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Review Article

MicroRNAs in Cardiovascular Regenerative Medicine: Directing Tissue Repair and Cellular Differentiation

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MicroRNAs (miRNAs) are a class of short noncoding RNA molecules, approximately 22 nucleotides in length, which regulate gene expression through inhibition of the translation of target genes. It is now generally accepted that miRNAs guide processes and cellular functions through precise titration of gene dosage, not only for a single gene but also controlling the levels of a large cohort of gene products. miRNA expression is altered in cardiovascular disease and may thereby limit and impair cardiovascular repair responses. Increasing evidence of the essential role of miRNAs in the self-renewal and differentiation of stem cells suggests the opportunity of using the modulation of miRNA levels or their function in directing cell transplantation, cell behavior, and thereby organ healing. In this paper, an overview of miRNA biogenesis and their way of action and different roles that miRNAs play during the myocardial responses to injury and upon cell transplantation will be provided. We focused on cardiomyocyte survival, angiogenesis, extracellular matrix production, and how miRNAs can direct cell plasticity of injected cells and thus drive differentiation for cardiovascular phenotypes, including vascular differentiation and cardiomyocyte differentiation.

1. MicroRNAs: An Introduction

Until recently, cellular gene control was considered to occur via a relatively simple mechanism: DNA is transcribed into mRNA, mRNA is translated into protein, and the protein carries out cellular tasks. However, the discovery of a new class of noncoding RNAs revealed a change in this paradigm, in which many small RNA molecules are not translated into protein (Figure 1). MicroRNAs (miRNAs) are an abundant class of short, noncoding, RNA molecules, approximately 22 nucleotides (nt) in length, which regulate gene expression through inhibition of the translation of target genes [1, 2]. The discovery of the abundance of miRNAs in diverse multicellular species raised many questions, including, perhaps most intriguingly, what these tiny noncoding RNAs may be doing in the cell. The first miRNA *lin-4* was identified in 1993 [3] where *lin-4* RNA had a sequence complementarity to multiple sites within the 3′-untranslated region (UTR) of the *lin-14* mRNA. The UTR is a particular section of messenger RNA (mRNA), which immediately follows the stop codon of the coding region [4]. The impact of these basic discoveries was highlighted by the early assignment of the Nobel Prize for physiology and medicine in 2006 to Prof. Andrew Z. Fire and Craig C. Mello. miRNAs were not recognized as a distinct class of biological regulators with conserved functions until 2001, when these small noncoding regulatory RNAs were first termed miRNAs [1, 2]. These miRNAs were identified as members of a whole new class of abundant, highly conserved posttranscriptional regulators of gene expression and triggered an exciting, and still ongoing, decade of research into the physiological and pathological roles of miRNAs. Each individual miRNA is identified by a unique number according to chronological discovery [5] and belongs to a class of small RNAs, including small interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs), but differs from these other classes of small RNAs since they derive from transcripts that fold back on themselves to form hairpin structures [6]. The power of this new regulatory mechanism lies in the unique ability of miRNAs to guide processes and cellular functions through precise titration of

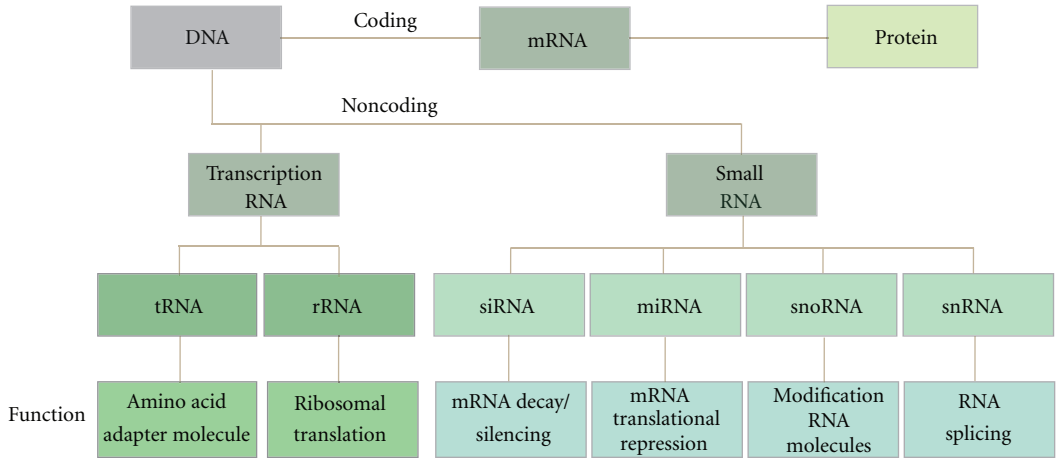

Figure 1: The classical dogma of cellular gene control was relatively simple; DNA is transcribed into mRNA, which is translated into protein and performs cellular tasks. However, the discovery of noncoding RNA molecules changed this classical view and made cellular control more complex.

gene dosage, and the ability of a single miRNA to control the levels of a large cohort of gene products. Due to their way of action, it also reasonable that they are involved or can be used in numerous processes and areas, including regenerative medicine.

Regenerative medicine is an emerging interdisciplinary field whose goal is to repair, replace, or regenerate cells, tissues, or organs that are lost or damaged due to disease, injury, or ageing. Especially the increasing evidence of the essential role of miRNAs in the self-renewal and differentiation of stem cells, including embryonic stem cells, suggests the opportunity of using the modulation of miRNA levels or their function in directing cell transplantation, cell behavior, and thereby organ healing [7].

2. Cardiac Response to Injury and the Role of Cell Therapy

Myocardial infarction (MI) is one of the main contributors to ischemic heart disease, caused by the erosion or rupture of a vulnerable atherosclerotic lesion, and leading to interruption of blood supply and the initiation of a cascade of events [8, 9]. The acute oxygen deprivation causes cardiomyocyte death via both apoptosis and necrosis, [10] which will trigger an inflammatory response [11] that contributes to the removal of cell debris by proteolysis and phagocytosis. Subsequently, cardiac fibroblasts infiltrate the damaged tissue to increase the myocardial tensile strength via production of extracellular matrix in the infarct area and border zone. New blood vessels are formed in the border zone by angiogenesis and vasculogenesis, and preexisting collateral arteries are enlarged by arteriogenesis [12, 13], resulting in partial restoration of blood flow. Because adult cardiomyocytes have a very low intrinsic proliferation [14] rate and cannot replace the substantial cardiomyocyte cell loss, a collagen-rich scar is formed. While scarring results in a relative quick preservation of myocardial integrity, the noncontractile scar impedes restoration of cardiac output. To compensate for the loss of function, the heart will undergo ventricular remodeling and cardiomyocyte hypertrophy, ultimately leading to severely impaired cardiac function and heart failure with limited treatment options.

In recent years, cardiac cell transplantation therapy has been suggested to be a new treatment modality to enhance myocardial regeneration by stem and/or progenitor cell injection and thereby replace the damaged myocardial cells or enhance the endogenous repair mechanisms of the heart [15, 16]. Although for heart failure patients, one aims for true myocyte replacement therapy, in order to increase myocardial muscle mass, current consensus on achieved results is more related to myocardial salvage and stimulation of the endogenous repair mechanisms. For myocardial stem cell therapies many cell sources are suggested [17] and used for their paracrine effects, ranging from bone marrow to adipose derived cells. Cells used for their true myogenic potential are embryonic stem cells and cardiac derived progenitor cells [18, 19] that can develop potentially into autologous human cardiomyocytes. An exciting new development in the field of cardiovascular regenerative medicine is the ability to reprogram adult somatic cells into pluripotent stem cell lines (induced pluripotent stem cells, iPSCs) and to differentiate them into functional cardiomyocytes [20], or even reprogram the fibroblasts directly into functional cardiomyocytes *in vitro* [21] or *in vivo* [22].

Furthermore, emerging data suggest that in cardiovascular disease altered miRNA expression may limit and impair cardiovascular repair responses, including differentiation and function of stem/progenitor cells. Hence, understanding of the physiological and pathophysiological regulation and role of miRNAs in stem and progenitor cells will lead to an improved understanding of the pathophysiology of cardiovascular disease and is likely to provide novel therapeutic targets to promote endogenous cardiovascular repair processes. In this paper, the role of miRNAs that contribute to the myocardial responses to injury and upon cell transplantation is discussed. Here, a focus on cardiomyocyte

survival, angiogenesis, and extracellular matrix production is presented (see Figure 4) and how miRNAs can direct cell plasticity of injected cells and thus drive differentiation for cardiovascular phenotypes, including vascular differentiation and cardiomyocyte differentiation (see Figure 5).

3. MicroRNA

3.1. Biogenesis and Function. MiRNAs are noncoding single-stranded RNAs that target mRNAs through Watson-Crick base pairing between the miRNA seed region, referring to positions 2 to 8 from the 5′ side of the miRNA, and the 3′ untranslated region (UTR) sequences of their target mRNAs [6]. See, for a visualization of miRNA biogenesis, Figure 2. MiRNAs are endogenously expressed from different genomic regions and they may be genomically located between coding genes (intergenic, >1 kb away from annotated/predicted genes), between exons of a single mRNA (intronic), and even cotranscribed from a single locus (polycistronic). The expression of miRNAs is highly regulated and whereas some are ubiquitously expressed, some have a tissue- and cellular-specific expression patterns. This suggests their involvement in a variety of unique biological processes. MiRNAs are generally transcribed by RNA polymerase II into primary miRNA precursor transcripts of a few hundred to thousands of nucleotides, termed pri-miRNAs. Pri-miRNAs are characterized by the presence of one or more imperfect complementary hairpin structures with a stem of approximately 33 base pairs [6]. Approximately half of the known miRNAs are clustered in the genome and transcribed as large primary transcripts containing more than one miRNA, whereas the rest are expressed as individual transcripts from intergenic or intronic locations. After transcription, pri-miRNAs are processed in the nucleus into approximately 70 nt precursor miRNAs (pre-miRNAs) by the double-stranded RNA-specific type III endoribonuclease Drosha, and the double-stranded RNA binding protein DiGeorge syndrome critical region gene 8 (DGCR8 or Pasha in invertebrates) [23, 24]. The nuclear export factor exportin 5 binds correctly processed pre-miRNAs and transports them to the cytoplasm [25] where they are processed by the endoribonuclease III Dicer into a miRNA duplex of approximately 22 base pairs [26]. Dicer is a highly conserved protein that is found in almost all eukaryotic organisms and is assisted by Dicer-interacting proteins that have various roles in miRNA stability, effector complex formation, and action. Once cleaved by Dicer, the duplex unwinds and one strand of the duplex, named the mature miRNA or guide strand, is selected by the Argonaute protein and loaded into the RNA-induced silencing complex (RISC) [27]. Depending on the degree of complementarity between the miRNA seed region and its mRNA target sequence [28] this results in translational blockage or mRNA degradation [29, 30]. In addition to the guided strand, known as the "mature" miRNA, which accumulates to higher levels, there is a less abundant partner that is referred to as the miRNA* or "star" strand. Although not well studied, many miRNA* strands are well conserved and exhibit regulatory capacities as well [31]. In addition to this well-defined mechanism that governs the maturation of most miRNAs in animals, via stepwise cleavage of precursor hairpin transcripts by the Drosha and Dicer RNase III enzymes, several alternative miRNA biogenesis pathways were elucidated in which Drosha cleavage was substituted with splicing, [32–34] but all are aimed at the generation of pre-miRNA hairpins, whose subsequent steps of nuclear export, Dicer cleavage, and loading into Argonaute complexes are shared [32]. Surprisingly, also a role of Drosha cleavage of coding mRNAs was indicated, thereby providing an intersection of different RNA worlds [32]. The molecular processes underlying miRNA-directed translational repression and mRNA destabilization, which include inhibition of translation initiation and poly(A) shortening, are complex and summarized elsewhere [35]. Recently, it was suggested that for most of the decreased protein levels (≥84%), having both ectopic and endogenous miRNA regulatory interactions, a lower mRNA levels could be accounted [29], this indicates that destabilization of target mRNAs is the predominant reason for reduced protein levels.

The use of deep-sequencing technologies has greatly accelerated miRNA discovery and, to date, over 1500 mature miRNAs have been identified in the human genome, many of which are highly conserved among species [36]. MiRNA gene regulatory networks are highly complex, as each specific miRNA can target up to several hundred distinct molecules of mRNA, and each mRNA can be targeted by many different miRNAs, which individually may have multiple target sites in the specific mRNA [37, 38] (see Figures 3(a) and 3(b)). Several open-source software packages are available using different algorithms, based on different assumptions on miRNA-mRNA binding conditions that try to predict recognized miRNA target sequences. The best known examples are mirBase, [39] miRanda, [40] and TargetScan [41]. These tools are based on the extent that when sites are conserved more than would be expected by chance, they are judged to be under selective pressure and therefore biologically functional. However, due to the imperfect nature of binding between miRNAs and targeted mRNAs, these predictions usually provide hundreds of potential targets, but only a fraction of those could be experimentally validated. Although predictions are challenging and every algorithm uses a different concept, some general items can be found. (1) miRNA-mRNA interaction requires conserved Watson-Crick pairing to the 5′ region of the miRNA centered on nucleotides 2–7, which is called the miRNA "seed" and markedly reduced the occurrence of false-positive predictions (see Figure 3(c)). (2) Conserved pairing to the seed region can be sufficient on its own for predicting conserved targets above the noise of false-positive predictions. (3) Highly conserved miRNAs have very many conserved targets [28]. These guidelines are far from complete yet and other features have been discovered that might boost site predication efficacy in the future, including (a) positioning of the miRNA within the 3′ UTR at least 15 nt from the stop codon, (b) positioning away from the center of long UTRs, (c) AU-rich nucleotide composition near the site or other measures of site accessibility, and (d) proximity to sites for coexpressed miRNAs [28]. Although the drawbacks due to the lack of knowledge of miRNA target

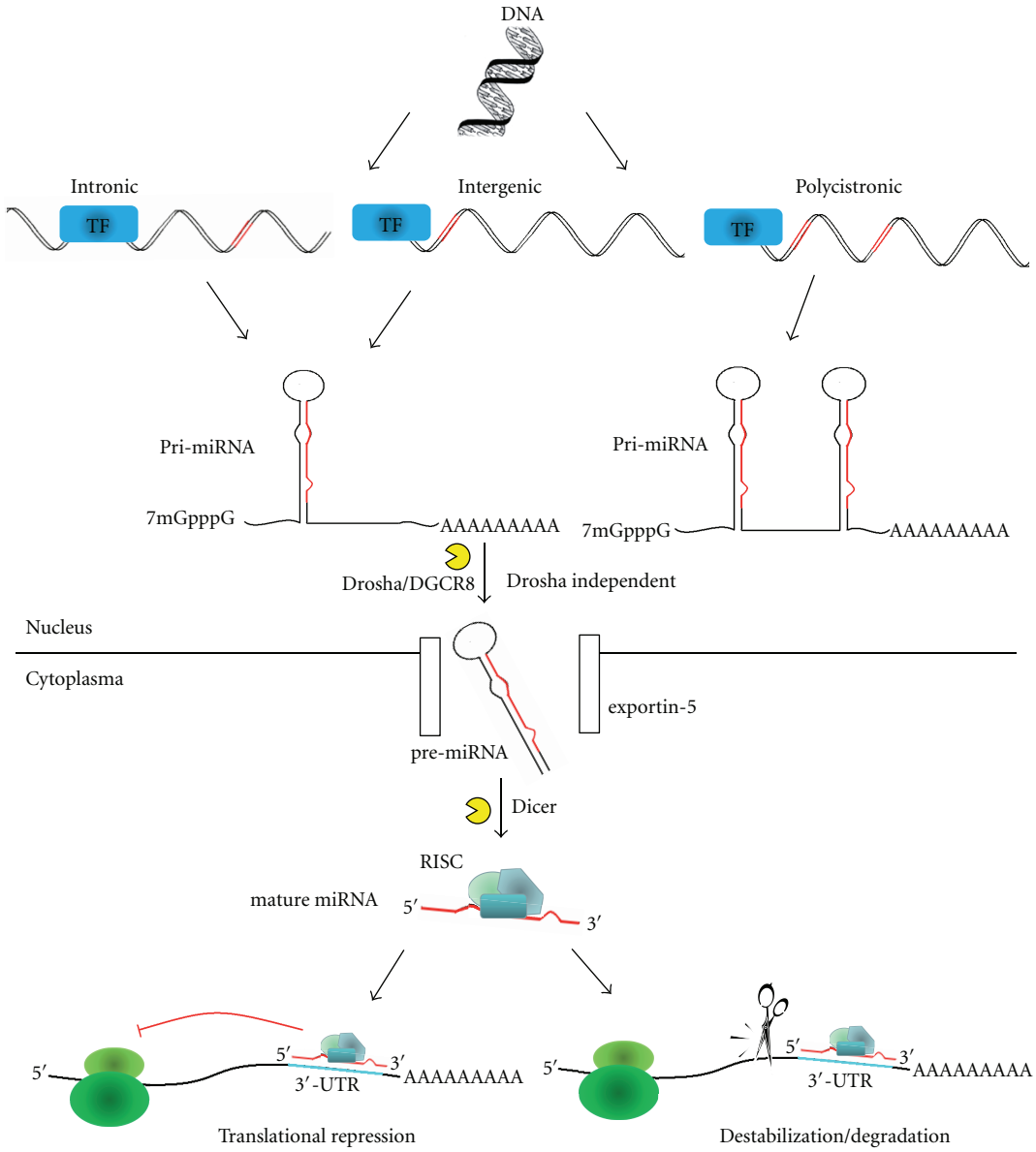

Figure 2: MiRNA biogenesis. miRNA molecules are endogenously transcribed within the nucleus of a cell, located between exons of a single mRNA (intronic), between coding genes (intergenic), or co-transcribed from a single locus (polycistronic); these transcripts are called pri-miRNAs. After cleavage into hairpin-shaped pre-miRNA molecules by Drosha/DGCR8, they are transported out of the nucleus by exportin-5. Here, Dicer will cleave the molecule into the mature single stranded miRNA that can be loaded into the RISC complex leading to translational repression or mRNA destabilization.

selection, many researchers were successful in identification of true experimental validated targets, mostly *in vitro*. To be able to identify and prove target binding and functional effects, several overviews were recently published [42, 43].

MiRNAs provide broad and robust transcriptional regulation that can be governed by individual miRNAs or the combined action of multiple miRNAs (see Figure 3(a)). The action of an individual miRNA can lead to a cumulative reduction in expression of multiple components of one specific functional network, and several miRNAs may cooperatively target various mRNAs whose protein products are part of the same molecular pathway. MiRNAs may target both positive and negative regulatory components, providing well-balanced pathways. It has also been shown that intronic miRNAs may regulate the same cellular processes as the protein encoded by the host gene [44]. Undoubtedly, miRNA gene regulation is sophisticated and highly orchestrated and has an impact on many, if not all, biological processes in humans. Above all, evidence has accumulated suggesting particularly important roles for miRNAs in disease, including cardiovascular disease.

3.2. MiRNAs in Cardiovascular Repair. The first evidence and suggestion for the essential general role of miRNAs

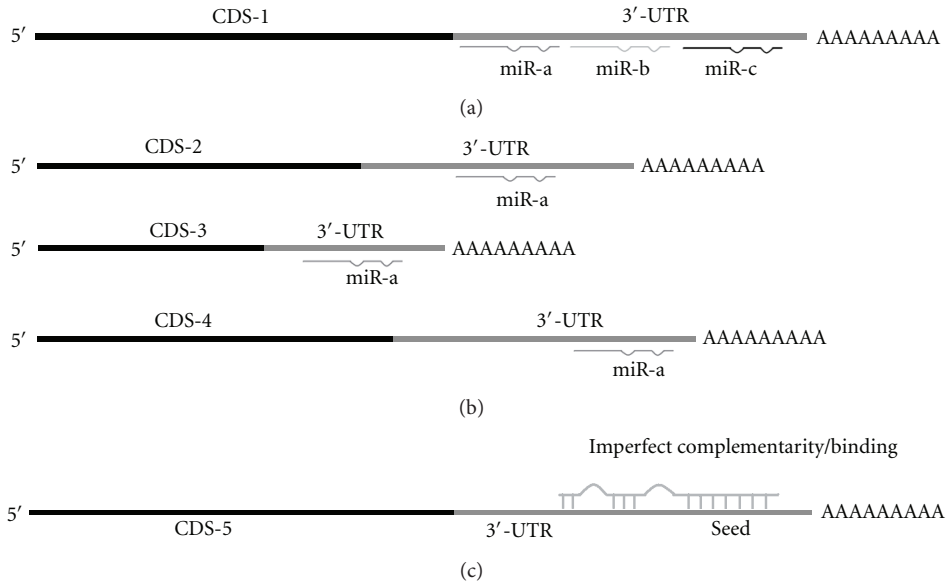

Figure 3: (a) A full length coding mRNA, finally translated into protein, has an untranslated portion ending its 3′ coding sequence (CDS), called the untranslated region (3′-UTR). Several different miRNAs (different colors) can bind to a single mRNA (CDS-1), whereas (b) a single miRNA can also bind to different target mRNA sequences (CDS2-4). (c) Mature miRNA binds to in an imperfect manner, meaning that not the full nucleotide length binds to the corresponding sequence but only parts. The nucleotides 2–7 of the miRNA are called the seed region.

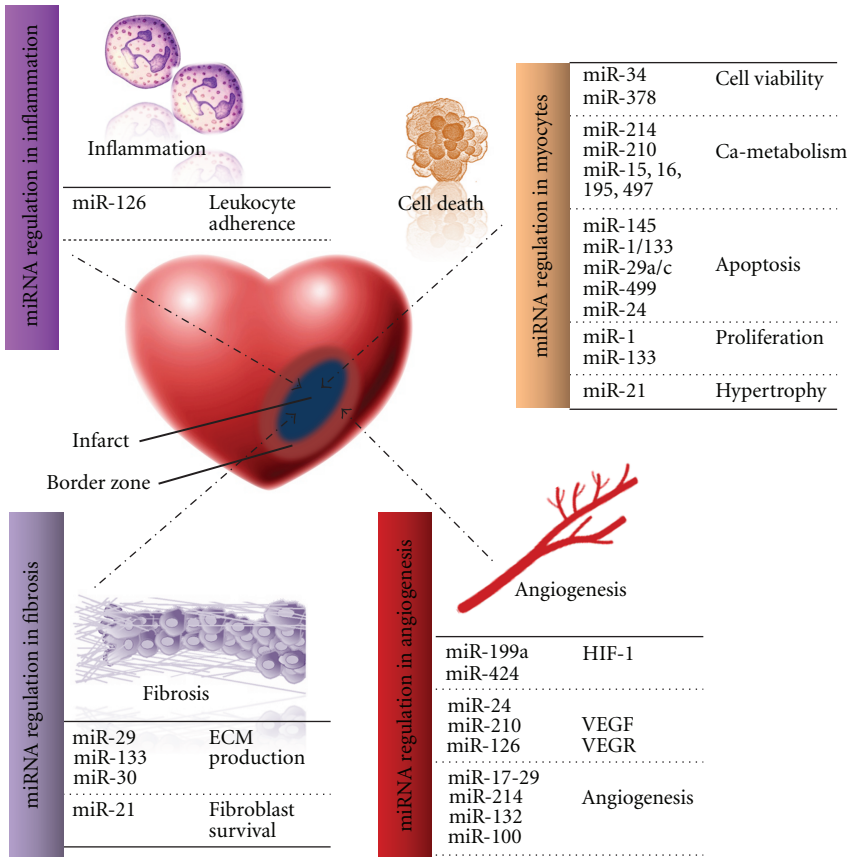

Figure 4: Upon cardiac injury and during its repair, miRNAs play a role in inflammation, in myocyte homeostasis, during angiogenesis, and in fibrosis.

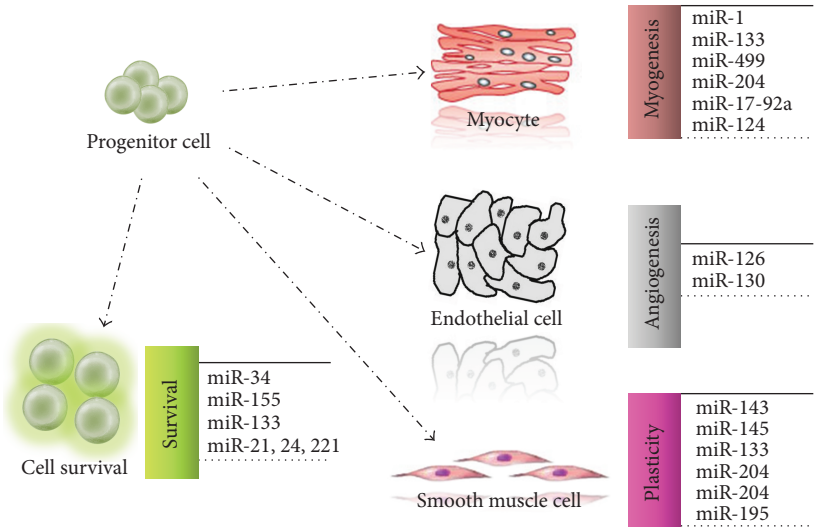

Figure 5: MiRNAs can direct cellular behavior and differentiation in cell transplantation therapies, for example, during cell survival, myocyte differentiation, endothelial differentiation, and smooth muscle cell plasticity.

in normal mammalian development came from analysis of mice lacking the miRNA processing enzyme Dicer, which resulted in embryonic lethality between E12.5 and E14.5 [45]. Dicer deficiency resulted in severe vascular developmental defects due to impaired blood vessel formation, which was confirmed by conditional ablation of Dicer from endothelial or vascular smooth muscle cells that resulted in defective blood vessel development [46, 47], thereby also suggesting a role of miRNAs for vascular development. Also during cardiogenesis miRNA processing is very important, since generating a cardiac progenitor (nkx2-5) conditional knockout of Dicer developed into embryos exhibited pericardial edema and a poorly developed ventricular myocardium, leading to cardiac failure and death before E12.5 [48]. Even postnatal, myocardial (alpha myosin heavy chain (α-MHC)) conditional deletion of Dicer or Dgcr8 led to rapidly progressive dilated cardiomyopathy, heart failure, and postnatal lethality after birth [49, 50] or provoked spontaneous and severe cardiac remodeling with high mortality rates in adult [51]. Interestingly, Dicer protein levels were decreased in human dilated cardiomyopathy and failing hearts, but are elevated in recovering hearts after installation of a left ventricular assist device (LVAD) [49]. Clearly, the knockout of Dicer or Dgcr8 and subsequent loss of all miRNA production have lethal consequences but only limited specific miRNA knockouts have been found that resulted in complete embryonic lethality in mice. This redundancy in miRNA function probably reflects the cooperative functions of many miRNAs. Nevertheless, single miRNA knockouts have been shown to lead to a high degree of lethality caused by significant defects in vascular development, as was shown for miR-126, [52, 53] and defects in cardiac developmental for miR-1 and miR-133a [48, 54].

The contribution of miRNAs in differentiation and function of, for example, embryonic stem cells has initially been elucidated by using the above mentioned miRNA-processing knockouts, that is, deletion of Dicer or the Drosha cofactor, DGCR8 [55, 56], underscoring the critical role of miRNAs for cell differentiation into specific cell lineages. The role of miRNAs in the cardiac response to injury and especially miRNAs that play a role in the myocardium upon cell transplantation or during cardiovascular cellular specification and differentiation will be discussed below. Although in an attempt to give a complete overview for the potential of miRNA therapeutics, several major players had to be highlighted since literature is rapidly expanding.

4. Cell Transplantation

Cell transplantation therapy with bone marrow cells (BMCs) is suggested as a therapeutic option to treat patients with heart disease; however the impaired functionality of patient-derived cells remains a major challenge. Xu et al. identified miR-34 to be increasingly expressed in patient-derived BMCs upon MI, as compared to healthy controls, which resulted in reduced cell survival upon transplantation *in vivo*. Reducing the expression of the proapoptotic miR-34a improves the survival of BMCs *in vitro*, via the antiapoptotic proteins Sirt1 and Bcl2 and the cell cycle regulatory cyclins and CDKs, and thereby enhanced the therapeutic benefit of cell therapy *in vivo* in mice after acute myocardial infarction as identified by improved cardiac function by high resolution echocardiography [57]. This highlights a role of miRNAs in the outcome of cell transplantation therapy by modulating cell survival and retention. However, their role is not limited to this aspect of cell therapy and here several areas of cardiovascular regenerative medicine where miRNAs play a role are suggested, ranging from cardiomyocyte survival, proliferation, and differentiation, endothelial cell (EC) specification and activation, smooth muscle cell (SMC) behavior, and finally the role in matrix remodeling and inflammation.

4.1. MiRNAs in Cardiomyocyte and Transplanted Cell Survival. During, for example, acute myocardial infarction or the reperfused heart, loss of cardiomyocytes is mostly caused by apoptosis and necrosis. Several signal-transduction pathways are involved and many pharmacological targets are studied to limit ischemic cell death [10], for which oxidative stress and its effect on mitochondria, especially the mitochondrial permeability transition pore (MPTP), are very important. Apoptosis is a well-defined process by which the cell undergoes cell death following a variety of different stimuli, finally resulting in the activation of caspases, whereas during regulated necrosis, or necroptosis, MPTP opening plays a central role. MPTP opening itself is mainly triggered by Ca^{2+}, but opening can also occur without a dramatic rise in intracellular calcium levels as many factors (including oxidative stress) influence MPTP sensitivity towards the available amount of Ca^{2+} [10]. Although many therapeutic strategies with proven efficacy in experimental studies on the processes described above have failed in the clinical setting, new options still appear via, for example, miRNA therapeutics [58]. Interestingly, similar mechanisms that occur during ischemia and reperfusion injury probably also play a role in the effect cell therapy has on resident myocytes in danger.

Intramyocardial delivery of bone marrow cells (BMCs) in infarcted mice regulates the expression of cardiac present miRNAs and, among others, significantly downregulates the proapoptotic miR-34a in cardiomyocytes. Thus in addition to its role in BMC survival, reduction in miR-34 levels also benefits myocytes, which are probably regulated via the release of IGF-1 by BMC and thereby improve myocyte survival [59]. IGF1 also reduced miR-378 expression in cardiomyocytes, thereby protecting cardiomyocytes from cell death by promoting IGF1R expression and downstream Akt signaling cascades, whereas overexpression of miR-378 enhanced apoptosis of cardiomyocytes [60]. This paracrine regulation of cardiac miRNAs by transplanted BMCs contributes to the protective effects of cell therapy [59]. The role of miR-34 in cell cycle, cell differentiation, and apoptosis is studied extensively and not only limited to cardiomyocytes and BMCs. Several families of targets, depending on the cellular context and environmental cues, have been identified and recently reviewed [61].

Also therapeutic modulation of Ca^{2+} handling provides some cardioprotection, and the observed increase of miR-214 during IR injury lead to a cardioprotective role, mediated via repression of the sodium/calcium exchanger 1 (Ncx1), a key regulator of Ca^{2+} influx, and thereby several downstream effectors of Ca^{2+} signaling that mediate cardiomyocyte cell death [62]. When oxygen is available on a limited basis, metabolic shift from mitochondria respiration to glycolysis takes place; miR-210 inhibits mitochondrial metabolism by targeting a number of proteins, thereby blocking mitochondrial energy production, impairing the oxygen consumption, inducing lactate accumulation, altering mitochondrial membrane potential, and disrupting mitochondrial structure [63].

The miR-15 family, containing miR-15a and b, miR-16, miR-195, and miR-497, is also upregulated in the infarcted region of porcine cardiac tissue, and by inhibiting them infarct size was reduced and cardiac function improved in response to ischemia [64]. MiR-15b may decrease mitochondrial integrity by targeting Arl2 in the heart and thereby decreasing intracellular ATP levels [65]. However, knockdown of the miR-15 family in neonatal mice with locked nucleic acid-modified anti-miRNAs was associated with an increased number of mitotic cardiomyocytes via derepression of Chek1 [66], thereby, although controversial maybe also explaining promising effects upon myocardial injury in the adult myocytes.

Also downstream apoptotic pathways could be affected by miRNAs, influencing directly or indirectly the caspase activation. MicroRNA-145 protected radical exposed cardiomyocytes through targeting Bnip3, an initiator for the mitochondrial induced apoptotic pathways [67], whereas miR-1 and miR-133 are involved in regulating apoptosis, via repression of HSP60 and HSP70 or caspase-9, respectively [68]. Pioglitazone (PIO), a peroxisome proliferator-activated receptor (PPAR)-gamma agonist, protects against myocardial ischemia-reperfusion (IR) injury via suppression of miR-29a and c levels and a decrease in caspase-3 activity, leading to an increased cell survival. Antagomirs against miR-29a or -29c significantly reduced myocardial infarct size and apoptosis in hearts subjected to IR injury, probably via an increased presence of Mcl-2, an antiapoptotic Bcl-2 family member [69]. Finally, modulation of microRNA-499 (miR-499) levels affected apoptosis and the severity of myocardial infarction and cardiac dysfunction induced by ischemia reperfusion. MiR-499 targets both the α- and β-isoforms of the calcineurin catalytic subunit and inhibits cardiomyocyte apoptosis through dephosphorylation of dynamin-related protein-1 (Drp1), thereby decreasing Drp1 accumulation in mitochondria and Drp1-mediated activation of the mitochondrial fission program [70]. MiR-24 (miRNA-24) expression is downregulated in the ischemic border zone of the murine left ventricle after MI. MiR-24 suppresses cardiomyocyte apoptosis, in part by direct repression of the BH3-only domain-containing protein Bim, which positively regulates apoptosis. Accordingly, induction of microRNA-24 by HIF-1 protects against ischemic injury in rat cardiomyocytes [71]. *In vivo* expression of miR-24 in a mouse MI model inhibited cardiomyocyte apoptosis, attenuated infarct size, and reduced cardiac dysfunction [72].

In addition to the effect miRNAs have on myocytes survival, several miRNAs have been identified that affect transplanted cell survival and retention. Human cardiomyocyte progenitor cells (hCMPCs) can be efficiently isolated and propagated from fetal heart and patient biopsies and differentiated into functional mature cardiomyocytes [73, 74]. Transplantation of these cells preserved long-term function of the infarcted mouse myocardium up to 3 months after MI, but cell survival was limited to only ~3% of injected cells. By targeting RIP1, miR-155 could repress necrotic cell death of the CMPCs, independent of the activation of Akt prosurvival pathways, and thereby provides the opportunity to block necrosis and improvement of cell engraftment for cell therapy

[75]. Additionally, miR-155 also efficiently inhibited CMPC cell migration via a reduction in MMP-2 and -9 activities by directly targeting MMP-16, thereby potentially improves local retention of hCMPCs after Intramyocardial delivery [76]. In this regard, mouse cardiac progenitor cells (CPCs) were transduced with lentivirus carrying the precursor of miR-21, miR-24, and miR-221, which lead to a higher viability by targeting Bim, a critical apoptotic activator, and, *in vivo*, upon Intramyocardial injections bioluminescence imaging (BLI) showed that miRNA cocktail-treated CPCs survived significantly longer after transplantation. Subsequently this leads to a marginal improvement in cardiac function, as compared to their control transfected cells [77].

4.2. MiRNAs in Cardiomyocyte Proliferation. An alternative way for restoring the number of lost myocytes by cell transplantation is the endogenous stimulation of cardiomyocyte proliferation, thereby increasing myocardial muscle mass. It has long been thought that mammalian cardiomyocytes are terminally differentiated and unable to proliferate. However, myocytes in more primitive animals such as zebrafish are able to dedifferentiate, proliferate, and regenerate amputated cardiac muscle. Zebrafish regenerate cardiac muscle after severe injuries through the activation and proliferation of spared cardiomyocytes, but little is known about factors that control these events or why this is only happening artificially in cultured myocytes and not frequently in adult mammalian hearts [78]. Recently, Yin and colleagues observed a diminished expression of miR-133 during regeneration in the zebrafish. MiR-133 depletion enhanced cardiomyocyte proliferation, mediated via cell cycle factors like mps1, cdc37, and PA2G4, and cell junction components cx43 and cldn5, revealing a dynamic regulation of miRNAs during heart regeneration, at least in the zebrafish heart, and indicates that the muscle-specific miR-133 restricts injury-induced cardiomyocyte proliferation [79].

During embryonic development, miR-1 is transcriptionally regulated via muscle transcription factors including serum response factor (SRF), MyoD and Mef2. MiR-1 is specifically expressed in cardiac and skeletal muscle (precursor) cells and regulates the pool of proliferating ventricular myocytes by inducing a premature exit from the cell cycle, thereby suppressing cardiac growth via the inhibition of Hand2 [58, 80], a basic helix-loop-helix protein involved in ventricular myocyte expansion. Postnatal mouse cardiomyocytes usually exit the cell cycle after the first 10 days of life; however, miR-1-null adult mice have an increase in mitotic cardiac myocytes along with cardiac hyperplasia. A genome-wide profiling of miR-null adult mouse hearts demonstrated an upregulation of cell cycle genes and downregulation of tumor suppressors [48], but if these changes promote cardiac repair after injury remains to be determined.

4.3. MiRNAs in Myogenic Differentiation. MiR-1 and miR-133 were among the first miRs that had been identified as major regulators of muscle lineage commitment and were shown to be critical regulators of muscle proliferation and differentiation. MiR-1 and miR-133 are clustered on the same chromosomal loci and transcribed together in a tissue-specific manner during development. However, they have distinct roles in modulating skeletal muscle proliferation and differentiation. MiR-1 promotes myogenesis by targeting histone deacetylase 4 (HDAC4), a transcriptional repressor of muscle gene expression, and miR-133 enhances myoblast proliferation by repressing serum response factor (SRF) [81]. MiR-1 genes are transcriptionally regulated by muscle differentiation factors, such as serum response factor, MyoD and Mef2. Increasing miR-1 levels in the developing heart leads to a decreased pool of proliferating ventricular cardiomyocytes, by targeting Hand2, and thereby controls the balance between differentiation and proliferation during cardiogenesis [80]. MiR-1 and miR-133 promote mesoderm formation in mouse embryonic stem cells but have opposing functions during further differentiation into cardiac muscle progenitors [82]. Interestingly, cardiogenic differentiation of hMSCs was promoted by targeting epidermal growth factor receptor using microRNA-133a [83], whereas the role of miR-1 for driving cardiac differentiation and suppressing cell proliferation was confirmed in human cardiomyocyte progenitor cells (CMPC) and human ESCs. Forced expression of miR-1 reduced proliferation and induced their differentiation into cardiomyocytes by repressing HDAC4 [84]. Additionally, another muscle-specific miRNA, miR-499, was able to drive differentiation as well by lowering sex determining region Y-box 6 (Sox6) levels [84], which could be reproduced in rat MSCs, by targeting wnt/β-catenin signaling [85], and in other human CSCs. The latter study showed that miR-499 translocates from myocytes to coupled hCSCs via gap junctional communication favoring their differentiation into functionally competent cells. By transplanting miR-499 overexpressing hCSCs in a mouse model of myocardial infarction, ventricular function improved via enhanced myocyte differentiation and the restoration of myocardial mass and function in the infarcted heart [86, 87]. MiR-1 and -499 play different roles in cardiac differentiation, at least in hESCs; while miR-499 promoted ventricular specification, miR-1 served to facilitate electrophysiological maturation [88]. Interestingly, a human 3′ miR-499 mutation altered the expression and function of cardiac mRNAs [89]. Using a transgenic mouse model, elevated miR-499 levels caused cellular hypertrophy and cardiac dysfunction, by affecting immediate early stress response genes (Egr1, Egr2, and Fos), β-myosin heavy chain (Myh7), and skeletal muscle actin (Acta1) and thereby may titrate the cardiac response to stress [90]. MiR-1 drives cardiac myocyte differentiation from transplanted ES cells but also and inhibits apoptosis post-MI, via activation of p-AKT and inhibition of caspase-3, phosphatase and tensin homolog, and superoxide production, ultimately giving rise to enhanced cardiac repair, regeneration, and function [91].

MiRNA-204 inhibition promoted hCMPC proliferation without affecting cell viability and the level of apoptosis and necrosis, and forced miRNA-204 expression stimulated hCMPC differentiation partly by targeting ATF-2 [92], a

member of the ATF/CREB bZip family of transcription factors, whereas ATF-2 overexpression promoted hCMPC proliferation as well [92]. Although controversial whether BMSCs can differentiate into myocytes, enhanced miR-124 expression could suppress myogenic differentiation of BMSCs via targeting STAT3 mRNA [93].

Although the precise roles of the miR-17-92 cluster in heart and lung development remain unclear, disruption of the miR-17-92 cluster resulted in smaller embryos and immediate postnatal death of all animals, likely due to hypoplastic lungs and ventricular septal defects in the hearts [94]. Moreover, miR-92 is a critical regulator of endoderm formation and left-right asymmetry during early zebrafish development by regulating gata5 [95].

4.4. MiRNAs in Angiogenesis. Angiogenesis is the process of new blood vessel and capillary network formation in the body. A large number of miRNAs are involved in angiogenesis and are expressed in ECs, but are also indispensable for angiogenesis to occur. In addition to the earlier stated observations that miRNAs have a crucial role in vascular development [45], a reduction of endothelial miRNAs by Cre-dependent knockout of Dicer in endothelial cells resulted in impaired angiogenesis in distinct models of post-natal angiogenesis [47]. Moreover, silencing of Dicer in endothelial cells *in vitro* caused an impairment of capillary sprouting, tube formation, and migration capacity [96, 97]. In both normal endothelium homeostasis [97] and angiogenesis [98, 99] miRNAs play a crucial role. Members of the let-7 family, mir-21, mir-126, mir-221, and mir-222 are highly expressed in endothelial cells, whereas let-7 regulates the expression of thrombospondin-1 and miR-222/221 endothelial nitric oxide synthase protein levels.

Over decades, induction of protein coding genes by low oxygen has been dominated the focal point of hypoxia research. One of the most sensitive physiological sensors of hypoxia is HIF. HIFs control the cellular response to hypoxia by regulating genes that are involved in metabolism, angiogenesis, erythropoiesis, cell proliferation, differentiation, and apoptosis. This regulation is under the control of specific hypoxia-inducible miRs also termed as "hypoxamiRs." Hypoxia-induced microRNA-424 expression in human endothelial cells regulates HIF-α isoforms and promotes angiogenesis [100]. MiR-424 targeted cullin 2 (CUL2), a scaffolding protein critical to the assembly of the ubiquitin ligase system, thereby stabilizing HIF-α isoforms. Downregulation of miR-199a derepresses hypoxia-inducible factor-1 and sirtuin 1 and recapitulates hypoxia preconditioning in cardiac myocytes [101]. MiR-199a is sensitive to low oxygen levels and is rapidly reduced to undetectable levels, thereby releasing Hif-1α and Sirtuin (Sirt) 1 mRNA of its inhibitory effect. Additionally, miR-199a-5p negatively regulates angiogenic responses by directly targeting v-ets erythroblastosis virus E26 oncogene homolog 1 (Ets-1). Thus, downregulation of miR-199a-5p is involved in the induction of wound angiogenesis through derepressing of the Ets-1-MMP1 pathway [102]. This suggests that miR-199a is a regulator of a hypoxia-triggered pathway but also involved in preconditioning of cells against hypoxic damage.

MiR-210 represents a master hypoxia-inducible miRs [63], induced by HIF, and inhibits cell proliferation via targeting E2F3 [103] and FGFR members. MiR-210 improved MSC cell survival after myocardial engraftment by ischemic preconditioning of the cells, thereby suppressing FLASH/Casp8ap2 [104]. MiR-210 progressively increased upon exposure to hypoxia in endothelial cells and by inhibiting the receptor tyrosine kinase ligand Ephrin-A3 that affects cell survival, migration, and differentiation [105]. It drives the expression of VEGF and VEGFR2 in endothelial cells and thereby supports the angiogenic response and facilitates microcirculation [106]. Additionally, miR-210 expression was significantly upregulated in VEGF treated CD34 cells, thereby enhancing the angiogenic property of expanded cells for the treatment of ischemic vascular disease [107]. MiRNA-126 was demonstrated to be important for vascular integrity and angiogenesis, regulating the response of endothelial cells to VEGF via SPRED1 and PIK3R2 [52]. MiR-126 knockout in mice is partially embryonic lethal, and surviving miR-126 knockout mice have defective cardiac neovascularization after myocardial infarction [53] miR-126 is located in the intron of epithelial growth factor like domain containing protein 7 (EGFL7), an endothelial-specific protein involved in development of the vasculature. Interestingly, recent work demonstrated that miR-126 levels are responding to a mechanosensitive factor, Klf2, thereby also activating VEGF signaling [108]. In addition to its proangiogenic role, miR-126 is involved in suppressing endothelial inflammation signals, since VCAM-1 is a direct target of miR-126 and thereby suppresses leukocyte adherence to ECs [109]. The administration of angiogenic early outgrowth cells or CD34- cells has been suggested to improve cardiac function by promoting neovascularization. Interestingly, a loss of miR-126 and -130a in these cells from patients with chronic heart failure was observed, and thereby limiting their capacity to improve cardiac neovascularization upon transplantation. By miR-126-mimic transfection the angiogenic capacity of the cells could be increased, [110] which was also the case for transplantation of MSCs transfected with miR-126 due to stimulation of the AKT/ERK-related pathway [111].

The miR17-93 cluster, consisting of miR-17, -18a, -19a, -20a, -19b-1, and -92a, is highly expressed in endothelial cells [98]. Forced overexpression of miR-92a resulted in blocked angiogenesis *in vitro* and *in vivo*, whereas blocking miR-92a *in vivo* via an antagomir approach restored vessel growth and functional recovery of tissue, both in cardiac ischemic and in limb ischemic models [98]. Transplantation of MSCs inhibits lung inflammation, vascular remodeling, and right heart failure and potentially reverses hypoxic pulmonary hypertension in experimental models of disease. The intravenous delivery of MSC-derived exosomes reduced vascular remodeling and pulmonary hypertension, whereas exosome-depleted media or fibroblast-derived exosomes had no effect, probably by suppressing STAT3 and the upregulation of the miR-17 superfamily [112].

In addition to its expression in myocytes, miR-24 is also enriched in cardiac endothelial cells and upregulated

after cardiac ischemia. MiR-24 induces endothelial cell apoptosis, abolishes endothelial capillary network formation on matrigel, and inhibits cell sprouting from endothelial spheroids, through targeting of the endothelium-enriched transcription factor GATA2 and the p21-activated kinase PAK4. Blocking of endothelial miR-24 limited myocardial infarct size of mice via the prevention of endothelial apoptosis and enhancement of vascularity, which led to preserved cardiac function and survival [113].

MiR-214 is expressed in all major vascular cell types and modulation of its levels in endothelial cells significantly affected angiogenesis, both *in vitro* as *in vivo*. The antiangiogenic effect of miR-214 is mediated through the downregulation of Quaking, since direct targeted and knockdown of Quaking reduced endothelial cell sprouting similar to miR-214 overexpression, probably via a reduction in proangiogenic growth factor expression [114]. Thus where miR-214 is beneficial for *in vivo* cardioprotection of myocytes [62], it is detrimental for the subsequently repair process of the heart by repressing the angiogenic response. MiR-132 was highly upregulated in a human embryonic stem cell model of vasculogenesis and present in activated endothelium, but undetectable in normal endothelium. MiR-132 acts as an angiogenic switch by suppressing endothelial p120RasGAP expression, leading to Ras activation and the induction of neovascularization, whereas the application of anti-miR-132 inhibits neovascularization by maintaining vessels in the resting state [115]. The use of saphenous vein-derived pericyte progenitor cells (SVPs) in a mouse myocardial infarction (MI) model, reduced myocardial scar, cardiomyocyte apoptosis, and interstitial fibrosis improved myocardial blood flow and neovascularization and attenuated vascular permeability. MiR-132 was constitutively expressed and secreted by these cells and could stimulate endothelial tube formation and reduce myofibroblast differentiation *in vitro* studies, whereas miR-132 decreased their capacity to improve contractility, reparative angiogenesis, and interstitial fibrosis in infarcted hearts [116].

MiR-100 modulates proliferation, tube formation, and sprouting activity of endothelial cells and migration of vascular smooth muscle cells and functions as an endogenous repressor of the serine/threonine protein kinase mammalian target of rapamycin (mTOR). Accordingly, miR-100 inhibition by specific antagomirs *in vivo* stimulated angiogenesis and resulted in functional improvement of perfusion after femoral artery occlusion in mice. In contrast, treatment with the mTOR inhibitor rapamycin had the opposite effect. miR-100 has therefore an antiangiogenic function and represses mTOR signaling in endothelial and vascular smooth muscle cells [117]. Moreover, downregulation of miR-100 in breast cancer cells, led to an upregulation of the proliferation- and survival-promoting oncogene insulin-like growth factor (IGF) 2, whereas stable overexpression of miR-100 could strongly reduce IGF2 expression and thereby inhibited tumor growth [118]. Inhibition of miR-100 could therefore be a novel approach for the modulation of blood vessel growth and other mTOR-dependent processes.

Although some miRNAs were highlighted here, an ongoing list of publications is growing and several others are reviewed elsewhere, including miR-34, miR-217, miR-424, miR-503, the miR-23-24-27 cluster, miR-16, miR-221, miR-222, miR-378, and miR-130 [119, 120].

4.5. MiRNAs in Matrix Production and Remodeling. Several miRNA are also reported to have an effect on myocardial remodeling and matrix turnover or production, as reviewed recently [58]. Several members of the miR-29 family are dysregulated in the heart during post-MI remodeling and are predicted to regulate several mRNAs that are involved in ECM production and fibrosis [121]. Upon cardiac injury, fibroblasts are activated by TGF-beta, resulting in repression of miR-29, and consequently increase levels of matrix production. Downregulation of miR-29 via antagomir approaches resulted in increased fibrotic responses, suggesting that maintaining miR-29 levels after MI could result in reduced fibrosis, and thereby reducing cardiac stiffness, improving contractility and conductance. Interestingly, downregulation of miR-29 by antagomirs also protected the heart against IR injury by reducing myocardial infarct size and apoptosis [69].

MiR-21 is one of the most highly and consistently upregulated miRNA in cardiac diseases, but its role in different cardiac cell types is still not clear. Increased levels of miR-21 were present in cardiac diseased models, mainly displaying hypertrophic responses. Blocking miR-21 in hypertrophic myocytes [122] resulted in diminished hypertrophy; however, miR-21 has also been reported to negatively regulate cardiac hypertrophy in myocytes [123]. Interestingly, the same authors report that miR-21 protects against H(2)O(2)-induced injury on cardiac myocytes via its target gene, Programmed Cell Death 4, thereby repressing myocyte death [124].

However, upon cardiac stress, a miRNA-21-mediated ERK-MAP kinase activity is also stimulated by means of an effect on SPRY1, which in turn positively regulates cardiac fibroblast survival, leading to fibrosis, hypertrophy, and cardiac dysfunction [125]. Inhibiting miR-21 with antagomirs during aortic banding resulted in improved cardiac performance and reduced fibrosis; however, in another approach by Patrick et al. in which they used both genetic knockout of miR-21 as well as pharmacological inhibition of miR-21 by tiny LNAs (seed-targeting 8-mer locked nucleic acid oligonucleotides) no functional benefits were observed upon pressure overload as myocardial infarction [126]. Although puzzling this might be caused by the use of different miR-21 inhibitor chemistries [127]. Furthermore, miR-21 also regulates MMP-2 expression in cardiac fibroblasts of the infarct zone after ischemia reperfusion via a PTEN pathway [103]. These different reports suggest that a single miRNA, in these cases miR-21 and miR-29, can have distinct functions and effects in different cell types, depending on localization and timing of expression, and of course the presence of potential target sites.

In addition to the roles of miRNA-21 and 29 in matrix production, effects of miRNA-133 and miR-130 on matrix production after cardiac injury were recently demonstrated. MiR-133 and miR-30 downregulate CTGF, a key profibrotic

protein, thereby affecting collagen production and controlling structural changes in the extracellular matrix of the myocardium [128].

4.6. MiRNAs in Smooth Muscle Cell Plasticity. Vascular smooth muscle cells have the unique capacity to oscillate between a contractile phenotype and a less differentiated, more synthetic state in response to external queues. This phenotypic modulation is a major component of the vascular repair process and diseases such as atherosclerosis. Not surprisingly, it involves dramatic changes in the smooth muscle gene program, which has recently been found to be heavily influenced by particular miRNAs. Control of vascular smooth muscle cell (VSMC) phenotype is also essential in the development and maintenance of a healthy vasculature. Recently, the role of miRNAs in differentiation and function of vascular smooth muscle cells (VSMCs) has been elucidated by generating a VSMC-specific Dicer knockout mouse. Mutant mice died between embryonic day 16 and 17 due to extensive hemorrhage and a thinned wall of the aorta caused by a decreased proliferation of VSMCs [46]. Furthermore, a loss of contractile function and differentiation of VSMCs could be observed, whereas transfection with mimic miR-145 could partly rescue the impaired contractile function and differentiation of VSMCs. MiR-143 and miR-145 are co-transcribed and are crucial for the fate of VSMCs, regulating their differentiation, phenotype, and function [129]. These miRNAs are co-transcribed and are controlled by Nkx2-5 (NK2 transcription factor related, locus 5) and the serum response factor with its coactivator myocardin. MiR-145 and miR-143 regulate smooth muscle cell fate and plasticity and myocardin-induced reprogramming of adult fibroblasts into smooth muscle cells. Furthermore, miR-145 and miR-143 cooperatively targeted a network of transcription factors, including Klf4 (Kruppel-like factor 4), myocardin, and Elk-1 (ELK1, member of ETS oncogene family), to promote differentiation and repress proliferation of smooth muscle cells. They were also downregulated in injured or atherosclerotic vessels containing proliferating, less differentiated smooth muscle cells. In miR-145−/− mice, and to a lesser extent in miR-143−/− mice, neointima formation was reduced after vessel injury [130], whereas overexpression of miR-143 and miR-145 decreased neointima formation [131], suggesting that miR-143/145 expression tightly controls the smooth muscle cell response of vessels after injury. Interestingly, blood pressure was reduced and atherosclerotic lesion development was enhanced in miR-143/145-deficient mice. These findings demonstrate that miR-145 can direct the smooth muscle fate and that miR-145 and miR-143 function to regulate the quiescent versus proliferative phenotype of smooth muscle cells [129].

MiR-133 is robustly expressed in vascular smooth muscle cells (VSMCs) *in vitro* and *in vivo*, whereas miR-1 vascular levels are negligible, and its expression is regulated by extracellular signal-regulated kinase 1/2 activation. When VSMCs are proliferating *in vitro* and following vascular injury *in vivo*, miR-133 levels decrease, whereas it increases when VSMCs are coaxed back to quiescence. MiR-133 specifically suppresses the transcription factor Sp-1 expression *in vitro* and *in vivo* and thereby exacerbates VSMC proliferation and migration [132].

Other interesting miRNAs are miR-204 and miR-195. Whereas miR-204 contributes to β-glycerophosphate-induced VSMC calcification through regulating Runx2 and thereby regulating VSMC calcification and potentially medial artery calcification [133], miR-195 plays a role in the cardiovascular system by inhibiting VSMC proliferation, migration, and proinflammatory biomarkers and thereby potentially reduces neointimal formation in patients receiving stenting or angioplasty [134].

5. Conclusions

MicroRNAs have been identified as critical regulators of cell behavior via repression of gene patterns, not only for regulating cellular homeostasis but also in stem cell self-renewal and differentiation, and thereby contributing to lineage commitment. Most knockouts of single miRs do not affect embryonic development, maybe by compensation of family members or due to their limited role only for fine-tuning cell behavior and not in decision making. As provided above, specific miRNAs have been suggested to play a role in the behavior and differentiation of cardiovascular cell lineages, like vascular smooth muscle, endothelial cell, and cardiomyocyte differentiation. Moreover, since altered expression profiles in circulating progenitor or resident tissue stem/progenitor cells may impair the endogenous cardiovascular repair capacity, we might be able to change functional capacities of impaired patient-derived cells via miRNA therapeutics before using them for cell transplantation in myocardial repair.

Interesting several miRNAs have also been reported to promote reprogramming of fibroblasts into induced pluripotent cells (iPSCs) [135], which is particularly important for regenerative medicine since more safe generation of iPSCs could be achieved. Appealing are new discoveries in which direct reprogramming of cardiac fibroblasts to cardiomyocytes could be achieved, both *in vitro* and *in vivo* [22], but also reached via modulating miRNA levels [136]. This suggests a concept of generating new myocyte mass within the infarcted myocardium as a novel approach to repair the myocardium from within.

As miRNA-targeted therapies are entering the clinical arena, a detailed understanding of the role of miRNAs in stem/progenitor cell differentiation and functions, as well as the alterations in cardiovascular disease, is required. By further understanding the role of specific miRNAs in regulating or limiting endogenous cardiovascular repair responses during cardiac injury and repair, in both progenitor cell cardiovascular lineage differentiation and endogenous cardiac cell responses, this may lead to novel therapeutic strategies to activate further functional potential of progenitor cells and thereby promote cardiovascular repair.

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