and 0.87 vs. 0.88, respectively; (3) Type of miRNA reference used for normalization (RNU or Cel-miRNA): the SROC values were 0.93 vs. 0.90, the pooled sensitivity and specificity were 0.86 vs. 0.82 and 0.89 vs. 0.85, respectively; (4) different study

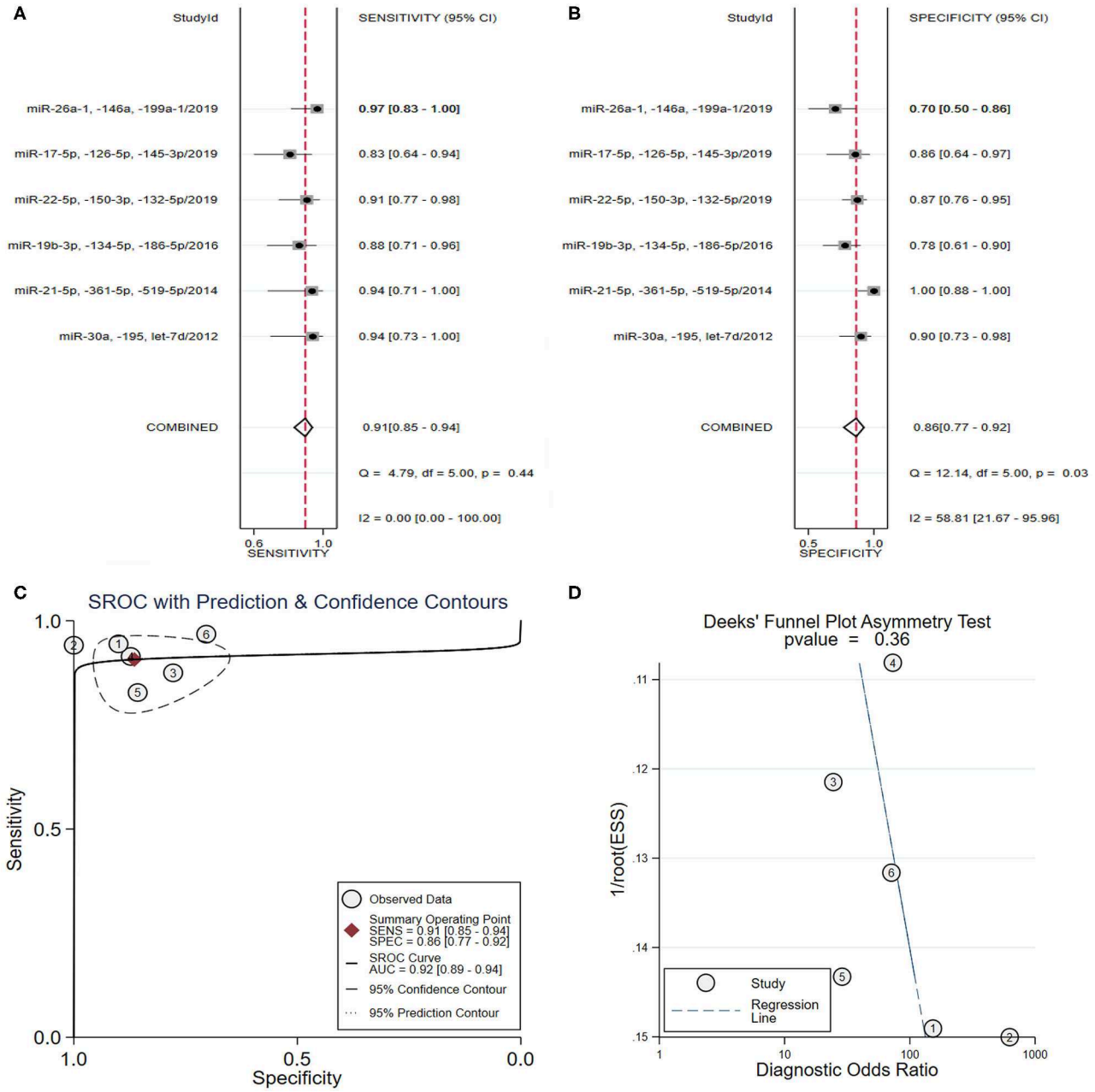

FIGURE 4 | The sensitivity, specificity, summary receiver operator characteristic (SROC) curve with area under curve (AUC), and funnel graph of the combination of three miRNAs in the diagnosis of acute myocardial infarction. **(A)** Sensitivity. **(B)** Specificity. **(C)** SROC curve with AUC. **(D)** Funnel graph.

sizes (sample size ≥ 100 vs. sample size < 100): the SROC values were 0.89 vs 0.93, the pooled sensitivity and specificity were 0.79 vs. 0.85 and 0.86 vs. 0.87, respectively; (5) Different populations (Caucasian vs. East Asian): the SROC values were 0.95 vs. 0.89, the pooled sensitivity and specificity were 0.86 vs. 0.80 and 0.94 vs. 0.84, respectively. A summary of the sensitivity analysis results is shown in **Table 4**. According to compared diagnostic values from each of the above groups, no major improvement or differences were found in the diagnostic accuracy values.

Meta-Analysis by Types of miRNAs

miRNA-1

In total, 11 studies identifying the diagnostic value of miRNA-1 in AMI were included in the meta-analysis (Ai et al., 2010; Corsten et al., 2010; Wang et al., 2010; Gidlöf et al., 2011, 2013; Long et al., 2012a; Li C. et al., 2013; Li Y. Q. et al., 2013; Li L. M. et al., 2014; Liu et al., 2015, 2018) (**Table 3**). As shown in **Figures 5A,B**, the pooled sensitivity and specificity estimates were 0.78 (95% CI: 0.71–0.84) and 0.86 (95% CI: 0.77–0.91), respectively. The area under the SROC curve for miRNA-1 was

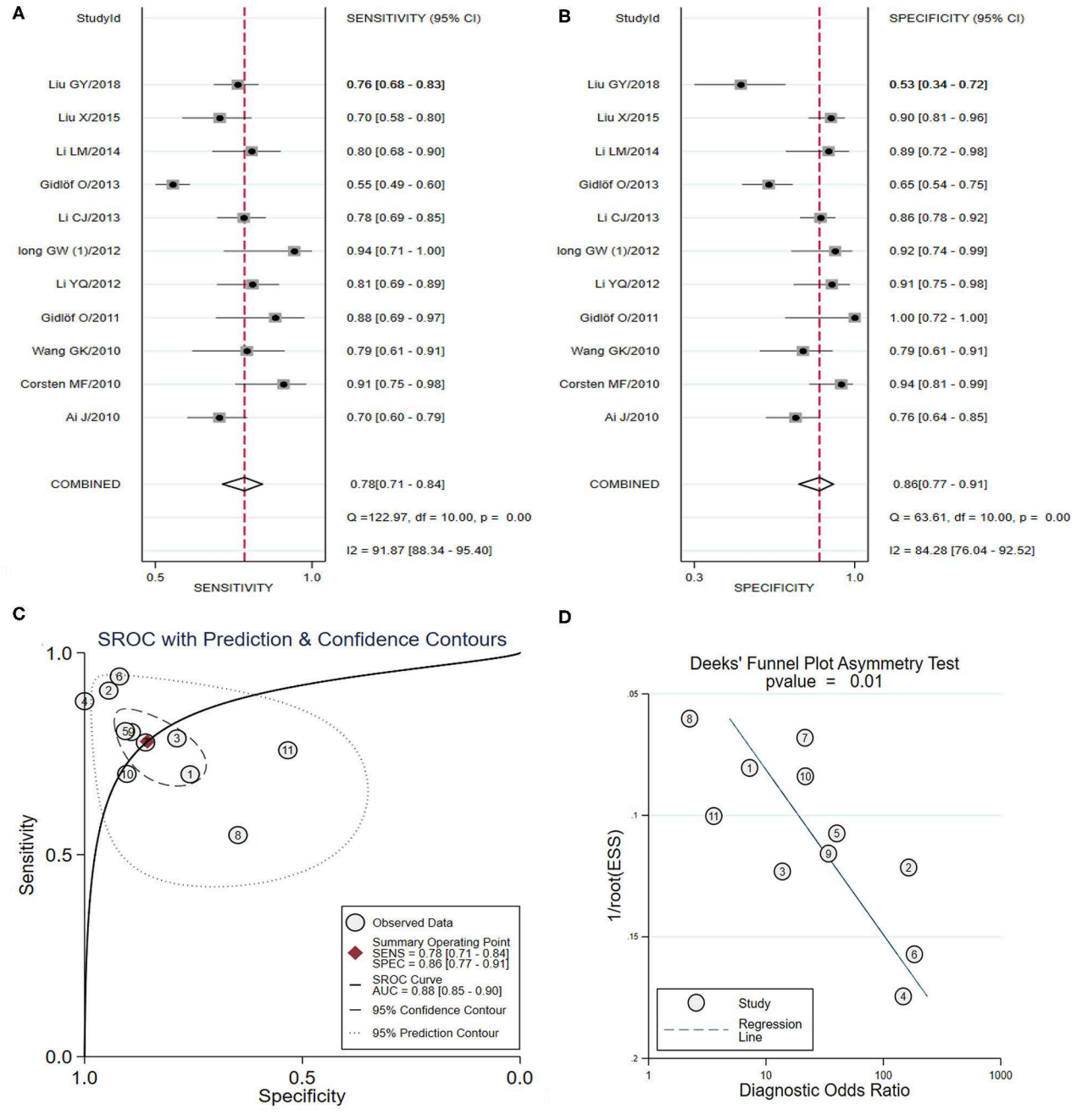

FIGURE 5 | The sensitivity, specificity, summary receiver operator characteristic (SROC) curve with area under curve (AUC), and funnel graph of miRNA-1 in the diagnosis of acute myocardial infarction. **(A)** Sensitivity. **(B)** Specificity. **(C)** SROC curve with AUC. **(D)** Funnel graph.

0.88 (95% CI: 0.85–0.90) (**Figure 5C**). The pooled PLR was 5.41 (95% CI: 3.18–9.19), and the pooled NLR was 0.26 (95% CI: 0.18–0.36). The DOR was 21.07 (95% CI: 9.17–48.38). Additionally, the Deeks' test suggested that publication bias may have some effect on the summary estimates (p-value = 0.01) (**Figure 5D**). Representative results from the above analyses are shown in **Table 4**.

miRNA-133

Nine studies that focused on the diagnostic accuracy of miRNA-133 (including miRNA-133a/b) in AMI were included in the meta-analysis (Corsten et al., 2010; D'Alessandra et al., 2010; Wang et al., 2010, 2011, 2013; Gidlöf et al., 2011; Li Y. Q. et al., 2013; Olivieri et al., 2013; Peng et al., 2014; Ji et al., 2015; Ke-Gang et al., 2016; Liu et al., 2018) (**Table 3**). As

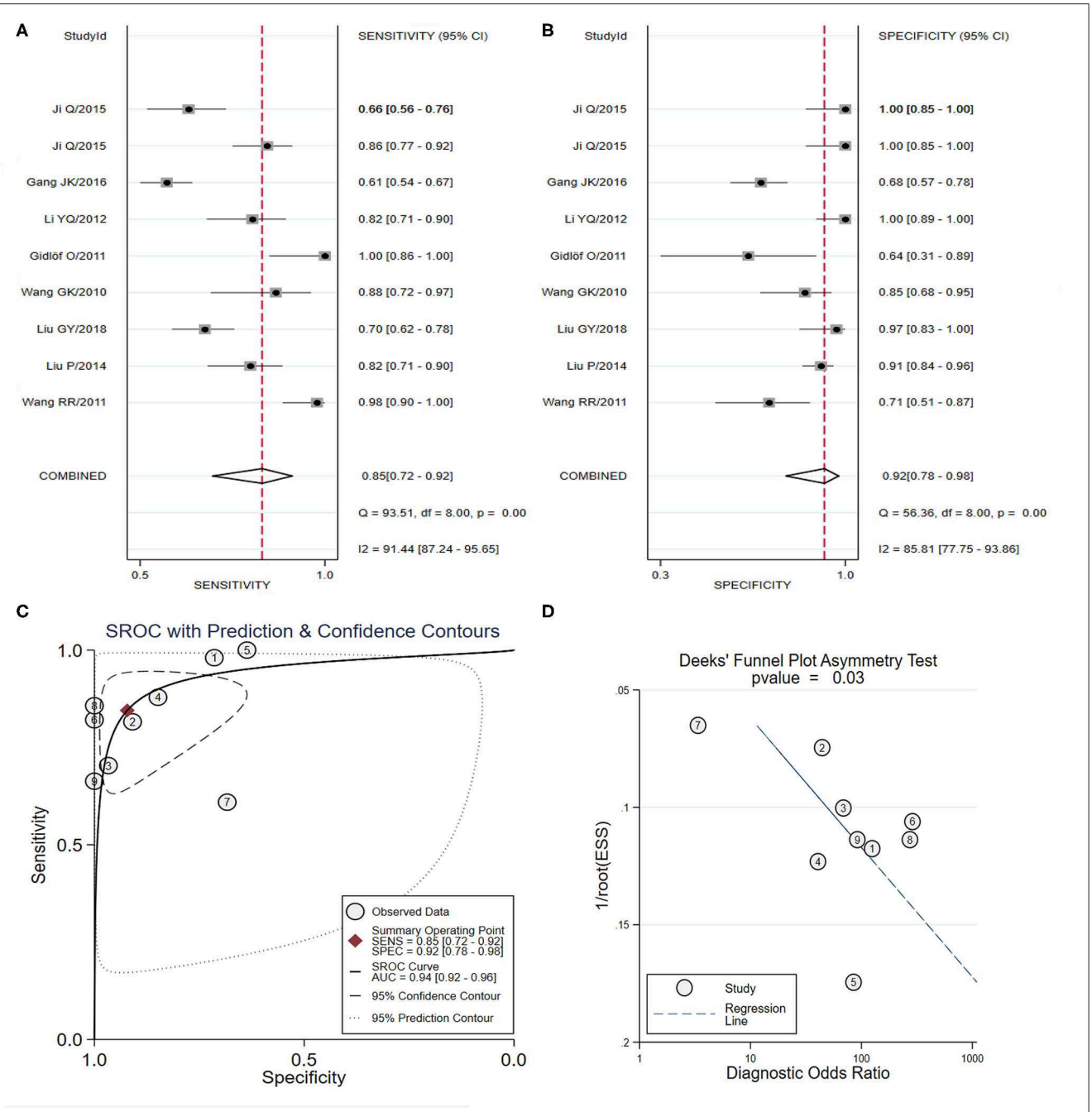

FIGURE 6 | The sensitivity, specificity, summary receiver operator characteristic (SROC) curve with area under curve (AUC), and funnel graph of the miRNA-133 family in the diagnosis of acute myocardial infarction. **(A)** Sensitivity. **(B)** Specificity. **(C)** SROC curve with AUC. **(D)** Funnel graph.

shown in **Figures 6A,B**, the pooled sensitivity and specificity estimates with 95% CI were 0.85 (95% CI: 0.72–0.92) and 0.92 (95% CI: 0.78–0.98), respectively. The area under the SROC curve for miRNA-133 was 0.94 (95% CI: 0.92–0.96) (**Figure 6C**). The pooled PLR was 10.79 (95% CI: 3.63–32.09) and the pooled NLR was 0.17 (95% CI: 0.09–0.31). The DOR was 64.18 (95% CI: 19.03–216.49). The Deeks' test suggested a potential publication bias (p-value = 0.01) (**Figure 6D**). Further subgroup analysis was conducted on miRNA-133a in the diagnosis of AMI and the SROC value, the pooled sensitivity, specificity, and DOR in the five studies (Wang et al., 2010; Gidlöf et al., 2011; Li Y. Q. et al., 2013; Ji et al., 2015; Ke-Gang et al., 2016) were 0.93 (95% CI: 0.91–0.95), 0.85 (95% CI: 0.69–0.94), 0.92 (95% CI: 0.61–0.99), and 66.15 (95% CI: 7.41–590.92). Representative results from the above analyses are shown in **Table 4**.

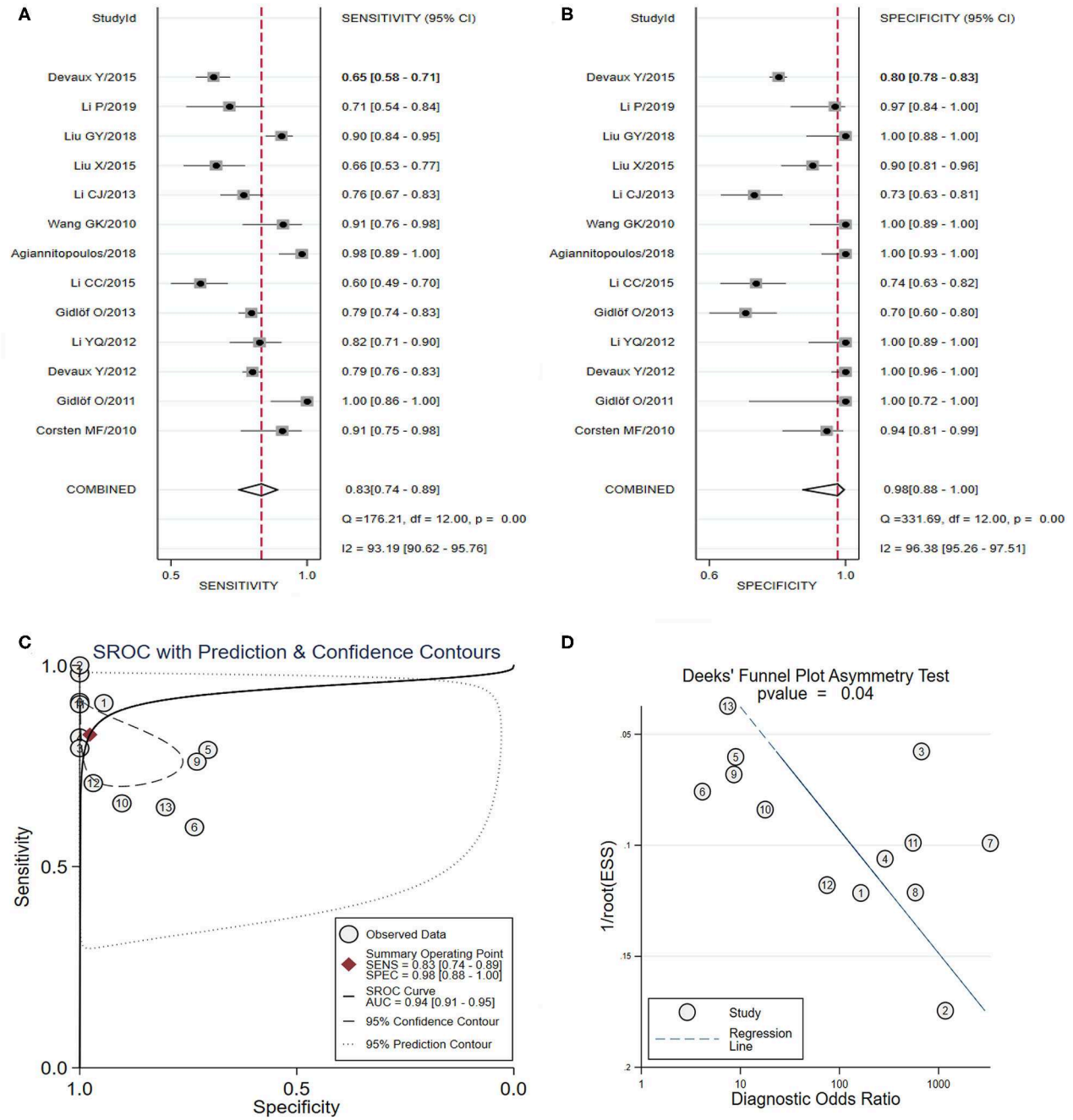

FIGURE 7 | The sensitivity, specificity, summary receiver operator characteristic (SROC) curve with area under curve (AUC), and funnel graph of the miRNA-208 family in the diagnosis of acute myocardial infarction. **(A)** Sensitivity. **(B)** Specificity. **(C)** SROC curve with AUC. **(D)** Funnel graph.

miRNA-208

Thirteen studies evaluated the diagnostic value of miRNA-208 (including miRNA-208a/b) in AMI and were included in the meta-analysis (Corsten et al., 2010; Wang et al., 2010; Gidlöf et al., 2011, 2013; Devaux et al., 2012, 2015; Li C. et al., 2013; Li Y. Q. et al., 2013; Li et al., 2015; Liu et al., 2015, 2018; Agiannitopoulos et al., 2018; Li P. et al., 2019) (**Table 3**). As shown in **Figures 7A,B**, the pooled sensitivity and specificity estimates with 95% CI were 0.83 (95% CI: 0.74–0.89) and 0.98 (95% CI: 0.88–0.99), respectively. The area under the SROC curve for miRNA-208 was 0.94 (95% CI: 0.91–0.95) (**Figure 7C**). The pooled PLR was 35.45 (95% CI: 5.90–212.88), and the pooled NLR was 0.18 (95% CI: 0.11–0.28). The DOR was 201.13 (95% CI: 24.36–1660.71). The Deeks' test suggested a possible publication bias (p-value = 0.04) (**Figure 7D**). In the subgroup analysis of miRNA-208b in the diagnosis of AMI, the SROC value, the pooled sensitivity,

specificity, and DOR in the seven studies (Corsten et al., 2010; Gidlöf et al., 2011, 2013; Devaux et al., 2012, 2015; Li Y. Q. et al., 2013; Li et al., 2015) were 0.91 (95% CI: 0.88–0.93), 0.80 (95% CI: 0.69–0.88), 0.96 (95% CI: 0.77–0.99), 92.61 (95% CI: 9.07–945.66). Representative results from the above analyses are shown in **Table 4**.

miRNA-499

Seventeen studies involving 3,976 individuals investigated the diagnostic accuracy of miRNA-499 as a novel biomarkers for AMI (Corsten et al., 2010; Wang et al., 2010; Gidlöf et al., 2011, 2013; Devaux et al., 2012, 2015; Li C. et al., 2013; Li Y. Q. et al., 2013; Olivieri et al., 2013; Liu et al., 2015, 2018; Zhang L. et al., 2015; Zhang R. et al., 2015; Shalaby et al., 2016; Agiannitopoulos et al., 2018; Fawzy et al., 2018; Li P. et al., 2019) (**Table 3**). As shown in **Figures 8A,B**, the pooled sensitivity and specificity estimates with 95%CI were 0.85 (95% CI: 0.77–0.91) and 0.95 (95% CI: 0.89–0.98), respectively. The area under the SROC curve for miRNA-499 was 0.96 (95% CI: 0.94–0.97) (**Figure 8C**). The pooled PLR was 16.27 (95% CI: 7.31–36.22), and the pooled NLR was 0.16 (95% CI: 0.10–0.26). The DOR was 103.54 (95% CI: 31.08–345.01). The Deeks' test suggested a low possibility of publication bias (p-value = 0.12) (**Figure 8D**). Representative results from the above analyses are shown in **Table 4**.

DISCUSSION

This systematic review and meta-analysis of 58 manuscripts that utilize blood circulating miRNAs (including plasma- or serum-based) in the diagnosis of AMI identified 51 significantly dysregulated miRNAs between AMI cases and controls. Additionally, this review assessed the feasibility of using these miRNAs as novel biomarkers for the diagnosis of AMI patients. Sixteen of the abnormally expressed miRNAs were investigated by more than one study, including thirteen upregulated miRNAs: miRNA-1, miRNA-19b-3p, miRNA-21, miRNA-122-5p, miRNA-126, miRNA-133a/b, miRNA-134, miRNA-150, miRNA-186, miRNA-208a/b, miRNA-486, miRNA-499, and miRNA-663b, and three downregulated miRNAs: miRNA-26a, miRNA-191, and miRNA-375. A further 34 dysregulated miRNAs were only reported by one study. The overall pooled diagnostic data of total miRNAs expression were as follows: SROC curve with AUC: 0.91, sensitivity: 0.82, specificity: 0.87, showing that circulating miRNAs might be suitable for use as potential biomarkers of AMI. Furthermore, the present meta-analysis was conducted via subgroup analyses based on type of miRNAs, including miRNA-1, miRNA-133a/b, miRNA-208a/b, and miRNA-499. MiRNA-499 had the highest diagnostic value (sensitivity: 0.85, specificity: 0.95, SROC curve with AUC: 0.96), followed by miRNA-133a (sensitivity: 0.85, specificity: 0.92, SROC curve with AUC: 0.93), and miRNA-208b had better specificity (0.96) than sensitivity (0.80). These results indicate a relatively high diagnostic accuracy for AMI based on significantly dysregulated miRNAs.

It is well-known that an early, accurate diagnosis and effective revascularization therapy play vital roles in reducing morbidity and mortality in patients with AMI (Yeh et al., 2010). At present, cardiac troponin, creatine kinase-MB (CK-MB), and myoglobin are the most widely used biomarkers in the diagnosis of AMI (de Winter et al., 1995). A good standard for the early diagnosis of AMI is an increase of cTn level (Celik et al., 2011). However, these markers are also likely to be elevated in patients with other diseases, whether or not CAD is also present (French and White, 2004). The detection of cTn has time constraints, as significant levels are reached 4–8 h following the onset of ischemia symptoms. Thus, novel genetic and molecular biomarkers of myocardial damage that have high sensitivity and specificity are still urgently needed.

Significant advances have occurred in the field of cardiovascular disease and miRNAs since their first discovery in the blood (Karakas et al., 2017). A growing number of studies indicate that the abnormal expression of miRNAs plays a critical role due to their various pathological functions in the presence of myocardial infarction (Gurha, 2016; Moghaddam et al., 2019). MiRNAs are steadily present in bodily fluids (including plasma, serum, urine, and saliva) due to protection from RNase via binding to argonaute proteins and the ability to be released from cells via microvesicles, exosomes, or bound to proteins (Meister, 2013). Moreover, recent studies have showed that cTn is more difficult to detect than miRNAs in patients with MI during the earlier acute stage, as it is usually below the cut-off value (Gidlöf et al., 2013; Zhang L. et al., 2015). This suggests a difference between these two types of biomarkers in the physiological process of myocardial infarction. Cardiac troponin releases into the blood during necrosis and during the pathological process of myocardial hypoxia and ischemia (Wu and Ford, 1999). However, miRNAs can be released in response to several forms of cellular stress occurring earlier then cell necrosis such as anoxia, lactic acidosis, and cellular edema (Edeleva and Shcherbata, 2013). Thus, experts may consider the expression levels of dysregulated miRNAs at an earlier stage of AMI, as they might be reliable candidate biomarkers for the diagnosis of AMI (Li C. et al., 2013).

In this study, the summary of single miRNAs showed that miRNA-1, miRNA-133, miRNA-208, and miRNA-499 are potential candidates for the detection of AMI, as they were most frequently detected in the previous studies. Among these individual miRNAs, circulating miRNA-499 might be an effective candidate biomarker of AMI. Some studies have demonstrated that miRNA-499 was specifically expressed in the myocardium and skeletal muscle of mammals (Xue et al., 2019a) and played a critical role in the recovery process following cardiac injury (Hosoda et al., 2011). Based on the present study, miRNA-499 has a higher sensitivity and specificity in identifying patients with AMI, and these results were similar to or better than previous studies (Cheng et al., 2014; Zhao et al., 2019). Previous studies were based on relatively small sample sizes and did not include studies published in recent years. Thus, the results of the present study are more reliable and convincing. Furthermore, we strictly considered the precise setting of specific miRNAs in the diagnosis of AMI and that assessing their diagnostic performance in combination with other biomarkers, especially highly sensitive troponin immunoassays, may be warranted. A study by Olivieri et al. showed a significant correlation between miRNA-499 and cTnT, and that the diagnosis value of this combination was

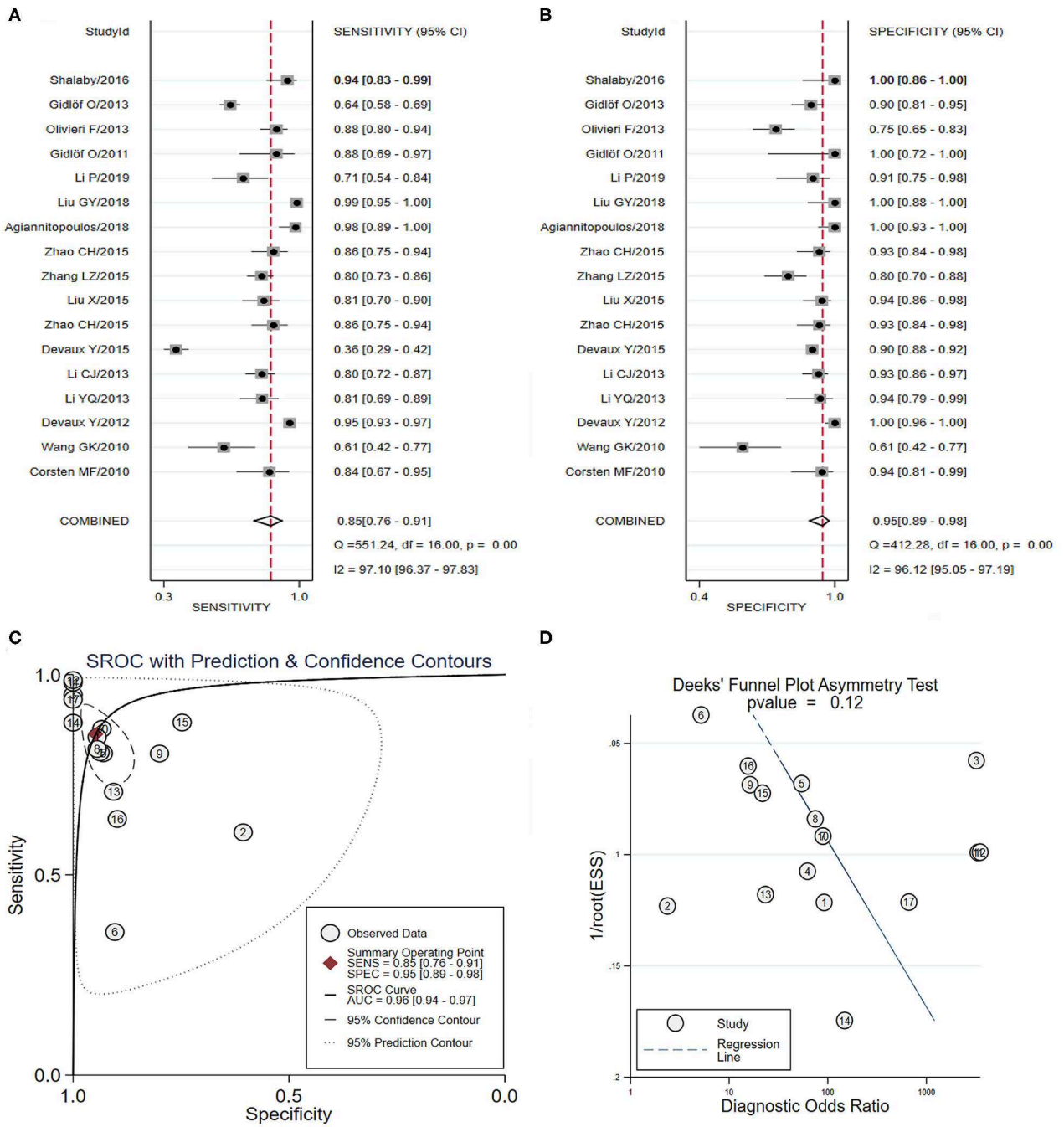

FIGURE 8 | The sensitivity, specificity, summary receiver operator characteristic (SROC) curve with area under curve (AUC), and funnel graph of miRNA-499 in the diagnosis of acute myocardial infarction. **(A)** Sensitivity. **(B)** Specificity. **(C)** SROC curve with AUC. **(D)** Funnel graph.

superior to either one alone (Olivieri et al., 2013). However, another study reported that the diagnostic value of miRNA-499 and hs-cTnT combined was not better than either them alone (Devaux et al., 2012). Thus, due to the limited sample sizes of these studies, further research is required.

There are only a small number of high-quality studies with large sample sizes focused on the diagnostic value of miRNAs in AMI. To the best of our knowledge, this study has included the largest sample size of dysregulated miRNAs as novel biomarkers for AMI for summary and evaluation. Further high-quality studies should be performed to acquire more reliable data for use in formulating standard diagnostic criterion and to determine optimal cut-off values. Moreover, due to the different diagnostic values of miRNAs, our study demonstrated that a panel of 2

or 3 miRNAs might be superior for diagnostic accuracy. The combination of 3 miRNAs trended toward a higher sensitivity than others for use as biomarkers in the diagnosis of AMI. Therefore, to further improve the feasibility of clinical diagnosis, future research should explore the most effective combination of multiple miRNAs, especially those confirmed to have a higher utilization value in the single miRNA groups.

Aside from the many studies investigating miRNA profiles in the detection of AMI, researchers should pay closer attention to the search for the technology to detect miRNAs quickly and accurately. Methods of RNA detection tend to be time-consuming, expensive, require sophisticated techniques, and are difficult to implement for urgent testing, especially in some developing countries (Lippi et al., 2013). However, novel detection technologies developed in recent years may provide a solution to these tissues, which would also support the clinical application of miRNAs in the future. Examples of new technologies include isothermal reactions based on cleavage with DNAzyme and signal amplification, which can simultaneously amplify and detect RNA (Zhao et al., 2013). This method is thought to be immune to genomic DNA pollution and has a relatively reliable sensitivity and specificity. We believed that new, precise methods of diagnosis that can detect miRNAs very rapidly and inexpensively need to be continuously improved.

In addition, the heterogeneity of the results of this study were substantial and could not be completely resolved. We found that the sources of heterogeneity included the following: quality of included studies, age, gender proportion, regional and environmental factors, and sampling criteria. Despite the heterogeneity, we believe the results of this study are worthwhile and valuable. Results of the overall and stratified analysis of different subgroups trended toward satisfactory values of miRNAs as novel biomarkers in the diagnosis of AMI. Furthermore, it was remarkable that some publication bias was found in the present study and might imply that the potential negative results were less likely to be published.

Although this study had a satisfactory result regarding the use of miRNAs for AMI detection, conclusions should be drawn cautiously due to several limitations: (1) there was a lack of standardization due to different normalization procedures across the included studies; (2) the exclusion of non-English articles may have caused important studies to be overlooked and publication bias due to significant results being more easily published; (3) the combined analysis of multiple miRNAs for a panel were insufficient, and we were unable to conduct a meta-analysis based on the data from limited studies; and (4) the results may have been affected by the impact of inevitable clinical heterogeneity, including the general condition of the included individuals, effects of medication, and medical history.

CONCLUSION

The current systematic review identifies numerous miRNAs associated with AMI and suggested that miRNAs may be used as a potential biomarker for the detection of AMI. For single, stand-alone miRNAs, miRNA-499 had better diagnostic accuracy than other miRNAs. A panel of two or three miRNAs might be superior for diagnostic accuracy. To develop a diagnostic test for AMI diagnosis, we suggest that a panel of miRNAs with high sensitivity and specificity should be tested. For this purpose, large scale, high-quality studies are still required to validate the clinical application of miRNAs for AMI diagnostics, as well as to identify the precise setting of dysregulated miRNAs in patients with AMI.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. Datasets are available through the corresponding author upon reasonable request.

ETHICS STATEMENT

All analyses were based on previous published studies; thus no ethical approval or patient consent were required. All previous published studies were approved by Ethics Committee, respectively.

AUTHOR CONTRIBUTIONS

HC and CZ designed the study. CZ carried out the statistical analysis and participated in most of the study steps. CZ, RL, JC, KH, and MA prepared the manuscript and assisted in the study processes. YH, JZ, YZ, LW, and RZ assisted in the data collection and helped in the interpretation of the study. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00691/full#supplementary-material

Supplementary Figure 1 | Methodological quality of studies in the meta-analysis using the Quality Assessment of Diagnostic Accuracy Studies 2 score system, including risk of bias and applicability concerns. The items were scored with "yes," "no," or "unsure".

Supplementary Figure 2 | Forest plots of the total miRNAs in the diagnosis of acute myocardial infarction among the studies included in the meta-analysis. **(A)** Sensitivity. **(B)** Specificity.

Supplementary Figure 3 | Summary receiver operator characteristic (SROC) curve with area under the curve (AUC) and funnel graph of the total miRNAs in the diagnosis of acute myocardial infarction. **(A)** SROC curve with AUC. **(B)** Funnel graph.

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MicroRNA Expression Profiling of Bone Marrow–Derived Proangiogenic Cells (PACs) in a Mouse Model of Hindlimb Ischemia: Modulation by Classical Cardiovascular Risk Factors

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Background: Classical cardiovascular risk factors (CRFs) are associated with impaired angiogenic activities of bone marrow–derived proangiogenic cells (PACs) related to peripheral artery diseases (PADs) and ischemia-induced neovascularization. MicroRNAs (miRs) are key regulators of gene expression, and they are involved in the modulation of PAC function and PAC paracrine activity. However, the effects of CRFs on the modulation of miR expression in PACs are unknown.

Aims and Methods: We used a model of hindlimb ischemia and next-generation sequencing to perform a complete profiling of miRs in PACs isolated from the bone marrow of mice subjected to three models of CRFs: aging, smoking (SMK) and hypercholesterolemia (HC).

Results: Approximately 570 miRs were detected in PACs in the different CRF models. When excluding miRs with a very low expression level (<100 RPM), 40 to 61 miRs were found to be significantly modulated by aging, SMK, or HC. In each CRF condition, we identified downregulated proangiogenic miRs and upregulated antiangiogenic miRs that could contribute to explain PAC dysfunction. Interestingly, several miRs were similarly downregulated (e.g., miR-542-3p, miR-29) or upregulated (e.g., miR-501, miR-92a) in all CRF conditions. *In silico* approaches including Kyoto Encyclopedia of Genes and Genomes and cluster dendogram analyses identified predictive effects of these miRs on pathways having key roles in the modulation of angiogenesis and PAC function, including vascular endothelial growth factor signaling, extracellular matrix remodeling, PI3K/AKT/MAPK signaling, transforming growth factor beta (TGFb) pathway, p53, and cell cycle progression.

Conclusion: This study describes for the first time the effects of CRFs on the modulation of miR profile in PACs related to PAD and ischemia-induced

neovascularization. We found that several angiogenesis-modulating miRs are similarly altered in different CRF conditions. Our findings constitute a solid framework for the identification of miRs that could be targeted in PACs in order to improve their angiogenic function and for the future development of novel therapies to improve neovascularization and reduce tissue damage in patients with severe PAD.

Keywords: microRNA (miR), proangiogenic cell (PAC), cardiovascular risk factors, hindlimb ischemia, neovascularization

INTRODUCTION

Peripheral artery disease (PAD) is a major health problem. It affects more than 200 million men and women worldwide and is the third leading cause of cardiovascular morbidity after coronary artery disease and stroke (Criqui and Aboyans, 2015). PAD is associated with intermittent claudication and impaired mobility. In its more severe form, PAD leads to chronic ischemia with rest pain, non-healing ulcers, tissue necrosis, gangrene, and lower limb amputation. Improving the natural capacity of the organism to develop new blood vessels (neovascularization) is an attractive strategy to promote perfusion of affected muscles, reduce pain, and avoid ischemic tissue damage and limb amputation (Losordo and Dimmeler, 2004; Cooke and Losordo, 2015). Neovascularization involves the development of collateral vessels (arteriogenesis), as well as the activation, proliferation, and migration of mature endothelial cells that will extend the preexisting vascular network (angiogenesis) (D'Amore and Thompson, 1987). However, it has now been demonstrated that postnatal neovascularization also depends on the action of bone marrow–derived proangiogenic cells (PACs) (Asahara et al., 1997; Asahara et al., 1999). PACs, initially described as early outgrowth endothelial progenitor cells (EPCs), were shown to reach sites of ischemia where they can promote neovascularization mainly through paracrine secretion of angiogenic factors and cytokines (Urbich and Dimmeler, 2004).

The administration of autologous mononuclear cells, including PACs, represents a possible therapeutic option for PAD patients with critical limb ischemia (CLI) (Cooke and Losordo, 2015). However, although positive results have been reported in preclinical studies and small clinical trials, several placebo-controlled randomized studies showed no advantage from bone marrow mononuclear cell administration over placebo (Peeters Weem et al., 2015). One possible explanation for these disappointing results is that the administered autologous cells might be dysfunctional in PAD patients who present several comorbid conditions. In fact, it has been demonstrated that classical cardiovascular risk factors (CRFs) involved in the development of atherosclerosis and PAD are also associated with impaired number and/or functional activities of PACs in humans (Vasa et al., 2001; Tepper et al., 2002; Hill et al., 2003; Chen et al., 2004; Michaud et al., 2006). However, the specific mechanisms leading to PAC dysfunction in these pathological conditions are poorly understood.

MicroRNAs (miRNAs or miRs) represent a novel class of endogenous non-coding small RNA molecules (20–25 nucleotides) that regulate a wide range of physiological and pathological processes, including angiogenesis (Urbich et al., 2008; Suarez and Sessa, 2009; Landskroner-Eiger et al., 2013). miRs can impact on stem/progenitor cell differentiation and function during cardiovascular repair responses (Jakob and Landmesser, 2012). In addition, miRs can be released by PACs to protect against ischemia and stimulate neovascularization in different tissues (Cantaluppi et al., 2012; Ranghino et al., 2012). Therefore, dysregulation of miR expression could contribute to explain the impaired functional activities of PACs in PAD patients with CRFs. Here, in a mouse model of PAD, we used next-generation sequencing (NGS) to study PAC miR expression profile in the context of three key CRFs: smoking (SMK), hypercholesterolemia (HC), and aging. Our results indicate that CRFs lead to important modulations in the expression of miRs in PACs, including several miRs predicted to be involved in the response to ischemia, angiogenesis, and postnatal neovascularization. Moreover, overlaps in the modulation of miRs by different CRFs might define a set of miRs with angiogenic/antiangiogenic properties that could be targeted in order to improve the function of PACs and promote therapeutic neovascularization in PAD patients with CLI.

MATERIALS AND METHODS

Murine Hindlimb Ischemia and Classical CRF Models

The protocol was approved by the Comité Institutionnel de Protection des Animaux of the Center Hospitalier de l'Université de Montréal. Unilateral hindlimb ischemia was surgically induced by removing the femoral artery under anesthesia with 2% isoflurane (Desjarlais et al., 2017; Desjarlais et al., 2019) and PACs were isolated from the bone marrow 2 days after ischemia. Four groups of mice were studied. The effect of aging was studied in 16-month-old C57BL/6 mice (aging). Young 2- to 3-month-old C57BL/6 mice were used as controls (CTL). The effect of SMK was studied in 2- to 3-month-old C57BL/6 mice exposed or not (CTL) to cigarette smoke (two cigarettes, twice a day) via an SMK machine starting 14 days prior to surgery (Dhahri et al., 2017). Commercial cigarettes (Player's Plain, tar: 17 mg, nicotine: 1.5 mg, carbon monoxide: 12 mg) were used. The effect of HC was studied in 2- to 3-month-old hypercholesterolemic ApoE$^{-/-}$ mice (C57BL/6 background) purchased from Jackson Laboratory (Bar Harbor, ME, United States) and put on a Western-type diet (1.25% cholesterol, 15% cocoa butter, 0.5% sodium cholate; Teklad

90221) beginning 5 weeks before the surgery (Desjarlais et al., 2017). Two- to 3-month-old normocholesterolemic C57BL/6 mice were used as CTL.

PAC Isolation and Characterization

Two days after hindlimb ischemia, bone marrow mononuclear cells were isolated from the femora and tibiae by flushing the bone marrow cavities using culture medium and kept on fibronectin-coated (Sigma, St. Louis, MO, United States) plates. After 4 days in culture, non-adherent cells were removed by thorough washing with phosphate-buffered saline (PBS). Adherent cells were stained with DAPI (0.5 mg/mL; Life Technologies); 1,10-dictadecyl-3,3,30,30 acetylated low-density lipoprotein (DiI-acLDL, 2.5 mg/mL for 1 h; Life Technologies); and fluorescein isothiocyanate (FITC)–labeled lectin BS-1 (*Bandeiraea simplicifolia*, 10 mg/mL for 1 h; Sigma). Bone marrow PACs were characterized as adherent cells that were positive for both DiI-acLDL uptake and lectin binding as previously described (Desjarlais et al., 2017; Dhahri et al., 2017; Desjarlais et al., 2019).

PAC Migration Assay

Proangiogenic cells migration was assessed using a modified Boyden chamber assay. Twenty thousand cells in growth factor–deprived medium were added to the upper chamber of a Transwell insert (pore size 8 μm; Corning, Corning, NY, United States) coated with 0.1% gelatin. The inserts were placed in a 24-well plate containing medium 200 with 50 ng/mL vascular endothelial growth factor (VEGF). After incubation for 6 h at 37°C, the cells that did not migrate were removed by wiping the upper surface with an absorbent tip. The migrant cells were fixed for 10 min with 3.7% formaldehyde and stained with hematoxylin. The number of cells that migrated was counted in three different representative high-power (200×) fields per insert. All experiments were performed in duplicate.

PAC Adhesion to an Endothelial Monolayer

A monolayer of human umbilical vein endothelial cells (HUVECs) (passages 4–6) was prepared in 24-well plates. HUVECs were pretreated for 16 h with tumor necrosis factor α (1 ng/mL; BD Biosciences), fixed and stained with DAPI (0.5 mg/mL; Life Technologies). PACs were labeled with DiI-AcLDL, and 15,000 cells were added to each well (2 wells/mouse) and incubated for 3 h at 37°C. Non-attached cells were gently removed with PBS, and adherent PACs were fixed with 2% paraformaldehyde and counted in three random fields per well.

miR Isolation and NGS Analyses

PACs were isolated from the different groups of mice 2 days after surgery, and total RNA was extracted from PACs after 4 days in culture using the Ambion mirVana^{TM} miR isolation kit (Life Technologies) according to the manufacturer's protocol. RNA quality was validated with the BioAnalyzer Nano (Agilent) using an RNA pico chip, and all samples had an RIN greater than eight. The amount of RNA we could recover from PACs isolated from one mouse was limited. Therefore, in order to optimize the quantity of RNA and also to avoid excessive cost, we pooled the RNA samples in each group before performing NGS. This strategy was recently shown to be a good option to optimize the cost and maintain the power for differential gene expression analysis (Assefa et al., 2020). Equal amounts of RNA samples (4–6/group) were pooled in each experimental condition: CTL, aging, SMK, and HC. Final RNA concentrations were then determined in each group, and an equal quantity of total RNA was used for library preparation. Library preparation was done with the QIAseq miR Library Kit (Qiagen). Sequencing was performed on a NextSeq 500 (Illumina), obtaining approximately 5 million reads per sample. The QIAseq miRNA sequencing files were uploaded to the GeneGlobe® Data Analysis center. To annotate the insert sequences, a unique sequence set was made for all readsets/samples. Following this, a sequential alignment strategy was followed to map to different databases (perfect match to miRBase mature, miRBase hairpin, non-coding RNA, mRNA and otherRNA, and ultimately a second mapping to miRBase mature, where up to two mismatches are tolerated) using bowtie (bowtie-bio.sourceforge.net/index.shtml). At each step, only unmapped sequences pass to the next step. Read counts for each RNA category (miRBase mature, miRBase hairpin, piRNA, tRNA, rRNA, mRNA, and otherRNA) were calculated from the mapping results (miRNA_Reads, hairpin_Reads, piRNA_Reads, etc.). miRBase V21 was used for miRNA. A mouse-specific miRBase mature database was used, and all remaining unmapped sequences were aligned to the mouse genome (Genome Reference Consortium GRCm38). Raw read counts obtained in each library are presented in **Supplementary Table 1**. Normalization by library size was then performed, and values expressed as reads per million reads mapped (RPM). We focused on miRs, with an expression level of at least 100 RPM, and differential gene expression levels were expressed as fold change between the CRF condition and the CTL (CRF/CTL). miRs were considered to be significantly upregulated or downregulated if the fold change value was greater than 1.6 for upregulated miRs and lower than 0.8 for downregulated miRs. Next-generation sequencing (NGS) data have been deposited and are available on NCBI Gene Expression Omnibus, GSE151609.

Identification of Predictive Targets/Pathways of miRs Modulated by CRFs in PACs

Predicted and validated target genes involved in angiogenesis, inflammation, oxidative stress, cell survival, apoptosis, senescence, and other pathways potentially linked to PAC dysfunction were identified in the different CRF conditions using the bioinformatic algorithms of miRSystem database ([1]; version 20160513), which is an integrated system used to characterize the enriched functions and pathways of miRNA targets (Lu et al., 2012). In target prediction, the miRSystem database integrates two experimentally validated databases, TarBase (version 7.0) and miRecords (April 27, 2013, release), and seven target gene

[1] http://mirsystem.cgm.ntu.edu.tw/index.php

prediction algorithms, including DIANA-microT (version 5.0), miRanda (August 2010 release), miRBridge (April 2010 release), PicTar (March 2007 release), PITA (August 2008 release), RNA22 (version 2.0), and Targetscan (version 7.1). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and dendrogram clusters of predictive biological Gene Ontology (GO) process-connectome of selected miRs were analyzed using DIANA tool program[2] (Vlachos et al., 2015).

Statistical Analysis

Results for PAC number and function (**Figure 1**) are presented as mean ± SEM. Statistical significance was evaluated by a one- or two-way analysis of variance followed by a Bonferroni *post hoc* test. $P < 0.05$ was interpreted to denote statistical significance.

RESULTS

Overview of miR Expression Profile in PACs Isolated From Aging, SMK, and HC Mice After Hindlimb Ischemia

To study how modulation of miRs could affect the function of PACs in pathological conditions, we used NGS to perform a complete profile of miRs in PACs isolated from mice subjected to three models of CRFs: aging (Groleau et al., 2011), SMK (Dhahri et al., 2017), and HC (Desjarlais et al., 2017; **Figure 1A**). PACs were isolated from the bone marrow of the different groups of mice 2 days after hindlimb ischemia surgery and cultured *ex vivo* for 4 days. Total RNA was extracted to perform NGS miR analyses. As shown in **Figures 1B,C**, the number of PACs and their chemotactic migration and adhesion properties were significantly decreased compared to CTL in aging, SMK, and HC mice. Next, we quantified the number of miRs in PACs exposed to the different CRFs and detected ∼570 miRs in each group, with a similar distribution of their level of expression (abundance): 73 to 76% low expression, 13 to 16% medium expression, and 9 to 10% high expression (**Figure 2A**). We then focused on the most abundant and modulated miRs by establishing an arbitrary expression cutoff level of 100 reads per million of reads mapped (RPM) and a modulation cutoff level of 0.8-fold versus CTL for downregulated miRs and 1.6-fold versus CTL for upregulated miRs. We identified 40 to 61 modulated miRs in PACs in the different CRF conditions (61 miRs in aging, 40 miRs in SMK, 59 miRs in HC) with a similar number of upregulated and downregulated miRs (**Figure 2B**).

Effect of Aging on the Modulation of miRs in PACs in the Context of Hindlimb Ischemia

We specifically looked at the expression profile of miRs in PACs isolated from the bone marrow of aging (16 months old) versus young (2 months old) mice at day 2 after hindlimb ischemia. **Figure 3A** confirms that several miRs are modulated by aging in PACs. The modulation is often seen in miRs showing moderate (100–1,000 RPM) or high (1,000–10,000 RPM) expression levels compared to those with very high expression levels (>10,000 RPM). For example, the five miRs exhibiting levels of expression greater than 100,000 RPM (miR-21a-5p, miR-16-5p, let-7f, miR-29a-3p and let-7i-5p) are not modulated by aging (**Figures 3A,B**). We next focused on miRs that showed the highest level of modulation (upregulation or downregulation) by aging (**Figure 3C**). Among downregulated miRs, four miRs (miR-31-p, miR-218-5p, miR-143-5p, and miR-10b-5p) showed greater than 70% suppression. On the other hand, three miRs were upregulated by 300% or more (miR-501-3p, miR-1198-5p, and miR-23b-3p). To better understand the potential biological effects of miR modulation by aging, we used KEGG analysis to identify predicted pathways involved in 10 highly expressed/highly modulated miRs (red arrows in **Figure 3C**). As seen in **Figure 3D**, the bioinformatic algorithm suggests that 14 pathways/processes are affected by these miRs. Interestingly, most of these pathways are involved in carcinogenesis, a condition that is linked to angiogenesis. Other key angiogenic pathways such as PI3K/AKT and transforming growth factor beta (TGFb) signaling are also predicted to be modulated by these miRs (**Figure 3D**). In order to begin understanding the role that individual miR might have on PAC function in the context of aging, we identified eight modulated miRs with predicted targets involved in angiogenesis or related processes such as inflammation, senescence, apoptosis, and oxidative stress. As seen in **Figure 3E**, downregulation of miR-34c and miR-126a-3p, two proangiogenic miRs, could negatively regulate VEGF signaling through upregulation of RRAS (Sawada et al., 2015) and SPRED1 (Fish et al., 2008), respectively, resulting in decreased angiogenic properties of PACs. Downregulation of miR-130a-3p could lead to stem cell dysfunction via increased KLF7 expression (Schuettpelz et al., 2012), whereas downregulation of miR-143-3p could decrease PAC survival by increasing the level of the proapoptotic factor AIFM1 (Bano and Prehn, 2018). On the other hand, upregulation of miR-30d and miR-23b could promote cell senescence and oxidative stress levels in PACs by decreasing the levels of CCNE2 (a promitotic factor) (Caldon et al., 2013) and GSH (an antioxidant enzyme) (Forman et al., 2009), respectively. The upregulation of miR-361 and miR-501 could decrease the angiogenic paracrine activity of PACs by targeting VEGF (Dal Monte et al., 2013) and MMP-13 (Kudo et al., 2012), respectively.

Effect of Cigarette Smoke Exposure on the Modulation of miRs in PACs in the Context of Hindlimb Ischemia

We compared the expression profile of miRs in PACs isolated from the bone marrow of mice exposed (SMK) or not (CTL) to cigarette smoke at day 2 after hindlimb ischemia. **Figure 4A** confirms that several miRs are modulated by SMK in PACs. The modulation is more often seen in miRs showing moderate (100–1,000 RPM) or high (1,000–10,000 RPM) expression levels compared to those with very high expression levels (>10,000 RPM). The miRs exhibiting the highest levels of expression are not modulated by SMK (**Figures 4A,B**). However, in opposition to aging, few miRs were significantly reduced

[2]http://www.microrna.gr/miRPathv3

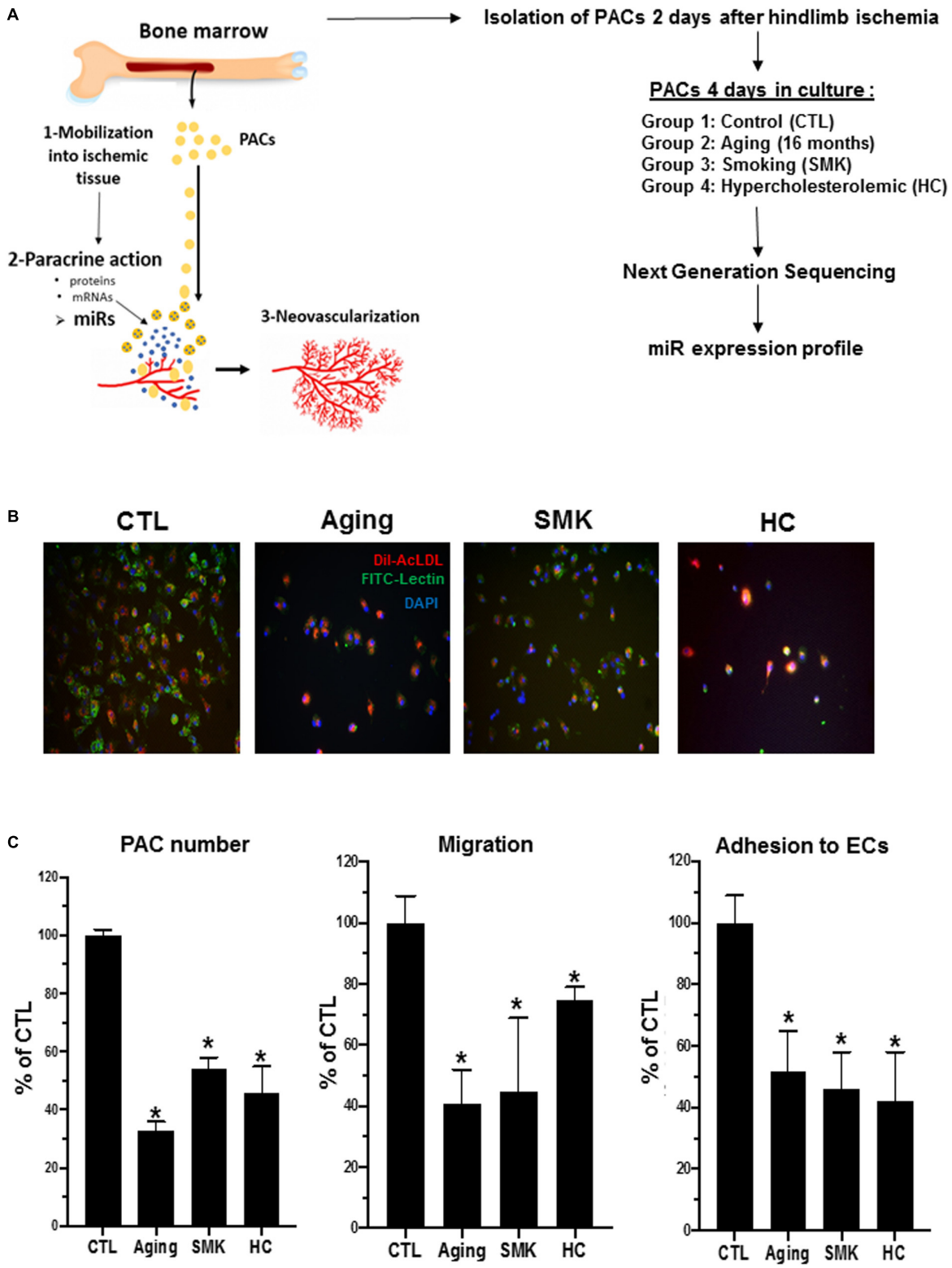


FIGURE 1 | Experimental design and effect of aging, smoking (SMK), and hypercholesterolemia (HC) on the number and functional activities of PACs. **(A)** Schematic representation of PAC angiogenic function (left) and experiment design used for miR profiling in PACs (right). **(B)** Representative pictures of PACs isolated from control (CTL), aging, SMK, and HC mice at day 2 after hindlimb ischemia. PACs were cultured *ex vivo* for 4 days before staining with DAPI (blue), BS-1 lectin-FITC (green), and DiI-acLDL (red). **(C)** Quantitative analysis of PAC number, PAC migration capacity, and PAC adhesion to endothelial cells (ECs) in the different groups of mice. Data are mean ± SEM (n = 4–6/group). *$P < 0.05$ versus CTL.

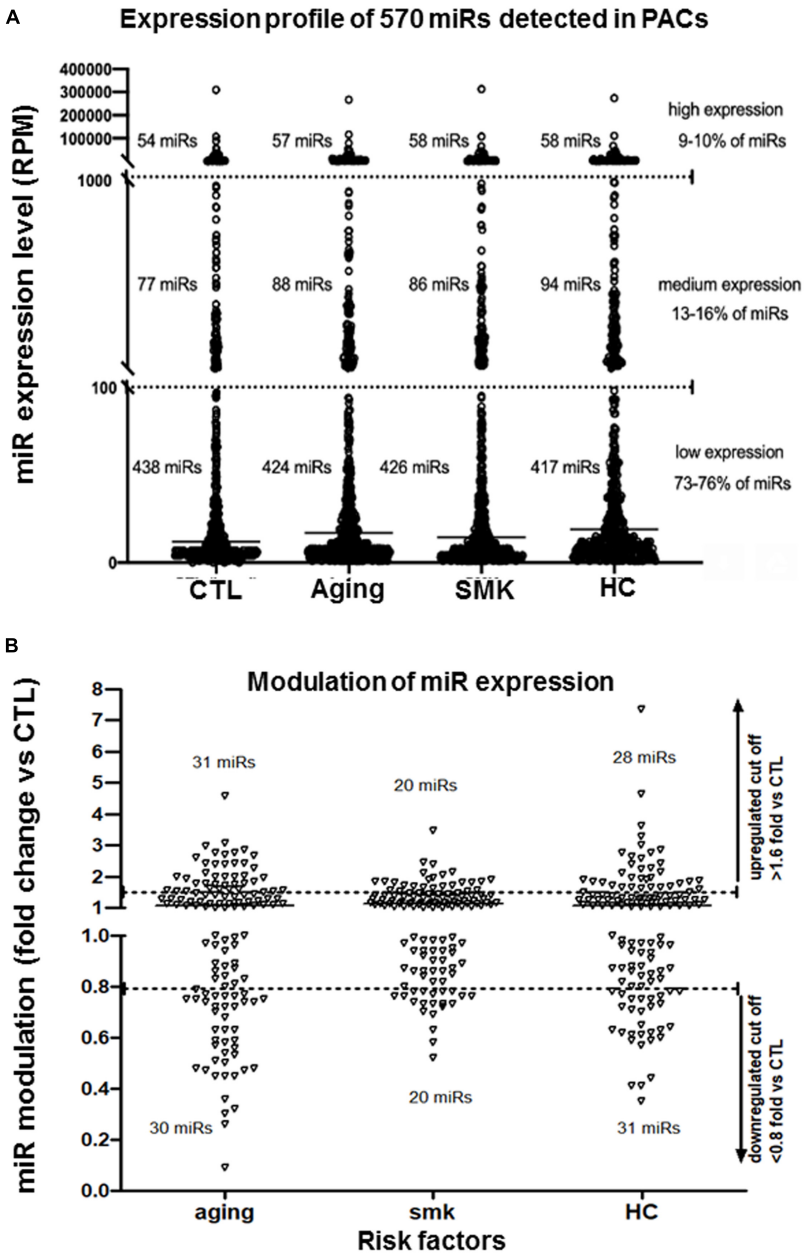
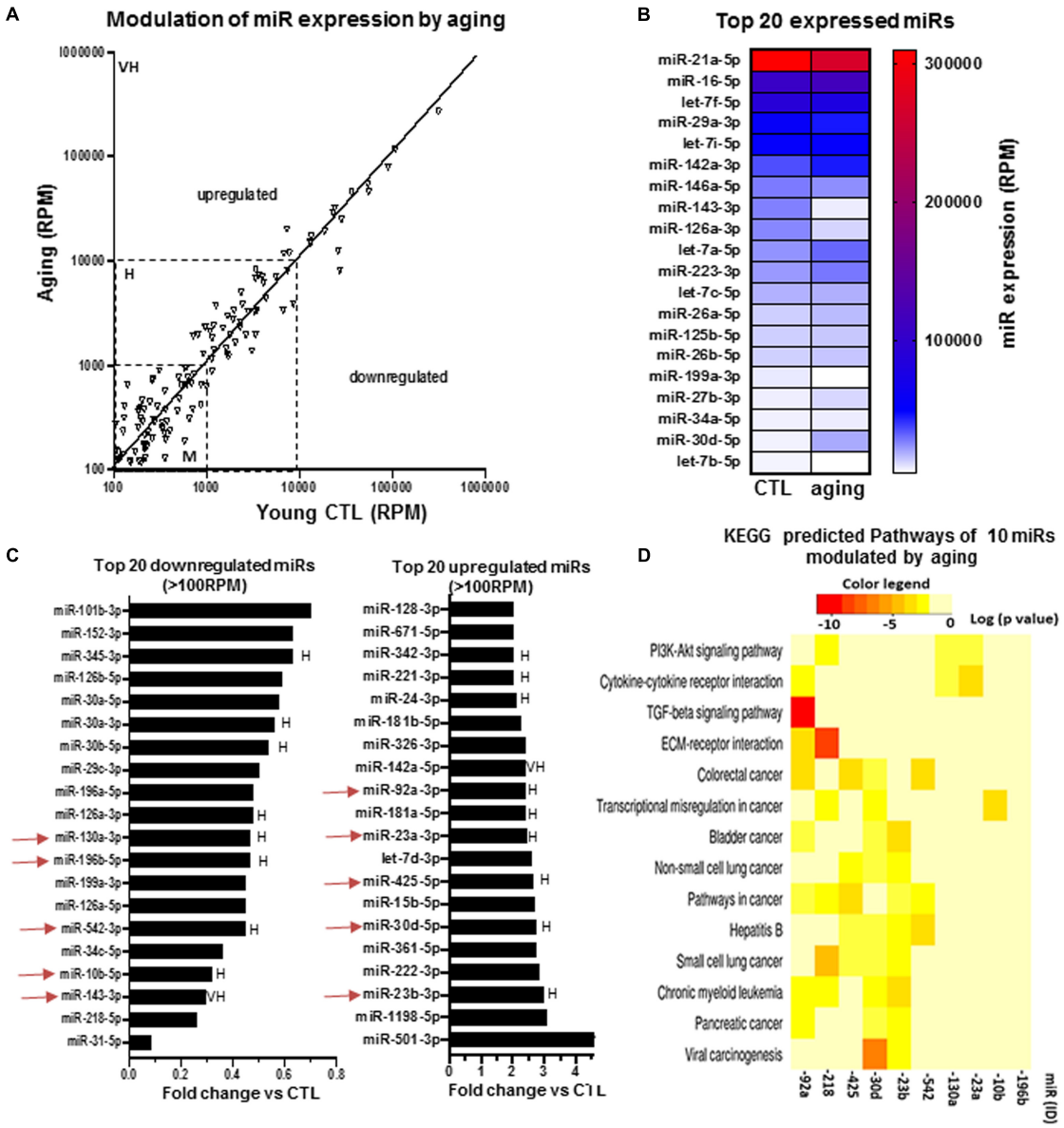


FIGURE 2 | Effect of aging, smoking (SMK), and hypercholesterolemia (HC) on the global expression levels of miRs in PACs. **(A)** Global evaluation of miR expression levels [reads per million of reads mapped (RPM)] in PACs isolated 2 days after hindlimb ischemia in aging, SMK, and HC mice compared to control mice (CTL). **(B)** Specific effect of aging, SMK, and HC on the modulation of miR expression. miRs with an expression level of at least 100 RPM are shown in each condition (pool of four to six mice per condition). Arbitrary cutoff levels of 0.8-fold (for downregulated miRs) and 1.6-fold (for upregulated miRs) are indicated.

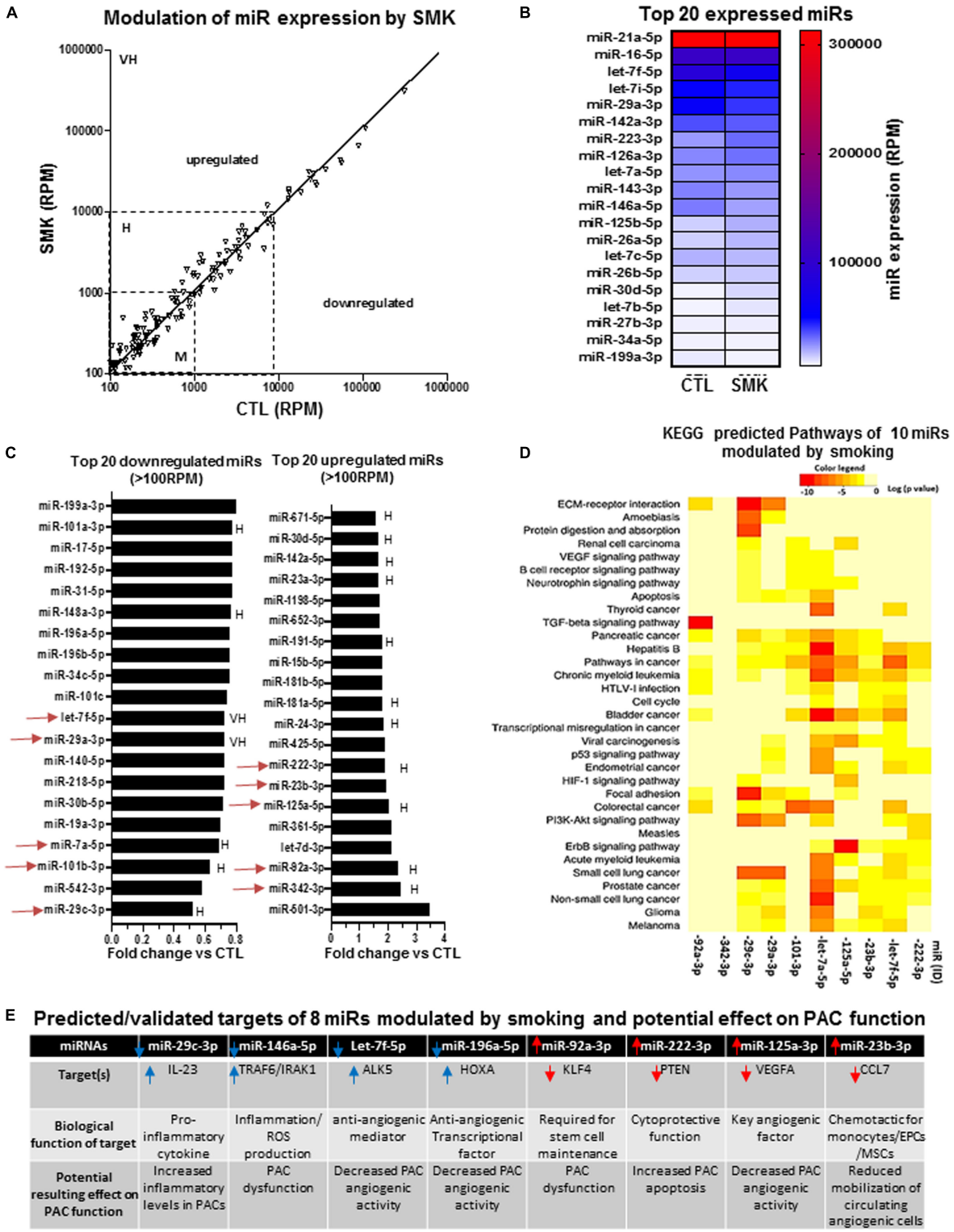
by SMK in PACs. Only three miRs were downregulated by >30% (miR-29c-3p, miR-542-3p, miR-7a-5p), and no miR was downregulated by more than 60% (**Figure 4C**). On the other hand, three miRs were increased by >225%: miR-501-3p, miR-342-3p, and miR-92a-3p. KEGG analysis was performed in 10 highly expressed/highly modulated miRs (red arrows in **Figure 4C**). As seen in **Figure 4D**, the bioinformatic algorithm suggests that 34 pathways/processes are affected by these miRs. Interestingly, key angiogenic signaling pathways such as VEGF, hypoxia-inducible factor 1 (HIF1), and TFGb are predicted to be regulated by these miRs. Individually, two miRs (miR-29c-3p and miR-146a-5p) that are downregulated by SMK have proinflammatory targets such IL-23 (Botta et al., 2018) and TRAF6/IRAK1 (Saba et al., 2014), which could increase inflammation and induce PAC dysfunction (**Figure 4E**). In addition, reduced expression of miR-196a-5p and let-7f-5p could, respectively, increase the levels of HOXA-5 (Liu et al., 2012; Cuevas et al., 2015) and ALK5 (Dhahri et al., 2017), two



E **Predicted/validated targets of 8 miRs modulated by aging and potential effect on PAC function**

miRNAs	↓ miR-34c-5p	↓ miR-126a-3p	↓ miR-130a-3p	↓ miR-143-3p	↑ miR-30d	↑ miR-23b	↑ miR-361	↑ miR-501
Target(s)	↑ RRAS	↑ SPRED1	↑ KLF7	↑ AIFM1	↓ CCNE2	↓ GSH	↓ VEGFA/STAT3	↓ MMP13
Biological function of target	Inhibit VEGFR2 phosphorylation	Negative regulator of VEGF signaling	Suppression of stem cell function	Promoting cell death	Mitosis cell cycle; G1	Anti-oxydant enzyme	Promote vasculogenesis and angiogenesis	ECM degradation/ angiogenic mediator
Potential resulting effect on PAC function	Decreased angiogenic activities	Decreased migration/ proliferation	PAC dysfunction	PAC apoptosis	PAC senescence	Increased ROS levels in PACs	Reduced PAC mobilization and function	Reduction of PAC paracrine activities

FIGURE 3 | Specific effect of aging on miR expression profile in PACs and bioinformatic analyses of predictive targets/pathways potentially involved in PAC dysfunction. **(A)** Overview of miR expression distribution in PACs isolated from aging versus young (CTL) mice. **(B)** Heat map showing the effect of aging on the expression of the 20 most abundant miRs in PACs. **(C)** Overview of the top 20 downregulated and upregulated miRs in PACs in the context of aging. **(D)** KEGG analysis to identify predicted pathways involved in 10 highly expressed/highly modulated miRs [red arrows in (C)]. **(E)** Predicted/validated targets of eight miRs modulated by aging and potentially associated with PAC dysfunction. M, H, and VH indicate miR with medium (100–1,000 RPM), high (1,000–10,000 RPM), and very high (>10,000 RPM) expression, respectively.



E **Predicted/validated targets of 8 miRs modulated by smoking and potential effect on PAC function**

miRNAs	miR-29c-3p	miR-146a-5p	Let-7f-5p	miR-196a-5p	miR-92a-3p	miR-222-3p	miR-125a-3p	miR-23b-3p
Target(s)	↑ IL-23	↑TRAF6/IRAK1	↑ ALK5	↑ HOXA	↓ KLF4	↓PTEN	↓ VEGFA	↓CCL7
Biological function of target	Pro-inflammatory cytokine	Inflammation/ ROS production	anti-angiogenic mediator	Anti-angiogenic Transcriptional factor	Required for stem cell maintenance	Cytoprotective function	Key angiogenic factor	Chemotactic for monocytes/EPCs /MSCs
Potential resulting effect on PAC function	Increased inflammatory levels in PACs	PAC dysfunction	Decreased PAC angiogenic activity	Decreased PAC angiogenic activity	PAC dysfunction	Increased PAC apoptosis	Decreased PAC angiogenic activity	Reduced mobilization of circulating angiogenic cells

FIGURE 4 | Specific effect of smoking (SMK) on miR expression profile in PACs and bioinformatic analyses of predictive targets/pathways potentially involved in PAC dysfunction. **(A)** Overview of miR expression distribution in PACs isolated from SMK versus control (CTL) mice. **(B)** Heat map showing the effect of smoking on the expression of the 20 most abundant miRs in PACs. **(C)** Overview of the top 20 downregulated and upregulated miRs in PACs in the context of smoking. **(D)** KEGG analysis to identify predicted pathways involved in 10 highly expressed/highly modulated miRs [red arrows in **(C)**]. **(E)** Predicted/validated targets of eight miRs modulated by smoking and potentially associated with PAC dysfunction. M, H, and VH indicate miR with medium (100–1,000 RPM), high (1,000–10,000 RPM), and very high (>10,000 RPM) expression, respectively.

antiangiogenic factors that could negatively affect PAC function. On the other hand, four upregulated miRs could impair PAC survival and PAC mobilization. For example, miR-92a-3p targets KLF4 (which is required for stem cells maintenance) (Yu et al., 2011; Fang and Davies, 2012), miR-222 targets PTEN (a cytoprotective factor for stem cell differentiation) (Garofalo et al., 2009; Lyu et al., 2015), miR-125a-3p targets VEGF (Yang et al., 2018), and miR-23b targets the chemotactic factor CCL7 (Zhang et al., 2018; **Figure 4E**).

Effect of Hypercholesterolemia on the Modulation of miRs in PACs in the Context of Hindlimb Ischemia

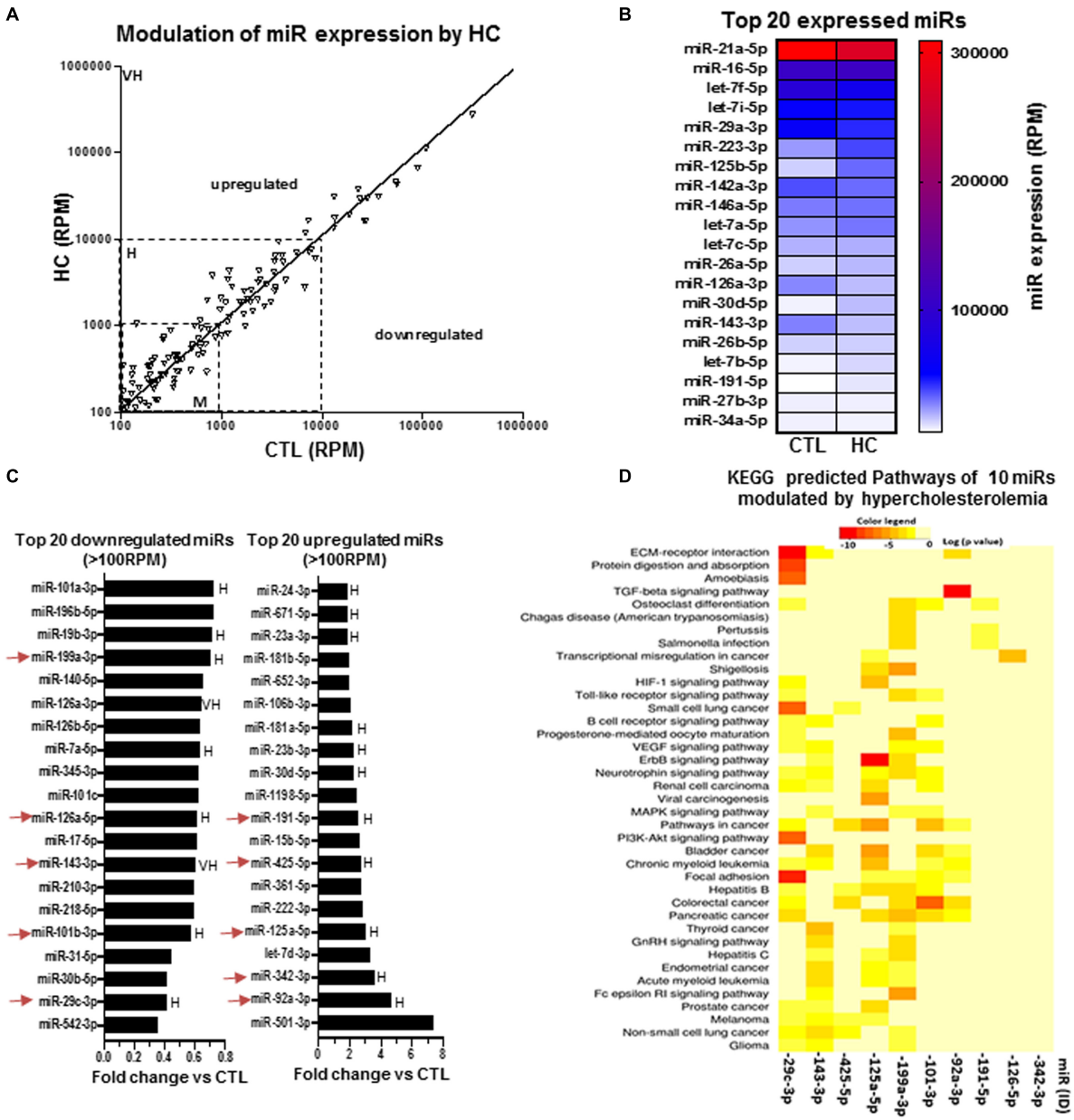
We next compared the expression profile of miRs in PACs isolated from the bone marrow of hypercholesterolemic (HC) or normocholesterolemic (CTL) mice at day 2 after hindlimb ischemia. **Figure 5A** confirms that several miRs are modulated by HC in PACs. Interestingly, contrary to aging and SMK, we identified miRs with very high expression levels that were modulated by HC. Among the top 15 miRs in abundance, two miRs (miR-223-3p and miR-125b-5p) were strongly upregulated, whereas three miRs (miR-142a-3p, miR-126a-3p, and miR-143-3p) were strongly downregulated in PACs exposed to HC conditions (**Figures 5A,B**). Among the top 20 miRs modulated by HC (**Figure 5C**), three miRs were downregulated by greater than 60% including miR-542-3p, miR-30b-5p, and the highly expressed miR-29c-3p. Four miRs were upregulated by greater than 300% including the highly expressed miR-92a-3p and miR-342-3p and the moderately expressed let-7d-3p and miR-501-3p (>700% upregulation). KEGG analysis was performed in 10 highly expressed/highly modulated miRs (red arrows in **Figure 5C**). As seen in **Figure 5D**, the bioinformatic algorithm suggests that 39 pathways/processes are affected by these miRs including VEGF, HIF1, and TFGb pathways. Individually, three miRs that are downregulated by HC and five miRs that are upregulated could contribute to PAC dysfunction (**Figure 5E**). For example, reduced expression of miR-542-3p could inhibit VEGF signaling by increasing IGFBP1 (a negative regulator of VRGFR2 activation) (Zhang et al., 2012; Tochigi et al., 2017). Reduced expression of miR-17-5p can increase the levels of BAMBI (Duan et al., 2019), a negative regulator of AKT signaling that is involved in PAC migration and proliferation (Zheng et al., 2007). Likewise, reduced expression of miR-31-5p could increase Satb2 levels and promote apoptotic death (Lian et al., 2018). On the other hand, upregulation of miR-15b-5p, miR-342-3p and miR-425-5p could impair PAC angiogenic properties by inhibiting the proangiogenic factors FGF-2, FOXM1, and IGF-1, respectively (Li et al., 2014; Liu et al., 2015; Schelch et al., 2018).

Integrated Model of the Effect of CRFs on miR Modulation in PACs Related to Their Angiogenic Function

To evaluate the cumulative effect that different CRFs might have on miR modulation and PAC function, we identified miRs that were modulated similarly (upregulation or downregulation) in two or more CRF conditions. As shown in **Figure 6A**, seven miRs were downregulated in two or more CRF conditions, including two miRs (miR-29c-3p and miR-542-3p) that were downregulated in all three CRF conditions. We used cluster dendrogram predictive analysis (**Figure 6B**) to better appreciate the resulting physiological and/or pathological effects of the seven miRs that were downregulated in two or more CRF conditions. We found 23 different pathways that could be affected, including several linked to angiogenesis such as extracellular matrix (ECM)–receptor interaction, focal adhesion, PI3K/AKT, and mTOR signaling (Ramjaun and Hodivala-Dilke, 2009; Karar and Maity, 2011; Mongiat et al., 2016). On the other hand, as shown in **Figure 6C**, 13 miRs were upregulated in two or more CRF conditions, including two miRs (miR501-3p and miR-92a-3p) that were robustly upregulated in all three CRF conditions. Here, cluster dendrogram analysis to evaluate the global effects of the 13 miRs that were upregulated in two or more CRF conditions (**Figure 6D**) predicted that 19 different pathways could be affected, including several linked to angiogenesis and/or cell cycle such as TGFb, PI3K/AKT, p53, and MAPK signaling (Wu, 2004; Karar and Maity, 2011; van Meeteren et al., 2011). To gain further insight into the interrelated effects of miR modulation by CRFs in PACs, we perform a connectome analysis based on VEGF signaling, a crucial physiological pathway involved in both angiogenesis and postnatal neovascularization (**Figure 7**). Interestingly, several miRs modulated by CRFs in PACs can directly target VEGFA (miR-15, miR-361, and miR-181) or the downstream oncogene KRAS (miR-143, miR-181), which is involved in RAS/MAPK signaling pathway. In addition, other miRs can indirectly modulate downstream factors involved in VEGF signaling or mobilization/function of PACs (**Figure 7**).

DISCUSSION

To our knowledge, this is the first comprehensive analysis of miR expression in bone marrow–derived PACs following hindlimb ischemia. It is also the first study describing the effect of CRFs on PAC miR expression profile in the context of PAD and ischemia-induced neovascularization. Although miRs are increasingly recognized as critical regulators of numerous biological and cellular processes such as growth, apoptosis, inflammation, metabolic activity, and angiogenesis (Wu, 2004; O'Connell et al., 2010; Karar and Maity, 2011; van Meeteren et al., 2011; Caporali and Emanueli, 2012; Paul et al., 2018), their specific role for the response to tissue ischemia in the context of vascular diseases is largely unknown. Moreover, how CRFs can modulate miR expression and global function in the context of angiogenesis and ischemia-induced neovascularization remains to be determined. Defining miR expression profile and modulation could be especially important in the case of bone marrow–derived PACs, whose potent angiogenic activities could be harnessed to develop novel therapies aimed at improving neovascularization and reducing tissue damage in patients with PAD. miRs could have a dual role for the modulation of PAC angiogenic properties. On the one hand, miRs have been shown to be involved in the differentiation and the functional activities of stem/progenitor cells during cardiovascular healing responses



E

Predicted/validated targets of 8 miRs modulated by HC and potential effect on PAC function

miRNAs	↓miR-542-3p	↓miR-17-5p	↓miR-31-5p	↑miR-191-5p	↑miR-15b-5p	↑miR-342-3p	↑miR-425-5p	↑miR-92a
Target(s)	↑IGFBP6	↑BAMBI	↑Satb2	↓EGR1	↓FGF-2	↓FOXM1	↓IGF-1	↓HO-1
Biological function of target	Inhibits VEGF-dependent angiogenesis	Negative regulator of AKT signaling	Pro-apoptotic	Promotes angiogenesis	Proangiogenic factor	Stimulator of cells migration	Angiogenic factors	Cytoprotective enzyme against ROS
Potential resulting effect on PAC function	Decreased PAC mobilization/ function	Decreased PAC migration/ proliferation	Increased PAC apoptosis	Decreased PAC angiogenic activity	Decreased PAC paracrine function	Decreased PAC mobilization	Decreased PAC paracrine function	Increased ROS levels in PACs

FIGURE 5 | Specific effect of hypercholesterolemia on miR expression profile in PACs and bioinformatic analyses of predictive targets/pathways potentially involved in PAC dysfunction. **(A)** Overview of miR expression distribution in PACs isolated from hypercholesterolemic (HC) versus control (CTL) mice. **(B)** Heat map showing the effect of hypercholesterolemia on the expression of the 20 most abundant miRs in PACs. **(C)** Overview of the top 20 downregulated and upregulated miRs in PACs in the context of hypercholesterolemia. **(D)** KEGG analysis to identify predicted pathways involved in 10 highly expressed/highly modulated miRs [red arrows in **(C)**]. **(E)** Predicted/validated targets of eight miRs modulated by hypercholesterolemia and potentially associated with PAC dysfunction. M, H, and VH indicate miR with medium (100–1,000 RPM), high (1,000–10,000 RPM), and very high (>10,000 RPM) expression, respectively.

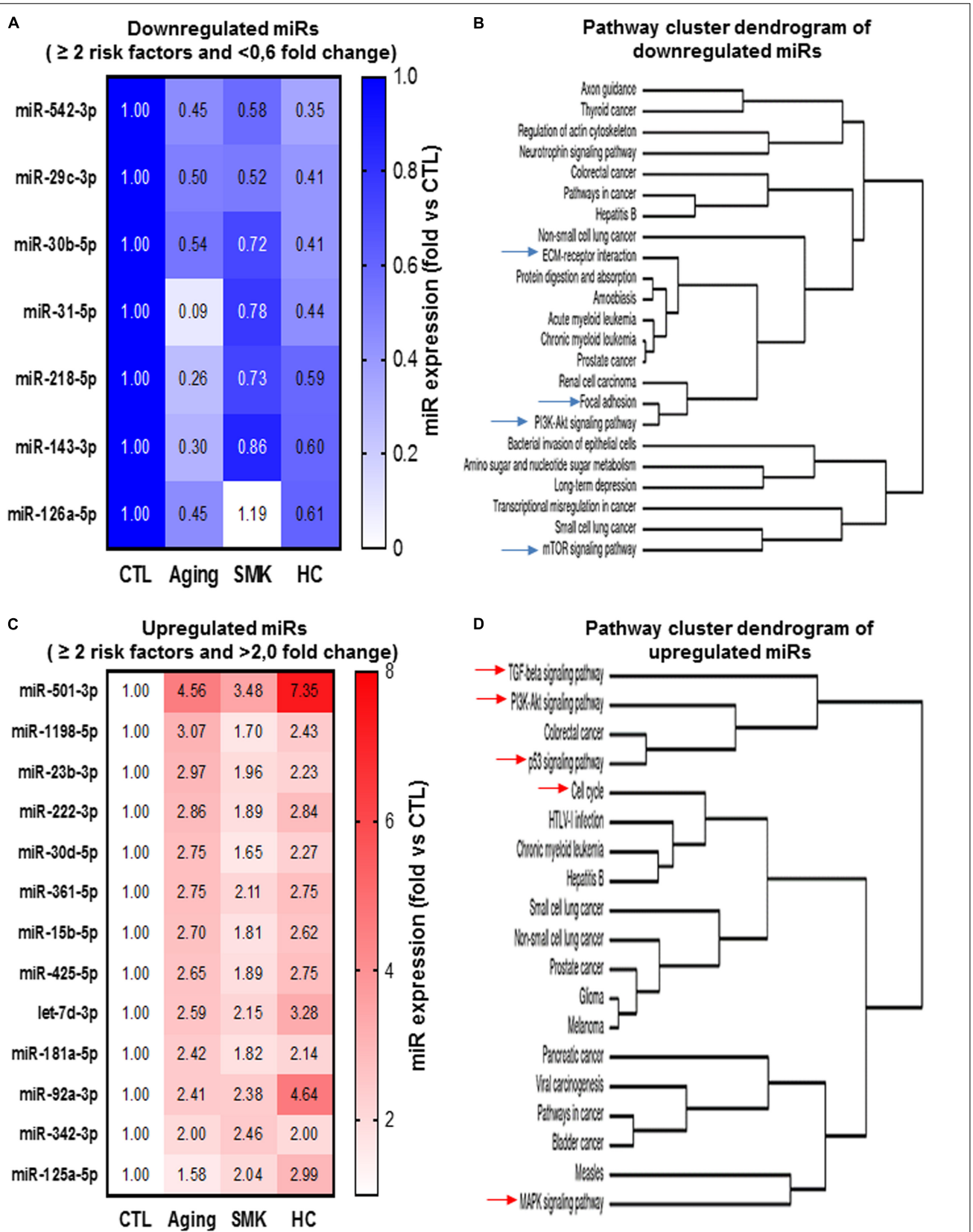


FIGURE 6 | Convergent modulation effect of different CRFs on the expression of miRs in PACs. Heat maps **(A,C)** and pathway cluster dendogram analyses **(B,D)** of miRs that are downregulated (<0.6-fold versus CTL) or upregulated (>2.0-fold versus CTL) by two risk factors or more in PACs.

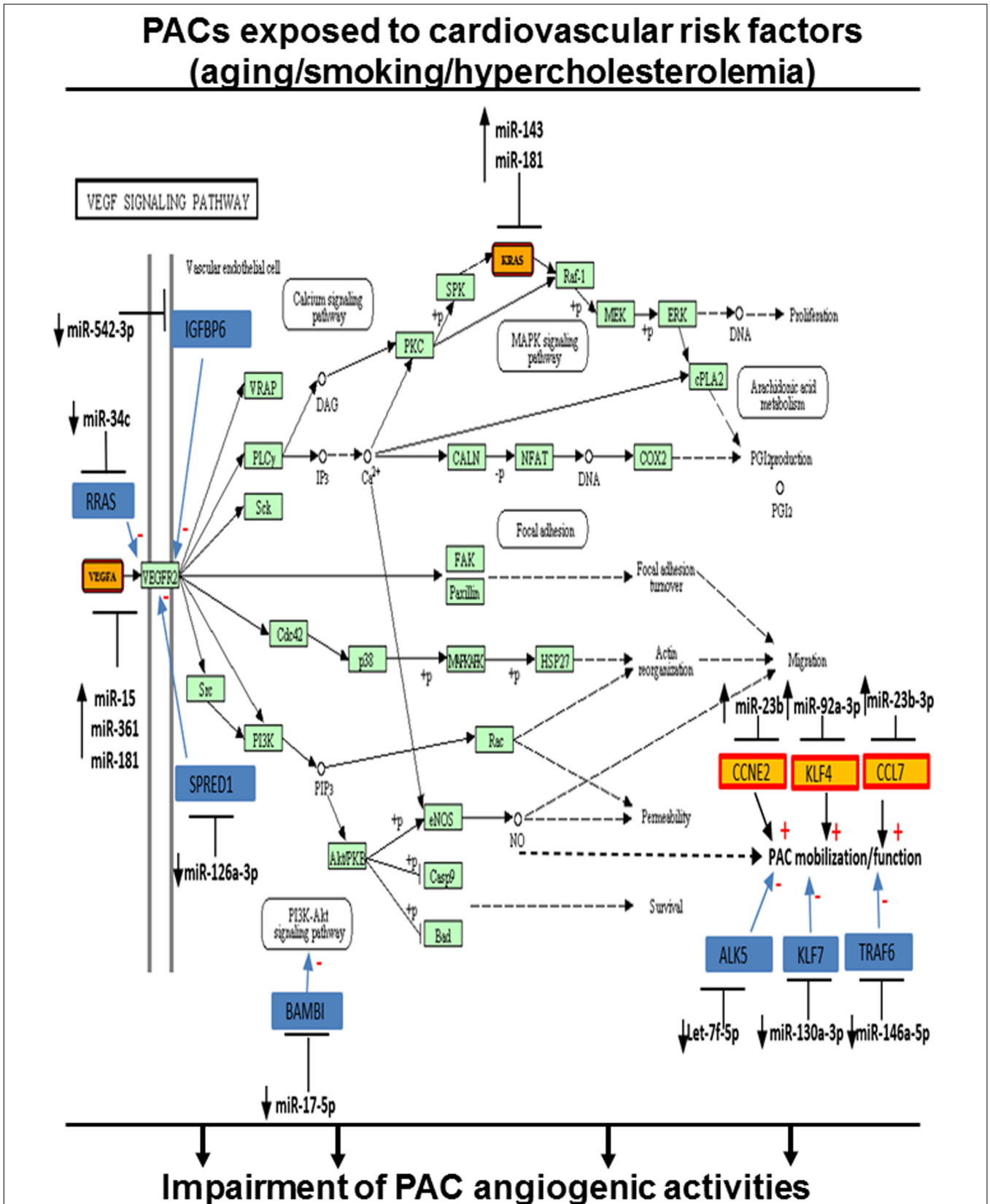


FIGURE 7 | Integrated model of the effect of CRFs on miR modulation in PACs related to their angiogenic function. Connectome of predicted (yellow) and validated (blue) targets of miRs modulated by CRFs in PACs and involved in VEGF signaling and/or PAC function, based on bioinformatic algorithms (miRSystem database).

(Jakob and Landmesser, 2012). On the other hand, the angiogenic properties of PACs have been linked to their paracrine activity. In response to stress conditions such as hypoxia, PACs can release extracellular vesicles that transfer proangiogenic miRs to endothelial cells (Cantaluppi et al., 2012; Ranghino et al., 2012). Therefore, it is possible that CRFs deregulate PAC miR content and extracellular vesicle miR cargo, so that impaired angiogenic signals are transmitted in ischemic tissues. Accordingly, it becomes very important to determine miR expression profile in PACs during healing processes and investigate the modulating effects of different pathological conditions, including CRFs.

We used a well-described model of hindlimb ischemia (Limbourg et al., 2009) to investigate the effects of CRFs on the number, function, and miR expression profile of PACs. Our results demonstrate that the number and the functional activities (migration, adhesion) of PACs are impaired in CRF conditions such as aging, SMK, and HC (**Figures 1B,C**). We used NGS to perform a global and unbiased evaluation of PAC miR expression profile in the context of CRFs (Pritchard et al., 2012; Goodwin et al., 2016). Among 570 miRs that were found to be expressed in PACs, we focused our attention on miRs with an expression level of at least 100 RPM that showed more than 20% downregulation or 60% upregulation in a given CRF condition. We found that 40 to 61 miRs are significantly modulated by CRFs. This level of modulation is comparable to that of previous reports on the modulation pattern of miRs by CRFs in different biological fluids or tissues (Huan et al., 2018; Izzotti et al., 2018; Xu et al., 2019). For example, a recent study in human blood showed that 127 miRs (of 1,000 miRs) are differentially expressed during aging (Huan et al., 2018). Another study based on microarray analysis (a technology that does not detect all miRs) showed that 22 miRs are significantly modulated in the serum of hyperlipidemic patients (Xu et al., 2019). Also, in a mouse model, exposure to cigarette smoke was associated with a variable number of modulated miRs depending on the organ studied (Izzotti et al., 2018). Overall, these studies suggest that the number of miRs that are modulated by CRFs can vary depending on the tissue/organ studied and the detection method used. Here, although we found that the overall level of miR modulation was similar in different CRF conditions, particular effects of specific CRFs were also observed. For example, miR modulation by aging and SMK was most frequently observed in miRs with medium or high expression levels, whereas HC was also associated with the modulation of miRs that were expressed at very high levels. HC also led to more important upregulation of miRs, five miRs showing more than threefold increase compared to one and two miRs for SMK and aging, respectively. We also found that the modulation of miRs by SMK was less robust compared to that of aging and HC and that SMK was mostly associated with miR upregulation versus downregulation. Admittedly, the differences that we observed here need to be interpreted with caution, as miR modulation could depend on the intensity and/or the duration of CRF conditions. Moreover, whether the modulation of a miR that is highly expressed has more functional consequence compared to that of a miR with lower expression is currently unknown.

Our miR profiling approach in PACs identified several miRs that have previously been associated with CRFs and/or involved in related pathological conditions. For example, we found that miR-31was the most downregulated miR during aging. Interestingly, miR-31 has recently been proposed to be a valuable plasma-based biomarker for aging (Weilner et al., 2016), and it is also associated with cardiovascular diseases and hypertension (Sekar et al., 2017). Moreover, miR-31 was shown to protect against reactive oxygen species (ROS) (Stepicheva and Song, 2016). A loss of the adaptive response to oxidative stress is one of the major characteristics of aging (Finkel and Holbrook, 2000), and systemic increases of ROS levels are associated with impaired PAC function (Haddad et al., 2009; Groleau et al., 2010). In that matter, we found that miR-23b, which targets the antioxidant enzyme superoxide dismutase (Long et al., 2017), was increased by 300% in PACs during aging. Therefore, age-dependent increase of miR-23b levels could lead to defective antioxidant activity, higher ROS levels, and impaired angiogenic activities of PACs. In fact, in each CRF condition, we identified downregulated proangiogenic miRs and upregulated antiangiogenic miRs that could contribute to explain PAC dysfunction in the context of aging, SMK, and HC (**Figures 3E**, **4E**, and **5E**). Taking into account both the abundance and the degree of modulation of selected miRs in a given CRF condition, KEGG cluster connection analyses identified several pathways that could impact on PAC function (**Figures 3D**, **4D**, and **5D**). Interestingly, all CRF conditions were associated with modulation of miRs involved in cancer (Lee and Dutta, 2009) and ECM remodeling (Piccinini and Midwood, 2014), both conditions being closely linked to angiogenesis (Sottile, 2004). The central HIF1–VEGF angiogenic pathway was also shown to be targeted, together with important downstream pathways involved in VEGF signaling such as MAPK and PI3K (Ferrara et al., 2003). TGFb pathway, which regulates cellular migration, proliferation, and angiogenesis (Bertolino et al., 2005), was also shown to be involved in the three CRF conditions.

An interesting finding in the current study is that a group of miRs appears to be similarly altered in PACs exposed to different CRF conditions. For example miR-542-3p and miR-29 were decreased in all three CRF conditions, being also the two most downregulated miRs in SMK and HC conditions. Recently, miR-542-3p has been shown to contribute to the angiogenic properties of EPCs by stimulating the expression of ANGPT2 (Lu et al., 2019). On the other hand, although downregulation of miR-29c has been shown to promote ischemic brain damage via derepression of its target DNMT3a (Pandi et al., 2013), the targets of miRs can be modulated according to cell types and microenvironmental conditions. Therefore, the specific effect of miR-29c downregulation for the modulation of PAC function in PAD remains to be determined. We also identified several miRs that were upregulated in different CRF conditions. For example, miR-501-3p was the most upregulated miR in all three CRF conditions. Although the role of miR-501-3p in ischemic vascular diseases and angiogenesis has not been investigated, it is interesting to note that overexpression of miR-501-3p was shown to inhibit proliferation, migration, and invasion in cancer cells (Luo et al., 2018). Another miR that was strongly increased

in all CRF conditions is miR-92a. Mir-92a has been shown to inhibit angiogenesis *in vitro* and *in vivo*, whereas inhibition of miR-92a expression can improve neovascularization and protect against ischemia in mouse ischemic hindlimbs (Bonauer et al., 2009) and after myocardial infarction in pigs (Hinkel et al., 2013). Moreover, inhibition of miR-92a can also protect PACs (EPCs) from hypoxia- or hyperglycemia-induced injury (Huang et al., 2019). We used pathway cluster dendrogram analyses to evaluate the potential resulting biological effects of miRs that are modulated in several CRF conditions. Our results indicate that the predictive pathways that are affected have key roles for the modulation of angiogenesis and PAC function, including ECM remodeling, PI3K/AKT/MAPK signaling, TGFb pathway, p53, and cell cycle progression. When looking specifically at VEGF pathway, which is crucial both for developmental and postnatal neovascularization (Ferrara et al., 2003), we found that several miRs that are modulated by CRF conditions can negatively influence VEGF-dependent angiogenesis and PAC function (**Figure 7**). The mechanism by which different CRFs can similarly modulate miRs involved in the angiogenic function of PACs remains to be determined. A common feature of all these CRFs is increased oxidative stress (Kunsch and Medford, 1999), which has also been associated with impaired ischemia-induced neovascularization and PAC function (Haddad et al., 2009; Groleau et al., 2010). Additional studies are needed to determine the specific role of oxidative stress for the modulation of miR expression in the context of PAC function, angiogenesis, and ischemia-induced neovascularization.

The current study has important limitations. Using NGS, we identified several miRs that were modulated in PACs in the context of CRFs, but it was not possible to confirm each miR (i.e., quantitative reverse transcription–polymerase chain reaction) in biological replicates exposed to the different conditions. In addition, targets and pathways modulated by miRs were identified using bioinformatic analyses, but we could not confirm all these results at the mRNA and protein levels in the different experimental conditions. Accordingly, the present work should be taken as a hypothesis-generating study, and the specific miRs and targets we describe in each specific experimental condition will need to be confirmed by additional experiments in future studies. We chose to study the effect of aging, SMK, and HC because those are important classical CRFs associated with impaired neovascularization and PAC dysfunction. Additional studies are needed to address the potential role of other CRFs (e.g., diabetes, hypertension), which could also modulate the expression of miRs related to PAC function. Finally, although we propose that modulating miR expression in PACs could improve their angiogenic activity and promote neovascularization in patients with PAD and CLI, it is important to note that the same miRs could have opposite and/or detrimental effects in patients who also present with other pathologies, such as cancer. Therefore, several questions remain to be answered before this type of angiogenic therapy can be developed, including which miRs to target in PACs and how to administer these "engineered" PACs in terms of dosing and delivery method (local versus systemic).

In conclusion, this study describes for the first time the effects of CRFs on the modulation of miR profile in PACs related to PAD and ischemia-induced neovascularization. We found that a significant proportion of miRs are modulated in PACs exposed to different CRFs. In each CRF condition, we identified specific proangiogenic miRs that were downregulated and also antiangiogenic miRs that were upregulated. Moreover, a group of angiogenesis-modulating miRs was found to be similarly altered in all CRF conditions. Our findings constitute a solid framework for the identification of miRs that could be targeted in PACs in order to improve their angiogenic function. In novel therapeutic strategies, these reprogrammed PACs could eventually serve as potent angiogenic vectors in order to improve neovascularization and reduce tissue damage in patients with severe PAD and CLI.

DATA AVAILABILITY STATEMENT

Next Generation Sequencing (NGS) data have been deposited and are available on NCBI Gene Expression Omnibus, GSE151609.

ETHICS STATEMENT

The animal study was reviewed and approved by Comité Institutionnel de Protection des Animaux (CIPA) of the Centre Hospitalier de l'Université de Montréal (CHUM).

AUTHOR CONTRIBUTIONS

MD and AR conceptualized and designed the study. MD prepared the initial draft of the manuscript and figures. JR and AR revised the manuscript and figures. MD and SD conducted the experiments. MD, SD, and AR analyzed the data. SC provided expert advice and recommendations. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.00947/full#supplementary-material

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REVIEW
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Extracellular Vesicle miRNAs in the Promotion of Cardiac Neovascularisation

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Cardiovascular disease (CVD) is the leading cause of mortality worldwide claiming almost 17. 9 million deaths annually. A primary cause is atherosclerosis within the coronary arteries, which restricts blood flow to the heart muscle resulting in myocardial infarction (MI) and cardiac cell death. Despite substantial progress in the management of coronary heart disease (CHD), there is still a significant number of patients developing chronic heart failure post-MI. Recent research has been focused on promoting neovascularisation post-MI with the ultimate goal being to reduce the extent of injury and improve function in the failing myocardium. Cardiac cell transplantation studies in pre-clinical models have shown improvement in cardiac function; nonetheless, poor retention of the cells has indicated a paracrine mechanism for the observed improvement. Cell communication in a paracrine manner is controlled by various mechanisms, including extracellular vesicles (EVs). EVs have emerged as novel regulators of intercellular communication, by transferring molecules able to influence molecular pathways in the recipient cell. Several studies have demonstrated the ability of EVs to stimulate angiogenesis by transferring microRNA (miRNA, miR) molecules to endothelial cells (ECs). In this review, we describe the process of neovascularisation and current developments in modulating neovascularisation in the heart using miRNAs and EV-bound miRNAs. Furthermore, we critically evaluate methods used in cell culture, EV isolation and administration.

Keywords: extracellular vesicles (EV), microRNA (miR), neovascularisation, angiogenesis, cardiac, myocardial infarct, exosome (EXO), regeneration

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INTRODUCTION

CVD is the leading cause of mortality worldwide with the World Health Organization (WHO) reporting that in 2016, 17.9 million people died from CVDs. Of those deaths, an estimated 7.4 million were attributed to CHD alone (WHO Cardiovascular diseases CVDs, 2017). CHD is characterised by narrowing of the coronary arteries due to the gradual formation and the subsequent rupture of plaque within the vessel walls. Blockage of these arteries results in oxygen and nutrient deprivation of the downstream tissue. Consequently, ischaemic damage and cardiomyocyte death occur in the affected region of the heart, a phenomenon known as MI (Thygesen et al., 2019). Various methods, including stem cell (SC) injection, growth factor (GF)

delivery, and miRNA treatment, have been proposed to reduce cardiomyocyte death; nonetheless, several limitations have restricted their use. The idea of restoring the lost myocardium was proven to be inefficient since adult mammal cardiomyocytes are terminally differentiated and in contrast to other vertebrate species, such as zebrafish, adult mammal cardiac regenerative capacity is limited (Ye et al., 2018). For this reason, SC injection was proposed as a promising strategy for cardiac repair. The initial hypothesis was that transplanted SCs would differentiate into cardiomyocytes, integrate into the host myocardium, and augment cardiac function through synchronised electromechanical coupling (Garbern and Lee, 2013; Lemcke et al., 2018). Despite some encouraging results, this approach has proven to be largely unsuccessful to date due to several issues, including poor engraftment, immune rejection, genetic instability, and possible teratocarcinoma formation. Importantly, the engrafted cells have also demonstrated relatively poor electrical coupling with the host myocardium, leading to the development of additional safety issues (Liu et al., 2018; Menasché et al., 2018; Romagnuolo et al., 2019). Recently, Vagnozzi et al. (2020), showed that the improvement in cardiac function following SC transplantation may be due to an acute inflammatory wound-healing response, rather than incorporation of delivered cells or production of new cardiomyocytes.

Aiming to improve recovery after MI and, therefore, rescue as many cardiomyocytes as possible, research interest is focused on promoting neovascularisation to create new myocardial blood vessel networks. Paracrine cell communication plays a critical role in the control of this process (Gnecchi et al., 2008; Mirotsou et al., 2011; Hodgkinson et al., 2016). Cell communication in a paracrine manner is regulated by several mechanisms, including EVs. EVs carry and transfer various bioactive molecules, such as small non-coding RNAs, proteins, and lipids, that moderate signalling pathways in the recipient cells. These functional contents depend on several factors, including the cellular origin and the (patho)physiological state of the cells during EV packing and secretion (Raposo and Stoorvogel, 2013). Recently, EV-miRNAs have gained immense interest in the control of cell behaviour in the recipient cells. miRNAs are small non-coding RNAs of approximately 22 nucleotides and have been recognised as critical regulators of gene expression. While most miRNAs regulate gene expression by binding to the 3′ untranslated region of their messenger RNA (mRNA) target, there are also sporadic reports of miRNAs that inhibit protein translation by binding to the 5′ untranslated region of their target. Under certain conditions, miRNAs can also control transcription or activate translation (O'Brien et al., 2018). Nonetheless, there is great controversy regarding the functional relevance of miRNAs in EVs. Several reports suggest that the majority of extracellular miRNAs are protected from plasma ribonucleases by forming complexes with proteins, such as Ago2 and that only a few copies of each miRNA are found in EVs (Arroyo et al., 2011; Chevillet et al., 2014). Moreover, in a non-peer-reviewed study, it was recently argued that miRNAs are not effectively delivered to target cells in a functional manner (Albanese et al., 2020). On the other hand, numerous studies have demonstrated that protected from plasma ribonucleases by their EV carriers, functional miRNAs can be delivered and internalised into recipient cells, acting as novel regulators of gene expression by inhibiting their targets (Johnson et al., 2019; Qiao et al., 2019; Cavallari et al., 2020). Despite this controversy, preclinical studies have demonstrated that EVs hold promise in the regulation of complex processes such as neovascularisation. In this review, we describe the process of neovascularisation and current developments in modulating neovascularisation in the heart using miRNAs and EV-bound miRNAs. Furthermore, we critically evaluate methods used in cell culture, EV isolation and routes of EV administration.

POST-ISCHAEMIC NEOVASCULARISATION

After the onset of myocardial ischaemia due to coronary artery occlusion, there is an inadequate blood supply to the heart muscle, which results in a pathological change in electrical, contractile or biochemical function of the heart (Grover, 1995). Therefore, post-ischaemic neovascularisation is essential to support the metabolic needs of cardiac cell populations. Angiogenesis and arteriogenesis are regulated by distinct, but partially overlapping, cellular and molecular mechanisms and are responsible for tissue repair and remodelling in acute and chronic ischaemic diseases (Persson and Buschmann, 2011). Angiogenesis refers to the formation of new blood vessels from pre-existing vessels and can be classified as intussusceptive or sprouting angiogenesis (Caduff et al., 1986; Burri and Tarek, 1990; Risau, 1997). Intussusceptive angiogenesis is a dynamic splitting process in which elements of interstitial tissues invade existing blood vessels forming a cylindrical microstructure that spans the lumen of capillaries and small vessels (Short, 1950; Caduff et al., 1986).

As implied by its name, sprouting angiogenesis is characterised by EC sprouts, which usually grow toward an angiogenic stimulus. Hypoxia is one of the key drivers of this process. The primary mechanism of hypoxia-induced neovascularisation involves an increase in hypoxia-inducible factor (HIF-1a) levels (**Figure 1**). When oxygen-sensing mechanisms detect low oxygen levels, new blood vessels are required to satisfy the metabolic requirements of parenchymal cells. Most of these cells respond to hypoxic stimuli by secreting a critical proangiogenic growth factor, vascular endothelial growth factor (VEGF-A). A particular type of EC, known as tip cells, leads sprouting angiogenesis (Gerhardt et al., 2003). The filopodia of endothelial tip cells are endowed with VEGF-A receptors (VEGFR2) that allow them to sense changes in VEGF-A concentrations and drive them to align with the VEGF gradient. Neighbouring cells, known as stalk cells, divide as they follow behind a tip cell, elongating the new vessel. Maturation and stabilisation of the vessels involve the recruitment of pericytes and ECM deposition along with mechanical stimuli (Stratman and Davis, 2012). This process is highly conserved and regulated by very specific molecular pathways, such as the Notch signalling pathway. In response to VEGF-A, the expression of the ligand Dll4 increases on the surface of tip cells. As a result, these

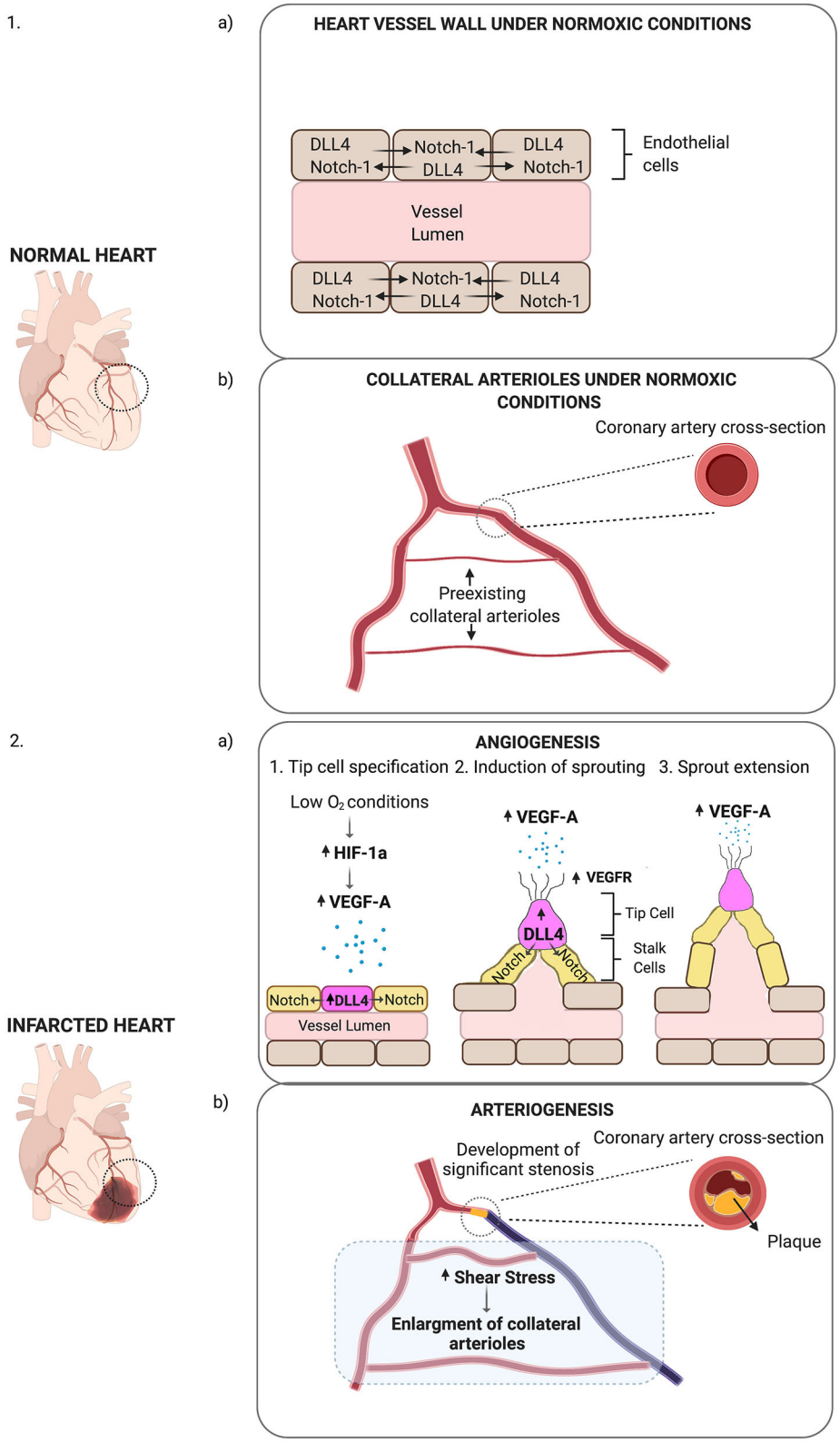

FIGURE 1 | Schematic representation of post-ischaemic neovascularisation. **(1a)** Under normoxic conditions, DLL4-Notch-1 signalling is balanced in ECs. **(1b)** Under normoxic conditions, small collateral arterioles may connect main arteries. **(2a)** Low O_2 conditions increase HIF-1a levels, which in turn stimulates the

(Continued)

FIGURE 1 | expression of VEGF-A (represented as small blue dots). In response to VEGF-A, Dll4 expression increases on the surface of tip cells (purple cell). Dll4 receptors on tip cells bind to Notch receptors on the surface of stalk cells (yellow cells) activating Dll4/Notch signalling pathway. Dll4—Notch-1 signalling directs ECs into migrating tip or proliferating stalk cells. In response to VEGF-A, VEGFR expression increases on the filopodia of tip cells (represented as protrusions on the tip cell), which then migrate towards VEGF-A with the stalk cells proliferating behind them. This results in the expansion of the vessel lumen and the formation of sprouts on the vessel wall (angiogenesis). **(2b)** When a coronary artery is occluded due to the formation of plaque within the arterial wall, there is increased shear stress in the collateral arterioles. In response to shear stress, collateral arteriole diameter increases in size (arteriogenesis).

ligands bind to Notch1 receptors of adjacent cells and activate Notch signalling pathway that suppresses tip cell fate and induces the stalk cell phenotype in these cells (Del Toro et al., 2010).

While angiogenesis is stimulated by low-oxygen concentrations and leads to the creation of new capillaries, arteriogenesis is induced by physical forces; mainly fluid shear stress. Arteriogenesis is an adaptive response to transient, repetitive occlusion of a large main artery and is characterised by an enlargement of the collateral arterioles (**Figure 1**). Once a main artery is occluded the arterial pressure behind the stenosis site decreases, and the blood is redistributed via the collateral arterioles. Thus, collateral arterioles now connect a high-pressure with a low-pressure region (Schaper and Pasyk, 1976). While hypoxia-induced pathways, as well as alterations in haemodynamic forces of the vascular wall, have been proposed as critical regulators of vessel growth after an ischaemic event, emerging evidence supports the idea that tissue-resident EPCs may also be involved in cardiac neovascularisation post-ischaemia (Fujisawa et al., 2019). A better understanding of the cellular frameworks and molecular mechanisms that drive these processes is crucial for the development of new therapeutic neovascularisation strategies.

The use of animal models is essential for a better understanding of the neovascularisation process post-MI at a cellular level. Aiming to identify the mechanism of EC proliferation post-ischaemia, Manavski et al. (2018), utilised Confetti fl/wild type (wt) cadherin 5 (Cdh5)- CreER^{T2} mice and revealed that ischaemia-induced vascularisation post-acute myocardial infarction (AMI) is mediated by clonally expanded ECs. At day 7 post-AMI 28% of the cardiac sections showed significant clonal expansion of Cdh5-expressing ECs, and this was increased to 33% on day 14. Since Cdh5 is also expressed in bone marrow, the origin of these clonally expanded cells remained inconclusive. Recently, Li et al. (2019) utilised a Pdgfb-iCreER -R26R-Brainbow2.1 mouse. In this system, the expression of *Cre* is driven by a Pdgfb promoter, which is specific to ECs (Claxton et al., 2008). Using this mouse, they demonstrated that vessel formation and clonal expansion of cardiac ECs was mediated by a subpopulation of resident cardiac ECs with progenitor-like properties.

Genetic lineage tracing has significantly improved our understanding of the neovascularisation process in the post-ischaemic heart. Nonetheless, cardiac neovascularisation potential is limited and does not appear to effectively promote myocardial regeneration. Recently, Kocijan et al. (2020), used an Apln-CreER;R26mT/mG mouse model to compare the angiogenic potential of the heart and skeletal muscle. Apln is highly expressed in ECs during embryonic development and is down-regulated in adulthood. However, in response to hypoxia, under tissue ischaemia or in the context of a tumour, the expression of Apln is reactivated, particularly in tip cells. Using this system, the authors showed that different pro-angiogenic stimuli activated Apln in skeletal muscle, resulting in angiogenic sprouts that could be incorporated into arteries. In the heart, however, Apln+ cells failed to give rise to new vessels. To confirm these data, the authors implanted cancer cells in different organs and showed that the angiogenic response in the heart was reduced. These data confirm that the inherent angiogenic response of the cardiac muscle is limited, emphasising the need for new therapeutic approaches to promote endogenous neovascularisation.

MicroRNAs in Therapeutic Neovascularisation

Over the past few years, miRNAs have gained widespread attention for their role in vascular health and disease, including in neovascularisation. MiRNAs are small (18–22 nucleotide, nt) endogenous non-coding RNA molecules that negatively regulate gene expression by targeting specific mRNAs. Most target sites on mRNAs only share a partial complementarity with their corresponding miRNAs, and thus, a single miRNA can target multiple mRNAs, contributing to biological and pathophysiological processes (Huntzinger and Izaurralde, 2011).

Emerging evidence suggests that miRNAs are critical regulators of both adaptive and maladaptive vascular remodelling and angiogenesis. **Table 1** contains a list of all known miRNAs that play a role in cardiovascular neovascularisation as well as their experimentally confirmed targets. Some of these have been extensively studied. MiR-126, for instance, is one of the most abundantly expressed miRNAs in ECs and has a prominent role in controlling angiogenesis by repressing negative regulators of the VEGF pathway, such as the Sprouty-related protein SPRED1 and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2/p85-beta) (Fish et al., 2008; Wang et al., 2008; Schober et al., 2014). Wang et al. (2008), showed that targeted deletion of miR-126 in mice leads to leaky vessels, haemorrhage and embryonic lethality due to defective vascular integrity. Half of the animals survived 1 week post-MI, while almost all died within 3 weeks post-MI. Another miRNA with angiogenic properties is miR-210. MiR-210 upregulation is a principal element of EC response to hypoxia (Fasanaro et al., 2009). Hu et al. (2010), demonstrated that overexpression of miRNA-210 post-MI in mice increased post-ischaemic neovascularisation by inhibiting ephrin-A3 and improved cardiac function 8 weeks post-MI. miR-23-24-27 cluster has also been reported to play a critical role in the regulation of neovascularisation. Knock-out of miR-27b, a component of this cluster impaired capillary branching in zebrafish embryos by targeting Dll4 and Sprouty (Spry)-2

TABLE 1 | miRNAs playing a role in cardiovascular neovascularisation.

miRNA	Model	Effect	Experimentally confirmed molecular targets	References
miRNAs THAT IMPROVE NEOVASCULARISATION				
miR-10a/10b	miR-10a and miR-10b deficient & WT zebrafish embryos	Impaired blood vessel outgrowth in miR-10a and miR-10b deficient zebrafish embryos compared with controls	mindbomb E3 ubiquitin protein ligase 1 (mib1)	Wang et al., 2016
miR-21	Cardiac microvascular endothelial cells (CMVECs) Sprague-Dawley rats -MI induction by LAD coronary artery ligation Chicken chorioallantoic membrane (CAM)	Increased EC tube formation, proliferation and decreased apoptosis post-miR21 overexpression *in-vitro.* Improved cardiac function post-miR-21 overexpression in rats. Opposite effects post-miR inhibition *in-vivo.* Implantation of matrigel plugs containing miR-21-overexpressing DU145 cancer cells onto the CAM of a chicken embryo resulted in increased microvessel formation compared to controls	Phosphatase and tensin homolog (PTEN)	Liu et al., 2011; Yang et al., 2016
miR-26b	HUVECs Male CD-1 mice-HLI induction post-left femoral artery ligation	Increased EC proliferation, migration and tube formation post-miR overexpression *in-vitro*. Decreased EC proliferation and tube formation post-miR-inhibition *in-vitro*. Increased microvessel formation in a Matrigel plug model. Overexpression of miR-26b in a mouse HLI model improved capillary survival in the ischaemic muscles	PTEN	Martello et al., 2018
miR-23-24-27 cluster *(miR-23a/b, miR-27a/b, miR-27a-3p)*	HUVECs EC spheroids miR-27b knock-out & WT zebrafish embryos aortic rings from athymic nude mice FVB mice-HLI induction C57/Bl6 mice- MI induction by LAD coronary artery ligation	Decreased EC sprouting in aortic rings *ex-vivo* post-miR-27b knock-down. Impaired capillary branching in miR-27b knock-out zebrafish embryos compared with controls. Increased capillary density and reperfusion post-miR-27b injection in mouse HLI and MI models compared with controls. Inhibition of miR-23a/b and miR-27a/b in HUVECs significantly impaired their tube formation ability. Aortic ring treatment with anti-miR-23 or anti-miR-27 resulted in impaired EC sprouting. Opposite results post miR-23b and miR-27b overexpression. Overexpression of miR-27a-3p in HUVECs significantly increased tube formation. Opposite results post-miRNA inhibition. Overexpression of miR-27a or miR-27b in EC spheroids, resulted in significantly increased EC sprouting compared to controls. MiR-27a/b inhibition led to opposite results.	Spry-2 (target of both miR-23 and miR-27) Dll4 (target of miR-27b) Semaphorin 6A (SEMA6A) (target of both miR-23 and miR-27)	Zhou et al., 2011; Biyashev et al., 2012; Veliceasa et al., 2015; Rao et al., 2019
miR-29a	HUVECs	Increased EC tube formation and proliferation post-miR overexpression *in-vitro.* Opposite effects were observed post-miR inhibition in EC *in-vitro*	HMG-Box Transcription Factor 1 (HBP1)	Yang et al., 2013
miR-30a	Transgenic (Tg) friend leukaemia integration 1 transcription factor (fli1): enhanced green fluorescent protein (EGFP) zebrafish	MiR-30a loss of function decreased arteriolar sprouts compared to controls. miR-30a gain of function increased arteriolar branching	Dll4	Jiang et al., 2013
miR-31 miR-720	EPCs from patients with CHD nude mice- HLI induction by ligation of both proximal and distal portion of the right femoral artery, as well as the distal portion of saphenous artery	Increased tube formation and migration of EPC post-miR-31/−720 overexpression. Opposite effects were observed post-miR inhibition. Increased blood flow by intramuscular injection of EPCs overexpressing miR-31	Thromboxane A2 receptor (miR-31 target) Vasohibin-1 (miR-720 target)	Wang et al., 2014
miR-101	HUVECs Aortic rings from female C57BL/6J mice Female C57Bl/6J mice- HLI induction by double ligation of the superficial femoral artery proximal to the deep femoral artery and distal femoral artery	Increased EC tube formation, proliferation and migration *in-vitro* post-miR-101 overexpression *in-vitro.* Increased EC sprouting *ex-vivo* post-transfection with mir-101-expressing lentivirus. Increased capillary density and limb perfusion in miR-101 injected mice compared with controls	CUL 3	Kim et al., 2014
miR-106b-93-25 cluster *(miR-106b)*	Primary bone marrow stromal cells (BMSCs) from Female WT & miR-106b∼25 knock-out mice Aortic rings from mir-106b∼25 wild-type or knock-out mice Female WT & miR-106b∼25 KO mice—HLI induction by femoral artery ligation	Increased tube formation ability and survival of BMSCs from WT compared to BMSCs from miR-106b∼2 knock-out mice. Increased capillary sprouting in WT compared to miR-106b∼2 knock-out mice. Significantly increased blood flow and number of lectin-positive capillaries in the WT compared with the KO mice on Day 7 & Day 14 post-ischaemia	Unknown	Semo, 2013

(Continued)

TABLE 1 | Continued

miRNA	Model	Effect	Experimentally confirmed molecular targets	References
miR-126	Human aortic endothelial cells (HAECs) HUVEC Aortic rings from miR-126$^{-/-}$ mice Male NMRI nude mice- MI induced by LAD coronary artery ligation miR-126$^{-/-}$ mice- MI induced by LAD coronary artery ligation	Improved HAEC tube formation post-miR-126 transfection *in-vitro*. Opposite effects post-miR inhibition *in-vitro*. Improved HUVEC tube formation post-miR-126-expressing adenovirus (Ad-miR-126) transfection *in-vitro*. Opposite effects were observed post-miR inhibition *in-vitro.* Impaired EC sprouting in aortic rings from miR-126$^{-/-}$ mice *ex-vivo.* Improved cardiac function and capillary density post-miR-126 mimic injection in NMRI nude mice post-MI. Opposite effects post-miR inhibition *in-vivo.* Reduced survival of miR-126$^{-/-}$ mice post-MI (half of the mice died 1week post-MI, and nearly all of them died within 3 weeks post-MI)	SPRED1 PIK3R2	Fish et al., 2008; Wang et al., 2008; Jakob et al., 2012
miR-130a	HUVECs	HUVEC co-transfection with pcDNA3.1-miR-130a and pcDNA3.1-growth-arrest-homeobox-transcription-factor (GAX) showed that miR-130a antagonised GAX-induced inhibition of HUVEC tube formation and migration, increasing tube formation and migration *in-vitro.* miR-130a inhibitor reversed antagonism of GAX activity	GAX	Chen and Gorski, 2008
miR-132	HUVECs	Increased HUVEC tube formation ability and proliferation post-miR overexpression. Opposite effects were observed post-miR inhibition in EC *in-vitro*	p120 Ras GTPase activating protein (p120RasGAP)	Anand et al., 2010
miR-150	HUVECs exposed to oxidised low-density lipoprotein (LDL) Apolipoprotein E-deficient (ApoE$^{-/-}$) mice-HLI induction by femoral artery ligation	Overexpression of miR-150 rescued the decreased tube formation ability of HUVECs exposed to LDL. Intramuscular injection of miR-150 mimic increased capillary and arteriolar (arteriogenesis) densities compared with controls	SRC Kinase Signalling Inhibitor 1(SRCIN1) (previously identified target)	Desjarlais et al., 2017
miR-210	Human umbilical vein endothelial cells (HUVECs), Aortic rings from female C57BL/6 mice, miR-210 overexpressing & WT mice subjected to cardiac ischaemia/reperfusion or permanent LAD coronary artery ligation	Increased HUVEC tube formation post-miR210 overexpression *in-vitro*. Opposite effects were observed post-miR inhibition *in-vitro.* Increased EC sprouting in aortic rings from miR-210 overexpressing mouse hearts *ex-vivo.* Increased reperfusion, capillary density and improved cardiac function in miR-210 overexpressing mice compared with controls both after ischaemia-reperfusion and MI induction	Ephrin-A3	Hu et al., 2010; Arif et al., 2017
14q32 cluster *(miR-329, miR-487b, miR-494, and miR-495)*	Male C57Bl/6 mice-HLI induction by electroagulation of the left femoral artery proximal to the superficial epigastric arteries (single ligation-model for effective arteriogenesis), or by electroagulation of the distal femoral artery proximal to the bifurcation of the popliteal and saphenous artery (double ligation-model for severe peripheral arterial disease)	Silencing of miRNAs was induced by gene silencing oligonucleotides (GSO). Treatment with all 4 GSO improved blood flow recovery post-ischaemia *in-vivo.* GSO-495 and GSO-329 treatment increased perfusion 3 days post-ischaemia. Treatment with GSO-329 only, increased perfusion 7 days post-ischaemia. Increased collateral artery diameters (arteriogenesis) and capillary densities post-GSO treatment in mice compared with controls	Myocyte enhancer factor 2A (MEF2a) (target of miR-329) FGFR2, VEGF-A, ephrin-2 (targets of miR-494)	Welten et al., 2014
miR-424	HUVECs	Increased tube formation, migration and proliferation of EC *in-vitro* post-miRNA overexpression. Opposite effects were observed post-miR inhibition in EC *in-vitro*	Cullin 2 (CUL2)	Ghosh et al., 2010
miR-503	HUVECs Human microvascular endothelial cells (HMVECs)	Increased tube formation and migration of EC *in-vitro* post-miRNA overexpression. Opposite results were observed post-miR inhibition in EC *in-vitro*	Cyclin E1 (CCNE1) Cell division cycle 25 A (cdc25A)	Caporali et al., 2011
let-7f	HUVECs	Decreased tube formation and migration of EC *in-vitro* post-miRNA inhibition	Unknown	Kuehbacher et al., 2007
miRNAs THAT INHIBIT NEOVASCULARISATION				
miR-15a/ miR-16-1 cluster *(miR-15a, miR-16)*	HUVECs EC-selective MiR-15a Transgenic (EC-miR-15a TG) & WT mice- HLI induction by femoral artery ligation Male CD1 mice- LI induction by left femoral artery ligation	Increased HUVEC tube formation and migration post-miR inhibition *in-vitro*. Opposite results post-miR-overexpression *in-vitro*. Decreased number of capillaries and blood perfusion in EC-miR-15a TG mice compared with controls. Inhibition of miR-15a/16 in mice post HLI improved capillary density and blood perfusion	FGF2 VEGF	Yin et al., 2012; Besnier et al., 2019

(Continued)

TABLE 1 | Continued

miRNA	Model	Effect	Experimentally confirmed molecular targets	References
miR-15b-5p	HUVEC Male C57BL/6 mice- HLI induction by left femoral artery ligation	Decreased HUVEC tube proliferation and migration post-miR overexpression *in-vitro.* Decreased blood flow recovery, capillary and arterial density in HLI mice post-miR-15b-5p overexpression	AKT Serine/Threonine Kinase 3 (AKT3)	Zhu et al., 2017
miR-16 miR-426	HUVECs	Decreased EC tube formation and migration post-miR overexpression *in-vitro*	VEGF receptor-2 (VEGFR2) (target of both miRNAs) Fibroblast growth factor receptor-1 (FGFR1) (target of both miRNAs)	Chamorro-Jorganes et al., 2011
miR-22	Fluorescent-labelled transgenic zebrafish Tg(fli1:EGFP) embryo expressing GFP in ECs	MiR-22 injection in zebrafish embryos resulted in defective vascular development which was rescued with miR-22 and VE-cadherin co-injection. miR-22 negative control (NC) injection in zebrafish embryos resulted in normal vessel development	Vascular endothelial (VE)-cdh	Gu et al., 2017
miR-23-24-27 cluster *(miR-24)*	HUVEC Tg(kdrl:eGFP)s843 zebrafish embryos Male C57BL/6 mice- MI induction by LAD coronary artery ligation	Decreased HUVEC tube formation, proliferation and migration post-miR overexpression *in-vitro*. Increased vascular defects in miR-24–overexpressing zebrafish compared with controls. Increased capillary and arteriolar density post-miR-24 inhibition in a MI mouse model	PAK4 GATA2	Fiedler et al., 2011
miR-23-24-27 cluster *(miR-24-3p)*	HUVECs Male CD1 mice-HLI model induction by left femoral artery ligation	Decreased EC survival, tube formation and proliferation post-transfection with pre-miR-24-3p *in-vitro*. Opposite results post-miR inhibition *in-vitro*. Increased capillary density post-miR inhibition *in-vivo,* but decreased blood perfusion since the new vessels appeared disorganised and twisted	Notch1 Dll1	Marchetti et al., 2020
miR-26a	HUVECs flk:eGFP zebrafish embryos Male C57BL/6 mice- MI induction by LAD coronary artery ligation	Decreased HUVEC tube formation and migration post-miR overexpression *in-vitro.* Overexpression of miR-26a in zebrafish impaired the development of caudal vein plexus (CVP) axial vein. Reduced infarct size and increased number of $CD31^+$ cells after miR-26a inhibition in mice post-MI	SMAD Family Member 1 (SMAD1)	Icli et al., 2013
miR-34	Male C57BL/6 mice- MI induction by LAD coronary artery ligation	Improved cardiac function, capillary density and left ventricle remodelling post-inhibition of miR-34 *in-vivo* compared with controls	Unknown	Bernardo et al., 2012
miR-17-92 Cluster *(miR-92a)*	Pig-ischaemia/reperfusion induction (percutaneous transluminal coronary angioplasty balloon was placed in the LAD artery distal to the first diagonal branch for 49-60 minutes MiR-92a–deficient mice- MI induction by LAD coronary artery ligation C57Bl/6 mice-MI induction by LAD coronary artery ligation & HLI induction by ligation of the superficial and deep femoral artery and vein	Increased capillary density and reduced cardiac inflammation and post-mir-92α inhibition in mouse and pig MI models and in a HLI mouse model compared with controls	INTGA5	Bonauer et al., 2009; Hinkel et al., 2013; Bellera et al., 2014
miR-100	HUVECs C57/Bl6J mice- HLI induced by double ligation of the superficial femoral artery proximal to the deep femoral artery and distal femoral artery	Increased HUVEC tube formation, proliferation and migration post-miR inhibition *in-vitro*. Opposite effects were observed post-miR-overexpression *in-vitro*. Increased perfusion and capillary and arterial density post-miR-100 inhibition *in-vivo* compared with controls	Unknown	Grundmann et al., 2011
miR-124	HUVEC Male C57BL/6 mice- thoracic aorta constriction (TAC)	Increased HUVEC tube formation, proliferation and migration post-miR inhibition *in-vitro*. Opposite effects were observed post-miR-overexpression *in-vitro*. Impaired cardiac function and blood flow in intravenous adeno-associated virus (AAV)-miR-124-injected mice compared with controls	CD151	Zhao et al., 2018

(Continued)

TABLE 1 | Continued

miRNA	Model	Effect	Experimentally confirmed molecular targets	References
miR-132/212	HUVEC Mice- HLI induced by double ligation of the superficial femoral artery proximal to the deep femoral artery and distal femoral artery	Decreased tube formation and migration of EC *in-vitro* post-miRNA overexpression. Opposite effects were observed post-miRNA inhibition. Increased capillary and arterial density post-miRNA inhibition *in-vivo*	RAS p21 protein activator 1 (Rasa1) (previously identified target of miR-132) SPRED1 (target of both miRNAs) Spry1 (target of both miRNAs)	Lei et al., 2015
miR-135-3p	HUVEC	Increased HUVEC tube formation, proliferation and migration post-miR inhibition *in-vitro*. Opposite effects were observed post-miR-overexpression *in-vitro*	Huntingtin-interacting protein 1 (HIP1)	Icli et al., 2019b
miR-142a-3p	Tg(fli1:EGFP)y1 zebrafish embryos expressing GFP in ECs	Loss of vascular integrity, haemorrhage and vascular remodelling post-injection of miR-142a-3p in zebrafish embryos. Normal primary vasculature but defective intersegmental vessels (Se) and abnormal remodelling in miR-142a-3p knock-out embryos	Cdh5 (VE-cdh)	Lalwani et al., 2012
miR-143/145 cluster *(miR-143, miR-145)*	HUVECs	Increased HUVEC tube formation and proliferation post-miR inhibition. Opposite results post-miR-143/-145 overexpression *in-vitro*	Hexokinase II (HKII) (miR-143 target) Integrin β (miR-145 target)	Climent et al., 2015
miR-183 cluster *(miR-96, miR-182, miR-183)*	Mouse cardiac endothelial cells (MCECs) HCMECs C57BL/6N mice -MI induction by LAD coronary artery ligation miR-96/miR-183 knock-out mice -MI induction by LAD coronary artery ligation	Overexpression of miR-96 and/or miR183 reduced tube formation and proliferation, but no migration of neonatal MCECs *in-vitro.* Inhibition of miR-183 cluster improved tube formation, proliferation and migration of adult MCECs *in-vitro.* Inhibition of miR-96 and miR-183 increased tube formation and proliferation of adult MCEC *in-vitro*, whereas miR-182 inhibition did not affect these measures. Similarly, overexpression of miR-96 and miR-183 in HCMECs reduced tube formation and proliferation, whereas dual inhibition of these miRs increased both parameters. Injection of miR-96 and miR-183 mimics in neonatal mice post-MI decreased vascularisation around the fibrotic tissue and increased the retention of scar tissue. MI induction in miR-96/miR-183 knockout mice resulted in increased capillary and arteriole densities, nut cardiac function and fibrosis did not change significantly compared to the WT controls.	Anillin (ANLN)	Castellan et al., 2020
miR-185	HMVECs	Decreased HMVEC tube formation, proliferation and migration post-miR overexpression. Opposite results post-miR-inhibition *in-vitro*	Stromal interaction molecule 1 (STIM1)	Hou et al., 2016
miR-199a-5p	Bovine aortic endothelial cell (BAEC)	Increased EC tube formation post-miRNA inhibition *in-vitro*	VEGFA (target in HEK293 cells) Calcineurin (target in HEK293 cells) SOD1 (target in HEK293 cells	Joris et al., 2018
miR-214	EPCs HUVECs Mice-HLI induction by right femoral artery and the distal portion of saphenous artery ligation C57BL/6 mice- TAC restriction	Increased EPC tube formation post-miR inhibition. Increased HUVEC tube formation, proliferation and migration post-miR inhibition. Opposite results post-miR-214 overexpression *in-vitro*. Increased blood flow by transplantation of EPC, in which miR-214 was inhibited, to the ischaemic limb tissue. Improved cardiac function and increased number of capillaries post-AAV9-anti-miR-214 injection compared with controls	X-box binding protein 1 (XBP1)	Duan et al., 2015; Jin et al., 2015

(Continued)

TABLE 1 | Continued

miRNA	Model	Effect	Experimentally confirmed molecular targets	References
miR-217	HUVECs HAECs HCAECs	Decreased EC tube formation and migration post-miR overexpression *in-vitro*. Opposite results post-miR-inhibition *in-vitro*	Silent Information Regulator 1 (SirT1) (target of miR-217)	Menghini et al., 2009
miR-221/222	HUVECs	Decreased HUVEC tube formation and migration post-miR overexpression *in-vitro*	Signal transducer and activator of transcription 5A (STAT5A) (miR-222 target)	Poliseno et al., 2006; Dentelli et al., 2010
miR-342-5p	HUVEC mouse aortas from endothelial-specific Notch-activating mice	Decreased HUVEC tube formation and migration post-miR overexpression *in-vitro*. Decreased EC sprouting in aortic rings post-miR mimic transfection ex-*vitro*	Endoglin	Yan et al., 2016
miR-483-5p	HUVECs	Decreased HUVEC tube formation and migration post-miR overexpression. Opposite results post-miR-inhibition *in-vitro*	Serum response factor (SRF)	Qiao et al., 2011
miR-615-5p	HUVEC C57BL/6 mice-HLI induced by femoral artery ligation	Increased HUVEC tube formation, proliferation and migration post-miR inhibition *in-vitro*. Opposite effects were observed post-miR-overexpression *in-vitro*. Improved blood flow recovery and capillary density post-miRNA inhibition *in-vivo*	IGF-2 Ras-associating domain family member 2 (RASSF2)	Icli et al., 2019a
miR-665	HUVECs Human cardiac microvascular endothelial cells (HCMVECs) Male C57BL/6 mice- TAC restriction	Decreased HUVEC tube formation, proliferation and migration post-miR overexpression *in-vitro*. Opposite results post-miR-inhibition *in-vitro*. Increased coronary microvessel density and improved heart function post-miR-665 inhibition *in vivo*	Ago2	Fan et al., 2018
miR-939	HUVECs	Decreased HUVEC tube formation, proliferation and adhesion, but increased migration post-miR overexpression *in-vitro*. Opposite results post-miR-inhibition *in-vitro*	γ-catenin	Hou et al., 2017
miRNAs WITH OPPOSITE *IN-VITRO* AND *IN-VIVO* FUNCTIONS				
miR-146a	HUVEC Balb/c mice-femoral artery ligation	Impaired EC tube formation and proliferation post-miRNA inhibition *in-vitro. In vivo,* miRNA inhibition post-femoral artery ligation did not affect capillary density but significantly increased collateral artery diameter (arteriogenesis)	Unknown	Heuslein et al., 2018
miR-155	HUVECs Aortic rings from miR-155$^{-/-}$ mice C57/BL6J mice- HLI induction by double ligation of the superficial femoral artery proximal and distal to the deep femoral artery miR-155$^{-/-}$ mice- HLI induction by double ligation of the superficial femoral artery proximal and distal to the deep femoral artery	Increased HUVEC tube formation and proliferation post-miRNA inhibition *in-vitro*. Decreased EC sprouting in aortic rings from miR-155$^{-/-}$ mice. Decreased blood flow recovery post-ischaemia in miR-155-deficient mice compared to controls	Angiotensin II receptor type I (AGTR1)	Pankratz et al., 2015

(Biyashev et al., 2012). Veliceasa et al. (2015), also showed that overexpression of miR-27b in a mouse MI model increased capillary density and reperfusion, and improved cardiac function with an approximately 2-fold increase in ejection fraction over the control 14 days post-MI, and significantly reduced fibrosis at day 28.

On the other hand, several miRNAs have an inhibitory role in post-ischaemic neovascularisation. Overexpression of another component of cluster miR-23-24-27, miR-24, significantly impaired angiogenesis in zebrafish embryos by targeting p21 activated kinase (PAK4) and global transcription factor binding protein 2 (GATA2). Inhibition of this miRNA with chemically engineered cholesterol-conjugated single-strand RNA analogues (antagomirs) in a MI mice model increased capillary and arteriolar density, and improved cardiac function 14 days post-MI (Fiedler et al., 2011). The miRNA 17-92 cluster also has a prominent role in the regulation of post-ischaemic neovascularisation. Overexpression of the miR-92a component of this cluster has been reported to block angiogenesis *in-vitro* and *in-vivo*. Among the verified targets of miR-92a, integrin alpha 5 (ITGA5) critically influences EC proliferation and migration (Bonauer et al., 2009; Doebele et al., 2010; Daniel et al., 2014). Interestingly, antimir-92a (MRG-110) was evaluated in clinical trials; the results showed increased angiogenesis and blood perfusion following intradermal injection at the site of a small skin wound in healthy volunteers, as well as reduced alpha-smooth muscle actin (α-SMA) expression, which is correlated with activation of myofibroblasts (Safety Tolerability Pharmacokinetics, 2018).

EVs IN NEOVASCULARISATION

Over the past few years, EVs have emerged as novel regulators of therapeutic processes, including cardiac neovascularisation due to their ability to transfer molecules, such as miRNAs, in ECs. EVs are defined as heterogeneous plasma membrane vesicles and are released in the extracellular space under normal and pathological conditions (Raposo and Stahl, 2019). According to their size and biogenesis pathway, they can be classified into three main types: exosomes, microvesicles and apoptotic bodies.

Exosomes are a class of cell-derived EVs of endosomal origin that are typically 30–150 nm in diameter and contain various macromolecules derived from the cell of origin. These include miRNAs, proteins, lipids and mRNAs (Isola and Chen, 2016). Exosome biogenesis starts from the intraluminal budding of endosomal compartments. This forms intraluminal vesicles (ILVs) in the endosomal compartments, which are known as multivesicular bodies (MVBs). MVBs can either fuse with lysosomes for subsequent degradation or fuse with the plasma membrane releasing the ILVs, in the extracellular space as exosomes. In contrast to exosomes, microvesicles, are typically 100–1,000 nm in size and are formed by the outward blebbing of the plasma membrane. During the blebbing process, disruption of the actin cytoskeleton and membrane reorganisation occurs (Cocucci and Meldolesi, 2015). The modification of membrane asymmetry results in the redistribution of aminophosholipids to the outer part of the cell membrane. Interestingly, microvesicle formation seems to occur in lipid-rich microdomains of the plasma membrane, such as lipid-rafts or caveolae domains (Del Conde et al., 2005; Morel et al., 2009). On the other hand, apoptotic bodies are exclusively formed during the last steps of apoptosis and range from 800 nm to 5 μm (Caruso and Poon, 2018). Investigating the mechanisms of EV release and uptake by ECs is critical to understand their role in neovascularisation under pathological conditions, such as MI. Recently, a new population of non-membranous nanoparticles ∼35 nm in size, termed "exomeres" was identified (Zhang H. et al., 2018; Zhang et al., 2019). Zhang H. et al. (2018), recently showed that exomeres have a distinct protein, lipid, DNA and RNA profile to exosomes, and demonstrate unique organ distribution patterns, suggesting different biological functions. In contrast to EVs, exomere biogenesis remains unclear.

EV Release by ECs

EV release is a complex process that involves cytoskeletal proteins (actin and microtubules), molecular motors (kinesins and myosins), as well as molecular switches (small GTPases) and the fusion machinery (SNAREs and tethering factors) (Raposo and Stoorvogel, 2013). Ras-related proteins in brain (RAB) family, including Rab11, Rab27a/27b, and Rab35 coordinate membrane trafficking events, and have emerged as essential components of exosomal release in several cell types (Savina et al., 2002; Hsu et al., 2010; Ostrowski et al., 2010). Rab7 is another member of RAB family that mediates the maturation of late endosome and mediates their fusion with lysosomes (Vanlandingham and Ceresa, 2009). Jaé et al. (2015), showed that Rab7 and Rab27b regulate the secretion of endothelial miRNA through EVs. Moreover, Ostrowski et al. (2010), demonstrated that knocking down the Rab27a/27b effectors synaptotagmin-like 4 (SYTL4) (also known as Slp4) and exophilin 5 (EXPH5) also inhibits exosome secretion in HEK293 cells. Interestingly, Slp4 was also shown to have a central role in Weibel-Palade body (WPB) exocytosis from ECs (Bierings et al., 2012).

Although our understanding of the exact mechanism of EV-release from EC is lacking, several factors have been shown to trigger the release of EVs from ECs. EC injury is a critical part of the development of CHD and significantly affects the levels of EC-derived EVs (Werner et al., 2006). In general, EC-EVs are present at lower concentrations under physiological conditions and, upon activation, are released from ECs (Koga et al., 2005). Elevated levels of plasma EC microparticles have been reported in several CVD, including CHD (Koga et al., 2005; Werner et al., 2006; Nozaki et al., 2009). Moreover, clinical studies in heart failure patients have revealed that the number of circulating EVs from ECs and EPCs greatly depends on the severity of heart failure. Berezin et al. (2016), for instance, showed that heart failure patients with preserved ejection fraction had an increased number of apoptotic EC-derived EVs and less activated EC-derived EVs than patients with heart failure with reduced injection fraction. Interestingly, EC-derived EVs have been proposed as novel biomarkers of EC dysfunction and may determine the risk of acute heart failure (Horstman, 2004).

EVs of endothelial origin have been found to play a versatile role in neovascularisation since their effect seems to be affected

by the dose used. Lacroix et al. (2007), for instance, showed that low amounts of microparticles (2×10^3 particles/well, in a 96-well plate) from TNFa-stimulated HMVECs could increase tube formation of endothelial progenitor derived cells (EPDCs) *in-vitro*. In contrast, high amounts of microparticles (2×10^5 particles/well, in a 96-well plate) had an inhibitory effect. Liang et al. (2018), showed that hypoxic HUVEC EVs could also inhibit EC migration and angiogenesis probably due to increased levels of miR-19b in these EVs (5×10^4 EVs/well, in a 6-well plate were used). The ability of ECs to stimulate angiogenesis may involve the transfer of miR-214 (Balkom et al., 2013). Interestingly, Chen et al. (2018), showed that EPC-derived EVs could increase angiogenesis but not proliferation *in-vitro* (7×10^9 EVs, in a 24-well plate). The intramyocardial delivery of EPC-derived EVs incorporated into shear-thinning hydrogels in Wistar rats, could increase angiogenesis, preserve ventricular geometry and improve haemodynamic function post-MI (9.33×10^9 EVs were delivered via 5×20 μl injections around the border zone of the infarcted area). Ou et al. (2011), however, demonstrated that endothelial microparticles from HUVECs in increased concentrations (higher than 10^5 microparticles/ml) could inhibit angiogenesis in mouse heart sections. These data suggest that EC release EVs with diverse roles in neovascularisation.

EV Uptake by ECs

EVs transfer information to the recipient cells by various mechanisms, including clathrin-mediated endocytosis, micropinocytosis, phagocytosis, caveolin-mediated endocytosis, and lipid raft mediated endocytosis (Mulcahy et al., 2014). The glycoproteins [e.g., Heparan sulphate proteoglycans (HSPG) (Christianson et al., 2013)] and proteins [e.g., integrins (Morelli et al., 2004), tetraspanins (Hemler, 2005)] on the surfaces of EVs and their target cells are recognised as critical factors that determine the uptake mechanism. Nazarenko et al. (2010), showed that treating exosomes with antibodies against tetraspanin-8, integrin CD49d, and vascular cell adhesion molecule-1 (VCAM-1, CD106) significantly reduced exosome uptake by RAECs. However, the precise mechanisms of EV uptake by ECs is still unknown. Some EVs can deliver their content to ECs through clathrin-mediated endocytosis. Dynamin is a GTPase essential for membrane fusion during this process. Blocking dynamin activity is a common strategy to study this mechanism (Singh et al., 2017). Chiba et al. (2018), showed that pancreatic cancer cell exosomes may be transferred to ECs through dynamin-dependent clathrin-mediated endocytosis, resulting in increased angiogenesis *in-vitro*. This was verified by blocking dynamin activity with a small inhibitor, called dynasore. Moreover, Svensson et al. (2013), reported that internalisation of exosomes derived from glioblastoma cells by ECs is significantly decreased post-methyl-β-cyclodextrin (MβCD) treatment. MβCD is a water-soluble oligosaccharide able to remove cholesterol from cell membranes, suggesting that lipid-raft endocytosis is a critical mechanism of EV uptake.

The most commonly used method for detecting EV uptake by ECs *in-vitro* and *in-vivo* is by using fluorescent lipid membrane dyes that stain EV membranes, such as PKH67 (Balkom et al., 2013), PKH26 (Lopatina et al., 2014; Lombardo et al., 2016; Zou et al., 2016; Mao et al., 2019) and DiI (Liang et al., 2016). Lopatina et al. (2014), for instance, showed that PKH26-stained MSC-EVs could be internalised into HMVECs and promote angiogenesis *in-vitro* and *in-vivo*. Moreover, Liang et al., demonstrated that the uptake of DiI-labelled MSC-exosomes by HUVECs increased tube formation *in-vitro*. To assess the role of MSC exosomes in EC angiogenesis *in-vivo,* the authors performed Matrigel plug assays in mice subcutaneously injecting HUVECs mixed with MSC-exosomes and HUVEC alone, and consistently with their *in-vitro* data, they showed that MSC-exosomes resulted in increased angiogenesis *in-vivo*. One potential issue with lipid-bound dyes is the leaching of the fluorescent molecules onto the cellular membranes, which can lead to a pattern of internalisation due to membrane recycling rather than EV uptake. To distinguish between surface-bound and internalised fluorescent EVs, the surface of the cell can be treated with trypsin (Franzen et al., 2014). A further limitation of these dyes is that the presence of the fluorescent molecules may affect the physical properties and thus, the normal behaviour of EVs. Membrane permeable dyes, such as carboxyfluorescein succinimidyl ester (CFSE) and 5(6)-carboxyfluorescein diacetate (CFDA), that are confined to the cytosol and fluoresce as a consequence of esterification, as well as Calcein AM are also used to study EV uptake by ECs (Teng et al., 2015; Li et al., 2018; Merckx et al., 2020). Radionuclides and magnetic particles have also been exploited to label EVs. Lee et al. (2020), for instance, recently showed that nanovesicles derived from iron oxide NP-incorporated MSCs could be effectively targeted to the myocardium of rats promoting cardiac function and angiogenesis. Finally, by fusing Gluc to a protein enriched in the membrane of exosomes, such as lactadherin, exosomes can emanate a strong luminescent signal when a Gluc substrate is present (Zhang K. et al., 2018). EVs can also be labelled with GFP by expressing a DNA construct coding for EV markers such as CD63, CD81, and CD9 fused to GFP in parent cells. This way, EVs can be tracked or purified to study their cargo (Garcia et al., 2015; Ribeiro-Rodrigues et al., 2017). Nonetheless, visualising GFP-labelled EV uptake by conventional fluorescent microscopy techniques is challenging due to the small nature of EVs. For this reason, the use of advanced technologies, including electron microscopy and atomic force microscopy are useful in EV characterisation (Mondal et al., 2019).

Recently, Cre-loxP system was also introduced as a very promising strategy to study EV uptake by cancer cells (Zomer et al., 2016). Adapting this system, de la Cuesta et al. (2019), visualised direct transfer of human pulmonary artery smooth muscle cells (HPASMCs) EVs to human pulmonary arterial endothelial cells (HPAECs). In particular, donor HPASMCs were transduced with a Cre recombinase lentiviral vector and HPAECs with a reporter lentiviral vector, that carried DsRed and eGFP separated by a loxP site. Cells were co-cultured in Boyden chambers, being physically separated by a membrane to prevent direct cell-cell contact. Zomer et al. (2015), previously showed that in this system, only *Cre* mRNA and not Cre protein is transferred into EVs. By performing qRT-PCR de la Cuesta et al. (2019) confirmed efficient loading of *Cre* mRNA into HPASMC-EVs. Using this system, the authors showed that HPASMC-EV-mediated Cre recombination resulted in a colour

switch from red to green in reporter$^+$ HPAECs, effectively evidencing communication via EVs. Therefore, the advantage of this approach is that EV uptake can be visualised in a more sensitive manner since it results in the fluorescent labelling of whole cells instead of small EVs.

Animal Models to Study EV Trafficking

One of the greatest limitations in using purified labelled EVs from cell culture supernatants into the circulation of animal models is the difficulty to translate the *in-vivo* implications of EVs at a functional level. The variations observed in studies using EVs from different cell sources, the number of particles injected, the route of administration as well as the fact that most studies are implemented in immunodeficient animals, are all important variables in the design and analysis of experimental data. For this reason, genetically engineered mouse models (GEMM) could provide a promising approach to outreach current limitations allowing tracing of EVs in living organisms. As previously mentioned, a new strategy that allows the study of EV transfer *in-vitro* and *in-vivo* using the Cre/loxP system was reported (Zomer et al., 2016). This approach involves the fluorescent labelling of Cre-reporter cells that take up the EVs released from cells which express Cre recombinase. In this method, prior to cell injection into mice ubiquitously expressing the Cre-LoxP reporter tdTomato (tdTomato B6 mice), cells were transfected with a plasmid carrying Cre recombinase and cyan fluorescence protein (CFP). In this way, donor cells expressing Cre recombinase were CFP positive (blue), and EVs derived from these cells carried *Cre* recombinase mRNA. Upon injection, EVs were taken up by recipient reporter cells that translated *Cre* mRNA into protein. This resulted in the recombination of the reporter gene and activation of GFP expression leading to a colour switch from red to green fluorescence exclusively in cells that had taken up functional EVs *in-vivo* Zomer et al. (2015), developed this system to study EV communication between tumour cells. EV-miRNA profiling revealed that malignant tumour cells contain mRNAs involved in tumour migration and metastasis. More importantly, intravital imaging, showed that EVs released by malignant cells can be internalised by less malignant cells within the same or distal tumours and that the less malignant tumour cells display enhanced migratory behaviour and metastatic capacity. The exact mechanism of how the *Cre* mRNA is transferred into exosomes remains unclear, but it is probably a simple reflection of increased cellular expression and sufficient EV loading. Interestingly, the authors verified that the colour switch was due to *Cre* mRNA containing EVs, and not by other mechanisms, such as free *Cre* mRNA or protein.

Other strategies involve the controlled expression of *Cre* recombinase under specific promotors. For example, *Cre* mRNA can be expressed selectively in haematopoietic cells under the vav1 promoter and sorted into EVs released into the bloodstream. Upon entering a target cell, this mRNA is translated to a functional protein, leading to excision of the stop-loxP site and induction of marker gene expression in transgenic mice. Based on this, Kur et al. (2020), proved that neuronal activity triggers the uptake of haematopoietic extracellular vesicles *in vivo*. In particular, they showed that after the induction of peripheral inflammation by intraperitoneal (IP) injection of lipopolysaccharide (LPS), there were frequent recombination events in the hippocampus, substantia nigra, and other regions of the brain as observed by yellow fluorescent protein (EYFP) expression. EYFP expression was mediated by Cre recombinase activity, indicating that haematopoietic cell EVs were transferred and internalised in these regions of the brain. Although most studies utilising GEMM to assess EV uptake are focused on cancer or neuroscience research, the adaptation of these animal models could greatly increase our knowledge in EV communication in cardiovascular disease, including MI. The use of the Cre-loxP system has significantly increased our understanding of EV communication. However, the fact that *Cre* mRNA can be loaded in and transferred through EVs may doubt the validity of approaches using the Cre-loxP system in a tissue-specific manner, since upon activation of *Cre* expression in specific cells, *Cre* mRNA or protein can be transferred to other cell types through EVs, leading to unfavourable recombination events.

Recently, McCann et al. (2020), generated a reporter mouse bearing a CD63-emGFP$^{loxP/stop/loxP}$ knock-in cassette that enables the labelling of cell type-specific EVs *in-vivo*, without prior *in vitro* manipulation. Upon crossing with a lineage-specific Cre recombinase driver mice, this system enables the specific labelling of circulating CD63$^+$ vesicles from the cell type of interest. By crossing the mice bearing the CD63-emGFP$^{loxP/stop/loxP}$ knock-in cassette with Cdh5-Cre^{ERT2} mice, the authors generated CD63emGFP+ vasculature and showed that following tamoxifen administration to pregnant females the developing vasculature of the embryos was marked with emerald GFP (emGFP). Most importantly, whole plasma-purified EVs contained a subpopulation of emGFP$^+$ vesicles that co-expressed EV markers, including CD9 and CD81, and EC markers, like CD105.

Altogether, the recent development of genetically modified mouse models to study EV trafficking *in-vivo* holds great promise as valuable tools for unravelling the *in-vivo* relevance of EVs in physiological and pathophysiological processes such as cardiac neovascularisation after cardiac ischemia.

EVs in Therapeutic Neovascularisation

EVs possess inherent tissue repair properties that make them ideal candidates for regenerative medicine therapeutics. Several studies have demonstrated the ability of EVs to promote neovascularisation post-ischaemia. Gallet et al. (2017), showed that intramyocardial delivery of cardiosphere-derived cell (CDC) exosomes in a pig MI model increased vessel density and cardiomyocyte hypertrophy, while preserving left ventricular (LV) and left ventricular ejection fraction (LVEF) volumes and reducing scar size. Moreover, Potz et al. (2018), showed that intramyocardial injection of human mesenchymal cell-derived EVs in a swine MI model could increase blood flow, capillary and arteriolar density 5 weeks post-left circumflex artery ligation. Chen et al. (2020), aimed to investigate the role of exosomes derived from remote ischemic conditioning (RIC) in cardiac remodelling and function. Exosomes were isolated from the plasma of rats subjected to HLI and injected

to the caudal vein of rats once every 3 days post-MI. The authors showed that RIC and RIC exosomes significantly improved cardiac function and blood vessel formation, and decreased collagen deposition 28 days post-MI. Wu et al. (2020), demonstrated that the intramyocardial injection of EVs from ESC-derived cardiovascular progenitor cells (CVPCs) cultured under normoxia or hypoxia could significantly improve cardiac function, vascularisation and cardiomyocyte survival, and reduce fibrosis at 28 days in a mouse MI model. More importantly, EVs secreted from ESC-CVPC cultured under hypoxia had a better benefit in improving cardiac function post-MI.

EVs possess several advantages over cell-based therapeutics and conventional delivery systems. A major advantage is that EVs may be less immunogenic than their parental cells, probably due to the presence of less membrane-bound proteins like MHC complexes on their surface (Ong and Wu, 2015). The number of MHC molecules on EV surface highly depends on the cell of origin and the EV subtype (Wahlund et al., 2017). Recently, Kompa et al. (2020), used a subcutaneously implanted TheraCyte device for sustained delivery of the secretome of human cardiac stem cells (hCSCs) in a rat MI model. Cells can be enclosed in the TheraCyte device, being protected by the host's immune system while allowing the therapeutic secreted products to freely diffuse from within the device. The authors showed that hCSC secretome could preserve LV ejection fraction and cardiac function, reduce fibrotic scar tissue, interstitial fibrosis and cardiomyocyte hypertrophy, while increasing vascular density. To visualise the EV transfer from hCSC to the myocardium, they used CSCs expressing plasma membrane reporters and confirmed that EVs from $W8B2^{+}$ CSCs could be transferred to the heart and other organs 4 weeks post-implantation. A further advantage of EVs is that they can be easily stored, retaining their function over prolonged periods, overcoming many limitations of the use of viable cells in regenerative medicine. Moreover, EVs are naturally occurring lipid nanoformulations that, compared to other synthetic drug delivery systems, may be promising carriers of therapeutic molecules, exhibiting less toxicity and increased stability under both physiological and pathological conditions. EVs might also be combined with other strategies to optimise therapeutic agent delivery. Liang et al. (2017), showed that exosomes could naturally envelope AAV-vectors (AAVExo) and protected from plasma neutralising antibodies, AAVExo could transduce cardiomyocytes with higher efficiency than free AAVs in a mouse MI model. For these reasons, EVs have emerged as ideal carriers for the delivery of therapeutic molecules, such as miRNAs, to ECs for the promotion of neovascularisation post ischaemia.

EV-bound miRNAs in Neovascularisation

EVs exert their action through the transfer of molecules, such as small RNAs and proteins, able to control molecular pathways in the recipient cells once transferred. For this reason, they have gained immense interest as therapeutic vehicles. Among their multidimensional role, several studies have demonstrated the ability of EVs to stimulate vascular growth and maturation by delivering pro-angiogenic miRNA molecules to ECs. **Table 2** contains a list of EV-bound miRNAs as reported in EV-based neovascularisation studies. However, since our intention was to identify EV-bound miRNAs with a role in neovascularisation, this table contains a simple summary of our findings with an emphasis on the miRNAs, and due to space constraints, not all EV-producing cell types are reported.

EV-bound miRNA molecules can be internalised in the cytoplasm of recipient cells and activate molecular pathways controlling cell behaviour (**Figure 2**). Barile et al. (2014), for instance, showed that EVs from human cardiac progenitor cells (hCPCs) were enriched in the angiogenic and cardioprotective miR-210, miR-132, and miR-146a-3p. As a result, EVs derived from these cells could significantly increase cardiac angiogenesis in a MI rat model. Wang et al. (2017), demonstrated that MSC-derived EVs were also enriched in miR-210. Treatment with MSC-EVs significantly increased HUVEC tube formation, proliferation and migration *in-vitro* and capillary density of matrigel plugs implanted in a mouse MI model by targeting Efna3. Silencing of miR-210 in MSC-EVs significantly impaired the *in-vitro* and *in-vivo* angiogenic effects. Adamiak et al. (2018), showed that treatment with iPSC-derived EVs, promoted murine cardiac EC tube formation, migration and antiapoptotic properties. Injection of iPSCs-EVs in a mouse MI model significantly increased capillary density & reduced LV remodelling and hypertrophy compared to the controls. EV microRNA expression profiling was performed by miRNA array and revealed that several miRNAs were upregulated in these EVs, including miR-16, miR-17-92, miR-19b, miR-20a, miR-34, miR-126-3p, miR-130a-3p, miR-210-3p, miR-294.

Specific conditions, such as hypoxia greatly affect gene expression and favour the production of miRNAs with angiogenic properties. As a result, several studies have confirmed that hypoxia leads to the release of angiogenic EVs. Ribeiro-Rodriquez et al. for instance, demonstrated that exosomes secreted by H9c2 myocardial cells and primary cardiomyocytes cultured under hypoxic conditions significantly increased the formation of new functional vessels post-MI in rats in comparison with exosomes cultured under normoxic conditions. An analysis of the miRNA profile of these exosomes revealed that the pro-angiogenic miR-222 and miR-143 were significantly increased in the hypoxic exosomes providing evidence that these miRNAs may contribute to post-ischemic neovascularisation (Ribeiro-Rodrigues et al., 2017). Similarly, Zhu et al. (2018), showed that exosomes derived from hypoxic MSCs augmented neovascularisation in a MI mouse model. Exosome RNA profiling revealed 145 genes that were upregulated in the hypoxic exosomes compared to the normoxic ones, with miR-210 being one of the most abundant miRNAs. To investigate the role of miR-210 in hypoxic-EV induced angiogenesis, the authors used a miR-210 inhibitor to block the activity of miR-210 in MSCs and revealed that exosomes derived from these cells failed to induce angiogenesis *in-vitro*. Moreover, overexpression of miR-210 in ECs resulted in improved tube formation similar to that achieved by hypoxic exosomes. Gray et al. (2015), reported that treatment with hypoxic EVs derived from CPCs significantly increased rat cardiac EC tube formation compared to treatment with normoxic EVs. Injection of hypoxic EVs in a mouse MI model significantly improved cardiac function and reduced fibrosis. EV microRNA

TABLE 2 | EV-bound miRNAs in the regulation of cardiovascular neovascularisation.

EV miRNA	Model	Effect	EV source	EV isolation method	References
miR-15b miR-17 miR-20a miR-103 miR-199a miR-210 miR-292	Rat cardiac endothelial cells Male Sprague-Dawley rats- MI induction by LAD coronary artery ligation	Increased EC tube formation post-hypoxic EV treatment *in-vitro*. Improved cardiac function and reduced fibrosis post-intramyocardial EV injection at 3 border zones. EV microRNA expression profiling by qPCR miRNA array revealed several upregulated miRNAs under hypoxic conditions	Normoxic/ hypoxic CPCs	Differential centrifugation ultracentrifugation	Gray et al., 2015
miR-16 miR-17-92 miR-19b miR-20a miR-34 miR-126-3p miR-130a-3p miR-210-3p miR-294	Murine cardiac ECs. C57BL6/J mice- MI induction by 30-min coronary occlusion followed by reperfusion	Increased EC tube formation, migration and antiapoptotic properties post-EV treatment *in-vitro*. Increased capillary density & reduced LV remodelling and hypertrophy post-intramyocardial injection of EVs at 5 sites at the border between infarcted and non-infarcted myocardium 48h post-reperfusion. EV microRNA expression profiling was performed by miRNA array and revealed several upregulated miRNAs.	iPSCs	Differential centrifugation ultracentrifugation	Adamiak et al., 2018
miR-17 miR-19a miR-19b miR-20a miR-30c miR-126	HUVECs	Increased EC tube formation, proliferation and migration post-exosome treatment *in-vitro.* EV microRNA expression profiling was performed by qPCR miRNA array and revealed increased levels of several miRNAs in these exosomes	Glucose starved H9C2 cardiomyocytes	Differential centrifugation ultracentrifugation	Garcia et al., 2015
miR-20b miR-27b miR-29b miR-42a miR-100 miR-125b miR-143 miR-195 miR-291b miR-497	CMVECs Aortic rings from Male Sprague-Dawley rats Male Sprague-Dawley rats- HLI induction by femoral artery ligation	Increased EC tube formation, proliferation and migration post-exosome treatment *in-vitro.* Increased EC sprouting post-exosome treatment *ex-vivo.* Increased capillary density and blood perfusion post-induced vascular progenitor cell (iVPC) exosome injection as compared with rat aortic endothelial cell (RAEC) exosomes and controls. EV microRNA expression profiling was performed by qPCR miRNA array and revealed increased levels of several miRNAs in these exosomes	iVPCs RAECs	Ultracentrifugation Ultrafiltration Size-exclusion chromatography	Johnson et al., 2019
miR-21-5p	HUVECs Female CD-1 mice- MI induction by LAD coronary artery ligation	Improved EC tube formation *in-vitro* post-treatment with EVs from patients with normal angiography results (NEXO) compared to patients with heart failure (FEXO). Increased capillary density, infarcted wall thickness and decreased infarct size post-NEXO EV treatment compared to FEXO EVs and PBS controls post-intramyocardial injection. Decreased miR-21-5p expression in FEXO compared to NEXO group identified by EV microRNA expression profiling by qPCR miRNA array. Silencing of miR-21-5p in NEXO group decreased tube-formation *in-vitro,* while upregulation of miR-21-5p in FEXO group promoted EC tube formation.	Explant-derived cardiac stromal cells from FEXO or NEXO patients	Ultracentrifugation	Qiao et al., 2019

(Continued)

TABLE 2 | Continued

EV miRNA	Model	Effect	EV source	EV isolation method	References
miR-21 miR-27a miR-29a miR-126 miR-130α miR-191 miR-210 miR-296-3p	HMVECs Human macrovascular endothelial cells (HMAVECs) SCID mice	Increased EC tube formation and proliferation post-treatment with EVs from obese individuals with type 2 diabetes (OD) compared with EVs from healthy individuals (H), patients with type 2 diabetes (D), obese individuals without T2DM (O), and patients with ischaemic disease (IC). EVs from patients of the above groups were divided as "effective" and "ineffective" depending on their effect on angiogenesis *in vitro*. Matrigel plug *in vivo* assays using EVs defined as "effective" in the *in vitro* assays, showed that these EVs resulted in an increased number of vessels in mice Matrigel plugs. EV microRNA expression profiling of "effective" and "ineffective" EVs of healthy individuals was performed by qPCR miRNA array and identified several angiogenic miRNAs	Human serum from H, D, OD, D, O, IC individuals	Ultracentrifugation	Cavallari et al., 2020
miR-31	HUVECs Aortic rings from male C57BL/6J mice	Increased EC tube formation and migration post-EV treatment *in-vitro.* Increased EC sprouting post-EV treatment *ex vivo.* EV microRNA expression profiling was performed by qPCR miRNA array and revealed increased levels of miR-31. EVs from cells transfected with anti-miR-31 decreased EC tube formation and migration *in-vitro*, whereas pre-miRNA transfection increased tube formation and migration.	Adipose-Derived Stem Cells (ADSCs)	Differential centrifugation Ultracentrifugation	Kang et al., 2016
miR-92a-3p	HCAEC	EV microRNA expression profiling was performed by qPCR miRNA array and revealed increased miR-92a-3p levels in oxidised low-density lipoprotein (oxLDL) and interleukin-6 (IL-6) stimulated HCAEC. Knock-down of this miRNA in HCAEC EVs decreased their ability to cause HCAEC migration, proliferation, and tube formation *in-vitro*.	oxLDL and IL-6 stimulated HCAEC	Differential centrifugation Ultracentrifugation	Liu et al., 2019
miR-125a-5p	HUVEC	Increased EC tube formation post-EV treatment *in-vitro*. EV microRNA expression profiling was performed by qPCR miRNA array. Overexpression of miR-125a in HUVECs, increased tube formation *in-vitro*. Matrigel plug *in vivo* assay results showed that the number of vascular structures was significantly higher in mice subcutaneously injected with HUVECs mixed with EVs than HUVECs alone	MSCs	Differential centrifugation Ultrafiltration Ultracentrifugation	Liang et al., 2016
miR-126-3p	HUVECs Immunocompromised BalbC mice- HLI induction by femoral artery ligation	Increased EC tube formation post-EV treatment *in-vitro*. EV microRNA expression profiling was performed by qPCR miRNA array. EVs from cells transfected with anti-miR-126-3p decreased HUVEC tube formation *in-vitro*. Improved blood flow and capillary density post-intramuscular injections of EVs at 4 different sites of the ischaemic limb.	$CD34^+$ SCs	Ultracentrifugation Density gradient centrifugation	Mathiyalagan et al., 2017
miR-126 miR-296	SCID mice- HLI induction by left femoral artery and the distal portion of saphenous artery ligation	Increased capillary density and blood perfusion post-MV injection. Presence of miR-296/-126 in the EVs was confirmed by qPCR	EPCs	Ultracentrifugation	Ranghino et al., 2012
miR-132 miR-146a-3p miR-181 miR-210	HUVECs Male Wistar rats- MI induction by LAD coronary artery ligation	Increased EC tube formation ability post-EV treatment *in-vitro*. EV microRNA expression profiling was performed by qPCR miRNA array. HUVECs transfected with a miR-132 mimic had increased tube formation. Opposite results were observed post-transfection with si-miR-132. *In-vivo* experiments revealed increased blood vessel density post-intramyocardial injection of CPC EV at 3 border zones.	hCPCs	ExoQuick™ precipitation solution (System Biosciences; SBI) Ultracentrifugation column precipitation using Exo-spin™ kits (Cell Guidance Systems, Cambridge, UK)	Barile et al., 2014

(Continued)

TABLE 2 | Continued

EV miRNA	Model	Effect	EV source	EV isolation method	References
miR-143 miR-222	MCECs HUVECs Rat aortic rings Fertilised chicken eggs Female C57BL/6 mice- MI induction by LAD coronary artery ligation	EVs from cells in ischaemic solutions increased EC tube formation, proliferation and protection against oxidative-induced lesion *in-vitro* and increased EC sprouting *ex-vivo*. Intramyocardial injection of hypoxic EVs increased capillary density, the number of CD31+ cells in the infarcted region and blood perfusion. EV microRNA expression profiling by qPCR miRNA array revealed increased miR-222/-143 expression in hypoxic EVs	H9c2 myocardial cells & primary rat cardiomyocytes from Wistar rat foetus cultured in appropriate media or ischaemia-mimetic solutions	Differential centrifugation Density gradient centrifugation	Ribeiro-Rodrigues et al., 2017
miR-210	HUVEC Male C57BL/6 mice- MI induction by LAD coronary artery ligation	Increased EC tube formation & decreased apoptosis post-hypoxic EV treatment *in-vitro*. EV microRNA expression profiling by qPCR miRNA array revealed increased miR-210 expression in hypoxic EVs. Transfection of HUVECs with miR-210 mimic yielded similar results to hypoxic EV treatment. Increased capillary and arteriole density and CM survival post-intramyocardial injection of hypoxic EVs at five sites around the border zone of infarcted hearts.	Normoxic/ hypoxic MSCs	Differential centrifugation Ultracentrifugation	Zhu et al., 2018
miR-214	HMVECs	Increased EC tube formation and migration post-EV treatment *in-vitro.* EV microRNA expression profiling was performed by qPCR miRNA array and showed increased miR-214 expression. EVs from cells transfected with anti-miR-214 decreased EC tube formation and migration *in-vitro*, whereas pre-miRNA transfection did not affect tube formation and migration.	HMVECs	Differential centrifugation Density gradient centrifugation	Balkom et al., 2013
miR-423-5p	HUVECs	Increased EC tube formation, proliferation and migration post-exosome treatment *in-vitro.* EV microRNA expression profiling by small RNA sequencing and qPCR miRNA array revealed increased levels of miR-423-5p in these exosomes. Overexpression of miR-423-5p in HUVECs increased their tube formation ability, proliferation and migration.	hADSCs	Differential centrifugation Ultracentrifugation	Xu et al., 2019
miR-939-5p	MCECs HUVECs C57BL/6 mice- MI by LAD coronary artery ligation & HLI by left femoral artery ligation	Increased EC tube formation, proliferation and migration post-ischaemic exosome treatment *in-vitro.* Increased capillary density and reduced scar size post-ischaemic exosome injection. EV microRNA expression profiling by qPCR miRNA array revealed decreased miR-939-5p expression in ischaemic exosomes. HUVEC transfection with miR-939-5p mimic decreased EC tube formation and migration *in-vitro*, whereas transfection with miRNA inhibitor increased tube formation and migration.	Coronary blood from patients with myocardial ischaemia and control group	Differential centrifugation Ultracentrifugation	Li et al., 2018
miR-1246	HUVECs	Incubation of HUVECs with DLD-1 cancer cell derived EVs significantly increased tube formation and migration. Mir-1246 was enriched in DLD-1-EVs. Overexpression of miR-1246 in HUVECs significantly increased their tube formation ability and migration.	DLD-1 cancer cells	Ultracentrifugation	Yamada et al., 2014
miR-4306	HCAECs	Decreased EC tube formation, migration and proliferation post-treatment with EVs isolated from the PCI group compared with those from the control group. Human monocyte-derived macrophage (HMDM) treatment with ox-LDL significantly increased their miR-4306 levels. Transfection of HCAECs with miR-4306 mimic significantly inhibited their tube formation ability and slightly suppressed HCAEC proliferation and migration.	HMDM from patients who had PCI performed within 12 hours (PCI group) and patients with chest pain syndrome with normal coronary artery findings post coronary angiography	Differential centrifugation Ultracentrifugation	Yang et al., 2019
let-7b-5p	HUVECs CD1 male mice-HLI induction by left femoral artery ligation	Increased EC tube formation, proliferation and reduced EC apoptosis post-exosome treatment *in-vitro.* EV microRNA expression profiling was performed by qPCR miRNA array and revealed increased levels of let-7b-5p. Exosomes restored the angiogenic capacity of DICER knock-out-ECs, but the reduction of exosomal let-7b-5p in exosomes failed to induce tube formation in recipient DICER-KD ECs *in-vitro.* Increased capillary density and blood flow recovery and reduced necrosis post-exosome injection *in-vivo*.	Human pericardial fluid (PF) exosomes	ExoQuick kit (System Biosciences)	Beltrami et al., 2017

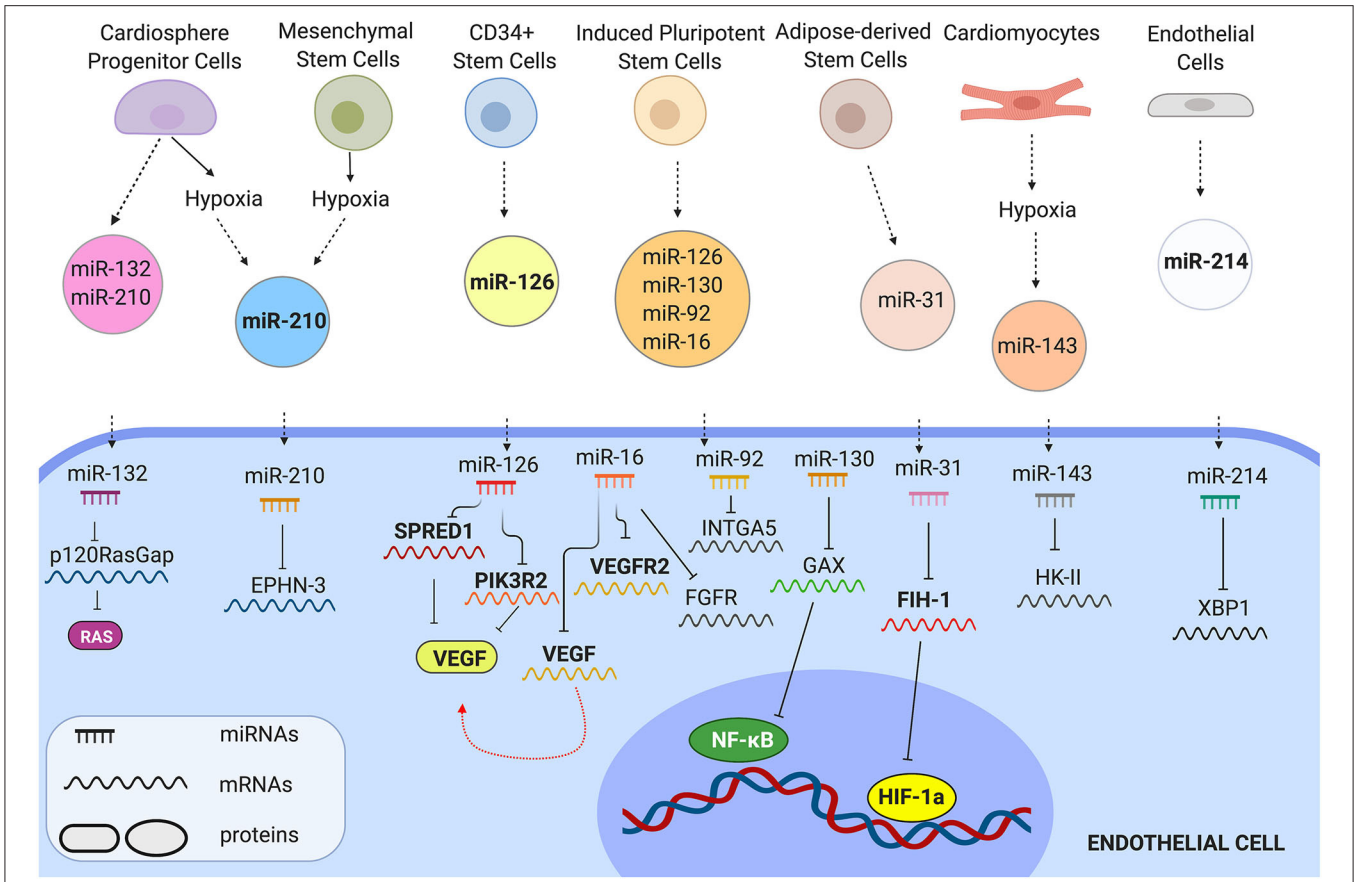

FIGURE 2 | Representative examples of EV miRNAs that control EC behaviour. EVs from different cells carry angiogenic miRNAs. Once transferred in ECs, miRNAs control molecular pathways by inhibiting their mRNA-targets.

expression profiling revealed several upregulated miRNAs under hypoxic conditions, including miR-15b, miR-17, miR-20a, miR-103, miR-199a, miR-210, miR-292.

Qiao et al. (2019), compared the effect of exosomes isolated from explant-derived cardiac stromal cells from patients with normal angiography results (NEXO) to exosomes from patients with heart failure (FEXO). They showed that intramyocardial injection of exosomes from the NEXO group in a mouse MI model significantly increased capillary density and decreased the infarct size, while injection of exosomes from the FEXO group exacerbated cardiac function and left ventricular remodelling. Exosomes from the FEXO group exhibited reduced ability to promote HUVEC tube formation *in-vitro*. EV microRNA expression profiling revealed decreased miR-21-5p expression in the FEXO compared to the NEXO group exosomes. As a result, silencing of miR-21-5p in NEXO group decreased HUVEC tube-formation, while upregulation of miR-21-5p in FEXO group restored HUVEC tube formation. Li et al. (2018) showed that EC treatment with exosomes from coronary blood of patients with myocardial ischaemia significantly increased EC tube formation, proliferation and migration *in-vitro*. Intramyocardial injection of exosomes in a mouse MI model significantly increased capillary density and reduced scar size. EV microRNA expression revealed decreased miR-939-5p expression in exosomes from coronary blood of patients with myocardial ischaemia compared to exosomes from healthy controls. Transfection of ECs with miR-939-5p mimic decreased EC tube formation and migration *in-vitro*, whereas transfection with miRNA inhibitor increased tube formation and migration.

Beltrami et al. (2017), showed that human pericardial fluid exosomes increased EC tube formation, proliferation and reduced EC apoptosis. EV microRNA expression profiling revealed that let-7b-5p was increased in these exosomes. Moreover, human pericardial fluid exosomes restored the angiogenic capacity of DICER knock-out-ECs *in-vitro*, but the reduction of exosomal let-7b-5p in exosomes failed to induce tube formation in the recipient DICER-KD ECs. Exosome injection in a mouse HLI model significantly increased capillary density and blood flow recovery and reduced necrosis. EVs from CD34+ stem cells also appear to be regulators of angiogenesis. In particular, Mathiyalagan et al. (2017), demonstrated that $CD34^{+}$ cell-derived exosomes are enriched in pro-angiogenic miRNAs, such as miR-126-3p. As a result, injection of these exosomes in an HLI mouse model increased miR-126-3p levels without affecting the endogenous synthesis of this miRNA, implying a direct transfer of exosomal miR-126-3p to the

ischaemic limb. Moreover, it was suggested that miR-126-3p enhanced angiogenesis by suppressing its known target, SPRED1. Ranghino et al. (2012), reported that the angiogenic miRNAs miR-126 and miR-296 were upregulated in EPC-derived EVs. Injection of EPC-derived EVs in a mouse HLI model significantly increased capillary density and blood perfusion to the ischaemic muscle. Recently, Johnson et al. (2019), showed that exosomes from induced vascular progenitor cell (iVPC) could significantly increase EC tube formation, proliferation and migration *in-vitro* and EC sprouting of mouse aortic rings. Exosome injection in a rat HLI model significantly increased capillary density and blood perfusion compared with rat aortic endothelial cell (RAEC) exosomes and controls. EV microRNA expression profiling revealed increased levels of several miRNAs in these exosomes, including miR-20b, miR−27b, miR-29b, miR-42a, miR-100, miR−125b, miR-143, miR-195, miR-291b, miR-497. Taken together, several studies have reported a role of EV-bound miRNAs in post-ischaemic neovascularisation. Nonetheless, a better understanding of their mechanism of action in the recipient cells is essential before clinical application.

CHALLENGES AND FUTURE PERSPECTIVES

The delivery of EVs, carrying therapeutic miRNA molecules, to the heart remains promising for the regulation of therapeutic neovascularisation post-MI, but there are several challenges to the field. Our knowledge of endogenous cell communication via EVs, for instance, both under physiological and pathological conditions is limited. Therefore, *in-vivo* visualisation of EV spatial and temporal release from the vascular wall, as well as of EV uptake by vascular ECs is of utmost importance for a better understanding of the biological function of EVs. To develop EV-based therapeutics, a more in-depth evaluation of EV pharmacokinetics *in-vivo* is also crucial. A critical factor that affects EV pharmacokinetics is the route of administration. Gallet et al. (2017), showed that CDC-derived exosomes resulted in improved vessel density and cardiac function post intramyocardial injection, but were ineffective after intracoronary injection. Therefore, preclinical models that allow for the study of endogenous EVs, as well as the biodistribution of exogenously administrated EVs, must be developed for a better understanding of their role in cardiac neovascularisation.

Moreover, clinical trials with EV products will only be possible with the improvement of isolation and purification techniques. The development of highly reproducible methods of isolating GMP-quality EVs, however, remains a great challenge in the field. Due to the lack of standardised cell culture and isolation methods, numerous studies, use relatively impure populations of EVs, which may affect angiogenesis assays, leading to wrong assumptions for their functionality. Lipoproteins are the most common contaminants from serum-containing medium (Yuana et al., 2014; Sódar et al., 2016). In this reason, the choice between culture medium containing EV-depleted serum and culture medium without serum needs to be considered. Serum starvation, however, has been reported to cause significant stress to the cultured cells, which subsequently leads to altered EV secretion (Witwer et al., 2013). Although this issue can be avoided by using EV-depleted serum, there are still several limitations associated with serum EV depletion strategies. Rigorous EV depletion of FBS using long ultracentrifugation protocols, for instance, is time-consuming and cannot remove small serum RNAs which can be mis-annotated as human RNAs (Wei et al., 2016). More recently introduced ultrafiltration-based strategies can overcome these limitations and result in solutions of extremely low EV and small RNA content (Kornilov et al., 2018). Another major dilemma in the EV field is the lack of a consensus methodology for the isolation of pure and intact EVs. Differential centrifugation is the most commonly used method for EV purification. Further purification from co-pelleted protein complexes and lipoproteins can be achieved by a density gradient. Nonetheless, this approach is associated with increased EV aggregation and usually results in fusion or disruption of the isolated EVs. Moreover, the optimal parameters of ultracentrifugation highly dependent on the type of centrifuge rotor used. Thus, it is essential that alternative approaches be used. Interestingly, a combination of ultrafiltration with size-exclusion chromatography has been proposed as a novel EV isolation approach that yields particles of improved purity and quality (Nordin et al., 2015).

Using standardised cell culture and EV isolation methods is extremely important in angiogenesis studies. Depending on the conditions that EVs have been generated and isolated, as well as the EV subtype and the dose used, they may exhibit a powerful proangiogenic or inhibitory effect. Moreover, since cell culture and EV isolation procedures affect the EV cargo, a more in-depth characterisation of the way that therapeutic molecules, such as miRNAs are sorted and released by EVs is essential before clinical application. EV-driven miRNA transfer post-MI is a new promising strategy for the promotion of neovascularisation. Numerous miRNAs including miR-126, miR-92a, miR-210, miR-27b, and miR-24 have been recognised as key regulators of post-ischaemic neovascularisation, but only a limited number have been identified in EVs. KRAS-MEK signalling has been proposed as an important regulator of miRNA sorting in EVs (McKenzie et al., 2016). However, the exact molecular mechanisms that drive miRNA transfer into EVs are not fully understood. Recently, a CRISPR-Cas-9 based system for single-cell detection of EV-mediated functional transfer of RNA was put forward (de Jong et al., 2020). This approach, termed CRISPR Operated Stoplight System for Functional Intercellular RNA Exchange (CROSS-FIRE), is based on activation of a fluorescent protein in recipient reporter cells upon functional delivery of specific sgRNAs, expressed in EV-donor cells. In general, there is a consensus that small RNA composition of cells differs from that in EVs, and emerging evidence supports that the presence of certain motifs on miRNAs may facilitate their transfer into EVs (Villarroya-Beltri et al., 2013; Koppers-Lalic et al., 2014).

Whether miRNA-Ago2 or miRNA-RISC complexes are present in EVs is of great interest to the EV field. Bound to Ago2, EV-miRNAs could efficiently assemble into functional RISCs for downregulation of their mRNA-target in the recipient cells. Interestingly, the entire RISC was reported to be present in EVs secreted from cancer cells, leading to efficient and rapid silencing of mRNA-targets at the recipient cells (Melo et al.,

2014). An argument against the presence of Ago2 in EVs is that serum, a typical cell culture additive, contains a significant amount of non-vesicular Ago2 which may contaminate crude EV pellets prepared by standard EV isolation techniques, including ultracentrifugation of the conditioned medium at 100,000 × *g* (Huang et al., 2013). Size exclusion chromatography or density gradient can be used to separate vesicular from non-vesicular components of cell culture media (Prieto-Fernández et al., 2019). Jeppesen et al. (2019) recently used high-resolution density gradients to separate a crude small EV pellet into non-vesicular and vesicular fractions. Western blot analysis revealed that argonauts, including Ago2, were present in both fractions in media from Gli36 glioblastoma cells, but only in the non-vesicular fraction of media from DKO1 or MDA-MB-231 cells. A possible explanation of these findings could be that both DKO1 and MDA-MB-231 cells carry KRAS mutations. In contrast to this, several pathogens appear to promote Ago2 trafficking in EVs (Bukong et al., 2014; Mantel et al., 2016). Therefore, the ability to detect Ago2 in EVs may be a result of multiple factors, including the cell of origin, experimental conditions and detection methods. Tracing miRNA transfer from the recipient to the donor cells via EVs would significantly increase our knowledge on the way that EV-bound miRNAs affect EC behaviour and therefore, promote neovascularisation post-cardiac ischaemia. Although, several approaches have been proposed (reviewed in Mateescu et al., 2017), the detection and tracing of specific miRNAs in EVs remain challenging due to several issues, including limited probe specificity, limited signal per EV and poor signal-to-background ratios.

Currently, interest has been shifted toward engineering EV surface proteins and cargo for improved targeting (e.g., by the inclusion of peptides) and functionality. In this way, synthetic EVs may be loaded with known angiogenic miRNAs and target ECs to promote neovascularisation. One of the most common strategies used includes the loading of EV-producing cells with exogenous miRNAs and the transfection with plasmids expressing peptides of interest. Thus, EVs derived from these cells may carry therapeutic miRNAs and express targeting peptides on their surface. Alternatively, exogenous cargo can be directly loaded into EVs by several methods, including electroporation, heat-shock or freeze-thaw procedures, detergent treatment or sonication (de Abreu et al., 2020). However, further investigation is needed to define the stoichiometry required for therapeutic effects observed post-EV-miRNA transfer.

Although the focus of this review is on miRNAs, to date, several types of non-coding RNAs have emerged as key regulators of angiogenesis. Long non-coding RNAs (lncRNAs) for instance, may also control EC proliferation (e.g., MALAT1, H19, GAS5) or angiogenesis (MEG3, MANTIS) (Kok and Baker, 2019; Simion et al., 2019). Increasing evidence suggests that the interactions of lncRNAs with miRNAs play a critical role in the regulation of angiogenesis (Zhao et al., 2020). Moreover, lncRNAs may be selectively packaged into EVs and transferred between cells, improving cardiac function and vascularisation and reducing fibrosis post-MI (Wu et al., 2020). Circular RNAs (circRNAs) have also emerged as novel regulators of EC function and angiogenesis (e.g., CANRIL, CZNF292, Circ_0010729,Circ_0003575, Circ_0054633, Circ_0000109) (Zhang and Huang, 2020). CircRNAs can also be transferred to ECs through EVs. In the context of post-ischaemic neovascularisation, Dou et al. (2020), showed that following femoral artery ligation in VSMC specific SIRT1 transgenic (SIRT1-Tg) mice, blood flow and capillary density is significantly decreased due to the delivery of exosomal cZFP609 from VSMCs to ECs. Interestingly, the authors reported that cZFP609 may inhibit angiogenesis via blockade of HIF-1a nuclear translocation and hypoxia-induced VEGF-A expression in ECs. Integrating knowledge of miRNAs with other non-coding RNAs and the way that they are transferred between cells will be critical to our understanding of the biological orchestration that controls neovascularisation in health and disease.

CONCLUSION

An increasing number of studies have documented the capacity of EV-bound miRNAs to modulate the angiogenic programs of ECs and to control neovascularisation following MI. EV cargo depends on several factors, including the cell source, the conditions in which EVs have been generated, and the isolation and purification techniques employed. These factors, along with the dose used, may determine the beneficial or detrimental effects of EVs on angiogenesis. EV-based therapeutic approaches, however, are still challenging since our knowledge of the mechanisms of miRNA sorting in EVs, EV release, EV-miRNA uptake and the stoichiometry required for the therapeutic effects of EV-miRNA treatment is limited. A broader understanding of these processes, together with the validation of accurate technologies for the clinical-grade quality control of EVs, would significantly delineate the benefit-risk balance, and open up new opportunities for therapeutic strategies.

AUTHOR CONTRIBUTIONS

DK, AB, MB, and AHB outlined concept and overview of review. DK wrote the manuscript. AB, AHB, MB, PdC, and LdW reviewed and edited the manuscript. DK designed and prepared the figures. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Circular RNAs in Cardiac Regeneration: Cardiac Cell Proliferation, Differentiation, Survival, and Reprogramming

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Circular RNAs (circRNAs) are classified as long non-coding RNAs (lncRNAs) that are characterized by a covalent closed-loop structure. This closed-loop shape is the result of a backsplicing event in which the 3' and 5' splice sites are ligated. Through the lack of 3' poly(A) tails and 5' cap structures, circRNAs are more stable than linear RNAs because these adjustments make the circular loop less susceptible to exonucleases. The majority of identified circRNAs possess cell- and tissue-specific expression patterns. In addition, high-throughput RNA-sequencing combined with novel bioinformatics algorithms revealed that circRNA sequences are often conserved across different species suggesting a positive evolutionary pressure. Implicated as regulators of protein turnover, micro RNA (miRNA) sponges, or broad effectors in cell differentiation, proliferation, and senescence, research of circRNA has increased in recent years. Particularly in cardiovascular research, circRNA-related discoveries have opened the door for the development of potential diagnostic and therapeutic tools. Increasing evidence links deviating circRNA expression patterns to various cardiovascular diseases including ischemic heart failure. In this mini-review, we summarize the current state of knowledge on circRNAs in cardiac regeneration with a focus on cardiac cell proliferation, differentiation, cardiomyocyte survival, and cardiac reprogramming.

Keywords: circular RNAs, cardiac regeneration, cardiac cell proliferation , cardiac reprogramming, ischemic heart failure, cardiovascular disease

INTRODUCTION

Approximately 98% of the human genome is comprised of non-coding RNA (ncRNA) transcripts (Bär et al., 2020). Nevertheless, protein-coding genes remain the most well-studied sequences in the mammalian genome (Esteller, 2011). However, it became apparent that ncRNAs are crucially involved in a wide array of physiological and pathophysiological processes (Brennecke et al., 2003; Xu et al., 2003; Abbaszadeh-Goudarzi et al., 2020; Hashemian et al., 2020; Yousefi et al., 2020). One recently re-discovered class of ncRNAs is circRNAs.

The first description of circRNA coding organisms stems from 1976, when Sanger et al. examined viroids, circular single-stranded RNA pathogens of higher plants (Sanger et al., 1976). In eukaryotes, circRNAs were first detected in immortalized cervical cancer HeLa cells in 1979 using electron microscopy (Hsu and Coca-Prados, 1979). Until the 1990s, circRNAs were widely considered to be little more than splicing by-products or splicing errors (Capel et al., 1993; Cocquerelle et al., 1993). Since 2012, advances in high-throughput RNA-sequencing technology have identified circRNAs to be widespread, abundant and conserved across different species (Salzman et al., 2012, 2013; Jeck et al., 2013; AbouHaidar et al., 2014). Several circRNAs have shown a high degree of orthology between species as different as mice, pigs, and humans (Jeck et al., 2013).

CircRNAs were previously assumed to be present in low intracellular concentrations compared to conventional splicing products. However, recent analyses detected circRNAs in several human cell types and even indicated that they constitute a significant portion of the total spliced genetic product, varying between different cell types (Salzman et al., 2012). For some genes, the circular variant was also found to be more highly transcribed than the linear version (Salzman et al., 2012; Lasda and Parker, 2016).

In this mini-review, we provide a short overview on circRNA biogenesis and their mechanism of action in general. Further, we will summarize the newest findings regarding their role in cardiovascular diseases (CVD), in cardiomyocyte proliferation, differentiation, survival, and cardiac reprogramming. In conclusion, we outline current approaches to give insights into circRNAs in diagnostic and therapeutic settings, while considering state of the art methods, their limitations, and future challenges.

TO COME FULL CIRCLE: BIOGENESIS OF CircRNAs

Canonical splicing is the default mode of splicing for linear pre-mRNA transcripts. In canonical splicing, an upstream (5') splice donor site is joined with a downstream (3') splice acceptor site (Ng et al., 2004). CircRNAs, however, are formed through non-canoncial splicing, where a downstream donor site is ligated with an upstream splice acceptor site (Braun et al., 1996). This type of splicing leads to the formation of a backsplice junction in circRNAs which therefore lack a 5' to 3' directionality. The resulting products are a covalently closed circRNA and an additional linear transcript with skipped exons that is subject to fast degradation (Chen and Yang, 2015).

CircRNAs have longer half-lives than linear RNAs because their closed-loop structure is resistant to exonucleases, which typically require a 3' or 5' end to initiate degradation (Jeck et al., 2013; Lasda and Parker, 2014; Enuka et al., 2016). The stability and the low degradation rates may imply that accumulation, rather than high production rates are accountable for the measured circRNA levels. The first sets of data regarding the expression patterns of circRNAs are becoming available. Although there is a general tendency for them to be expressed on a level similar to their linear counterpart, there are several exceptions, including the highly expressed circRNAs correlated to Titin (TTN) and ryanodine receptor 2 (RYR2; Tan et al., 2017). Further differences between circRNAs and linear RNAs are shown in **Table 1**.

MECHANISM OF ACTION

The length of circRNAs ranges from a few hundred to thousands of nucleotides (Chen, 2016) and their functions are versatile. Most studies on circRNAs focus on their ability to act as miRNA sponges, whereby they inhibit miRNA-mRNA binding (Hansen et al., 2013; Memczak et al., 2013). Further, study by Li et al. (2015) revealed that exon-intron circRNAs (EIcircRNAs) regulate gene expression in the nucleus by increasing the expression of their parental genes *via cis*-mediated mechanisms. In addition, intron-containing circRNAs (ciRNAs) have been shown to act as regulators of RNA polymerase II in cells by associating with the machinery responsible for the Polymerase II elongation and positively regulating its transcription (Zhang et al., 2013; Holdt et al., 2018). Even though they are generally classified as ncRNAs, studies have identifies a subset of circRNAs, which can be translated in a cap-independent manner (Legnini et al., 2017; Pamudurti et al., 2017). However, the exact mechanism of circRNA translation is yet to be fully elucidated. The majority of identified circRNAs are stably expressed, with cell- and tissue-specific expression patterns (Salzman et al., 2013; Ji et al., 2019). Because of the ability of cells to distinguish between endogenous and exogenous circRNAs based on the intronic sequence that initiates the circularization of the RNA during splicing (Chen et al., 2017), it follows that the recognition and degradation of invasive circRNAs is a regulated response of the immune system, with implications for autoimmune diseases (Chen et al., 2017; Zhong et al., 2019). Further pathologic conditions, in which their regulatory mechanisms have been thoroughly investigated include cancer (Shabaninejad et al., 2019; Naeli et al., 2020), neurologic disorders (Memczak et al., 2013), diabetes (Abbaszadeh-Goudarzi et al., 2020), and CVD (Wang et al., 2016; Werfel et al., 2016).

TABLE 1 | Main differences between circular RNAs (circRNAs) and linear RNAs.

	CircRNA	Linear RNA	References
Deep sequencing required	Yes	Yes	Salzman et al., 2012; Cooper et al., 2018; Das et al., 2019
Stability	Yes	No	Jeck et al., 2013; Lasda and Parker, 2014; Enuka et al., 2016
Exonuclease resistant*	Yes	No	Suzuki et al., 2006; Vincent and Deutscher, 2006; Salzman et al., 2012
Backsplice junction	Yes	No	Das et al., 2019
5' cap structure	No	Yes	Holdt et al., 2018
3' poly(A)-tails	No	Yes	Holdt et al., 2018
Length in basepairs (bp)	>200	21 to >200	Ding et al., 2018

**Some circRNAs are not exonuclease resistant (Szabo and Salzman, 2016; Legnini et al., 2017).*

CircRNAs IN CVD

In recent years, the link between circRNAs and CVD has been studied intensively and indicates their involvement in the CVD pathogenesis (Viereck and Thum, 2017; Gurha, 2019; Huang et al., 2020). Since many circRNAs are highly conserved across species, animal models can be used to infer the role of circRNAs in human CVD (Werfel et al., 2016; Tan et al., 2017).

Two of the most well-known circRNAs with key functions in the heart, namely heart-related circRNA (HRCR) and CDR1as act as miRNA sponges (Huang et al., 2020). The conveyed effect depends on the "sponged" miRNA: while HRCR has an attenuating effect on hypertrophy by sponging miR-223 (Wang et al., 2016), CDR1as seems to amplify post-myocardial infarction (MI) ischemic damage in mice through sponging miR-7 (Geng et al., 2016). Interestingly, in a porcine model of MI increased expression of CDR1as was associated with reduced infarct size and increased left ventricular (LV) and right ventricular (RV) function (Mester-Tonczar et al., 2020). This might indicate interspecies differences in the role of CDR1as in CVD.

CircRNAs have been detected in peripheral fluids such as whole blood and plasma, where their increased chemical stability may be an advantage for their use as clinical biomarkers of diseases (Gurha, 2019). A study from 2019 established a circRNA-miRNA-mRNA network and identified circYOD-1 as a circulating biomarker for coronary artery disease (Miao et al., 2019). A study by Wu et al. (2019) identified three differentialy regulated circRNAs in pediatric patients with congenital heart disease: hsa_circRNA_004183, hsa_circRNA_079265 and hsa_circRNA_105039. Another study reported several circRNAs linked to hypertrophic cardiomyopathy (HCM) in humans. Three of the investigated circRNAs, DNAJC6, TMEM56, and MBOAT2 were found in serum and can help distinguish healthy from HCM patients. Furthermore, DNAJC6 and TMEM56 could serve as indicators for disease severity in patients with obstructive HCM (Sonnenschein et al., 2019). Identifying differentially regulated circRNAs through newly available sequencing technologies is the first step in planning further research with circRNAs.

CircRNAs IN CARDIAC REGENERATION: CARDIOMYOCYTE PROLIFERATION, DIFFERENTIATION, AND SURVIVAL

Despite essential advances in cardiac regenerative medicine, regeneration following myocardial ischemia still imposes many obstacles. Until recently, stem cell treatments, gene therapy, cell-based gene therapy, or the use of paracrine factors as regenerative cocktails of sorts represented the main focus of regenerative cardiology, yet the efficacy of such treatments failed to live up to expectations (Gyöngyösi et al., 2018). Regardless of the underlying etiology and the multitude of approaches for revascularizing or repairing ischemic tissue, reducing further damage outside the infarcted zone, and protecting the remaining healthy myocardium, the lack of clinically meaningful regenerative potential of mature cardiomyocytes and their replacement with non-functional scar results in an irreversible loss of functional tissue. Thus, new research directions focused on circRNAs in cardiac cell proliferation, differentiation, and cardiac survival should be examined.

The following part of the review focuses on current studies involving circRNAs within the context of the growing field of research on cardiac reprogramming. The natural starting point for any such investigations is the exploration of circRNA regulation in the growing and developing heart. Further investigations have focused on the differential regulation of circRNA transcripts in mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs) during their experimentally induced differentiation into cardiomyocytes. Lastly, we discuss the literature on circRNA expression following MI.

CircRNA Expression Patterns During Cardiac Development

In 2017, Li et al. examined the stage specific expression of lncRNAs and circRNAs in the undifferentiated mesoderm, cardiac progenitor, and definitive cardiomyocyte stages of cardiomyocyte development. The analysis uncovered important circRNA regulators, including circ-TTN, and a co-expression pattern of the circRNAs with genes such as MYL4, whose mutation has been linked to cardiac structural and electrical abnormalities (Li et al., 2017).

A 2017 study by Xu et al. measured the circRNA expression patterns in six human tissues, including the heart, and compared them to the expression patterns of the corresponding fetal tissue. As a general rule, they found circRNAs to be more highly expressed in the fetal stage of development. CircSLC8A1 was found to have a heart-specific upregulation pattern and might be a viable target for further research (Xu et al., 2017).

CircRNAs in MSCs

MSCs not only have the ability to differentiate into cardiomyocytes, but can also recruit resident cardiac stem cells and secrete a variety of factors that could be useful in terms of cardiac regeneration (Williams and Hare, 2011).

A total of 226 differentially regulated circRNAs were discovered regarding differentiation of umbilical cord-derived human MSCs (huMSCs) into cardiomyocytes, with the most highly differentially regulated circRNAs related to differentiation and proliferation pathways, including the Wnt pathway (Ruan et al., 2019).

Another circRNA involved in the transcriptional preservation of stem cell identity is circFOXP1, whose silencing reduces huMSC growth and proliferation. MiR-17-3p and miR-127-5p are among the miRNAs sponged by circFOXP1. MiR-17-3p and miR-127-3p have a role in epidermal growth factor receptor (EGFR) and canonical Wnt signaling, an axis that allows them to interact in growth and survival pathways, as well as influencing the amount of differentiated MSCs (Cherubini et al., 2019).

CircRNA CDR1as has also been found to be abundant in huMSCs. The increase of huMSC proliferation, secretion, and differentiation induced by 3,3'-diindolylmethane also increased

the abundance of CDR1as, whereas the knockdown of CDR1as reduces their proliferation and differentiation capacity, which are essential for their regenerative potential (Yang et al., 2019).

CircRNAs in iPSCs

Early attempts at inducing cardiac regeneration focused on the stimulation of stem cell and cardiac progenitor cell populations naturally residing in the heart (Cai and Molkentin, 2017; Tzahor and Poss, 2017). However, this approach is limited by the small number of these c-kit+ and Sca-1+ cells in the adult myocardium and their replicative and functional limitations in replacing cardiomyocytes and achieving significant physiological effects (van Berlo and Molkentin, 2014; Cai and Molkentin, 2017).

Other approaches focused on the application of autologous or allogeneic somatic or embryonic stem cells. However, immunogenicity, engraftment, differentiation, tumogenicity, and ethical concerns were common issues with these methods.

A new era in the field of regenerative research, particularly in cardiac regeneration, was ushered in by Takahashi (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). His first study revealed that differentiated somatic mouse cells can be reprogramed through the use of transcription factors to revert back to a pluripotent state (Takahashi and Yamanaka, 2006). The second study managed to replicate the same results in humans, inducing pluripotency in human dermal fibroblasts (Takahashi et al., 2007). These iPSCs became the focus of a multitude of studies and remain a very active field of research to this day. In a human induced pluripotent stem cell derived cardiomyocyte model, researchers investigated the differential regulation of circRNAs in cardiac development and identified 384 circRNAs that are specific to the setting of developing cardiomyocytes. Furthermore, multiple circRNAs were identified, which are relatively abundant or depleted in relation to the respective linear transcript over the course of cardiomyocyte differentiation, exemplified by circCDYL and circ-SMARCA5 (Siede et al., 2017).

A breakthrough study by Ieda et al. (2010) demonstrated that transformation of fibroblasts into functioning cardiomyocytes was successful, leading to the concept that differentiated somatic cells can directly be programed into another cell type without transitioning through the pluripotent stage.

CircRNAs Following MI: A Story of Angiogenesis, Pluripotency, Proliferation, and Altered Outcomes

It is often difficult to speculate whether a single circRNA can convey a meaningful regeneration following a MI, since many circRNAs act on multiple pathways.

In 2019, Huang et al. identified super-enhancer associated circRNAs that could be relevant in regenerative pathways and further examined the role of circRNA Nfix in the regenerative capacity of the myocardium following a MI. On the one hand, circNfix knockdown not only induced increased cardiomyocyte proliferation in a murine model, but also resulted in improved angiogenesis, reduced infarcted and fibrotic area and improved cardiac functional parameters following an infarction. An induced overexpression of circNfix on the other hand decreased the ability of cardiomyocytes to proliferate *in vivo*. All of these potentially highly therapeutically relevant effects were found to be conveyed through promoting Ybx degradation and sponging miR-214 (Huang et al., 2019).

CircCDYL was the focus of a 2020 study on cardiac regeneration conducted by Zhang et al. This research group investigated circCDYL in adult mouse cardiomyocytes and in murine myocardium, and showed that overexpression of circCDYL *in vitro* promotes cardiomyocyte proliferation, whereas downregulation of circCDYL inhibits their proliferation. *In vivo* overexpression of circCDYL led to increase in ejection fraction (EF) in mice, whereas downregulation of circCDYL lowered the EF (Zhang et al., 2020b).

CircRNAs are also involved in post-infarction fibrotic remodeling that results in heart failure following MI in the long run. Zhu et al. (2019) investigated the role of circNFIB in cardiac fibrosis in a mouse model of MI and reported that circNFIB inhibition promotes adult fibroblast proliferation. Additionally, the sponge activity of circNFIB on miR-433 was also identified.

CircRNAs are often the topic of multidisciplinary research. CircHIPK3 is an example of a circRNA involved in cellular growth that has simultaneously been implicated in the growth, proliferation, and metastasis of cancer (Zheng et al., 2016; Zeng et al., 2018). CircHIPK3 is also relatively abundant in the fetal and neonatal myocardium of mice (Si et al., 2020, p. 3). This motivated an investigation into its potential regeneration-inducing capacity in the myocardium *in vitro* and *in vivo*. CircHIPK3 is perhaps the perfect example to illustrate the sheer multitude of effects that can be exhibited by a single circRNA. Si et al. (2020) demonstrated that the overexpression adenoviral vector-associated circHIPK3 induces coronary artery endothelial proliferation, increases cardiomyocyte proliferation, promotes angiogenesis, and decreases fibrosis in the zone surrounding the infarction area in adult mice. Whereas the pro-angiogenic properties seem to be conveyed through a miRNA-133a sponging effect, the effects on the cardiomyocytes were found to be achieved through an increase in the stability of the Notch1 intracellular domain. **Table 2** summarizes circRNAs involved in cardiomyocyte proliferation, differentiation, and cardiac reprogramming.

AREAS OF APPLICATION

As CircRNAs are involved in cell differentiation and proliferation as well as regulating transcription (Bose and Ain, 2018) and translation (Pamudurti et al., 2017), their potential applications are plentiful. CircRNAs are currently the focus of a number of biomarker studies (Ouyang et al., 2017; Zhao et al., 2017; Li et al., 2018), due to their chemical stability compared to other RNA molecules (Jeck et al., 2013). Wesselhoeft et al. (2018) demonstrated the use of circRNAs for robust and stable translation in eukaryotic cells. This innovation offers great possibilities for obtaining stable protein production for biotechnological use. Their development can be used as an alternative to mRNA translation as mRNAs display relatively short half-lives compared to circRNAs. Another area of application is the construction of

TABLE 2 | CircRNAs and their role in cardiac cell proliferation, differentiation, and cardiac reprogramming.

CircRNA	Regulation	Mechanism	Consequence	Type of sample (number of samples)	References
HRCR	Overexpression	miR-223 sponge	Repression of cardiac hypertrophy and HF in mice	Mice hearts (*n* = 4–8/group) Adult human hearts (*n* = 7–8/group)	Wang et al., 2016
CDR1as	Increased expression	Currently unknown	Reduced infarct size and positive influence on LV and RV function	Pig hearts (*n* = 5/group)	Mester-Tonczar et al., 2020
CDR1as	Overexpression	miR-7 sponge	Increased infarct size in a mouse model	Mice hearts (*n* = 10/group)	Geng et al., 2016
CDR1as	Knockdown	Currently unknown	Depleted proliferation and differentitation capacity of huMSCs and induced cell apoptosis	huMSCs	Yang et al., 2019
circFOXP1	Silencing circFOXP1	miR-17-3p sponge and miR-127-5p sponge resulting in the modulation of non-canonical Wnt and EGFR pathways	Reduction of huMSC growth and proliferation	huMSCs	Cherubini et al., 2019
circNfix	Downregulation	Promotes Ybx1 ubiquitin-dependent degradation and miR-214 sponge	Cardiomyocyte proliferation and inhibition of cardiomyocyte apoptosis after MI in mice	Mice hearts (*n* = 2–10/group) Adult human hearts (*n* = 2)	Huang et al., 2019
circCDYL	Overexpression	Currently unknown	Improved heart function after AMI	Myocardial tissue of mice (*n* = 6) Hypoxic cardiomyocytes	Zhang et al., 2020b
circNFIB	Overexpression	miR-433 sponge	Attenuation of cardiac fibroblast proliferation	Mice hearts (*n* = 5–6/group) TGF-beta treated cardiac mice fibroblasts	Zhu et al., 2019
circHIPK3	Overexpression	Increased N1ICD acetylation and miR-133a sponge	Induction of coronary artery endothelial cell proliferation, promoted cardiomyocyte proliferation and angiogenesis, and decreased fibrosis after MI	Mouse model of MI (*n* = 6)	Si et al., 2020

huMSCs, human mesenchymal stem cells; HF, heart failure; LV function, left ventricular function; MI, myocardial infarction; N1ICD, Notch1 intracellular domain; RV function, right ventricular function. The number of samples varies in these studies, because the research groups used varying numbers of animals for each of their experiments.

synthetic miRNA sponges. Liu et al. (2018) demonstrated a successful designing of a synthetic sponge constructed out of a circularized product targeting miR-21. As a consequence, gastric carcinoma cell proliferation was inhibited. In the past 3 years, other studies also reported the engineering of cost-effective artificial miRNA sponges (Rossbach, 2019; Wang et al., 2019). These examples illustrate the potential of circRNA for developing biomarkers, tools for circRNAs translation, and therapeutic potential for regulating cell proliferation.

CHALLENGES IN CircRNA RESEARCH

Since most circRNAs show low expression patterns (Zhang et al., 2020a), their detectability requires state of the art methods. For the detection of novel circRNAs deep-sequencing paired with bioinformatics algorithms specialized on detecting backsplice junctions is required. Several algorithms exist, each with its own advantages and disadvantages. Some algorithms like KNIFE, CIRCexplorer, and CIRI achieved the best sensitivity, whereas others like UROBORUS can detect low expression levels of circRNAs in rRNA depleted samples without RNase R digestion (Chen et al., 2020). No poly(A) enrichment should be performed before RNA-sequencing and a read length of at least 100 basepairs is recommended to accurately align the reads with the backsplice junction (Kristensen et al., 2019). Some laboratories propose performing an RNase R digestion for circRNAs, however, not all circRNAs are resistant to RNase R (Szabo and Salzman, 2016; Legnini et al., 2017). Instead of using RNase R in circRNA research, one could use divergent primers spanning the backsplice junction and confirm the circular transcript with Sanger sequencing. Similarly, RNA-sequencing data can be validated through Sanger sequencing as well.

CONCLUSION

In this review, we summarized some of the recent findings on the topic of circRNAs and their role in cardiomyocyte proliferation, differentiation, survival, and cardiovascular regeneration. Given the fact that circRNAs were only recently re-discovered compared to other ncRNAs, the full potential of circRNAs has yet to be elaborated.

AUTHOR CONTRIBUTIONS

JM-T wrote the manuscript and put the tables together. MG, EH, and AS helped in literature search and assisted with writing the manuscript. DT, NK, KZ, PE, NP, and GG edited the manuscript and the tables and helped to structure and revise the manuscript. All authors contributed to the article and approved the submitted version.

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Age-Related Pathways in Cardiac Regeneration: A Role for lncRNAs?

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Aging imposes a barrier for tissue regeneration. In the heart, aging leads to a severe rearrangement of the cardiac structure and function and to a subsequent increased risk of heart failure. An intricate network of distinct pathways contributes to age-related alterations during healthy heart aging and account for a higher susceptibility of heart disease. Our understanding of the systemic aging process has already led to the design of anti-aging strategies or to the adoption of protective interventions. Nevertheless, our understanding of the molecular determinants operating during cardiac aging or repair remains limited. Here, we will summarize the molecular and physiological alterations that occur during aging of the heart, highlighting the potential role for long non-coding RNAs (lncRNAs) as novel and valuable targets in cardiac regeneration/repair.

Keywords: lncRNAs, heart, regeneration, reprogramming, transdifferentiation

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INTRODUCTION

Worldwide, cardiovascular diseases are the leading cause of death, causing nearly 18 million deaths in 2017. Cardiovascular diseases comprise several pathological conditions, including heart failure (Yusuf et al., 2001; Lloyd-Jones et al., 2009, 2010; Mensah et al., 2019). Aging is probably the most important risk factor for heart failure (Li et al., 2020a). As opposed to the neonatal heart, adult mammalian hearts lose their capacity to fully regenerate after an exogenous or endogenous harm (Lam and Sadek, 2018). This may be mediated through several interconnected processes, including cellular senescence and secreted factors, telomere attrition, mitochondrial damage, cell death, or inflammation (for a comprehensive review on age-related pathways affecting the heart, see Li et al., 2020a). Although a partial myocyte turnover has been observed in adult heart after damage (e.g., myocardial infarction), it only partially and slightly restores heart function. For instance, it has been recently demonstrated that manipulation of telomere length through the expression of telomerase, whose expression is silenced in the mouse heart from day 5 to 7 (Blasco et al., 1995; Borges and Liew, 1997; Richardson et al., 2012), may be beneficial in heart healing and healthspan (Bernardes de Jesus and Blasco, 2011; Bar et al., 2014).

Long non-coding RNAs (lncRNAs) have emerged as important regulators of epigenetic modulation and gene expression. Although deprived from coding potential, lncRNAs have been associated with several biological processes, including dosage compensation, genomic imprinting, aging, and cell differentiation (Mercer et al., 2009; Rinn and Chang, 2012; Sousa-Franco et al., 2019; Yao et al., 2019). Furthermore, lncRNAs have been linked to several diseases including cardiovascular diseases (Hobuß et al., 2019; Abbas et al., 2020).

In this review, we will discuss the cardiac regeneration properties of neonatal and adult hearts. We will focus on lncRNAs and their potential role in cardiac regeneration briefly discussing the potential of fibroblasts as a source of cardiomyocytes for regenerative medicine purposes.

CARDIAC REGENERATION IN NEONATAL AND ADULT HEARTS

There is a general consensus on the capacity of neonatal hearts to regenerate, after distinct types of damage (**Supplementary Figure 1** – Porrello et al., 2011, 2013; Haubner et al., 2012, 2016; Jesty et al., 2012; Mahmoud et al., 2013, 2014, 2015; Rubin et al., 2013; Andersen et al., 2014, 2016; Aurora et al., 2014; Sadek et al., 2014; Bryant et al., 2015; Darehzereshki et al., 2015; Han et al., 2015; Jiang et al., 2015; Konfino et al., 2015; Aix et al., 2016; Blom et al., 2016; Kang et al., 2016; Tao et al., 2016; Valiente-Alandi et al., 2016; Xiong and Hou, 2016; Yu et al., 2016; Ai et al., 2017; Bassat et al., 2017; Malek Mohammadi et al., 2017; Zebrowski et al., 2017; Ahmed et al., 2018; Ingason et al., 2018; Sampaio-Pinto et al., 2018; Sereti et al., 2018; Cai et al., 2019; Elhelaly et al., 2019; Wang et al., 2019b; Fan et al., 2020; Pei et al., 2020; Li et al., 2020b, 2020c). A comprehensive overview of neonatal heart regeneration studies has been previously and elegantly detailed by Lam and Sadek (2018). Heart regeneration seems to be dependent on the type of injury that causes loss of cardiomyocytes. For example, cryoinjury does not induce the same level of regeneration as apical resection or myocardial infarction. Furthermore, it is likely that neonatal heart regeneration is mediated by the proliferation of pre-existing cardiomyocytes, and not by cardiac stem or progenitor cells. This regenerative state occurs in an extremely short time frame (<10days; Eschenhagen et al., 2017) and, just a few days after birth, cardiomyocytes exit the cell cycle resulting in a decline in heart regeneration capacity. This is accompanied by other alterations in the cardiomyocytes, including their metabolic needs or changes in the expression of both coding and non-coding genes. Subsequently, several strategies have been designed for regenerating the adult heart. Those approaches may include the forced re-entering in the cell cycle of the pre-existing cardiomyocytes, or may include cell transdifferentiation strategies, in which somatic cells can be converted into functional cardiomyocytes for cell replacement therapy (Qian et al., 2012; Addis and Epstein, 2013; Nam et al., 2013; Wada et al., 2013; Ghiroldi et al., 2017; Amin et al., 2018; Engel and Ardehali, 2018a).

A ROLE FOR lncRNAs IN HEART REGENERATION

The importance of lncRNAs in heart regeneration has been brought to light recently (Bar et al., 2016; Abbas et al., 2020). LncRNAs are a vast category of non-coding, poorly conserved, and tissue- and developmental stage-specific transcripts with distinct functions in several biological processes, including epigenetic, transcriptional, and post-transcriptional regulation. Regarding the role of lncRNAs in heart regeneration, we will discuss some recent studies describing lncRNAs directly acting (promoting or inhibiting) on heart regeneration (**Table 1**).

LncRNAs That Promote Cardiomyocyte Proliferation and Cardiac Regeneration

P7 mice subjected to LAD ligation and injected with adenovirus containing NR_045363 exhibited improved left ventricular ejection fraction and reduced infarct size compared to the control-injected group (Wang et al., 2019a). Mice overexpressing NR_045363 showed higher expression of cardiomyocyte mitotic markers, such as Ki67 and phosphorylated histone H3 (pH3), suggesting that improved heart function after MI was due to cardiomyocyte proliferation. The authors reported that NR_045363 acted as a competing endogenous RNA (ceRNA), binding to miR-216a (Wang et al., 2019a). miR-216a is known to repress JAK2, leading to decreased levels of phosphorylation of STAT3 (Hou et al., 2015). Furthermore, deletion of STAT3 was shown to impair cardiomyocyte proliferation after apical resection (Kurdi et al., 2018), suggesting that NR_045363 promoted cardiomyocyte proliferation by modulating the JAK2-STAT3 pathway. So, the absence of NR_045363 (which results in an upregulation of miR216a) led to reduced activity of the JAK2-STAT3, whilst NR_045363 overexpression (which leads to a downregulation of miR-216a) resulted in an increase of the phosphorylation

TABLE 1 | LncRNAs with reported roles in cardiac regeneration.

LncRNA	Reported role in cardiac regeneration	Reference
Negative regulators		
AZIN2-sv	↓ cardiomyocyte proliferation by sequestering miR-214 and leading to a decrease in the phosphorylation of Akt and Cyclin D	Li et al., 2018b
CAREL	↓ cardiomyocyte proliferation by sequestering miR-296 and activating Trp53inp1 and Itm2a	Cai et al., 2018
CPR	↓ cardiomyocyte proliferation by the recruitment of DNMT3A, leading to increased levels of methylation of the MCM3 promoter	Ponnusamy et al., 2019
CRRL	↓ cardiomyocyte proliferation by sequestering miR-199a-3p, leading to an increased expression of Hopx	Chen et al., 2018
LncDACH1	↓ cardiomyocyte proliferation by regulating PP1A/YAP1 signaling	Cai et al., 2020a
SARRAH	↑ cardiomyocyte apoptosis by increasing caspase activity	Trembinski et al., 2020
Positive regulators		
NR_045363	↑ cardiomyocyte proliferation *via* the miR-216a/JAK-STAT3 pathway ↓ cardiomyocyte apoptosis by blocking p53 activation	Wang et al., 2019a; Chen et al., 2020
ECRAR	↑ cardiomyocyte proliferation by promoting phosphorylation of ERK1/2 to activate Cyclins D1 and E1	Chen et al., 2019
Sirt1 antisense lncRNA	↑ cardiomyocyte proliferation and ↓ cardiomyocyte apoptosis by stabilizing Sirt1	Li et al., 2018a

levels of JAK2 and STAT3, thus promoting cardiomyocyte proliferation (Wang et al., 2019a). More recently, NR_045363 was associated with cardiomyocyte apoptosis. Chen et al. (2020) reported that loss of NR_045363 led to the activation of the p53 signaling pathway, promoting apoptosis. On the other hand, overexpressing NR_045363 inhibited apoptosis and improved cardiac function after MI, thus potentially mediating the cardiac functions observed after NR_045363 modulation.

Long non-coding RNA endogenous cardiac regeneration-associated regulator (ECRAR) was found to be upregulated in the fetal heart, and its expression gradually decreased in postnatal hearts. Overexpression of ECRAR in postnatal rat cardiomyocytes, both *in vitro* and *in vivo*, resulted in an increase of DNA synthesis, and an increase of cytokinesis (pH3 and aurora B kinase), suggesting a direct involvement in cardiomyocytes proliferation (Chen et al., 2019). Overexpression of ECRAR resulted in the phosphorylation of ERK1/2, their subsequent translocation to the nucleus and the transcription of cell proliferation and cell cycle-related genes (Chen et al., 2019). Li et al. (2018a) identified Sirt1 antisense lncRNA (Sirt1-as), whose expression was high during heart development. Overexpression of this lncRNA resulted in an increase of Ki67- and pH3-positive cardiomyocytes. On the other hand, silencing of Sirt1-as, both *in vitro* and *in vivo*, led to a decrease of Ki67- and pH3-positive cardiomyocytes, indicating a potential decline in cell division (Li et al., 2018a). Furthermore, overexpression of Sirt1-as after MI in adult mice resulted in an increased expression of cell-cycle specific factors Ki67 and pH3, thus suggesting a potential implication in cardiac health (Li et al., 2018a).

More recently, Wilson et al. (2020) described BANCR, a lncRNA exclusively expressed in primate fetal cardiomyocytes. BANCR promotes cardiomyocyte migration *in vitro* and ventricular enlargement *in vivo*. To elucidate the regulation of BANCR in cardiomyocytes, the authors suggested that TBX5 binding was responsible for the fetal heart-specific expression of BANCR. Additionally, the authors identified TEAD4 and YAP1 (two factors involved in the HIPPO pathway) in the same enhancer, promoting BANCR expression. Finally, the authors identified a role for BANCR in heart disease, demonstrating higher expression in pediatric but not adult dilated cardiomyopathy (Nelakanti and Xiao, 2020; Wilson et al., 2020). Other lncRNAs associated with aged hearts include lncRNA H19 (downregulated in aged or ischemic heart; Hofmann et al., 2019), and MALAT1 a lncRNA which, itself, is regulated by an antisense lncRNA transcript (TALAM1; Zong et al., 2016; Gomes et al., 2019), was also shown to be decreased in aged hearts (Bink et al., 2019; Gomes et al., 2019), and this decrease was shown to be involved in cardiac dysfunction (Zhu et al., 2019; Li et al., 2020a).

LncRNAs That Inhibit Cardiomyocyte Proliferation and Cardiac Regeneration

Cai et al. (2018) explored the role of lncRNAs during heart regeneration after ischemic injury, in both neonatal and adult mice. *CAREL*, a lncRNA whose expression gradually increased in the neonatal hearts from P1 to P10 mice, with P7 corresponding to the time point at which the heart regenerative capacity is lost in mice (Cai et al., 2018). Cardiac-specific overexpression of *CAREL* led to a decrease of cardiomyocyte proliferation and reduced heart regeneration in neonatal mice after injury. On the contrary, silencing *CAREL* promoted cardiac regeneration and improved heart functional parameters after myocardial infarction in neonatal and adult mice (Cai et al., 2018). *CAREL* was found to be a ceRNA, sequestering miR-296. It was suggested that the *CAREL-miR-296* interaction led to the activation of Trp53inp1 and Itm2a, leading to a decrease in cardiomyocyte proliferation, thus resulting in a reduction of regeneration. Intramyocardial administration of *CAREL* to p1 neonatal mice inhibited cardiomyocyte mitosis and increased the formation of cardiac scar and, on the other hand, overexpression of miR-256 promoted cardiomyocyte proliferation and cardiac regeneration after injury. Similarly, lncRNA cardiomyocyte proliferation regulator (CPR) was shown to be a negative regulator of cardiomyocyte proliferation and cardiac repair. Ponnusamy et al. (2019) observed that higher levels of CPR hampered cardiomyocyte proliferation, whilst silencing CPR resulted in cardiomyocyte proliferation in postnatal and adult hearts. CPR expression levels were found to be higher in the adult heart, which is consistent with their lack of regeneration. The authors reported that CPR recruits DNMT3A to several locus leading, in particular, to increased levels of methylation in the MCM3 promoter (Ponnusamy et al., 2019). In dividing tissues, MCM3 promotes the initiation of DNA replication and cell cycle progression (Lin et al., 2008), something halted by CPR in the heart and leading to the inhibition of cardiomyocytes proliferation.

Another lncRNA that negatively regulates cardiac regeneration is LncDACH1. This lncRNA was found to be gradually upregulated in postnatal hearts, which is in accordance with the loss of myocardial regenerative capacity soon after birth (Cai et al., 2020). The authors suggest that LncDACH1 binds protein phosphatase 1 catalytic subunit alpha (PP1A), reducing its dephosphorylation capacity, and increases the phosphorylation of yes-associated protein 1 (YAP1), preventing its translocation to the nucleus and, thus, the activation of cell proliferation-related genes (von Gise et al., 2012; Cai et al., 2020). Cardiac-specific overexpression of LncDACH1 resulted in the suppression of neonatal heart regeneration and aggravation of cardiac function after apical resection. These phenotypes were accompanied by a decrease in the number of cardiac-cells expressing proliferative markers (Cai et al., 2020). Cardiomyocyte regeneration-related lncRNA (CRRL) was also found to be involved in heart regeneration. CRRL silencing was associated with an increased expression of EdU, Ki67, and pH3 in P1 and P7 rat cardiomyocytes (Chen et al., 2018). Similar results were obtained in neonatal rats post-MI, concomitantly with better prognosis such as reduction of the fibrotic length of the infarct wall and fibrosis area in the non-infarct zone. Instead, overexpression of CRRL leads to a decrease in pH3-positive cardiomyocytes and inhibition of functional recovery post-MI. CRRL function seemed to be mediated through the binding to miR-199a-3p, resulting in an increased expression of *Hopx*, which is a negative regulator of cardiomyocyte proliferation (Trivedi et al., 2010).

LncRNA AZIN2-sv, a splice variant of the AZIN2 gene, was found to be upregulated in human adult hearts. AZIN2-sv was

reported to negatively regulate cardiomyocyte proliferation, both *in vitro* and *in vivo* (Li et al., 2018b). Overexpression of AZIN2-sv led to an anti-proliferative phenotype, marked by decreased levels of EdU-, Ki67-, pH3-, and Aurora-B. On the other hand, silencing AZIN2-sv promoted cardiomyocyte proliferation and improved cardiac function after MI. AZIN2-sv sequesters miR-214, leading to the release of its target PTEN, resulting in a decrease in the phosphorylation of Akt and Cyclin-D, therefore inhibiting cardiomyocyte proliferation. Reduced levels of AZIN2-sv allow miR-214 to repress PTEN, leading to increased levels of phosphorylated Akt and Cyclin-D1, thus promoting cardiomyocyte proliferation.

More recently, Trembinski et al. (2020) identified lncRNA SCOT1-antisense RNA regulated during aging in the heart (*SARRAH*), whose expression declines during aging. Inhibition of *Sarrah* induces caspase activity in mouse and human cardiomyocytes, promoting apoptosis. Gene set enrichment analysis after *SARRAH* silencing showed enrichment of apoptosis-related pathways, corroborating previous observations (Trembinski et al., 2020). *SARRAH* was also found to directly bind to the promoters through RNA-DNA triplex helix structures, suggesting that its binding may activate gene expression. Indeed, it was reported that *SARRAH* interacted with cardiac transcription factor cysteine-rich protein 2 (CRIP2) and p300, which acetylates histone H3 lysine 27 to activate transcription (Trembinski et al., 2020). On the other hand, overexpression of SARRAH led to a decrease in caspase activity. In adult mice a decline in apoptosis was observed after overexpressing SARRAH, suggesting that reduced expression levels of this lncRNA in aged mice might contribute to cardiomyocyte cell death *in vivo*. Furthermore, reduced levels of Sarrah were observed in the infarcted and border regions after acute MI (Trembinski et al., 2020).

Furthermore, several lncRNAs have been identified as promoters of cardiac fibrosis (Liang et al., 2018; Wang et al., 2018; Hao et al., 2019; Zhang et al., 2019a). Aged tissues accumulate signals that promote the epithelial-mesenchymal transition (EMT), inducing the transdifferentiation of epithelial cells to mesenchymal cells, such as fibroblasts, which are the main mediators of fibrosis through the deposition of extracellular matrix. In the heart, many fibroblasts derive from endothelial cells, leading to excessive deposition of extracellular matrix and causing cardiac fibrosis, which is common in patients with heart failure (reviewed by Santos et al., 2019). Thus, targeting these lncRNAs may also be considered for improving heart function.

REPROGRAMMING OF FIBROBLASTS INTO CARDIOMYOCYTES AS A POTENTIAL CELL REPLACEMENT THERAPY – A ROLE FOR lncRNA

Cell reprogramming has emerged as a novel strategy for regenerative medicine and cell-based therapy. The reprogramming of mouse and human fibroblasts into induced pluripotent stem cells (iPSCs) using transcription factors (TFs) known to play key roles in the maintenance of embryonic stem cell identity suggested that patient-derived iPSCs could be produced from somatic cells. This strategy allowed the conversion of fully differentiated cells into cells with the potency to be differentiated in tissues from different development lineages (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yamanaka, 2009; Abad et al., 2013). Additionally, many of the reprogramming barriers, such as the obstacles imposed by aging, have been addressed through the direct manipulation of tumor suppressor genes, p53 or Ink4a/ARF (Li et al., 2009; Marion et al., 2009), or the EMT-promoting factor ZEB2 (Bernardes de Jesus et al., 2018; Santos et al., 2019).

The reprogramming of fibroblasts into iPSCs opened doors to direct cell reprogramming. Direct cardiac reprogramming of fibroblasts into cardiomyocytes [usually termed induced cardiomyocytes (iCMs)] has emerged as an attractive strategy for replacing lost or damaged cells in the heart. Mouse postnatal cardiac and dermal fibroblasts have been transdifferentiated into iCMs through the combined expression of three different cardiac-specific TFs: *Gata4*, *Mef2c,* and *Tbx5* (GMT). The ectopic expression of GMT activates a cardiac-like gene expression program and promotes the conversion of fibroblasts into iCMs (Ieda et al., 2010; Qian et al., 2012). Comparative gene expression analyses reported that iCMs generated *in vitro* exhibited *bona fide* adult cardiomyocyte-like features, such as fatty acid oxidation or cell cycle exit (Muraoka et al., 2019). Remarkably, this approach has been adapted *in vivo*, where cardiac fibroblasts have been transdifferentiated into iCMs (Song et al., 2012; Zhang et al., 2019b, 2019c), bypassing the need to revert fibroblasts to a pluripotent state (Liu et al., 2017; Muraoka et al., 2019). Endogenous cardiac fibroblasts comprise about 50% of all the cells in the heart, making them a potential source of cardiomyocytes for regenerative therapy (Ieda et al., 2010). In fact, iCMs reprogrammed from endogenous cardiac fibroblasts enhanced cardiac function after myocardial infarction, fully demonstrating the potential of this strategy for cardiac repair (Miyamoto et al., 2018; Bektik and Fu, 2019; Lee et al., 2020).

Despite several encouraging results, current reprogramming methodologies remain somewhat inefficient, as very few fibroblasts are fully converted into functional iCMs. Differential expression patterns of lncRNAs have been observed in several developmental stages, including cardiogenesis, and involve the expression of lncRNAs *Braveheart*, *Fendrr,* and *Carmen*. In fact, lncRNA *ZEB2-NAT* has been modulated in order to improve the reprogramming of fibroblasts into iPSCs (Bernardes de Jesus et al., 2018). Having these concepts in mind, it seems reasonable to expect that modulating lncRNAs might improve the efficiency of direct cardiac reprogramming.

In vivo Therapeutic Delivery – Current Issues

Regarding phenoconversion of cardiac cells, it is important to mention, however, that many of the current protocols depend on viral vectors for gene delivery. There are a few safety issues associated with the use of lentiviral and retroviral vectors, as they integrate their genome in the host cell. They could potentially disturb endogenous gene expression and are associated

with the risk of insertional mutagenesis, hampering the clinical application of this method. However, non-integrative viruses, such as Sendai virus, and non-viral reprogramming systems have emerged as safer alternatives for clinical application (Engel and Ardehali, 2018b; Miyamoto et al., 2018; Tani et al., 2018; Chang et al., 2019).

CONCLUSION

As previously discussed, several lncRNAs are expressed during the development of the heart and during heart pathologies. Targeting lncRNAs may be a novel strategy against heart diseases (Bar et al., 2016). Technically, the development of specific and deliverable antisense transcripts (e.g., LNA-GapmeRs) has been proved powerful and efficient carriers for *in vivo* targeting and RNase H-mediated degradation of specific targets (Bernardes de Jesus et al., 2018). Similar approaches may be designed for expression of selected lncRNAs, downregulated in cardiac diseases. We have to face, however, that most human lncRNAs are non-conserved between species, making it extremely challenging to identify the functional lncRNAs *in vivo*. The lack of sequence conservation poses a challenge for the translational application of human lncRNAs. Since lncRNAs are species-specific, we often can only visualize their impact when studied in their specific system. This challenge may only be addressed through a humanized experimental model where the detailed function of non-conserved lncRNAs may be tested. In conclusion, understand lncRNAs specific profiles in dividing vs. non-dividing cardiomyocytes may allow the detection of potentially druggable targets for adult heart repair.

AUTHOR CONTRIBUTIONS

FS, MC, SN-P, and BB planned, wrote, and discussed the paper. SN-P and BB revised the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.583191/full#supplementary-material

Supplementary Figure 1 | Studies on neonatal heart regeneration depicting different methodologies. The studies with blue background observed heart regeneration whether the studies in red could not detect heart healing.

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Novel Insights Linking lncRNAs and Metabolism With Implications for Cardiac Regeneration

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Heart disease is the leading cause of mortality in developed countries. The associated pathology is typically characterized by the loss of cardiomyocytes that leads, eventually, to heart failure. Although conventional treatments exist, novel regenerative procedures are warranted for improving cardiac regeneration and patients well fare. Whereas following injury the capacity for regeneration of adult mammalian heart is limited, the neonatal heart is capable of substantial regeneration but this capacity is lost at postnatal stages. Interestingly, this is accompanied by a shift in the metabolic pathways and energetic fuels preferentially used by cardiomyocytes from embryonic glucose-driven anaerobic glycolysis to adult oxidation of substrates in the mitochondria. Apart from energetic sources, metabolites are emerging as key regulators of gene expression and epigenetic programs which could impact cardiac regeneration. Long non-coding RNAs (lncRNAs) are known master regulators of cellular and organismal carbohydrate and lipid metabolism and play multifaceted functions in the cardiovascular system. Still, our understanding of the metabolic determinants and pathways that can promote cardiac regeneration in the injured hearth remains limited. Here, we will discuss the emerging concepts that provide evidence for a molecular interplay between lncRNAs and metabolic signaling in cardiovascular function and whether exploiting this axis could provide ground for improved regenerative strategies in the heart.

Keywords: lncRNAs, metabolism, mitochondria, regeneration, heart

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INTRODUCTION

The World Health Organization (WHO) has been reporting every year that cardiovascular diseases (CVD) are the leading cause of death in the world. Although currently there are large range of pharmaceutical drugs and surgical options that prevent further deterioration or restore function to the failing heart, for end-stage heart failure, the only long-term selection is heart transplantation which presents several limitations (Hudson and Porrello, 2013). Therefore, the development of improved cardiac regenerative strategies is an area of growing interest.

Subsequent to cardiac injury, cardiomyocytes undergo necrotic and apoptotic cell death and cardiac fibroblasts are activated to produce collagen and other extracellular matrix components, leading to fibrosis and harmed cardiac function (Song et al., 2012; Hashimoto et al., 2018). The main

goal of regenerative cardiovascular medicine is to repair injured hearts by replacing cardiomyocytes and diminishing fibrosis. In order to suppress the outcomes of heart failure several regenerative strategies have been proposed, including post-injury activation of cardiomyocyte proliferation, recruitment of stem cells or progenitor cells, delivery of *de novo* cardiomyocytes from iPSCs, and direct reprogramming of resident cardiac fibroblasts (CFs) into induced cardiac-like myocytes (iCLMs) (Ieda et al., 2010; Song et al., 2012; Ghiroldi et al., 2018; Hashimoto et al., 2018). Although most strategies aim at recovering cardiac function post-injury, approaches that target mechanisms of heart regeneration at pre-injury and during injury events can also potentially be conceived (Uygur and Lee, 2016; Galdos et al., 2017; Tzahor and Poss, 2017).

Besides holding great promise, most cardiac regenerative strategies still lack effective clinical outcomes (Ghiroldi et al., 2017; Hashimoto et al., 2018). Therefore understanding the molecular mechanisms and players governing cardiac regeneration in the injured hearth is warranted for improving the efficiency of cardiac regenerative strategies. In this context, long non-coding RNAs (lncRNAs), a class of > 200 nucleotides-long ribonucleic acid sequences, are abundantly expressed in the cardiovascular system and are part of a complex regulatory network governing cardiovascular function in health and disease (Bär et al., 2016; Das et al., 2018; Hobuß et al., 2019). Essential roles for some lncRNAs in heart development have been described (Matkovich et al., 2014; Bär et al., 2016; Haemmig et al., 2017) and exploring the role of lncRNAs in cardiovascular function may facilitate the development of new therapeutics for treating cardiovascular disease (Bär et al., 2016; Hobuß et al., 2019).

Although the adult mammalian heart has limited regenerative capacity, with estimation of only ≈1% *de novo* cardiomyogenesis per year (Neidig et al., 2018), the neonatal heart is capable of substantial regeneration but this capacity is lost by postnatal day (P) 7 (Soonpaa et al., 1996). Interestingly, this lost in proliferative potential is accompanied by a shift in the main energetic metabolic pathway and fuels preferentially used by cardiomyocytes from embryonic glucose-driven anaerobic glycolysis to adult oxygen-dependent oxidative phosphorylation (OXPHOS) of pyruvate and fatty acids (FAs) in the mitochondria (Lopaschuk et al., 1992; Lehman and Kelly, 2002). Apart from energetic sources, metabolites are key regulators of gene expression programs by acting as essential substrates or cofactors for chromatin-modifying enzymes (Intlekofer and Finley, 2019). Interestingly, lncRNAs are emerging as master regulators of cellular and systemic carbohydrate and lipid metabolism with clear implications for cardiovascular function (Zhao, 2015; van Soligen, 2018; Mongelli et al., 2019), opening the possibility for a two-way communication between metabolism and lncRNAs in cardiac heart regeneration.

Here, we will discuss the emerging insights in the molecular interplay between lncRNAs and metabolism in the heart highlighting evidence for the impact of metabolic signaling in cardiac regeneration. Also, particular emphasis will be given to those lncRNAs regulating metabolic targets in the cardiovascular system and the potential modulation of the lncRNAs-metabolic axis for the development of innovative regenerative strategies.

CAN AN EMBRYONIC-LIKE METABOLIC PROGRAM PROMOTE HEART REGENERATION?

The fetal heart's environment is low in oxygen and FAs, thus fetal cardiomyocytes are highly dependent on glycolysis for ATP production (Lopaschuk et al., 1992). The heart suffers a major metabolic alteration driven by the physiological changes at postnatal stages, as enhanced workload and the demand for growth, that cannot be supported by glucose and lactate metabolism (Malandraki-Miller et al., 2018). The mammalian heart has to contract constantly thus, the need for an optimal energy fuel is imperative. During the early postnatal period, the number of mitochondria in cardiomyocytes increases dramatically (Mayor and Cuezva, 1985; Attardi and Schatz, 1988). Mitochondria is the organelle that coordinates the energy transduction function and it is responsible to produce more than 95% of ATP utilized by the heart (Doenst et al., 2013). Additionally, mitochondria regulates intracellular calcium homeostasis, signaling and apoptosis (Kolwicz et al., 2013). As a result, mammalian cardiomyocytes undergo extensive metabolic remodeling after birth. In order to adapt to the high-energetic demands of the postnatal life, cardiomyocytes suffer a metabolic switch and produce their energy via mitochondrial OXPHOS, a more efficient process than glycolysis (Lehman and Kelly, 2002; Vivien et al., 2016). Postnatal cardiomyocytes also revealed a shift in the energetic substrate utilization from pyruvate to FAs that are energetically more favorable (Lopaschuk et al., 1992; Lehman and Kelly, 2002). As the neonatal mammalian heart regenerative capacity is lost by P7, which corresponds with cardiomyocyte binucleation and cell-cycle arrest (Soonpaa et al., 1996; Porrello et al., 2011), one could hypothesize that the "fetal metabolic shift" would have a role in suppressing cardiomyocyte proliferation and heart repair (Martik, 2020). Currently many studies are focusing in understanding the role of mitochondrial metabolism in regulating cell-cycle arrest in postnatal cardiomyocytes with future implications in regenerative strategies.

Heart regeneration in zebrafish is incredibly effective and relies on the proliferation of pre-existing cardiomyocytes. Apart from cardiomyocytes, other cell types (such as epicardial, endocardial, immune cells and fibroblasts) respond to the heart injury and contribute for the healing process (Vivien et al., 2016; Honkoop et al., 2019). Cardiomyocytes from highly regenerative species such as zebrafish have a preference for glycolysis and increase OXPHOS activity promotes cardiomyocyte maturation and reduces the proliferative capacity (Vivien et al., 2016; Honkoop et al., 2019; Fukuda et al., 2019). Although the "fetal switch" to mitochondrial respiration has been associated to loss of the regenerative potential (Malandraki-Miller et al., 2018), the role of bioenergetics in regulating cardiogenesis remains unclear. Recent evidence suggest that hypoxia inducible factor 1 (HIF1) signaling, an important inducer of aerobic glycolysis and the Warburg effect in cancer cells (Kroemer and Pouyssegur, 2008),

controls the embryonic switch toward oxidative metabolism in the developing mouse heart (Menendez-Montes et al., 2016). In midgestational mouse heart, the compact myocardium downregulates HIF1α and switches toward oxidative metabolism. Deletion of the E3 ubiquitin ligase von Hippel-Lindau (VHL) results in HIF1α hyperactivation, blocking the midgestational metabolic shift and impairing cardiac maturation and function (Menendez-Montes et al., 2016). This study highlights the VHL-HIF-mediated metabolic program as an important axis for myocardial differentiation and its potential relevance for cardiac regeneration.

The adult mammalian heart cannot regenerate lost or damaged myocardium although it does present a limited myocyte turnover that reveals insufficient for restoring contractile dysfunction. The brief window of regenerative response following injury seems to be also driven by proliferation of pre-existing cardiomyocytes (Porrello et al., 2011; Elhelaly et al., 2019). Strikingly, increase production of mitochondrial-derived reactive oxygen species (ROS) and DNA oxidation leads to cell-cycle arrest in mouse postnatal cardiomyocytes through the activation of DNA damage response pathways (Puente et al., 2014). FAs oxidation is directly linked to high ROS production and cardiomyocyte cell-cycle arrest (Cardoso et al., 2020). Moreover, the constant use of FAs as an energetic fuel provokes a dependency on this substrate as the acetyl-CoA produced from FAs oxidation inhibits the mitochondrial enzyme pyruvate dehydrogenase (PDH) and therefore the reliance on glucose and its metabolites for energy (Rindler et al., 2013). Still, whether modulating substrate utilization can directly impact DNA damage and promote cardiomyocytes cell-cycle re-entry needs further clarification. Supplementation of FAs-depleted diets to mice prolongs the postnatal window for cardiomyocyte proliferation; however, it is associated with a marked hepatomegaly and steatosis due to a compensatory increase in hepatic *de novo* fatty-acid synthesis (Cardoso et al., 2020). Moreover, deletion of the dehydrogenase kinase isoform 4 (PDK4) in adult cardiomyocytes, the main enzyme responsible for PDH inhibition and FAs usage, results in a marked shift in myocardial substrate utilization with decrease FAs and enhanced pyruvate-driven glucose oxidation, resulting in less DNA damage and increased cardiomyocyte proliferation (Cardoso et al., 2020). Pharmacological activation of PDH through administration of dichloroacetate in mice also resulted in improved glucose utilization and cardioprotective features (Cardoso et al., 2020).

In sum, recent studies are beginning to dissect the intricate relationship between the "fetal metabolic switch" and loss of cardiomyocyte proliferation where several molecular axis (as HIF signaling, ROS and bioenergetic fuels) are emerging as key regulators. This raises important questions and opportunities in the field. For instance, cardiac regenerative strategies based on the generation of induced cardiac-like myocytes (iCLMs) from iPSCs or resident cardiac fibroblasts (CFs) (Hashimoto et al., 2018), could be improved by metabolic modulation and induction of the "fetal switch". Moreover, systemic metabolic shifts, as nutritional stages and diets, may impact cardiac regeneration post-injury in the mammalian heart (Malandraki-Miller et al., 2018). Further investigation in this field is warranted and may provide unique opportunities to boost cardiac regeneration and repair.

LncRNAs CONTROLLING METABOLIC PATHWAYS IN THE HEART

LncRNAs represent one of the most prominent but least understood transcriptome in the heart. Thousands of lncRNAs have been identified to be dynamically transcribed during development, differentiation, and maturation of cardiac myocytes (Devaux et al., 2015; He et al., 2016; Li et al., 2017; Beermann et al., 2018). Due to their unique regulatory action and tissue-specific expression, lncRNAs are attractive candidates for diagnosis of cardiovascular pathologies and regenerative strategies using several lncRNA-based therapeutic approaches (Bär et al., 2016; Bernardes de Jesus et al., 2018; Hobuß et al., 2019). lncRNAs are localized in the nucleus or the cytoplasm where they may regulate gene expression at transcriptional or posttranscriptional level, respectively, through diverse mechanisms; including epigenetic remodeling, transcriptional activation or repression (signal, decoy, guide, scaffold, or enhancer lncRNAs), formation and maintenance of sub-nuclear domains, posttranscriptional regulation and modulation of protein activity (Schonrock et al., 2012; Kornfeld and Brüning, 2014; Devaux et al., 2015; Thum and Condorelli, 2015; Muret et al., 2019).

LncRNAs are emerging as master regulators of cellular and organismal carbohydrate and lipid metabolism in adipose tissue and liver (Kornfeld and Brüning, 2014; Zhao, 2015; van Soligen, 2018; Mongelli et al., 2019; Muret et al., 2019). Alteration in serum lipid levels is one of the most relevant risk factor for CVD (Doggen et al., 2004). In the recent years, several studies have highlighted the complex contribution of lncRNAs in controlling systemic and cell-type-specific cholesterol, FAs, and triglyceride metabolism, with important implications for CVD. For instance, several lncRNAs, including *H19*, lncRNA HCV regulated 1 (*lncHR1*), *MALAT-1* and *lncARSR*, have been shown to regulate the expression of the sterol regulatory element binding protein 1c (*SREBP-1c*), a transcription factor that regulates lipid synthesis and uptake in the liver (Yan et al., 2016; Li et al., 2018; Liu et al., 2018; Zhang et al., 2018). Other examples are the liver-specific triglyceride regulator lncRNA Lancaster (*lncLSTR*) that regulates triglyceride plasma levels and energy metabolism (Li et al., 2015) and *AT102202* that inhibits cholesterol synthesis in the liver by targeting the rate limiting enzyme HMGCR (Liu et al., 2015). Whether lncRNAs-mediated control of systemic lipid metabolism has a direct impact in cardiac regeneration remains to be addressed.

As previously discussed, of particular interest are the lncRNAs controlling the "fetal metabolic switch" from embryonic glycolysis to adult mitochondrial respiration and the preferred usage of FAs as energetic fuel in differentiated cardiomyocytes. Although most of our knowledge in lncRNAs control of metabolism comes from studies in lipogenic tissues and/or cancer energetics (Gomes et al., 2019), some mechanistic insights in cardiac muscle development and function, particularly

concerning mitochondrial metabolism, are beginning to arise (**Table 1**). Due to the implication of mitochondrial-dependent FAs oxidation and ROS production in the loss of cardiomyocyte proliferation (Puente et al., 2014; Cardoso et al., 2020), lncRNAs regulating these pathways are particularly attractive for cardiac regeneration.

In heart and skeletal muscle aged tissue, the lncRNA *LINC00116* is among the most significantly downregulated gene (GEO: GSE362 and GSE674). Interestingly, a small region of the most predominant isoform is actively translated in human and mouse muscle and has been found to encode a highly conserved transmembrane microprotein, named mitoregulin (Mtln), also known as Micropeptide regulator of β-oxidation (MOXI), where it associates with the mitochondrial trifunctional protein (MTP), an enzyme complex that plays a critical role in fatty acid β-oxidation (Makarewich et al., 2018; Stein et al., 2018). Isolated heart and skeletal muscle mitochondria from MOXI knockout mice preferentially oxidize carbohydrates over fatty acids, while transgenic MOXI overexpression leads to enhanced β-oxidation. MOXI knockout mice also exhibit a profound reduction in exercise capacity, highlighting the role of MOXI in metabolic control (Makarewich et al., 2018). The impact of Mtln expression in cardiovascular disease and regeneration is still unclear but GTEx portal annotates the existence of common genetic variants that strongly associate with *LINC00116* expression in the human heart (Stein et al., 2018). *NEAT1* (nuclear enriched abundant transcript 1) is another lncRNA with increase expression in non-regenerative cardiomyocytes (**Table 1**). In skeletal muscle, *NEAT1* modulates myogenesis by accelerating myoblast proliferation and suppressing myoblast differentiation and fusion (Wang et al., 2019). *NEAT1* act by recruiting EZH2 to target gene promoters, decreasing the expression of the cyclin-dependent kinase inhibitor *p21* and suppressing the myoblast differentiation program. Strikingly, several mitochondrial regulators have been identified to associate to *NEAT1* in paraspeckles, a type of nuclear body with multiple roles in gene expression (Wang et al., 2018). Specifically, *NEAT1* depletion lead to profound effects on mitochondrial dynamics and function by altering the paraspeckles-specific sequestration of essential mito-mRNAs, including *CYCS* (cytochrome c), *NDUFA13* (NADH:Ubiquinone Oxidoreductase Subunit A13) and *CPT1A* (Carnitine Palmitoyltransferase 1A) (Wang et al., 2018) and *NEAT1*-depleted HeLa cells show reduced mitochondrial DNA, ATP production and proliferation rate (Wang et al., 2018).

Cardiac muscle is an extremely metabolically active tissue that undergoes significant changes in energy metabolism in disease. In mouse cardiomyocytes, cardiac apoptosis-related lncRNA (*CARL*) binds to and sequester microRNA-539, a microRNA found to target the mRNA of the PHB2 sub-unit of prohibitin, a protein localized to the inner mitochondrial membrane that regulates mitochondrial homeostasis (Wang et al., 2014). Downregulation of PHB2 during pathological insults was found to be dependent on upregulation of microRNA-539. *CARL* acts as the endogenous sponge for this microRNA suppressing mitochondrial fission and cardiomyocyte apoptosis (Wang et al., 2014), highlighting the therapeutic potential of modulating lncRNAs during myocardial infarction. The lncRNA *CDKN2B−AS1* (also known as *ANRIL*) has been described as a genetic risk factor for coronary artery disease (CAD) (Deloukas et al., 2013). *ANRIL* expression level is associated with left ventricular dysfunction after myocardial infarction (Vausort et al., 2014). Experimental manipulation in several human cell lines (including HEK and HeLa), revealed that *ANRIL* knock-down decreases the expression of *ADIPOR1* (adiponectin receptor 1), *TMEM258* (also known as *C11ORF10* for chromosome 11 open reading frame 10) and *VAMP3* (vesicle associated membrane protein 3), both at the transcript and protein level, which are important genes in the regulation of glucose and fatty-acid metabolism (Bochenek et al., 2013). However, the impact of *ANRIL*-mediated metabolic regulation in cardiomyocytes remains to be explored. Conversely, in patients with myocardial infarction the levels of the lncRNA hypoxia inducible factor 1A antisense RNA 2 *(HIF1A-AS2)* was found to be upregulated (Vausort et al., 2014). In humans, the HIF pathway is induced early in acute myocardial and remains activated in chronic human heart failure (Zolk et al., 2008). Due to the role of the HIF signaling in controlling myocardial metabolism and differentiation in the neonatal heart (Menendez-Montes et al., 2016) and the implication of the lncRNA *lincRNA-p21* in hypoxia-enhanced glycolysis (Yang et al., 2014), manipulation of the lncRNA/HIF regulatory network might constitute an attractive target to modulate cardiac regeneration.

Type 2 diabetes (T2D) is a multifactorial disorder characterized, among other aspects, by high blood glucose and lipid levels (hyperglycemia and hyperlipidemia) in association with insulin resistance and atherosclerosis (Bornfeldt and Tabas, 2011) and diabetic cardiomyopathy (DCM) is a critical complication of T2D (Jia et al., 2018). Studies suggest that lncRNAs that regulate metabolic targets are aberrantly regulated in DCM, thus targeting lncRNAs could have potential implications for DCM diagnosis and therapy. The mitochondrial long intergenic non-coding RNA predicting cardiac remodeling (*MT-LIPCAR*) is a lncRNA possibly transcribed from mitochondrial DNA that cross the membrane barrier being released into circulation (Dorn, 2014). Plasma levels of *MT-LIPCAR* were positively associated with left ventricular diastolic dysfunction in T2D patients with DCM showing prognostic value as an indicator of heart failure and patient mortality. *MT-LIPCAR* was the first proof that plasma lncRNAs might be used for cardiovascular disease prognostic (Kumarswamy et al., 2014). Despite the invaluable potential as a cardiac biomarker, *MT-LIPCAR* targets and metabolic impact remains unclear. Evidence suggest that the complete *MT-LIPCAR* sequence could map to the mitochondrial genes *CYTB* (Mitochondrially Encoded Cytochrome B) and *COX2* (Mitochondrially Encoded Cytochrome C Oxidase II) (Dorn, 2014), raising further questions regarding MT-LIPCAR biogenesis as a mitochondrial or nuclear pseudogene transcript. *H19* is a lncRNA transcribed from H19/insulin-like growth factor-II (IGF2) genomic imprinted cluster which accumulates in cardiomyocytes of the mature myocardium in humans and rodents (Pant et al., 2018; Viereck et al., 2020). Decrease expression of cardiac *H19* was reported in a rat model of

DCM (Li et al., 2016; Zhuo et al., 2017). Overexpression of *H19* in myocardial tissue was able to suppress oxidative stress, inflammation and improve left ventricular function leading to DCM amelioration. Mechanistically, *H19* serves as template for microRNA-675 expression from its first exon (Zhang et al., 2017; Pant et al., 2018). Since microRNA-675 has multiple biological targets, *H19* is able to regulate a number of mitochondrial functions including suppression of apoptosis by targeting voltage-dependent anion channel 1 (*VDAC1*) (Li et al., 2016), and inhibiting autophagy in cardiomyocytes exposed to high glucose through down-regulation of the GTP- binding protein Di-Ras-3 (*DIRAS3*) (Zhuo et al., 2017).

In sum, recent work on lncRNAs has started to shed light on their regulatory potential in controlling heart metabolism in health and disease, opening the possibility to explore lncRNAs-mediated metabolic control as a strategy to improve cardiac regeneration and heart function.

LncRNAs AND METABOLITES AS CENTRAL EPIGENETIC PLAYERS IN GENE EXPRESSION REGULATION

An hallmark function of lncRNAs is their ability to mediate epigenetic regulation and in the heart, lncRNAs have crucial roles in regulating cardiac chromatin structure during development and pathological remodeling (Schonrock et al., 2012). lncRNAs exhibit tissue-specific regulated expression patterns which are frequently lost during disease (Cabili et al., 2011). However, the regulation of lncRNAs expression during different stages of cardiac development and in disease is still under investigation. Strikingly, inhibition of epigenetic modifications was shown to impact the expression pattern of lncRNAs (Schonrock et al., 2012). Metabolites are emerging as key regulators of gene expression programs and epigenetic modifications, acting as essential substrates or cofactors for enzymes that deposit or remove chemical modifications in DNA and/or histones (Intlekofer and Finley, 2019). FAs and cholesterol have been shown to regulate lncRNAs expression in lipogenic tissues placing metabolism as a central regulator of epigenetic-driven lncRNAs transcription. For instance, the expression of the lncRNAs *H19* and *MALAT1* is upregulated by FAs exposure, coinciding with an increase in (SREBP)-1c in hepatic cells (van Soligen, 2018) and *HULC* is induced by cholesterol in hepatoma cells via the retinoic receptor RXRA, leading to lipogenesis (Cui et al., 2015). Recent evidence suggests that lipid metabolism also impact lncRNAs expression in the cardiovascular system. For instance, the lncRNA *CHROME, a* master regulator of cholesterol homeostasis, is upregulated in atherosclerotic vascular disease in non-human primates and conversely, *CHROME* expression is influenced by dietary and cellular cholesterol (Hennessy et al., 2019). Although evidence for the direct implication of nutritional signals in the epigenetic alterations that govern lncRNAs expression in the heart is still at its early days, it seems clear that lncRNAs and metabolic signaling can engage in a two-way communication road in the control of gene expression that impacts cellular and systemic metabolism. Moreover, nutritional cues have been shown to control the specification of skeletal cell fate, highlighting the possibility for a similar network to take place in cardiomyocyte progenitors. When lipids are scarce, skeletal muscle progenitors activate the expression of forkhead box O (FOXO) transcription factors leading to a Sox9-dependent suppression of FAs oxidation and chondrogenic commitment (van Gastel et al., 2020). Moreover, glucose metabolism is crucial for muscle stem cells (MuSCs) commitment. In proliferating MuSCs, glucose is dispensable for mitochondrial respiration and becomes available for maintaining high histone acetylation via acetyl-CoA, whereas differentiating MuSCs increases glucose oxidation and has consequently reduced acetylation (Yucel et al., 2019). PDH is pivotal for this

TABLE 1 | LncRNAs with characterized and/or potential metabolic targets in the heart.

LncRNA	Tissue/Cell type	Species	Mechanism of action	Target genes	Metabolic impact	References
LINC00116	Heart and skeletal muscle	Human, mouse	Mitochondrial-related sORFs	Mtln	Mtln enhances mitochondrial respiration and fatty acid β-oxidation	Makarewich et al., 2018; Stein et al., 2018
NEAT1	Heart and skeletal muscle, HeLa cells	Human, mouse	Establishment of paraspeckles	*CYCS*, *NDUFA13, CPT1A*	Induces dysfunction of mitochondrial respiration and fission	Wang et al., 2018, 2019
CARL	Heart, cardiomyocytes	Mouse	Decoy, sequester microRNA-539	PHB2	Suppress mitochondrial fission and apoptosis	Wang et al., 2014
CDKN2B-AS1 (ANRIL)	HEK, HeLa cells	Human	Scaffold	*ADIPOR1, TMEM258, VAMP3*	Impacts glucose and fatty acid metabolism	Bochenek et al., 2013
MT-LIPCAR	Plasma	Human	Unknown	*CYTB, COX2 (?)*	Unknown	Kumarswamy et al., 2014
H19	Heart and neonatal cardiomyocytes	Human, mouse, rat	MicroRNA-675 precursor	*VDAC1*, *DIRAS3*	Decreases oxidative stress, apoptosis and autophagy	Li et al., 2016; Zhuo et al., 2017

sORFs, small opening reading frames; Mitoregulin, Mtln; NEAT1, nuclear enriched abundant transcript 1; CYCS, cytochrome c; NDUFA13, NADH:Ubiquinone Oxidoreductase Subunit A13; CPT1A, Carnitine Palmitoyltransferase 1A; CARL, cardiac apoptosis-related lncRNA; PHB2, Prohibitin 2; ANRIL, antisense non-coding RNA in the INK4 locus; HEK, human embryonic kidney; ADIPOR1, adiponectin receptor 1; TMEM258, Transmembrane Protein 258; VAMP3, vesicle associated membrane protein 3; MT-LIPCAR, Mitochondrially Encoded Long Non-Coding Cardiac Associated RNA; CYTB, Cytochrome B; COX2, Cytochrome C Oxidase II; VDAC1, voltage-dependent anion channel 1; DIRAS3, DIRAS Family GTPase 3.

switch and determines the differentiation potential of myogenic progenitors during muscle regeneration (Yucel et al., 2019). Whether metabolic fuels also directly impinge cardiomyocyte cell fate decisions and dietary cues can modulate cardiac regeneration (for instance, by controlling lncRNAs expression) are exciting possibilities that require further investigation.

CONCLUDING REMARKS

Given the emerging regulatory potential of lncRNAs, it is undoubted that these molecules offer potential solutions in the pursuit for cardiac regeneration (Hudson and Porrello, 2013). In the recent years, several lncRNAs with characterized and/or potential metabolic targets in the heart have been identified (**Table 1**) and a link between metabolic pathways and cardiac proliferative potential has been established. But can we boost cardiac regeneration by modulating the lncRNAs-metabolic axis? Emerging evidence suggests that exploring the two-way communication road between lncRNAs and cardiac (or systemic) metabolism may offer new perspectives and opportunities for increasing the regenerative potential of the injured heart.

AUTHOR CONTRIBUTIONS

MC, BB, and SN-P planned, wrote, and discussed the manuscript. BB and SN-P revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Non-coding RNAs in Cardiac Regeneration

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The adult heart has a limited capacity to replace or regenerate damaged cardiac tissue following severe myocardial injury. Thus, therapies facilitating the induction of cardiac regeneration holds great promise for the treatment of end-stage heart failure, and for pathologies invoking severe cardiac dysfunction as a result of cardiomyocyte death. Recently, a number of studies have demonstrated that cardiac regeneration can be achieved through modulation and/or reprogramming of cardiomyocyte proliferation, differentiation, and survival signaling. Non-coding RNAs (ncRNAs), including microRNAs (miRNAs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs), are reported to play critical roles in regulating key aspects of cardiomyocyte physiologic and pathologic signaling, including the regulation of cardiac regeneration both *in vitro* and *in vivo*. In this review, we will explore and detail the current understanding of ncRNA function in cardiac regeneration, and highlight established and novel strategies for the treatment of heart failure through modulation of ncRNAs-driven cardiac regeneration.

Keywords: ncRNA, miRNA, lncRNA, cardiac regeneration, cardiomyocyte proliferation, cardiac differentiation, cardiac reprogramming, cardiomyocyte apoptosis

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INTRODUCTION

Despite improvements in interventional and pharmacological therapies, cardiovascular disease is still the leading cause of hospitalization and mortality in the industrial world. Heart failure, in particular, affects 64 million people worldwide and is a major cause of cardiovascular morbidity and mortality with tremendous impact on healthcare systems and economic productivity (Disease et al., 2017). Heart failure is classified into three primary indication groups depending on the left ventricular ejection fraction (EF): heart failure with reduced ejection fraction (HFrEF), heart failure with preserved ejection fraction (HFpEF), and heart failure with mid-range ejection fraction (HFmEF) (Ponikowski et al., 2016). Loss of cardiomyocytes is a key hallmark of heart failure, in both age- and myocardial infarction (MI)-related heart failure. During MI, around 25% of cardiomyocytes die and are replaced by fibrotic scar tissue (Murry et al., 2006). However, the remaining cardiomyocytes have very limited regenerative and repair capacity to restore the damaged tissue. Thus, these changes lead to cardiac remodeling and fibrosis, culminating in heart failure and mortality (He and Zhou, 2017). Currently, heart transplantation is the primary treatment of choice for end-stage heart failure as a means to renew impaired heart function. However, heart transplantation is restricted by the limited availability of donor organs, treatment costs and surgical complexities. Thus, the unmet need for new therapies in the treatment of heart failure remains very high, and cardiac regenerative strategies targeting the replenishment and replacements of lost cardiomyocytes hold great promise and potential.

Non-coding RNAs (ncRNAs) are functional transcripts that are mainly transcribed by RNA polymerase II and share several characteristics with coding messenger RNAs (mRNAs). ncRNAs can be divided into two main groups: the small ncRNAs (< 200 nt) and the long ncRNAs (lncRNAs, > 200 nt) (Garreta et al., 2017). Among the various types of ncRNAs, microRNA (miRNAs), lncRNAs and circular RNAs (circRNAs) are the three major classes of ncRNAs and have attracted increasing attention. Recently, many studies demonstrate that a large number of ncRNAs, including miRNAs, lncRNAs and circRNAs, have been identified to regulate genetic networks through modulation of transcriptional modulation and epigenetic, and sequentially govern cardiomyocyte fate and potentially mediate cardiac regeneration (Hobuss et al., 2019; Abbas et al., 2020; Braga et al., 2020). Gain- and loss-of-function approaches demonstrate a key role of ncRNAs in regulating cardiac regeneration in both *in vitro* and *in vivo* models.

In this review, we will focus on the current understanding of the roles of ncRNAs in the process of cardiac regeneration, including cardiomyocyte proliferation, cardiomyocyte survival, cardiac differentiation and cardiac reprogramming. Based on this scientific knowledge we will also discuss the strategy of targeting ncRNAs for cardiac regeneration therapy.

PART 1: CARDIAC REGENERATION

The mammalian heart cell is considered as being post-mitotic due to its negligible proliferative capacity (Doppler et al., 2017). It was previously thought that the total number of cardiomyocytes is established after birth and cardiomyocytes of adult mammals are permanently quiescent. However, an ever increasing body of evidence suggests that turnover of cardiomyocytes does in fact occur in the adult mammalian heart. It has been show that the hearts of 1-day-old neonatal mice are able to regenerate following partial cardiac resection, but this capacity is lost by 7 days of age (Porrello et al., 2011b). Similarly, neonatal human hearts have the regeneration capacity to repair myocardial damage after MI and completely recover cardiac function (Haubner et al., 2016). Furthermore, a study by Bergmann et al. (2009, 2015) revealed that cardiomyocyte annual turnover gradually decreases from a rate of 1% at the age of 20, to 0.3% at the age of 75, while other cell types, including endothelial and mesenchymal cells, maintain annual turnover rates of around 20%. This suggests that adult cardiomyocytes have very limited proliferative and regenerative capacity, and are thus incapable of replacing damage cardiomyocytes to restore normal cardiac function after myocardial injury. Thus, an urgent need has emerged for new therapeutic strategies to enhance the regenerative capacity of cardiomyocytes to restore the functional capacity of the diseased myocardium.

Despite tremendous advances in cardiac repair and cardiac regenerative medicine, current therapies for cardiac regeneration following myocardial infarction are limited and non-curative. Over the past several decades, cardiac regeneration has been the central target for restoring the injured heart and has been shown to be achievable through several approaches: (1) cell transplantation strategy, (2) direct or indirect reprogramming non-myocytes into cardiomyocyte-like cells, (3) enhance the proliferation of endogenous cardiomyocytes, (4) decrease cardiomyocyte apoptosis (Choong et al., 2017). For strategy 1, multiple studies have used induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) to generate new cardiomyocytes *ex vivo* for transcoronary delivery or transplantation into infarcted or injured regions of the heart (Xin et al., 2013b). For strategy 2, reprogramming of cardiac fibroblasts to the cardiomyocyte lineage has been proposed as an alternative cell-free approach for cardiac regeneration (Song et al., 2012). For strategies 3 and 4, enhancing proliferation of endogenous cardiomyocytes or improving cardiomyocyte survival in the face of pathologic stress has been proposed (Xin et al., 2013a; Hashimoto et al., 2018). In this review, we will elucidate a number of ncRNAs implicated in four aspects of cardiac regeneration: cardiomyocyte proliferation, differentiation, reprogramming and survival.

PART 2: MIRNAS AND CARDIAC REGENERATION

miRNA Biology

MicroRNAs (miRNAs) are small, ∼ 22 nucleotide non-coding RNAs that control patterns of gene expression by binding to 3′-untranslated region (3′-UTR) of their targeted mRNAs thereby promoting mRNA degradation or inhibiting mRNA translation (Filipowicz et al., 2008). miRNAs have been demonstrated to play essential roles in proliferation, apoptosis, differentiation and development of many cells and tissues including the heart (Porrello, 2013; Hodgkinson et al., 2015).

MicroRNAs biogenesis is initiated by generation of primary miRNA (pri-miRNA) transcripts. The pri-miRNA are transcribed from DNA sequences by RNA polymerase II and then processed into precursor miRNAs (pre-miRNAs) by the microprocessor complex, consisting of RNase III enzyme Drosha and dsRNA-binding protein DiGeorge Syndrome Critical Region 8 (DGCR8) (Denli et al., 2004). Thereafter, the pre-miRNAs are transported into the cytoplasm via the nuclear export factor exportin 5 (EXP-5)/RanGTP complex and then processed by the RNase III endonuclease Dicer to produce ∼ 22 nucleotide mature miRNA duplexes (Zhang et al., 2004). The mature miRNA duplexes can be loaded into the Argonaute (AGO) family of proteins (AGO 1-4 in humans) in an ATP-dependent manner, and then unwound into the single miRNA guide strand by the RNA-induced silencing complex (miRISC). Then the miRISC complexes specifically bind to the 3′-UTR of target mRNAs and inhibits their translation or leads to their degradation (Bartel, 2004).

Currently, many studies indicate that miRNAs are involved in various specialized biological processes during cardiac development, disease and ultimately cardiac regeneration and repair (Porrello, 2013; Wu et al., 2013; Hodgkinson et al., 2015). A large number of miRNAs have been shown to participate in regulating cardiac regeneration through controlling cardiomyocyte proliferation, cardiomyocyte differentiation,

cardiomyocyte reprogramming, and cardiomyocyte survival *in vitro* and *in vivo* (**Figure 1**).

miRNAs and Cardiomyocyte Proliferation

The proliferation capacity of mammalian cardiomyocyte is robust during the fetal period but is switched off early after birth (Gunthel et al., 2018). A number of miRNAs have been identified that induce or inhibit cardiomyocyte proliferation *in vitro* and *in vivo*. A high-throughput functional phenotypic screen using a whole-genome miRNA libraryidentifies 204 potential human miRNAs that are able to promote cardiomyocyte proliferation in neonatal rat cardiomyocytes as determined by 5-ethynyl-2′-deoxyuridine (EdU)-incorporation (Eulalio et al., 2012). Among the 204 miRNAs identified, miR-199a, miR-302b, miR-518, miR-590 and miR-1825 are shown to increase EdU-incorporation, Ki-67, and phospho-histone H3 (pHH3) in mouse or rat cardiomyocytes (Eulalio et al., 2012). Moreover, miR-199a and miR-590 were linked to activation of cell cycle induction and progression, and the enhancement of cardiomyocyte proliferation *in vitro* and *in vivo* (Eulalio et al., 2012). Furthermore, the induction of cardiomyocyte proliferation induced by these two miRNAs led to improved cardiac function and decrease cardiac fibrosis in response to myocardial infarction (MI) (Eulalio et al., 2012). An independent proliferation screen performed in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) identified 96 miRNAs as drivers of DNA synthesis and cell division in cardiomyocytes (Diez-Cunado et al., 2018). Additional chemical screening and computational approaches revealed that 67 of 96 miRNAs stimulated cardiomyocyte proliferation in a yes-associated protein (YAP)-dependent manner in hiPSC-CMs (Diez-Cunado et al., 2018).

Other studies have implicated additional miRNAs in cardiomyocyte proliferative induction, including the miR-17-92 cluster, miR-25, miR-31a, miR-204, miR-222, miR-294, miR-302-367 cluster, miR-499 and miR-1825. The miR-17-92 cluster, one of the best-characterized miRNA clusters, encodes six mature miRNA members including miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a (Mogilyansky and Rigoutsos, 2013). A recently study has shown that overexpression of the miR-17-92 cluster induces cardiomyocyte proliferation in embryonic, postnatal as well as in adult heart and rescues cardiac function after MI by inhibiting phosphatase and tensin homolog (PTEN) (Chen et al., 2013; Shi et al., 2017). In contrast, inhibition of miR-17 *in vivo* was shown to attenuate excise-induced cardiac growth and cardiomyocyte proliferation (Shi et al., 2017). The combination of miR-19a and miR-19b led to increased cardiomyocyte proliferation and protected

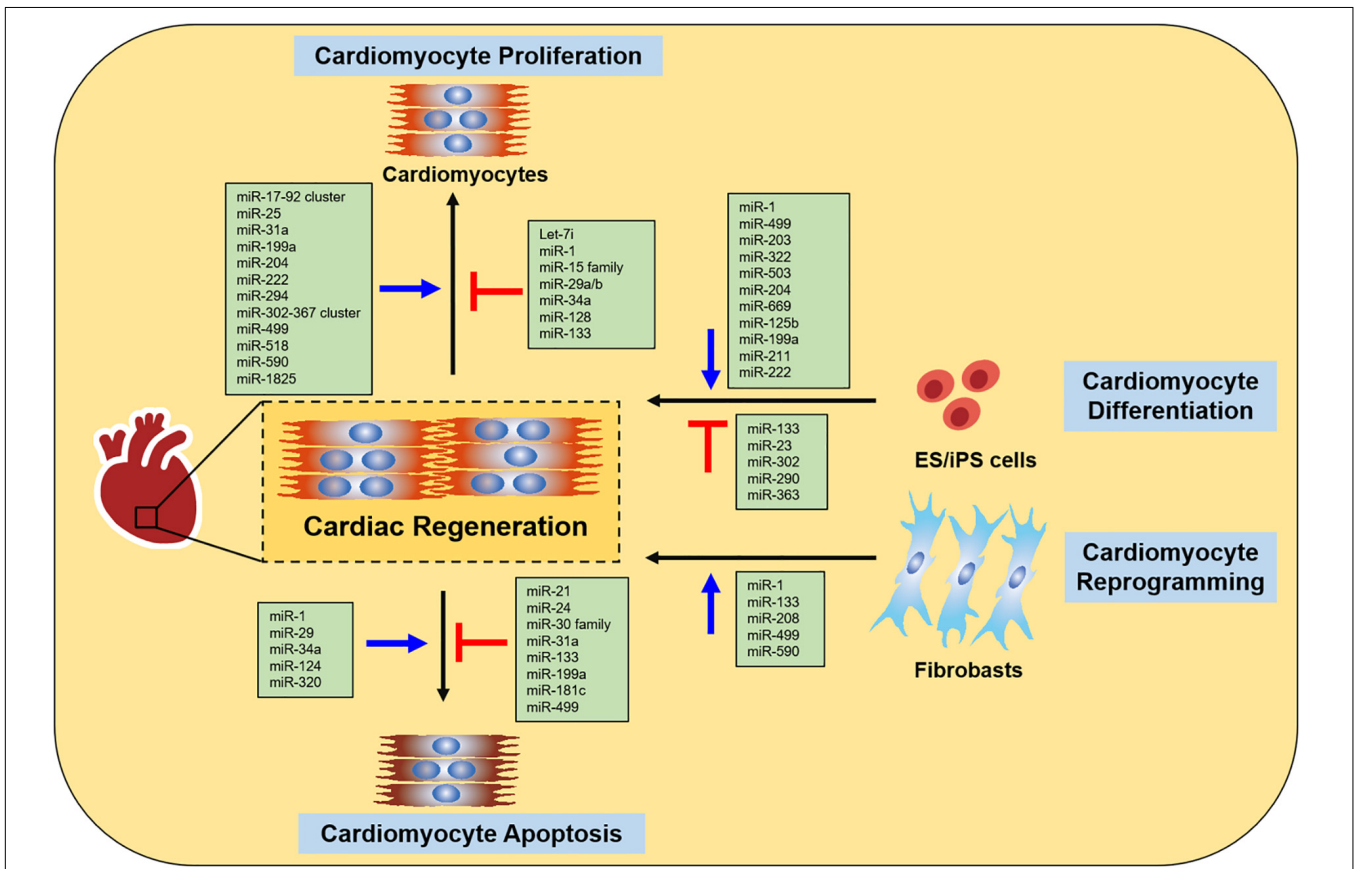

FIGURE 1 | miRNAs in cardiac regeneration. The figure summarizes the function of miRNAs in regulating cardiac regeneration, including cardiomyocyte proliferation, cardiomyocyte differentiation, cardiomyocyte reprogramming and cardiomyocyte apoptosis.

heart against MI in mice (Gao et al., 2019). miR-25, which has the same seed sequences as miR-92a, is also able to stimulate cardiomyocyte proliferation in neonatal rat cardiomyocytes (NRCs) by targeting the Bcl-like protein BCL2 like 11 (Bim) (Qin et al., 2019). Another study has also shown that miR-25 overexpression promotes cardiomyocyte proliferation in hPSC-CMs by targeting F-Box And WD Repeat Domain Containing 7 (FBXW7) (Wang et al., 2020). Overexpressing miR-204 improves cardiomyocyte proliferation via targeting Jarid2, resulted in the upregulation of cell cycle regulator Cyclin A, Cyclin B, cyclin D2, Cyclin E, CDC2 and PCNA *in vitro* and *in vivo* (Liang et al., 2015). miR-31a, is significantly upregulated in post-natal day 10 (P10) cardiomyocytes compared to P0, has also been shown to regulate cardiomyocyte cell cycle progression (Xiao et al., 2017). Overexpression of miR-31a in neonatal rat ventricular myocytes (NRVM) and in rat neonates induces cardiomyocyte proliferation by targeting Rho Related BTB Domain Containing 1 (RhoBTB1) (Xiao et al., 2017). In addition, overexpression of miR-222 in mice increased cardiomyocyte proliferation after ischemia/reperfusion (I/R) injury, whereas miR-222 inhibition reduced cardiomyocyte proliferation in response to physical exercise (Liu et al., 2015; Vujic et al., 2018). miR-302-367 cluster is expressed in the embryonic mouse heart and lost in the adult heart, has also been shown to regulate cardiomyocyte proliferation. Loss of miR-302-367 cluster led to decreased cardiomyocyte proliferation in mice, while overexpression of miR-302-367 cluster improved cardiomyocyte proliferation and decreased cardiac fibrosis after MI through repression of the Hippo pathway (Tian et al., 2015). miR-410 and miR-495 both belong to Gtl2-Dio3 miRNAs and have been reported to promote cardiomyocyte proliferation through suppression of CREB binding protein (CBP)/p300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 2 (Cited2) as determined by increased EdU incorporation and Ki67 in neonatal cardiomyocytes (Clark and Naya, 2015). miR-294, which is expressed in the heart during development, increased cardiomyocyte proliferation, improved cardiac function and decreased infarct size after MI via suppression of Wee1 *in vivo* (Borden et al., 2019). miR-499, a myocyte-specific miRNA (myomiR) is expressed within the one of the introns of β-myosin heavy chain (Myh7b) gene, could also promote neonatal cardiomyocyte proliferation by regulating SRY-Box Transcription Factor 6 (Sox6) and cyclin D1 (Li et al., 2013). A recent study demonstrated that miR-1825 increased both DNA synthesis and cytokinesis in adult cardiomyocytes and improve cardiac function after MI (Pandey et al., 2017).

In addition to miRNAs that promote cardiomyocyte proliferation, several miRNAs that are endogenously expressed in cardiomyocytes have been shown to suppress cardiomyocyte proliferation, including let-7i, miR-1, miR-15 family, miR-29a/b, miR-34a, miR-128, miR-128, miR-133 (Braga et al., 2020). Let-7 was one of the first miRNAs isolated and characterized in the nematode *C. elegans*. Overexpression of let-7i inhibits cardiomyocyte proliferation, whereas the suppression of let-7i induces cardiomyocyte proliferation and improves cardiac function in response to MI by enhancing E2F Transcription Factor 2 (E2F2) and Cyclin D2 (Hu et al., 2019). miR-1 and miR-133 are highly conserved and expressed in cardiac and skeletal muscle and are transcriptionally regulated by myogenic transcription factors MyoD, myocyte enhancer factor-2 (Mef2) and serum response factor (SRF) (Zhao et al., 2007). Overexpression of miR-1 in the developing hearts leads to decreased cardiomyocyte proliferation by targeting the heart and neural crest derivatives-expressed protein 2 (Hand 2) transcription factor (Zhao et al., 2005). Conversely, miR-1 deletion increases cardiomyocyte proliferation in the adult hearts (Zhao et al., 2007). Similar to miR-1, overexpression of miR-133a restricts cardiomyocyte proliferation in zebrafish (Yin et al., 2012), while miR-133a inhibition increases cardiomyocyte proliferation in adult hearts (Liu et al., 2008). The miR-15 family (including miR-15a, miR-15b, miR-16-1, miR-16-2, miR-195 and miR-497), modulates neonatal heart regeneration through inhibition of postnatal cardiomyocyte proliferation and repression of cell cycle genes, thus resulting in postnatal loss of cardiac regenerative capacity (Porrello et al., 2013). Inhibition of miR-15 family from the early postnatal period until adulthood promotes cardiomyocyte proliferation and enhances cardiac function in response to MI (Hullinger et al., 2012; Porrello et al., 2013). Overexpression of miR-195 in the embryonic heart suppresses cardiomyocyte proliferation and impairs the regeneration capability of 1-day-old mouse heart after MI through the repression of a number of cell cycle genes, including checkpoint kinase 1 (Chek1) *in vivo* (Porrello et al., 2011a, 2013). Inhibition of miR-26a increased proliferation of mouse neonatal cardiomyocytes *in vitro* and *in vivo* via regulation of cell cycle inhibitors (Crippa et al., 2016). Overexpression of miR-29a suppresses cardiomyocyte proliferation, while its inhibition enhances cell division by inducing cyclin D2 expression in NRCs (Cao et al., 2013). Similar to miR-29a, inhibition of miR-29b promotes cardiomyocyte proliferation by inactivation of notch receptor 2 (NOTCH2) function *in vitro* and *in vivo* (Yang et al., 2020). miR-34a, a regulator of age-associated physiology, also suppresses cardiomyocyte proliferation and its inhibition leads to enhanced cardiomyocyte proliferation and improves cardiac function in response to MI through targeting of silent information regulator factor 2 related enzyme 1 (Sirt1), B-cell lymphoma 2 (Bcl2) and Cyclin D1 (Yang et al., 2015). Inhibition of miR-128 promotes cardiomyocyte proliferation by activating cyclin E- and cyclin dependent kinase 2 (CDK2)-positive cell cycle regulators and improves cardiac function in response to MI (Huang et al., 2018).

miRNAs and Cardiac Differentiation

In addition to enhancing the proliferative capacity of endogenous cardiomyocytes, transplanting cardiac stem cells or cardiomyocytes derived from iPSCs or ESCs is another therapeutic approach in the field of cardiac regeneration and is considered a promising strategy to heart failure. Pluripotent stem cells provide great promise for regenerative medicine due to their self-renewal potential and ability to differentiate into multiple cell lineages or specific functional cell types. Several studies have shown that miRNAs play a key role in differentiation of stem cells, including miR-1, miR-23, miR-125b, miR-133,

miR-199a, miR-203, miR-204, miR-211, miR-222, miR-290, miR-302, miR-499, miR-669.

miR-1 and miR-499 are also highly enriched in cardiomyocytes and upregulated in differentiated cells. Studies have shown that miR-1 promotes cardiac differentiation from ESCs by the downregulation of Notch ligand Delta-like 1 (Chen et al., 2006). Moreover, overexpression of miR-1 itself is sufficient to promote cardiac differentiation mediated by upregulation of the cardiac differentiation genes nk2 homeobox 5 (NKX2.5) and myosin heavy chain beta (β-MHC) in mouse and human ESCs (Ivey et al., 2008). In addition, Sluijter and colleagues have also shown that miR-1 overexpression enhances human cardiomyocyte progenitor cells (hCMPCs) differentiation into cardiomyocytes by repression of Histone Deacetylase 4 (HDAC4) (Sluijter et al., 2010). Furthermore, transplantation of mouse ESCs overexpressing miR-1 (miR-1-ES cells) into the border zone of the infarcted hearts significantly promotes cardiac differentiation and protects against MI-induced apoptosis through activation of PI3K/Akt pathway (Glass and Singla, 2011). Similarly, miR-499 overexpression enhances differentiation of cardiac progenitors into cardiomyocytes in human CMPCs and ESCs by inhibition of Sox6 in human CMPCs (Sluijter et al., 2010). In addition, overexpression of miR-499 significantly enhances cardiac differentiation in human ESC-derived cardiomyocytes by upregulation of cardiac myosin heavy chain genes and cardiac transcription factor MEF2C (Wilson et al., 2010).

In addition to miR-1 and miR-499, overexpression of miR-203 improves efficient differentiation and maturation to cardiomyocytes by repression of DNA methyltransferase 3a/b (Dnmt3a/b) in mouse iPSCs (Salazar-Roa et al., 2020). miR-322 and miR-503 clusters have shown that participate in the specification into cardiomyocyte progenitors of the mesodermal cells by the repression of CUGBP Elav-like family member (Celf) family proteins (Shen et al., 2016). miR-204, miR-669 and miR-23 have also been shown to promote cardiac progenitor differentiation (Crippa et al., 2011; Xiao et al., 2012). Furthermore, recently another study shows that a combination of miRNAs (miR-125b, miR-199a, miR-221 and miR-222) promotes the maturation of both mouse and human cardiomyocytes differentiated from ESCs (Lee et al., 2015).

miR-1 and miR-133 are co-transcribed due to their polycistronic clustering on the same chromosome. However, they have antagonistic effects on lineage commitment and terminal differentiation, as miR-133 inhibits cardiomyocyte terminal differentiation and maintains them in a proliferative state (Chen et al., 2006). Overexpression of miR-133 suppresses the expression of cardiac markers in human and mouse ESCs, and inhibits differentiation of ESCs into CMs (Alfar et al., 2018), while miR-133 deletion promotes cardiomyocyte proliferation partly via activation of SRF and G1/S-specific cyclin D2 (Chen et al., 2006; Liu et al., 2008), thus highlighting the inhibitory role of miR-133 in both cardiomyocyte differentiation and proliferation. Recently, a study revealed that inhibition of miR-23 facilitated the transformation of bone marrow mesenchymal stem cells (BMSCs) into myocardial cells and conferred protection against Ischemia/reperfusion (I/R)-induced myocardial damage by activation of Wingless and Int-1 (Wnt) pathway (Lu et al., 2019). miR-302/miR-290 members are involved in the maintenance of pluripotency of murine ES cells, and the members of these families have been shown to suppress cell differentiation (Judson et al., 2009; Melton et al., 2010). In addition, miR-363 negatively regulates in cardiomyocyte specification, miR-363 inhibition leads to an enrichment of left ventricular ESC-derived cardiomyocytes by targeting Hand 1 (Wagh et al., 2014).

Taken together, these studies demonstrate that miRNAs regulate cardiomyocyte differentiation and help balance cardiomyocyte differentiation with proliferation, which could be further translated clinically by using miRNAs to induce transferred ESCs/iPSCs to differentiate into mature cardiomyocytes to replace damaged cardiomyocytes.

miRNAs and Cardiac Reprogramming

There are two methods for cardiomyocyte reprogramming: indirect reprogramming and direct reprogramming. The indirect reprogramming uses an intermediary step where the non-myocytes dedifferentiate into progenitor cells that then could be further programmed into the cardiomyocytes (i.e., iPSC reprogramming). The direct reprogramming directly converts the non-myocytes into cardiomyocytes without passing through the pluripotent state (Farber and Qian, 2020). Over the last decade, direct reprogramming from cardiac fibroblasts of the infarcted area into cardiomyocytes has been successful in the repair of damaged heart tissue, and has great promise for the clinic.

miRNAs are able to directly induce the cellular reprogramming of cardiac fibroblasts into cardiomyocytes *in vitro* and *in vivo* (Rao et al., 2006; Jayawardena et al., 2012; Joladarashi et al., 2014). Recently, Jayawardena et al. has identified and evaluated a specific combination of miRNAs (miR-1, miR-133, miR-208 and miR-499; miR combo) which is able to directly reprogram cardiac fibroblasts into cardiomyocyte-like cells *in vitro* and *in vivo* (Jayawardena et al., 2012). *In vitro*, the reprogrammed cardiomyocyte-like cells express cardiomyocyte-specific genes, and exhibit sarcomeric organization and functional properties characteristic of mature cardiomyocytes (Jayawardena et al., 2012). Furthermore, injection of lentivirus miR combo into the border zone of the infarcted heart also induced generation of new cardiomyocyte-like cells from lineage-traced non-cardiac myocytes by 4 weeks post myocardial injury (Jayawardena et al., 2012). In addition, miR-combo significantly decreases cardiac fibrosis and improves cardiac function, as indicated by the improvement of fractional shortening (FS) and ejection fraction (EF) following MI (Jayawardena et al., 2015). These data demonstrate that administration of miRNAs to the peri-infarct area of the infarcted heart *in vivo* is able to direct reprogramming of cardiac fibroblasts into functional cardiomyocytes.

Recent studies demonstrate that co-overexpression of the GATA binding protein 4 (Gata4), Mef2c, and T-box transcription factor 5 (Tbx5), termed GMT, leads to the reprogramming of cardiac fibroblasts into cardiomyocytes *in vitro* and *in vivo* (Ieda et al., 2010; Qian et al., 2012). Additionally, they observe that the combination of miR-133 and GMT further enhanced

cardiac reprogramming of mouse embryonic fibroblasts into cardiomyocytes in terms of kinetics and efficiency by directly binding to the snail family transcriptional repressor 1 (Snail 1) (Jayawardena et al., 2012). Interestingly, a study by Singh and colleagues reveal that miR-590, which is a key miRNA in cardiomyocyte proliferation, could also further enhance GMT-mediated reprogramming in human and pig fibroblasts by suppressing the Sp1 transcription factor (Sp1), that can inhibit the activity of cardiac-specific genes (Singh et al., 2016). In addition, another study shows that the combination of miR-1, miR-133 and Gata4, Mef2c, TBx5, Myocardin could further increase the direct reprogramming efficiency of human fibroblasts to cardiomyocyte-like cells (Nam et al., 2013).

miRNAs and Cardiomyocyte Survival

Cardiomyocyte survival is recognized as being a central feature of cardiac remodeling and progression in heart failure (Briasoulis et al., 2016). The underlying triggers of cell survival and development of novel treatment strategies to suppress cardiomyocyte apoptosis is important. Cardiomyocyte apoptosis can be triggered by the activation of two major signaling pathways: the intrinsic and extrinsic pathways. The intrinsic pathway is mediated by mitochondrial membrane permeabilization and the extrinsic pathway involving the activation of surface death receptor by death ligands (Bennett, 2002). Several miRNAs have been shown to pro-apoptotic and anti-apoptotic regulate apoptotic signaling pathways, which may have to effect on survival of cardiomyocytes. miR-21, miR-24, miR-30 family, miR-31a, miR-133, miR-138, miR-199a, miR-181c, and miR-499 are main miRNAs that could inhibit cardiomyocyte apoptosis. The miR-1, miR-29, miR-34a, miR-124, and miR-320 exhibit an pro-apoptotic effect in cardiomyocytes.

Many studies demonstrate that miR-21 promotes cardiac survival and attenuates cardiomyocyte apoptosis *in vitro* and *in vivo*. Overexpression of miR-21 protects against hydrogen peroxide (H_2O_2)-induced cardiac apoptosis via inhibition of programmed cell death protein 4 (pDCD4) and activator protein 1 (AP-1) pathway *in vitro* (Cheng et al., 2009). miR-21 is decreased in the infarct zone after MI, while miR-21 overexpression in rat hearts reduced cardiac fibrosis and decreased cardiomyocyte apoptosis in the infarct and border zone after AMI (Dong et al., 2009; Yan et al., 2015). Furthermore, overexpression of miR-21 alone significantly inhibits autophagic activity and alleviates hypoxic/reoxygenation (H/R)-induced cardiac apoptosis by regulation of PTEN/Akt/mTOR pathway (Huang et al., 2017). In addition, combination of miR-21 and miR-146 could further decrease ischemic/Hypoxic-induced cardiomyocyte apoptosis compared to either miR-21 or miR-146a alone in NRCs (Huang et al., 2016). Furthermore, simultaneous delivery of agomiR-21 and agomiR-146a in mice attenuates cardiomyocyte apoptosis and improves cardiac function following AMI by regulation of p38 MAPK (Huang et al., 2016). *In vivo* studies show that overexpression of miR-24 protects against cardiomyocyte apoptosis and mediated by repression of BH3-domain-caontaining protein (Bim) in response to MI in mice (Qian et al., 2011). miR-30 family members, including miR-30a, miR-30b and miR-30d, inhibits mitochondrial fission through suppressing the expression of p53 and downstream targets dynamin-related protein-1 (Drp1), leading to decreased cardiomyocytes apoptosis (Li et al., 2010). Furthermore, doxorubicin-induced apoptosis could be rescued by miR-30 expression in rat cardiomyocytes *in vivo* (Roca-Alonso et al., 2015). miR-31a is upregulated in the failing heart, overexpression of miR-31a protects against the angiotensin II (AngII)-induced apoptosis and caspase-3 activity by targeting Tp53 in the cardiac H9C2 cell line (Yan et al., 2018). Gain-of -function studies demonstrate that miR-133 overexpression inhibits cardiac apoptosis and enhances cardiac function following chronic pressure overload. miR-133 overexpression decreases I/R-induced apoptosis, whereas miR-133 deletion increases cardiac apoptosis mediated by regulating the expression of casp9 (He et al., 2011). miR-199a also plays a crucial role in hypoxia-induced apoptosis (Rane et al., 2009). Replenishing miR-199a during hypoxia decreases cardiomyocytes apoptosis through inhibition of hypoxia-inducible factor (Hif)-1α and its stabilization of p53, while miR-199a inhibition recapitulates hypoxia preconditioning by up-regulating Hif-1α and Sirt1 (Rane et al., 2009). miR-181c is suppressed in the heart tissue of doxorubicin (DOX)-induced heart failure animal model (Li et al., 2020). Study shows that miR-181c overexpression protects heart failure by impeding cardiomyocyte apoptosis through PI3K/Akt pathway (Li et al., 2020). Overexpression or knockdown of miR-499 decreases or increases the cardiomyocyte apoptosis *in vitro* (Li et al., 2016). In addition, overexpression of miR-499 decreases cardiac apoptosis and reduces myocardial infarct size in the rat AMI models by inhibiting PDCD4 (Li et al., 2016).

In addition to miRNAs that reduce cardiomyocyte apoptosis, there are several miRNAs that exert the opposite effect on cardiomyocyte apoptosis. miR-1, which has been shown to play an essential role in the regulation of cardiac proliferation and differentiation, also plays a critical role in cardiomyocyte apoptosis. Overexpression of miR-1 in mice increases cardiomyocyte apoptosis, while deletion of miR-1 reduces I/R-induced cardiomyocyte apoptosis and attenuates cardiac I/R injury by targeting protein kinase C epsilon (PKCε) (Pan et al., 2012). Overexpression of miR-29 promotes apoptosis, whereas inhibition of miR-29 reduces cardiomyocyte apoptosis and infarct size in hearts subjected to I/R injury by decreasing the expression of pro-apoptotic molecular Bcl-2-associated X protein (Bax) and increasing anti-apoptotic molecular Bcl2 (Ye et al., 2010). miR-34a is induced in the heart during aging in both mice and humans, and its deletion *in vivo* reduces age-induced cardiomyocyte cell death and fibrosis, and improves cardiac function after AMI by activation of serine/threonine-protein phosphatase 1 regulatory subunit 10 (PNUTS) (Boon et al., 2013). miR-124 is upregulated in a mice model of MI, inhibition of miR-124 decreases MI-induced cardiomyocyte apoptosis and infarct size and improves cardiac function in mice by upregulating signal transducer and activator of transcription 3 (STAT3) (He et al., 2018). miR-320 is significantly decreased in the hearts with I/R injury *in vivo* and *ex vivo*, and its overexpression enhances cardiomyocyte apoptosis and death in the hearts on I/R (Ren et al., 2009). Conversely, administration of antagomir-320

reduces I/R-induced cardiac injury and cardiomyocyte apoptosis by targeting heat-shock protein 20 (Hsp20) (Ren et al., 2009).

Together, these studies indicate that miRNAs can effectively stimulate cardiac regeneration by directly modulating target gene expression, and provide promising a therapeutic avenue for heart failure treatment. To date, a large number of miRNAs have been discovered to regulate cardiac regeneration. However, the molecular targets and mechanisms underlying miRNA function needs to be further investigated, and the miRNA-based preclinical and clinical trials need to be better and more stringently assessed for therapeutic efficacy.

PART 3: LNCRNAS AND CARDIAC REGENERATION

Long non-coding RNAs (LncRNAs) are defined as RNA transcripts over 200 nucleotides in length with no evidence for a protein-coding function (Mercer et al., 2009). There are our major classes of lncRNAs: Antisense lncRNAs, Bidirectional lncRNAs, Intergenic lncRNAs and Sense-intronic lncRNAs. The role of lncRNAs in controlling cardiac regeneration has only been studied in recent years.

Similarly to miRNAs, lncRNAs have been shown to regulate cardiomyocyte proliferation either by enhancing or suppressing cell cycle progression (**Table 1**). The Sirt1 antisense lncRNA can bind to and stabilize the 3′UTR of the Sirt1 mRNA, and gain-/ loss-of-function studies of Sirt1 antisense lncRNA demonstrate that it regulates cardiomyocyte proliferation *in vitro* and *in vivo*, and inhibition of Sirt1 antisense lncRNA suppresses cardiomyocyte proliferation (Li B. et al., 2018). Furthermore, Sirt1 antisense lncRNA overexpression promotes cardiomyocyte proliferation, improves cardiac function and decreases mortality rate after MI by interacting and stabilizing Sirt1 mRNA (Li B. et al., 2018). An lncRNA NR_045363, which is mainly expressed in cardiomyocytes and rarely non-cardiomyocytes, has been shown to stimulate cardiomyocyte proliferation and improve cardiac function in response to MI through interaction with miR-216a (Wang et al., 2019). Another recent work demonstrates that lncRNA endogenous cardiac regeneration-associated regulator (ECRAR), which is upregulated in human fetal heart, promotes DNA synthesis and cytokinesis in P7 as well as in adult rat cardiomyocytes (Chen et al., 2019). Moreover, Overexpression of ECRAR significantly stimulates cardiac regeneration and restores cardiac function after MI by targeting extracellular signal-regulated kinases 1 and 2 (ERK1/2) signaling pathway (Chen et al., 2019).

In addition to lncRNAs that promote cardiac regeneration, there are also lncRNAs that suppress cardiomyocyte proliferation. The antizyme inhibitor 2 (AZIN2)-sv lncRNA is a splice variant of the AZIN2 gene that represses endogenous cardiomyocyte proliferation *in vitro* and *in vivo* (Li X. et al., 2018). In contrast, loss of AZIN2-sv promotes cellular survival and proliferation, attenuates ventricular remodeling and

TABLE 1 | LncRNAs in cardiac regeneration.

LncRNAs	Genomic context	Target genes	Mechanisms	Related functions	References
AZIN2-sv	Antisense	miR-214	Increase the level of PTEN and inhibit Akt/PKB signaling pathway	Repress cardiomyocyte proliferation	Li X. et al., 2018
Braveheart	Intergenic	miR-143/145	Directly interact with SUZ12 during cardiomyocyte differentiation	Maintain cardiac fate in neonatal cardiomyocytes	Klattenhoff et al., 2013
CAREL	Intergenic	miR-296	A sponge for miR-296, repress miRNA-296, following by activate Trp53inp1 and Itm2a	Repress cardiomyocyte proliferation and differentiation	Cai et al., 2018
CARMEN	Intergenic	Unknown	Interact with SUZ12 and EZH2	Promote cardiac specification and differentiation	Ounzain et al., 2015
CARL	Intergenic	miR-539	Act as an endogenous miR-539 sponge that regulates PHB2 expression, mitochondrial fission and apoptosis	Suppress Cardiomyocyte apoptosis	Wang et al., 2014
CRRL	Intergenic	miR-199a-3p	Protect Hopx from degeneration of CRRL	Suppress cardiomyocyte proliferation	Chen et al., 2018
CPR	Intergenic	DNMT3A	Interact with DNMT3A to repress the level or MCM3, promotes its methylation and inhibits its expression	Repress cardiomyocyte proliferation	Ponnusamy et al., 2019
DACH1	Intronic	PP1A	Enhance YAP1 phosphorylation and reduce ist nuclear translocation by binding PP1A	Repress cardiomyocyte proliferation	Cai et al., 2020
ECRAR	Antisense	ERK1/2	Promote the expression of cyclin D1, cyclin E1, and E2F1 proteins via ERK1/2 pathway	Promote cardiomyocyte proliferation	Chen et al., 2019
H19	Intergenic	miR-675	Regulate the expression of proliferation-associated protein 2G (PA2G4) Regulate the expression of MyoD, Myf6 and Mier2	Inhibit cardiomyocyte apoptosis Repress cardiac differentiation	Zhang et al., 2017 Ragina et al., 2012
Meg3	Intergenic	miR-145	Direct bind with RNA-binding protein FUS	Promote cardiomyocyte apoptosis	Wu et al., 2018; Chen et al., 2019
NR_045363	Intergenic	miR-216a	Promote JAK2/STAT3 signaling pathway	Promote cardiomyocyte proliferation	Wang et al., 2019
Sirt1	Antisense	Sirt1	Deacetylate and inhibit the activity of Nkx2.5, stabilized and increase the Sirt1 mRNA expression	Enhance cardiomyocyte proliferation Decrease cardiomyocyte apoptosis	Li B. et al., 2018
ST8SIA3 (RoR)	Intergenic	Unknown	Regulate the expression of genes involved in P53 response	Enhance the reprogramming of fibroblasts to cardiomyocytes	Loewer et al., 2010

improves cardiac function after AMI by targeting phosphatase and tensin homology (PTEN), which blocked activation of the PI3K/Akt pathway (Li X. et al., 2018). A recent work indicates that CRRL lncRNA also suppresses cardiomyocyte proliferation by binding miR-199a-3p, while CRRL knockdown promotes neonatal rat cardiomyocyte proliferation both *in vitro* and *in vivo* (Chen et al., 2018). Loss of CRRL attenuates post-AMI remodeling and restores cardiac function in adult rats (Chen et al., 2018). Overexpression of lncRNA CAREL in mice reduces cardiomyocyte proliferation by directly binding to miR-296 (Cai et al., 2018). Conversely, loss of CAREL markedly promotes cardiac regeneration and improves cardiac function after AMI in both neonatal and adult mice (Cai et al., 2018). LncRNA cardiomyocyte proliferation regulator (CPR) overexpression remarkably suppresses neonatal cardiomyocyte proliferation and cardiac regeneration in mice (Ponnusamy et al., 2019). Conversely, CPR depletion significantly increases cardiomyocyte proliferation and restores cardiac function in postnatal and adult hearts in response to AMI in mice by directly interacting and recruiting DNMT3A to its promoter cysteine-phosphate-guanine sites (Ponnusamy et al., 2019). LncRNA dachshund homolog 1 (lncDACH1) is elevated in the postnatal hearts, and its overexpression suppresses cardiomyocyte proliferation *in vitro* and *in vivo* (Cai et al., 2020). In contrast, *in vivo* cardiac conditional knockout of lncDACH1 increases cardiomyocyte proliferation and promotes myocardial regeneration after AMI by directly binding to the protein phosphatase 1 (PP1) catalytic subunit (Cai et al., 2020). LncRNA H19 is highly conserved and downregulated in failing hearts (Viereck et al., 2020), and its inhibition promotes cardiomyocyte proliferation in P19CL6 cells during late-stage cardiac differentiation (Han et al., 2016).

Along with regulating cardiomyocyte proliferation, several lncRNAs also regulates cardiac differentiation and cardiac reprogramming. Downregulation of lncRNA H19 promotes differentiation of parthenogenetic embryonic stem cells (p-ESCs) into a higher percentage of beating cardiomyocytes (Ragina et al., 2012). Braveheart, a heart-associated lncRNA in mouse, maintains cardiac fate in neonatal cardiomyocytes by interacting with SUZ12 polycomb repressive complex 2 subunit (SUZ12), a component of polycomb-repressive complex 2 (PRC2) (Klattenhoff et al., 2013). LncRNA-ST8SIA3 (lncRNA-ROR), is a iPSC-enriched lncRNA, enhances the reprogramming of fibroblasts to cardiomyocytes (Loewer et al., 2010). Recent study shows that lncRNA CARMEN is an important regulator of cardiac differentiation, CARMEN knockdown inhibits cardiac specification and differentiation in human CPCs (Ounzain et al., 2015).

Long non-coding RNAs CARL, lncRNA maternally expressed 3 (Meg3) and lncRNA H19 are involved in cardiomyocyte apoptosis. Sirt1 antisense lncRNA not only regulates cardiomyocyte proliferation, but also regulates cardiomyocyte apoptosis. Overexpression of Sirt1 antisense lncRNA attenuates cardiomyocyte apoptosis and decrease mortality rate after AMI *in vivo* (Li B. et al., 2018). LncRNA CARL suppresses cardiomyocyte apoptosis by targeting miR-539 and PHB2 (Wang et al., 2014). LncRNA Meg3, is upregulated in the mouse heart after AMI, overexpression of Meg3 promotes cardiomyocyte apoptosis via its direct binding to RNA-binding protein FUS (Wu et al., 2018). Conversely, Meg3 deletion reduces AMI-induced cardiomyocyte apoptosis and improves cardiac function *in vivo* (Wu et al., 2018). Downregulation of lncRNA H19 inhibits cardiomyocyte apoptosis by regulating miR-19b and Sox6 in mouse P19CL6 cells (Han et al., 2016). Furthermore, knockdown of lncRNA H19 weakens cardiomyocyte apoptosis and improves myocardial function in adriamycin-induced dilated cardiomyopathy (DCM) by targeting miR-675 and Proliferation-associated protein 2G (PA2G4) (Zhang et al., 2017).

In summary, lncRNAs provide a new approach in the regulation of cardiac regeneration. However, little is known about the various and intricate regulatory mechanisms of the lncRNAs in cardiac regeneration and heart failure, which will be of particular interest in the near future.

PART 4: CIRCULAR RNAS AND CARDIAC REGENERATION

Circular RNA (circRNA) is a special subclass of ncRNAs characterized by a covalently closed loop structure, lacking 3′ poly(A) tails and 5′ cap structures (Mester-Tonczar et al., 2020). circRNAs are highly conserved across multiple speciesand participate in various biological processes of the cardiovascular system such as cardiomyocyte hypertrophy, cardiac regeneration and cardiac development, re, and apoptosis, which suggests that circRNAs may play a critical role in heart failure (Abbas et al., 2020). Although investigation of the role of circRNAs in cardiac regeneration has just commenced in recent years, some encouraging and promising data have already been collected, including circCDYL (Zhang et al., 2020), circ-Amot1 (Zeng et al., 2017), circFndc3b (Garikipati et al., 2019), and circNfix (Huang et al., 2019; **Table 2**).

Circular RNA circCDYL is downregulated in myocardial tissue after AMI and hypoxia myocardial cells, and also triggers cardiomyocyte proliferation. overexpression of circCDYL promotes cardiomyocyte proliferation *in vitro*, while circCDYL downregulation reduces cardiomyocyte proliferation (Zhang et al., 2020). Moreover, *in vivo* study shows that circCDYL also promotes cardiomyocytes proliferation and improves cardiac function after MI by interacting with miR-4793-5p (Zhang et al., 2020).

TABLE 2 | Circular RNAs in cardiac regeneration.

Circular RNAs	Mechanisms	Related functions	References
Amot1	Bind to PDK1 and AKT1	Attenuate cardiomyocyte apoptosis	Zeng et al., 2017
circCDYL	Interact with miR-4793-5p	Promote cardiomyocyte proliferation	Zhang et al., 2020
circNfix	Promote Ybx1 ubiquitin-dependent degradation and miR-214 sponge	Suppress cardiomyocyte proliferation Promote cardiomyocyte apoptosis	Huang et al., 2019
Fndc3b	Bind to FUS	Decrease cardiomyocyte apoptosis	Garikipati et al., 2019

Circ-Amot1 is highly expressed in neonatal human cardiac tissue, overexpression of circ-Amot1 protects against doxorubicin-induced apoptosis and cardiomyopathy by binding to PDK1 and AKT1 *in vivo* (Zeng et al., 2017). Circular RNA circFndc3b is downregulated in cardiac tissues of ischemic cardiomyopathy patients, and modulates cardiac regeneration (Garikipati et al., 2019). Cardiac overexpression of circFndc3b *in vivo* attenuates cardiomyocytes apoptosis and hereby ameliorates cardiac function after MI by interacting with FUS (Garikipati et al., 2019). Circular RNA circNfix is a super enhancer-associated circRNA that is highly conserved in the hearts of rodents and humans (Huang et al., 2019). Overexpression of circNfix inhibits cardiomyocytes proliferation *in vitro* and *in vivo*, whereas circNfix downregulation promotes cardiomyocytes proliferation and decreases cardiomyocytes apoptosis after MI, attenuating cardiac dysfunction by suppressing Y-box binding protein 1 (Ybx1) ubiquitin-dependent degradation and increasing miR-214 activity (Huang et al., 2019).

To date, the field of circRNAs research is still in its infancy, particularly in the cardiac regeneration. In the future, exploration into the effects and molecular mechanisms of circRNAs on cardiac regeneration may provide new insights and therapeutic possibilities for the treatment of heart failure.

PART 5: NCRNAS AS BIOMARKERS AND THERAPEUTIC TARGETS

The adult human heart has a limited capacity to regenerate new cardiomyocytes during myocardial infarction and aging. Therefore, the endogenous cardiomyocyte renewal rate is unable to restore lost cardiomyocyte and to preserve cardiac function under physiological and pathological conditions. Benefited from the molecular and cellular discoveries as well as promising preclinical outcomes in cardiac regeneration, give us optimism in terms of regenerative therapies for the failing human heart. With the development of bioinformatics and emerging technologies, a large number of ncRNAs have been identified to regulate cardiac regeneration and are considered to be potential therapeutic targets in heart failure over the past 10 years. However, the development of ncRNAs therapy in heart failure still remains challenging.

MicroRNAs are considered ideal therapeutic targets in heart failure (Elzenaar et al., 2013). Currently, there are two general strategies to manipulate miRNAs. One is delivery of miRNA mimics or viral vector-mediated miRNA overexpression to augment the effects of miRNAs, another is to suppress the function of miRNAs by injecting anti-miRs, including antagomiRs or locked nucleic acid (LNA)-based anti-miRs (Ooi et al., 2014). AntagomiRs are 3′-cholesterol-conjugated, 2′-O-Me, 2′-fluoro, or 2′O-methoxyethyl RNA oligos of about 20–25 nucleotides, with an additional phosphorothioate backbone (Krutzfeldt et al., 2005), while LNA-based anti-miRs are antisense RNAs with several nucleotides substituted by bicyclic RNA analogs in a "locked" conformation (Lundin et al., 2013). Various studies have convincingly showed that miRNAs may improve cell therapy or enhance endogenous cardiac repair processes, and LNA-based anti-miRs have already been used in large animal models and a first clinical trial. that. For example, LNA-based anti-miR-132 treatment improves ejection fraction (EF) and ameliorates cardiac dysfunction in response to MI in pig model (Foinquinos et al., 2020). Furthermore, recent a clinical study has been launched to test the safety of CDR132L, a synthetic LNA-based antisense oligonucleotide against miR-132 (anti-miR-132), directly in heart failure patients[1] (NCT04045405), and the results show that CDR132L is able to reverse heart failure *in vivo* (Foinquinos et al., 2020). In addition, study shows that anti-miR-92a increases cardiac regeneration, reduces infarct size and protects against Ischemia-reperfusion (I/R) injury in pig model (Hinkel et al., 2013). MRG-110 is a LNA-based antisense oligonucleotide that targets miR-92a. Recently, MRG-110 has been tested in healthy human adults and MRG-110 treatment reduces miR-92a level and de-represses the target genes in human peripheral blood cells (European Clinical Trials Database [EudraCT] No. 2017-004180-12) (Abplanalp et al., 2020). Currently, the miRNA-based LNAs treatment is in clinical trials and receives positive feedback on their potentials, but none of them have been reached in the pharmaceutical breakthrough yet. Therefore, the development of miRNA therapeutic tools is still a long process, and a multitude of companies will put more efforts on the miRNA therapeutics in the next several years to bring them from the bench to the market.

The potential for identifying novel lncRNAs involved in the regulation of cardiac regeneration is fairly high. Unfortunately, the identification of novel lncRNAs is more limited due to the lack of libraries to perform systematic screenings, since all the current lncRNAs regulating cardiac regeneration were identified in the last very few years. It has been shown that lncRNAs can be detected in the plasma or urine and display a dynamic alteration upon initiation and progression of heart failure. Recent studies show that LncRNA long intergenic non-coding RNA predicting cardiac remodeling (LIPCAR), smooth muscle and endothelial cell-enriched migration/differentiation-associated long non-coding RNA (SENCR) and myocardial infarction associated transcript (MIAT) can be detected in the plasma and serve as potential markers of heart failure (Hobuss et al., 2019). To date, the development of lncRNA therapy in heart failure is still in its infancy. However, lncRNAs potentially offer a promising new therapeutic approach to treat heart failure in the future. Therefore, to study the molecular mechanisms of lncRNAs and to identify novel lncRNAs in cardiomyocytes will provide a new insight into understanding of the lncRNAs in heart failure.

The number of ncRNAs implicated in the regulation of cardiac regeneration is certainly expectable to increase in the near future, making ncRNAs-based therapy is a very promising approach for the treatment of heart failure. However, an in-depth and thorough understanding of ncRNAs molecular targets and regulatory mechanisms are necessary to develop more efficient, efficacious, and safe therapeutic strategies for treatment of heart failure patients. Thus, there is an urgent need for further development of ncRNAs for the therapy of Heart failure in both

[1] https://clinicaltrials.gov

animal models and human clinic trials. One miRNA/lncRNA can bind up to several hundreds of mRNAs thereby affecting gene expression networks. In addition, one mRNA can be targeted by several miRNAs/lncRNAs highlighting the complex network of interactions. Therefore, a better understanding the detailed functions and molecular mechanisms of ncRNAs in cardiac regeneration need to be further explored. Additionally, repairing the failing human heart may necessitate a combination of multiple therapeutic approaches. Additionally, repairing the human failing heart may necessitate the use of combinatorial therapeutic approaches to overcome the many innate hurdles of cardiac regeneration. Thus, a combinatorial ncRNA targeting strategy needs to be considered in the path to novel MI therapeutics development.

AUTHOR CONTRIBUTIONS

TY wrote the manuscript and put the Figure and tables together. JK edited the manuscript, the figure and tables, and helped to structure and revise the manuscript. Both authors contributed to the article and approved the submitted version.

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