Noncoding RNAs in Ischemic Cardiovascular Disease and Repair Mechanisms

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Abstract

Ischemic cardiomyopathy is frequently a consequence of myocardial infarction resulting in a marked loss of cardiomyocytes, which is replaced by a scar. As endogenous cardiovascular repair mechanisms are not sufficient to compensate for the loss of heart muscle and subsequent adverse remodeling predisposes to development of heart failure, novel experimental therapies are intensely explored to reduce scar size, counteract adverse hypertrophy and promote repair of the heart muscle. These therapies include cell-based therapies, cardiomyocytes renewal or proliferation and trans-differentiation into cardiomyocytes. Importantly, microRNAs evolved as major modulators of pathophysiological mechanisms involved in heart failure progression as well as repair mechanisms. Identification of microRNAs contributing or counteracting these deteriorative mechanisms resulted in novel microRNA-targeted therapies in experimental studies. While this class of non-coding RNAs has already entered clinical translation, another subclass of non-coding RNAs - long-non-coding RNAs (lncRNAs) – is now intensely explored as well. Recent observations indicate that lncRNAs are important regulators of cardiovascular development and mechanisms involved

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in adverse cardiac remodeling and development of cardiomyopathy. Therefore lncRNAs, in addition to microRNAs, may also emerge as novel therapeutic targets in cardiovascular disease.

4.1 Targeting miRNAs to Enhance Cell-Based Therapies

The studies of cell-based therapies using bone marrow-derived cells in patients with acute myocardial infarction (Assmus et al. 2002) triggered a vast number of experimental and clinical investigations using bone marrow-, mesenchymal-, or cardiac-derived stem and progenitor cells (Jakob and Landmesser 2013). Recently, cardiac-derived stem cells were examined in clinical phase I/II trials (Bolli et al. 2011; Makkar et al. 2012). However, while some clinical trials using cell-based therapies after myocardial infarction observed an improvement of left ventricular function or clinical symptoms, others failed to show beneficial effects (Jeevanantham et al. 2012; Fisher et al. 2015; Gyongyosi et al. 2015). Importantly, as the majority of conducted clinical trials included only small number of patients and was mostly underpowered for mortality as an endpoint, a Pan-European clinical phase III trial investigating the effect of cell-based treatment in patients with acute myocardial infarction (MI) (http://www.bami-fp7.eu/) is now being conducted.

The modest effects observed in cell-based therapies may in part be attributable to isolation procedures (Assmus et al. 2010; Seeger et al. 2012), low survival (Li et al. 2009), limited homing (Chavakis and Dimmeler 2011), and highly limited differentiation into contractility contributing cardiomyocytes of the applied cell product (Tongers et al. 2011). Of note, cells derived from patients with cardiovascular diseases show an impaired cardiac repair capacity when compared to cells from healthy subjects (Jakob et al. 2012a; Heeschen et al. 2004). Mechanistic studies have shown that miRNAs are crucially involved in these processes. As a potential mechanism, deregulation of miRNAs in cells with repair capacity was observed (Mocharla et al. 2013; Jakob et al. 2012a; Xu et al. 2012). We and others observed deregulation of miRNA expression in early angiogenic outgrowth cells (EOCs) derived from patients with chronic heart failure (CHF) (Jakob et al. 2012b). Notably, overexpression of miR-126 that was reduced in EOCs from patients with CHF enhanced EOC-mediated cardiac repair capacity in vivo (Jakob et al. 2012b). MiR-126 was previously shown to stimulate angiogenesis (Wang et al. 2008) and is also involved in the prevention of experimental atherosclerosis (Schober et al. 2014). In addition, miR-21, that is upregulated in EOCs from patients with coronary artery disease, impairs their migratory potential through an increase in reactive oxygen species (Fleissner et al. 2010). Another approach is to prevent apoptosis of transplanted cells, a process thought to substantially decrease cardiac repair capacity after cell transplantation due to low survival of transplanted cells. Expression of miR-34a, a pro-apoptotic miRNA, was increased in bone marrow mononuclear cells (BMC) from patients with myocardial infarction (Xu et al. 2012). Pretreatment of BMCs with miR-34a inhibitors improved their capacity to restore cardiac function in a murine infarct model (Xu et al. 2012). Of note, miR-34a is also increased during ageing in the heart (Boon et al. 2013). Hu et al. applied a cocktail consisting of

miR-21, miR-24, and miR-221 to cardiac progenitor cells, which increased their survival after cardiac transplantation in an experimental myocardial infarct model and resulted in a better preserved cardiac function (Hu et al. 2011). Bim, an inducer of apoptosis, was repressed by these three miRNAs (Hu et al. 2011), demonstrating that multiple miRNAs can synergistically repress one target. Hence, miRNAs have the potential to improve impaired cardiac repair capacity of adult stem/progenitor cells, and miRNA modulation of adult stem/progenitor cells may serve as a strategy to enhance cardiac repair processes in cell-based therapies (Fig. 4.1).

As cell isolation procedures are labor intensive and expensive and improvement in cardiac function is mostly related to paracrine mechanisms (Gnecchi et al. 2008; Murry et al. 2004), recent studies focused on compounds released from progenitor/stem cells. In this context, exosomes, small secreted membrane-bound vesicles released from cells, evolve as a potential cell-free therapy for cardioprotection (Vicencio et al. 2015; Chen et al. 2013b). Interestingly, miRNA transferred via exosomes contributes to this intercellular communication system. Intramyocardial delivery of exosomes derived from mouse embryonic stem cells improved LV function after induction of myocardial infarction in mice (Khan et al. 2015). This was

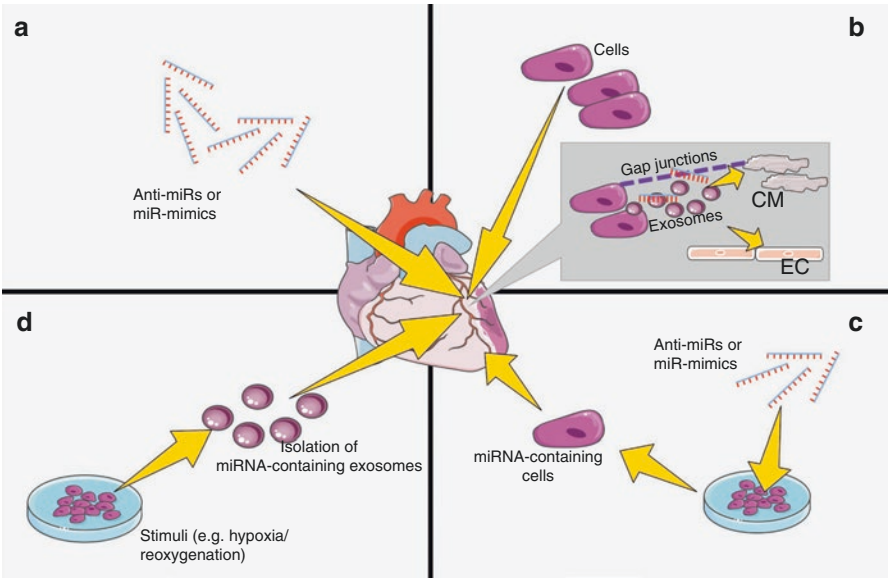

Fig. 4.1 Experimental (and clinical) strategies to improve cardiac function using cells with miRNA-mediated cardiac repair potential, cell-derived miRNA-containing components, or chemically modified synthetic miRNAs. Chemically modified synthetic miRNAs or viral constructs can be delivered directly (local or systemic) for therapeutic manipulation of miRNAs (**a**). Systemic or intramyocardial delivered cells enhance cardiac repair by the release of miRNAs (and other growth factors) to host cells through exosomes and gap junctions (**b**). MiRNA pretreatment of cells may enhance their cardiac repair potential and survival (**c**). MiRNA-containing exosomes of stem/progenitor cells can be isolated and delivered to improve cardiac repair capacity. This cell-free strategy avoids potential side effects that may arise after transplantation of stem cells (**d**). *CM* cardiomyocytes, *EC* endothelial cells, *miR* microRNA

related to an increase in proliferative myocytes and number of cardiac progenitor cells (CPCs, c-kit + cells) in vivo. Of note, miRNA profiling of exosomes revealed an enhanced expression of the cell cycle regulator cluster miR-290. Overexpression of one of the members, miR-294, in CPCs increased proliferation and decreased apoptosis in vitro (Khan et al. 2015). Similarly, hypoxia-induced release of exosomes in CPCs improved LV function in an experimental ischemia-reperfusion model, which is related to an increased exosome content of miRNAs involved in fibrosis pathways (Gray et al. 2015). In addition, exosomes from host tissue after cell therapy may alter function of the applied cell product. Ong et al. showed that CPCs co-delivered with a minicircle plasmid containing hypoxia-inducible factor 1 (HIF-1)-induced endothelial cells to secrete exosomes enriched of miR-126 and miR-210. Uptake of these miRs in CPCs leads to a higher tolerance against hypoxic stress in vitro which in turn enhances survival of CPCs after intramyocardial delivery (Ong et al. 2014). Interestingly, Hosoda et al. (2011) showed that miR-499 may also be transferred via gap junctions from myocytes to cardiac stem cells, thereby promoting differentiation via suppression of differentiation modulators Sox6 and Rod1. MiR-499 is highly expressed in differentiated cardiomyocytes and markedly reduces proliferation rates of cardiomyocyte progenitor cells (Sluijter et al. 2010).

4.2 Targeting miRNAs to Induce a Cardiac Phenotype in Pluripotent Stem Cells

Stimulation of cardiomyocyte lineage commitment was reported in embryonic stem cells (ESCs) and inducible pluripotent stem cells (iPSCs) as a potential strategy to promote cardiac regeneration. Human ESC-derived cardiomyocytes enhanced cardiac function in a rat myocardial infarction model (Laflamme et al. 2007). In addition, integration and survival of human ESC-derived cardiomyocytes after transplantation in nonhuman primates in an experimental myocardial infarct model were reported recently. Re-muscularization of substantial amounts of the infarcted monkey heart was observed, albeit with occurrence of nonfatal ventricular arrhythmias (Chong et al. 2014). Dynamic regulation of miRNA is involved in differentiation of ESCs toward a cardiomyocyte fate. In vitro, induction of miR-1 and miR-499 in the differentiation from human ECS and cardiac progenitor cells toward cardiomyocytes was observed. Forced expression of these miRNAs enhanced differentiation toward a cardiomyocyte fate (Wilson et al. 2010; Sluijter et al. 2010). In vivo, transplantation of hESCs treated with miR-1 mimics improved cardiac function and increased the number of donor-derived cardiomyocytes (Glass and Singla 2011). Of interest, cardiac apoptosis was decreased after treatment (Glass and Singla 2011) suggesting that miR-1 not only facilitates cardiomyocyte differentiation but also contributes to cardioprotection after cardiac injury via paracrine mechanisms. Using miRNA sequencing and bioinformatical analysis in hESCs-derived cardiomyocytes before and after (1 year) cardiac maturation revealed a pronounced upregulation of members of the let-7 family. Overexpression of let-7 in human ESC-CMs accelerated cardiac maturation as shown by enhanced morphological and functional characteristics. Profiling after overexpression of let-7 members showed upregulation of fatty

acid metabolism and downregulation of PI3/AKT/insulin signaling, suggesting that a metabolic switch enhances cardiomyocyte maturation (Kuppusamy et al. 2015). Together, these studies reveal important roles of miRNAs for cardiac lineage commitment and maturation of pluripotent stem cells.

4.3 Targeting miRNAs to Facilitate Cardiac Regenerative Pathways

A limited number of cardiac cells are able or regain the potential to reenter cell cycle (Bergmann et al. 2009). However, this cell renewal cannot compensate for cardiomyocyte loss after acute myocardial infarction or in the progression of chronic heart failure. Therefore, therapeutic strategies to induce cardiac regeneration are currently intensely investigated. As miRNAs are crucially involved in cardiac development, lineage commitment, differentiation, and maturation of cardiomyocytes, miRNAs were recently investigated for their potential to regenerate the heart, either by direct cardiac reprogramming or induction of cardiomyocyte proliferation (Fig. 4.2).

Direct cardiac reprogramming describes a process in which resident cardiac host cells are directly trans-differentiated into cardiomyocytes. Direct reprogramming therefore circumvents the step of dedifferentiation into pluripotent stem cells but

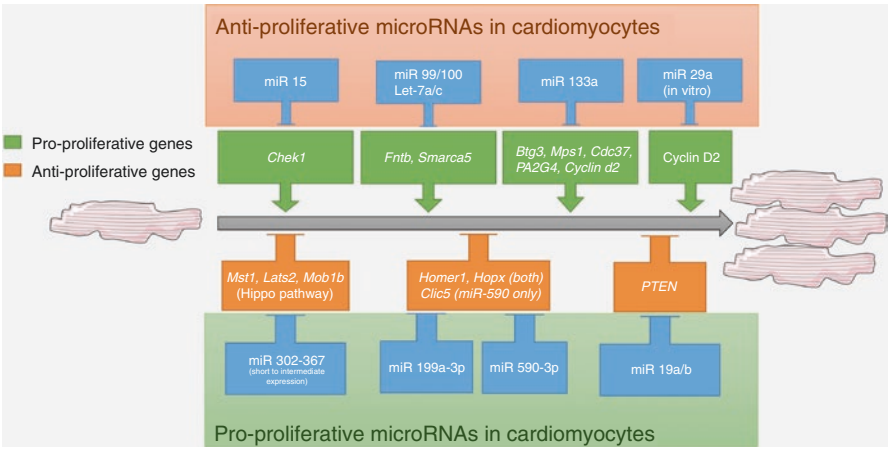

Fig. 4.2 Induction or inhibition of cardiomyocyte proliferation is regulated by miRNAs. Whereas neonatal mice show a robust cardiac repair response after cardiac injury, proliferative capacity of cardiomyocytes is (almost) lost in the adult heart of human and mice. Therapeutic targeting of miRNAs that suppresses genes involved in cell cycle reentry and mitosis results in an increased proliferation of cardiomyocytes. *Chek1* checkpoint kinase 1, *Fntb* beta subunit of farnesyltransferase, *Smarca5* SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily a, member 5, *Btg3* B-cell translocation gene 3, *Mps1* monopolar spindle 1, *Cdc37* cell division cycle 37, *PA2G4* proliferation associated protein, *mst1* mammalian STE20-like protein kinase 1, *Lats2* large tumor suppressor 2, *Mob1b* Mps one binder kinase activator 1B, *Hopx* HOP homeobox, *PTEN* phosphatase and tensin homolog

pursues reprogramming directly from endogenous non-cardiomyocytes (e.g., cardiac fibroblasts) toward functional cardiomyocytes. Ieda et al. (2010) reported recently reprogramming of *mouse* fibroblasts into cardiomyocytes. Delivery of three cardiac transcription factors (Gata4, Mef2c, Tbx5, or GMT) into fibroblasts derived from mouse hearts and skin resulted in cardiomyocyte-like cells with expression of cardiomyocyte-specific promoters and structures. The induction was also observed in a murine myocardial infarction model by using viral transfection of cardiac transcription factors (Qian et al. 2012; Song et al. 2012). Interestingly, the addition of miR-133a to GMT increased the number of directly reprogrammed cardiomyocytes and accelerated onset of beating cells by suppression of fibroblast signatures through SNAI1, a master gene of epithelial-to-mesenchymal transition (Muraoka et al. 2014). Jayawardena et al. (2012) extended these observations by using miRNAs involved in cardiac muscle development. Overexpression of miR-1, miR-133, miR-208, and miR-499 in mouse cardiac fibroblasts drives these cells toward cardiomyocytes with expression of cardiomyocyte markers and functions in vitro. Moreover, genetic tracing methods showed that intramyocardial injection of this set of miRNAs after experimental myocardial infarction converted cardiac fibroblast into cardiomyocytes-like cells (Jayawardena et al. 2012). In a follow-up study (Jayawardena et al. 2015), serial echocardiography in mice undergoing MI and injected with miR-1, miR-133, miR-208, and miR-499 showed an improvement in cardiac function over 3 months as compared to controls. Furthermore, reprogrammed rod-shaped cells exhibited similar physiological properties as mature adult ventricular cardiomyocytes. Fibroblasts from humans are more resistant to reprogramming techniques, as cardiac transcription factors (Gata4, Mef2c, Tbx5, and Hand2) used in mouse fibroblasts (Song et al. 2012) failed to reprogram human fibroblasts (Nam et al. 2013). However, addition of MyocD effectively induced cardiac gene expression. Interestingly, miR-1 and miR-133 together with four transcription factors (Gata4, Hand2, Tbx5, and MyocD) further enhanced reprogramming efficiency toward a cardiomyocyte phenotype (Nam et al. 2013). Though 19% of reprogrammed cells were cardiac troponin T-positive, functional characteristics of mature cardiomyocytes such as upregulation of cardiac genes, calcium transients, and beating cells were rarely observed.

Apart from direct reprogramming, induction of cardiomyocyte proliferation is an alternative different strategy to enhance cardiac function in injured hearts that likely underlies the capacity of zebra fish and postnatal mice to regenerate after cardiac injury. Cardiomyocyte proliferation is recognized for decades to be a prerequisite in embryogenesis and for lower vertebrates. However, Bergmann et al. showed that in adults, a low but relevant count of cardiomyocytes still proliferates (approx. 1% turnover rate/year) (Bergmann et al. 2009), which raises the possibility to enhance cell cycle in mature cardiomyocytes. In experimental studies, proliferation of cardiomyocytes after surgical injury in neonatal mice has been reported (Porrello et al. 2011b). These observations indicate postnatal regeneration of the heart. MiRNAs are required for modulation of proliferative and apoptotic processes in cardiomyocytes, as cardiac deletion of enzymes required in the biogenesis of miRNAs resulted in dilatation of the heart and premature lethality (Chen

et al. 2008; Rao et al. 2009). MiR-1 and miR-133 have been shown to regulate mitotic processes. MiR-1 is specifically expressed in the skeletal and cardiac muscle and consists of two miRNAs, miR-1-1 and miR-1-2 (Zhao et al. 2007). Mice lacking miR-1-2 die early due to ventricular septal defects (Zhao et al. 2007). Adult mice lacking miR-1 will result to overt cardiomyocyte hyperplasia. Molecular studies showed an increased expression of proteins involved in cardiac morphogenesis and development, such as Hand2 (Zhao et al. 2007). In contrast cardiac-specific overexpression of miR-1 leads to decreased ventricular cardiomyocyte proliferation (Zhao et al. 2005). MiR-133a is co-transcribed as a bicistronic construct with miR-1 and involved in cardiac development. Deletion of miR-133a-1/miR-133a-2 causes lethal ventricular septal defects in embryonic and neonatal stages and dilated cardiomyopathy in surviving adult mice (Liu et al. 2008). In these double-mutant mice, a disorganization of sarcomeres and an increased proliferation and apoptosis of cardiomyocytes were detected. Consistently, cell cycle genes were upregulated in double knockout mice. In zebra fish, downregulation of miR-133 was observed after resection of the cardiac apex. Transgenic overexpression of miR-133 suppresses cell cycle genes btg3, cdc37, PA2G4 and mps1, and connexin-43, a gap junction protein required for intercellular communication (Yin et al. 2012), as it was shown for miR-499 (Hosoda et al. 2011). MiR-133a therefore suppresses cardiomyocyte cell cycle and guides differentiation into cardiomyocytes. As changes in spatiotemporal expression of miRNA are observed, a study linked the transient regenerative capacity in postnatal murine hearts (Porrello et al. 2011b) to detect up- and downregulated miRNAs using a microarray approach (Porrello et al. 2011a). MiR-195, a member of the miR-15 family, is highly upregulated in mouse hearts between day 1 and 10 after birth. Delivery of anti-miRs targeting miR-15 family members in neonatal mice increased cardiomyocyte proliferation by de-inhibition of cell cycle genes (Porrello et al. 2011a). Porrello et al. (2013) further investigated the impact of miR-15 on cardiac regeneration after cardiac injury in postnatal mice. Postnatal MI at day 1 resulted in an extensive infarcted area. However, at day 21, a functional recovery can be observed (Porrello et al. 2013). Cardiac-specific overexpression of miR-195 (a member of the miR-15 family) in these mice impaired cardiac regenerative capacity with extensive fibrosis in the infarcted area and decreased proliferating cardiomyocytes (Porrello et al. 2013). Furthermore, pretreatment of postnatal mice with anti-miR-15 improved cardiac function after induction of myocardial infarction in adult mice (Porrello et al. 2013). Of note, transgenic overexpression of miR-195 results in cardiac growth and disassembly of cardiomyocytes (van Rooij et al. 2006), leading to dilated cardiomyopathy. However, inhibition of miR-195 was recently shown to increase elastin deposition in the aorta of mice (Zampetaki et al. 2014). Therefore, the role of miR-195 in cardiac extracellular matrix deposition has to be determined in future studies. Similar to miR-195, miR-29a is upregulated when comparing miRNA array expression data from cardiomyocytes derived from rats at postnatal day 2 when compared to postnatal day 28 (Cao et al. 2013). MiR-29a targets cell cycle genes (CCND2). In vitro, inhibition of miR-29a in neonatal cardiomyocytes enhances cardiomyocyte proliferation (Cao et al. 2013).

However, important regulators of regenerative processes can be missed in mammals when evolutionary conserved mechanisms are not activated upon heart injury. In zebra fish, heart amputation results in a downregulation of miR-99/100 and let--7a/c, which is not observed in mice after MI (Aguirre et al. 2014). However, intramyocardial delivery of an adenovirus encoding for anti-miR-99/100 and anti-let-7a/c in mice undergoing MI improved cardiac function and decreased scar formation. These effects were triggered by an increase in dedifferentiated and proliferation of cardiomyocytes and resembled the regenerative mechanisms observed in zebra fish (Aguirre et al. 2014).

These studies investigated miRNAs with antiproliferative effects on cardiomyocytes. In contrast, a recent study reported that miRNAs can also induce proliferation of cardiomyocytes (Eulalio et al. 2012). A functional high-throughput screening was performed to detect miRNAs involved in cardiomyocyte proliferation. Neonatal cardiomyocytes were transfected with a miRNA library consisting of 875 miRNAs (Eulalio et al. 2012). Remarkably, 204 miRNAs increased neonatal cardiomyocyte proliferation in vitro. Two pro-proliferative miRNAs – miR-199a and miR-590 – were further used for in vivo experiments. Overexpression of these miRNAs in neonatal rats revealed a thicker myocardium and increased cardiomyocyte proliferation. Moreover, intramyocardial overexpression of miR-199a and miR-590 in adult mice undergoing myocardial infarction induced cardiomyocyte proliferation in the peri-infarct area, reduced infarct size, and improved cardiac function (Eulalio et al. 2012).

The role of the miRNA cluster miR-17-92 for cardiac proliferative processes was also investigated. Cardiac-specific deletion of miR-17-92 leads to decreased cardiomyocyte proliferation in postnatal hearts (Chen et al. 2013a). Consistently, overexpression of miR-17-92 in embryonic and postnatal cardiomyocytes increased their proliferative capacity with a thickened myocardium due to hyperplasia. Intriguingly, induced cardiac expression of miR-17-92 in adult mice, where proliferative capacity of cardiomyocytes is almost lost, resulted in an increased cardiomyocyte proliferation. In addition, cardiac overexpression of miR-17-92 preserved cardiac function after myocardial infarction (Chen et al. 2013a). Another study investigated the miR-302-367 cluster in hearts due to its contribution in lung development. Cardiac-specific knockout resulted in decreased embryonic cell proliferation associated with a decreased cardiomyocyte differentiation (Tian et al. 2015). Target analysis after overexpression of miR-302-367 showed suppression of Mst1, Lats2, and Mob1b, which are all acting as contributors of the Hippo signaling pathway upstream of the Yes-associated protein (YAP). Phosphorylation of the transcriptional co-activator YAP results in suppression of cell proliferation. Consistently, transgenic cardiac or systemic transient overexpression of the miR-302-367 enhanced cardiomyocyte proliferation and improved cardiac function in a mouse myocardial infarction model. However, long-term overexpression of miR-302-367, consistent with the role of the Hippo pathway in the regulation of organ growth, leads to dilatation of the left ventricle, which favors a transient application of this miR cluster (Tian et al. 2015). These studies indicate that cell cycle reentry of cardiomyocytes can be induced by administration of pro-proliferative miRNAs.

4.4 MiRNAs in the Development and Progression of Heart Failure

Multiple mechanical and pathological stress triggers and cardiac injury evoke detrimental cardiac remodeling processes in the adult heart leading to chronic heart failure. It is not surprising that miRNAs are also involved in cardiomyocyte pathophysiological mechanisms (including cardiomyocyte hypertrophy, apoptosis, survival, and reactivation of the fetal gene program). However, as the heart comprises other important cell fractions, miRNAs altering functions of cardiac fibroblasts, which trigger extracellular matrix deposition and fibrosis and changes in endothelial-derived miRNAs that regulate angiogenesis, are also important mechanisms for heart failure development and progression. During heart failure development, many fetal genes, which are quiescent in the adult heart, are reactivated (Miyata et al. 2000; Nakao et al. 1997). As activation of gene programs in the fetal heart is regulated by miRNAs, miRNAs inducing fetal genes were also found in experimental and clinical studies. First insights came from a conditional knockout of Dicer, an enzyme needed for processing functional mature miRNAs. Knockout of Dicer led to cardiac remodeling processes and upregulation of fetal gene transcripts (viz., Acta1, Nppb, Myh7, and Nppa) (da Costa Martins et al. 2008). Comparison of miRNAs in experimental hypertrophic cardiomyopathy models showed >12 deregulated miRNAs as compared to sham-operated mice, which showed an overlap when comparing nonfailing versus end-stage heart failure tissues from humans (van Rooij et al. 2006). Seok et al. (2014) observed a downregulation of miR-155 in cardiomyocytes in a pressure overload model. Knockout of miR-155 in mice repressed cardiac hypertrophy, partly by targeting Jarid2, a key transcriptional regulator of cardiac development and function. Formerly, miR-155 was known as an abundantly expressed miRNA in macrophages and monocytes. The loss of miR-155 in macrophages was found to inhibit leukocyte infiltration and protect murine hearts from hypertrophy, mostly by inhibition of pro-inflammatory macrophage-derived factors and downregulation of adhesion molecules (Heymans et al. 2013). These studies imply that targeted therapy of one miRNA may protect different cell types from pathological mechanisms – either by inhibition of paracrine secretion of macrophages or from hypertrophic response in cardiomyocyte after pressure overload.

Comparison of miRNA expression profiles using left ventricular tissue samples from patients with heart failure, nonfailing hearts, and fetal human heart tissues revealed a profound alteration in miRNA expression of heart failure tissues as compared to healthy tissue samples. In addition, up- and downregulated miRNA showed a > 85% coverage when miRNA expression of heart failure tissues was compared with fetal tissue, indicating a close relationship in molecular miRNA-dependent mechanism with the reactivation of fetal gene programs (Thum et al. 2007). Another elegant study performed a deep-sequencing analysis of RNA of human left ventricular tissue samples derived from nonfailing human LV and failing human LV before and after left ventricular assist device (LVAD) support (Yang et al. 2014). More than 147 miRNAs were differentially regulated when comparing nonfailing

with heart failure LV samples; however, only two to five miRNAs returned to normal levels after LVAD support. These observations are consistent with a recent study where only subtle changes in miRNA expression between ischemic and dilated cardiomyopathy were detected, and miRNA profiling did not reveal differences before and after LVAD treatment (Akat et al. 2014). In contrast, >570 lncRNAs were found to be deregulated, mostly of mitochondrial origin, and approximately 10% of these lncRNAs normalized after LVAD support (Yang et al. 2014).

Changes in intracellular calcium handling are critical for heart failure, as they determine cardiac contractility. Gene therapy using an adenoviral vector containing sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2) led to a decrease in clinical symptoms and reverse remodeling in heart failure patients in a small clinical phase II study (Jessup et al. 2011). SERCA2 is a calcium-transporting ATPase, which enables calcium uptake in the sarcoplasmic reticulum during relaxation of cardiomyocytes. Wahlquist et al. (2014) used the 3′-UTR region of SERCA2 as a sensor construct to identify miRNAs targeting SERCA2. MiR-25 markedly inhibited SERCA2 expression and was upregulated in the myocardium derived from patients with heart failure. Treatment with anti-miR-25 in a transaortic constriction (TAC) model in mice blunted progression of cardiac dysfunction and improved survival. Interestingly, in a study investigating experimental Hand2-induced cardiac hypertrophy, inhibition of miR-25 resulted in impaired cardiac function using a TAC model (Dirkx et al. 2013). Although the two studies used the same experimental hypertrophy model, inhibition of miR-25 was started 3 months (Wahlquist et al. 2014) versus 3 days (Dirkx et al. 2013) after TAC operation and cardiac function were assessed at 5.5 months (Wahlquist et al. 2014) as compared to 1 month (Dirkx et al. 2013) after TAC. The findings therefore suggest that miR-25 has different functions in the subacute versus chronic heart failure phase, and miR-25 expression may be dynamic in the course of hypertrophy and heart failure. However, these questions have to be addressed in future studies. Dynamic expression of miRNAs upon a stress trigger has been shown in various studies, such as for miR-212/132 (Ucar et al. 2012), miR-208 (van Rooij et al. 2007), and miR-195 (van Rooij et al. 2006).

Cardiac fibroblasts contribute to adverse remodeling processes and progression of heart failure. MiRNAs have been identified as critical regulators in cardiac fibroblasts, thereby contributing to extracellular matrix modulation and cardiac fibrosis. MiR-29 targets multiple collagens and expression of extracellular matrix proteins. In an experimental model of MI, miR-29 expression was markedly reduced in the infarct region and mostly of fibroblast origin when comparing expression between fibroblasts and cardiomyocytes (van Rooij et al. 2008). Interestingly, transforming growth factor β (TGFβ), a key enhancer of cardiac fibrosis, decreased miR-29 expression in vitro, suggesting that upregulation of TGFβ represses miR-29 expression, resulting in an enhanced deposition of extracellular matrix proteins (van Rooij et al. 2008). MiR-29 downregulation was also identified as a pro-fibrotic mechanism in pulmonary (Cushing et al. 2011) and renal (Qin et al. 2011) fibrosis. However, miR-29 is also involved in the progression of aortic aneurysms. Reduced expression levels of miR-29a and inverse correlation with aortic size have also been

observed in patients with aortic aneurysm (Jones et al. 2011). Another miR-29 family member, miR-29b, was reported to be upregulated in patients and in experimental models of aortic aneurysm (no significant difference of miR-29a expression was observed in this study). Downregulation of miR-29 by delivery of LNA-modified antisense oligonucleotides in vivo resulted in enhanced expression of collagen members and reduction in aortic diameter (Boon et al. 2011). Therefore, whereas overexpression of miR-29 after myocardial infarction may reduce cardiac fibrosis, a reduced matrix deposition in miR-29-treated subjects may lead to the progression of aortic dilatation.

Another prominent fibrosis regulating miRNA is miR-21 that is upregulated in rodent models of ischemia-reperfusion (Roy et al. 2009) and hypertrophy (van Rooij et al. 2006). First shown as a pro-fibrotic miRNA in a pressure overload mouse model, which supports cardiac remodeling by an increase of ERK-MAP kinase activity in cardiac fibroblasts, it was later shown that upregulation of miR-21 after experimental ischemic preconditioning was protective in cardiac myocytes by inhibiting the expression of programmed cell death 4 (PDCD4) (Dong et al. 2009). In addition, sodium sulfide (Na2S), a donor for hydrogen sulfide, which is protective in various injury models of the heart, induces miR-21 in cardiomyocytes, indicating that the observed improvement in survival and decreased infarct size in an ischemia-reperfusion injury model are mediated by miR-21 through an inhibition of inflammasome function (Toldo et al. 2014). As miR-21 is expressed in both cardiomyocytes and cardiac fibroblasts, miRNA-mediated cell-to-cell communication was recently investigated. In this study, miR-21-3p (the star strand of miR-21, which is supposedly degraded) showed a high abundance in fibroblast-secreted exosomes and uptake of miR-21-3p in cardiomyocytes that resulted in cardiomyocyte hypertrophy via downregulation of SH3 domain-containing protein 2 (SORBS2) and PDZ and LIM domain 5 (PDLIM5) (Bang et al. 2014). These experimental studies imply that one miRNA may exert different functions in different cell types within the heart. In addition, miR-21-3p, a star strand that was earlier thought to be degraded, may also act as a functional miRNA. Moreover, the observation that miR-21 is not needed to induce cardiac hypertrophy in a knockout model and an experimental model using a different anti-miR (8-mer LNA miR-21) (Patrick et al. 2010) and different cardiac heart failure models leading to divergent findings of miR-21 action suggests that spatiotemporal expression of miRNAs and different methodological approaches are important determinants of miRNA functional activity.

4.5 Long Noncoding RNAs in Cardiovascular Disease

Long noncoding RNAs (lncRNAs) consist of a new group of noncoding RNAs emerging as genetic modifiers in cardiovascular disease. Whereas miRNAs belong to the short RNAs (i.e., <200 nucleotides), lncRNAs harbor more than 200 nucleotides. In addition, other than miRNAs, lncRNA interaction is not (almost) exclusively dependent on mRNA translational repression or mRNA degradation but rather activates or silences gene transcription through chromatin regulation and

transcriptional modulation (Geisler and Coller 2013; Wahlestedt 2013). In addition, transcriptional modulation of lncRNAs acts by *cis* (at the site of synthesis) or *trans* (at many different sites) fashion (Wahlestedt 2013; Mercer and Mattick 2013). LncRNA are not well preserved between species, and around 30% are specifically detected in primates (Derrien et al. 2012). Initial studies were performed in the oncology field, where investigated lncRNA was linked to cancer diseases (Gutschner et al. 2013; Yap et al. 2010). Some of these lncRNAs now gain also attention in the cardiovascular field, such as MALAT1 and ANRIL (Vausort et al. 2014; Burd et al. 2010). As gene transcription regulators, lncRNAs, like miRNAs, are involved in the regulation of cardiac development. One such lncRNA is Fendrr. Fendrr knockout models have been shown to impair embryonic cardiomyocyte proliferation, leading to hypoplasia and impaired cardiac function, resulting in embryonic death (Grote et al. 2013). Mechanistic studies observed that Fendrr modifies chromatin regulation through the binding of PRC2 and TrxG/MLL complexes (Grote et al. 2013). Deletion of Fendrr in another knockout model (Sauvageau et al. 2013) resulted in perinatal lethality. Ventricular septal defects and unstructured vessels were observed. In addition, reduced expression of Fendrr was observed in mutants of an endothelial-specific knockout model of Forkhead Box transcription factor F1 (FOXF1), a critical factor for vascular development (Ren et al. 2014). Another lncRNA involved in heart development is Braveheart, which is expressed in mice but lacks an orthologue in other species (Klattenhoff et al. 2013). Braveheart was found to interact in a gene network upstream of Mesp1 and is needed for activation of cardiac transcription factors, which drive mesodermal cells toward a cardiovascular phenotype (Klattenhoff et al. 2013). Braveheart was also detected as a cardiac-enriched lncRNA in a study investigating lncRNA expression in different mouse tissues (hearts, livers, and skin cells) using RNA sequencing (Matkovich et al. 2014). In adult mouse hearts, 152 lncRNAs showed high expression levels. Out of these lncRNAs, 48 lncRNAs are enriched in the heart as compared to liver and skin cells. Furthermore, RNA sequencing in cardiomyocytes and fibroblasts from adult mouse hearts indicates that most of these lncRNAs are enriched in the cardiomyocyte cell fraction (Matkovich et al. 2014). RNA sequencing allows also to search for differentially regulated lncRNA in cardiovascular disease models. Pedrazzini's research group investigated alterations in cardiac lncRNA profiles after myocardial infarction in mice (Ounzain et al. 2015). Analysis of the RNA sequencing revealed 988 annotated lncRNAs but also identified 1521 novel lncRNAs, of which 60% are heart specific according to computational analysis. Importantly, human orthologues were found in 73% of novel lncRNAs detected in mouse. Downregulation of an unannotated novel lncRNA, NovInc6, was further shown in patients with dilated cardiomyopathy, in concert with suppression of a predicted target Nkx2-5, a key transcription factor of cardiac development and cardiac gene program. Using a similar approach, Zangrando et al. (2014) screened for differentially expressed lncRNAs 24 h after induction of MI in C57/BL6 mice using an Agilent microarray with 55,681 probes. Ten and twenty lncRNAs were down- and upregulated more than twofold, with NR_028427 (named myocardial infarction-associated transcript 1 (MIRT1)) and ENSMUST000001005122 (named MIRT2) showing the highest fold changes

between groups. As a trend toward a correlation with LV remodeling parameters was observed, computational analysis of genes involved in remodeling processes identified strong correlation with 18 (for MIRT 1) and 17 (for MIRT 2) remodeling genes. However, no orthologues of MIRT1 and MIRT2 exist in humans.

As experimental models significantly affect lncRNA levels, it is of interest whether therapeutic interventions result in changes of lncRNA expression in humans. Yang et al. (2014) investigated differential expression of lncRNA in ischemic and nonischemic cardiomyopathy before and after LVAD support using RNA sequencing. Interestingly, cluster analysis revealed lncRNA signatures discriminating between ischemic and nonischemic cardiomyopathy. After LVAD support, a higher percentage of lncRNA show improved or normalized levels as compared to miRNA and mRNA expression profiles. LncRNA expression profiles furthermore were able to distinguish between before and after LVAD treatment, which indicates that lncRNA is involved in signaling pathways leading to reverse remodeling after LVAD support (Yang et al. 2014). Changes in transcriptome, including lncRNA expression, not only occur in the heart. A recent report by Deveaux's group (Vausort et al. 2014) assumed that MI alters lncRNA levels in the blood drawn from these patients. In a large sample group, five pre-specified lncRNAs associated with cardiovascular disease processes (hypoxia-inducible factor 1A antisense RNA 2 (aHIF), ANRIL, potassium voltage-gated channel, KQT-like subfamily, member 1 opposite strand/antisense transcript 1 (KCNQ1OT1), MIAT, and MALAT1) were analyzed in patients with acute MI and presumably healthy subjects. Levels of aHIF, MALAT1, and KCNQ1OT1 were higher, and expression of ANRIL is lower in patients with MI as compared to healthy subjects. Expression analysis of the five lncRNAs in subpopulations of mononuclear cells (in healthy subjects) showed that the distribution pattern of lncRNAs differs in the subpopulations. In addition, ANRIL and KCNQ1OT1 added prognostic information to a clinical model for LV dysfunction (LVEF <40%) at 4-month follow-up.

Genetic variants have been observed as predictors of cardiovascular diseases. Variations on chromosome 9p21 (Samani et al. 2007; Ye et al. 2008) increase the susceptibility of cardiovascular disease. Single nucleotide polymorphisms (SNPs) within this genomic region are associated with coronary artery disease and premature myocardial infarction (Abdullah et al. 2008; Samani et al. 2007). Interestingly chromosome 9p21 harbors the lncRNA ANRIL (antisense noncoding RNA at the ink4 locus or CDKN2BAS (antisense to CDKN2B)). Recently, ANRIL expression was correlated with variants associated with a higher risk for coronary artery disease, suggesting that ANRIL regulates chromatin modulation of coronary artery disease susceptibility genes like the INK/ARF locus (Burd et al. 2010). Overexpression of ANRIL in monocytic cell lines increased proliferation, cell adhesion, and blunts apoptosis (Holdt et al. 2013), potential mechanisms that trigger atherosclerosis. This raises the possibility that at least some lncRNAs are the missing link between SNP and risk of MI and CAD. Another example of SNPs causing a risk of myocardial infarction is the discovery of myocardial infarction associated transcript (MIAT). Subjects with a SNP in exon 5 of MIAT show a higher susceptibility for myocardial infarction in a large-scale case-control association study.

Numerous reports observed that MIAT is involved in splicing efficiency, which may explain the findings of the aforementioned study (Aprea et al. 2013; Tsuiji et al. 2011). Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1)/NEAT2 is another nuclear lncRNA with splicing ability (Hutchinson et al. 2007) and was found to enhance proliferation in human diploid fibroblasts and HeLa cells via this mechanism. However, in endothelial cells, where MALAT1 silencing impairs endothelial cell proliferation, expression of splicing-related genes is not altered, but cyclins and kinases were downregulated (Michalik et al. 2014). Together, these studies expand the knowledge of silencing and activation of gene networks in cardiovascular research and introduce lncRNA as new regulators in the complex molecular understanding. As lncRNAs are crucially involved in key features of cardiac injury, such as apoptosis, inflammation, impaired angiogenesis, and device treatment that lead to a change in lncRNA expression, lncRNAs may provide a future diagnostic and therapeutic clinical tool.

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Conflict of Interest Philipp Jakob has received research grants from Bayer Healthcare (Grants4Targets).

Compliance with Ethical Standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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