Heart and Metabolism is a quarterly journal focusing on the management of cardiovascular diseases. Its aim is to inform cardiologists and other specialists about the newest findings on the role of metabolism in cardiac disease and to explore their potential clinical implications. Each issue includes an editorial, followed by articles on a key topic. Experts in the field explain the metabolic consequences of cardiac disease and the multiple potential targets for pharmacotherapy in ischemic and nonischemic heart disease.

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Since they were first identified just over 20 years ago, microRNAs have emerged as major regulators of cellular physiology, as well as being identified as therapeutic targets for treating a number of cardiovascular diseases. MicroRNAs are small, non-coding RNA molecules that act as inhibitors of mRNA protein expression. MicroRNAs are highly conserved among species and have a critical role in regulating cellular physiology. Since the initial description of a microRNA in Caenorhabditis elegans, in excess of a thousand microRNAs have been identified, including numerous microRNAs involved in regulating the cardiovascular system. In addition, microRNAs have become a target for therapeutics in many types of cardiovascular diseases, with therapeutics aimed at modifying microRNA functions now entering clinical trials. This not only includes the use of microRNAs to inhibit protein expression, but also the use of inhibitors of microRNAs (such as antisense microRNAs) to overcome the inhibitory effects of microRNAs. Furthermore, microRNAs have become important biomarkers to identify cardiovascular disease. This issue of *Heart and Metabolism* consists of a number of key articles that address this exciting and emerging area of cardiovascular biology.

The article by Thomas Thum discusses the role of microRNAs in the cardiovascular system, including the role of microRNAs in fine-tuning and regulating proteins involved in many cardiovascular pathways, including cell signaling pathways, pathways involved in critical cellular functions, and developmental pathways. He also discusses the potential for long noncoding RNAs to regulate gene and protein expression. The article by John R. Ussher discusses how a number of microRNAs can undergo deregulated expression in response to numerous stresses including ischemia, heart failure, and pulmonary hypertension. This strengthens the concept of targeting microRNAs to treat cardiovascular disease.

The article by Eva van Rooij reviews the potential of regulating microRNAs in vivo for treating cardiovascular diseases and the therapeutic benefit of microRNA modulation. She also discusses the strategy of pharmacological modulation of individual microRNAs by modified antisense oligonucleotides (ie, antimiRs) to inhibit a microRNA function, as well as the clinical status of this promising approach. Louise R. Rodino-Klapac’s article discusses potential strategies to modulate the activity of microRNA activity using several different approaches, which includes either upregulating or blocking microRNA function. The article by Philipp Jakob and Ulf Landmesser also demonstrates the potential of microRNA targeting to regulate cardiac developmental processes and cardiomyocyte proliferation. They demonstrate the importance of microRNAs in cardiac repair, cardiac lineage commitment, and regulation of cardiomyocyte proliferation. The authors also discuss the potential of targeting microRNAs to reprogram cells to induce pluripotency and conversion of cells into cardiomyocytes.
that can be used to treat myocardial injury. The paper by Anna Zampetaki et al. discusses the potential use of microRNAs to design novel therapeutic approaches to cardiovascular disease, as well as the potential to use circulating microRNA signatures as disease biomarkers and as diagnostic and prognostic tools.

As microRNAs emerge as important regulators of the cardiovascular system, it becomes important to develop new molecular imaging tools to evaluate microRNAs. The article by Wanda Kloos et al. describes the state-of-the-art approaches being developed to assess microRNA expression, including sensitive and dynamic in vivo imaging capabilities. This includes the use of fluorescent proteins, bioluminescent enzymes, molecular beacons, and nanoparticles to monitor microRNA function.

While microRNAs have emerged as critical regulators of gene expression in cardiovascular disease, it is by no means the only way to alter gene expression in cardiovascular disease. This point is stressed in the article by Pericle Di Napoli who describes the nonmetabolic effects of trimetazidine in regulating gene expression and preventing adverse remodeling in ischemic heart disease.

In a very short period of time, microRNAs have emerged as a promising target for therapeutic intervention in treating many types of cardiovascular diseases. This has occurred despite only a decade having passed since the identification of the first human microRNA. The next decade should solidify microRNA biology as a major therapeutic approach to treat cardiovascular disease.

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MicroRNA therapeutics for cardiovascular disease

Eva van Rooij, PhD
Hubrecht Institute, KNAW and University Medical Center Utrecht, The Netherlands

Correspondence: Eva van Rooij, Hubrecht Institute, KNAW and University Medical Center Utrecht, 3584CT Utrecht, The Netherlands
E-mail: E.vanrooij@hubrecht.eu

Abstract
MicroRNAs (miRNAs) are short, single-stranded, noncoding RNAs that exert their function through annealing with complementary sequences in specific messenger RNAs (mRNAs) to inhibit their translation into proteins. Even though it was only a decade ago that the first human miRNA was discovered, therapeutics to regulate miRNA function are already entering the clinical space. The main reasons for this rapid transition into the clinic are their prominent roles during disease, their known and conserved sequences, and their heightened functions under conditions of disease. Based on lessons learned from antisense technologies, the opportunity to potently regulate miRNAs in vivo already exists and rapidly opened up the potential for miRNAs to become a new class of drugs, which is underscored by the wealth of recent animal and even human efficacy data indicating the therapeutic benefit of miRNA modulation. Also, for cardiac disease, important miRNA functions have been uncovered and preclinical efficacy studies using oligonucleotide chemistries to modulate miRNA levels have proven to be effective in targeting pathological miRNAs, resulting in therapeutic benefit.

Keywords: anti-miR; cardiovascular disease; microRNA; miRNA mimic; therapeutics

MicroRNAs (miRNAs) are short RNA molecules that act by inhibiting the expression of specific proteins encoded by messenger RNAs (mRNAs). The primary transcripts of miRNAs are transcribed as long precursor RNAs, called pri-miRNAs. Pri-miRNAs are sequentially processed in the nucleus and cytoplasm into 20 to 22 nucleotide strands of RNA, called mature miRNA. The mature miRNA associates with mRNAs within a multiprotein complex of Argonaute proteins, known as the RNA-induced silencing complex (RISC), to facilitate the interaction with target mRNAs. Through complementary base pairing, they anneal with protein coding mRNAs and inhibit their protein translation through mRNA decay or translational repression (Figure 1). It is now thought that the human genome contains approximately 1500 miRNAs, of which roughly 300 are detected in the heart.

An interesting aspect about miRNA activity is that while miRNAs are often moderate regulators under homeostatic conditions, their function becomes more pronounced under conditions of injury or stress. The heightened function of a miRNA during stress can be explained by the fact that stress, or “disease” signals, influences numerous aspects of miRNA biogenesis and function, such as the abundance of the miRNA, changes in expression of the mRNA targets, or differences in proteins regulating miRNA activity under stress. While the influence of stress is likely miRNA- and trigger-dependent, changes in miRNA expression and functionality under disease conditions imply
the existence of an active mechanism for differential miRNA activity under stress.

**MicroRNA-modulating drugs**

In contrast to a classic drug approach, anti-miR drugs are designed knowing that they will affect all genes that are under the control of the target miRNA. The pharmacological modulation of individual miRNAs can be achieved by modified antisense oligonucleotides, referred to as anti-miRs, to inhibit a miRNA or by a miRNA mimic to replace a miRNA (Figure 2). Of these two approaches, the anti-miRs are currently the most advanced.

The first in vivo mammalian study using modified cholesterol-conjugated oligonucleotides complementary to inhibit miR-122, a liver-specific miRNA, was published in 2005. Many follow-up pharmacokinetic studies have taught us that these compounds can be delivered subcutaneously and distributed to all organs, including the heart, with a preferential delivery to the kidney and liver, and that the in vivo half-life of anti-miRs is in the order of weeks. For anti-miRs to achieve effective pharmacological inhibition of disease-associated miRNAs, they have to show a high level of in vivo stability, specificity, and binding affinity to the miRNA of interest. Currently, several chemical modifications are used to increase

**Fig. 1** miRNA biogenesis and function. miRNA genes are usually transcribed by RNA polymerase II to form a miRNA precursor, termed a pri-miRNA, that forms a hairpin-shaped loop structure, called pre-miRNA, which is cleaved by an enzyme called Drosha. The pre-miRNA is exported from the nucleus into the cytoplasm, where it is further cleaved by the RNase III enzyme Dicer, yielding an imperfect miRNA:miRNA* duplex about 22 nucleotides in length. Only one strand is usually incorporated into the RISC to associate with mRNA targets. Association of a miRNA with its mRNA target results in degradation of the mRNA, as well as translational inhibition. Stress conditions can influence miRNA biogenesis on multiple levels.

**Abbreviations:** AAA, ATPases Associated with diverse cellular Activities; miRNA, messenger RNA; mRNA, microRNA; ORF, open reading frame; pri-miRNA, primary microRNA; RISC, RNA-induced silencing complex; RNase, ribonuclease.


**Fig. 2** miRNA-based therapeutics. Pharmacological modulation of individual miRNAs can be achieved by modified antisense oligonucleotides, referred to as anti-miRs, to inhibit a miRNA or as a miRNA mimic to replace a miRNA. A. An anti-miR can either be fully complementary to the mature miRNA sequence or harbor a truncated sequence targeting the 5’ region of the mature miRNA. Systemic delivery will allow the anti-miR to find and bind to the target miRNA and prevent it from functioning. B. Restoring the function of lost or downregulated miRNAs can be achieved by therapeutic mimicry or reexpression of a miRNA by using synthetic RNA duplexes designed to mimic the endogenous functions of the miRNA of interest. These miRNA mimics harbor chemical modifications that improve their stability and cellular uptake without interfering with its miRNA function. An example of a synthetic double-stranded miRNA mimic is shown.

**Abbreviations:** Chol, cholesterol; miRNA, microRNA.

MicroRNA therapeutics

van Rooij

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nuclease resistance, facilitate cellular uptake, and reduce clearance by glomerular filtration and urinary excretion.8,10,11 The first anti-miR drug has now entered the clinical arena as Santaris Pharma recently reported on both the safety and efficacy of their anti-miR against miR-122, miravirsen, in humans. The data indicated that miravirsen given as a 4-week monotherapy to hepatitis C (HCV) patients provided long-lasting suppression of viremia and a high barrier to viral infection.12

Restoring the function of lost or downregulated miRNAs can be achieved by therapeutic mimicry or reexpression of a miRNA by using synthetic RNA duplexes designed to mimic the endogenous functions of the miRNA of interest. These miRNA mimics harbor chemical modifications that improve their stability and cellular uptake without interfering with its miRNA function.7 The first phase 1 study of the liposome-formulated miR-34 mimic-based drug was recently announced to commence in patients with primary liver cancer or metastatic cancer with liver involvement. This is the first miRNA mimic to advance into the clinic and, thus, is an important milestone for the development of miRNA-based replacement therapeutics.13

These advancements in miRNA therapeutics toward the clinic support the enthusiasm for exploring opportunities in additional disease areas.

Cardiac microRNA therapeutics

For heart disease, miRNAs have been shown to be major players. The first evidence suggesting the involvement of miRNAs in heart disease came from studies demonstrating changes in expression of specific miRNAs in diseased hearts from mice and humans.14 By now, numerous miRNAs have been shown to influence cardiomyocyte hypertrophy, cardiomyocyte survival, changes in cardiac metabolism, and other processes associated with the progression of heart disease.15 The rapidly growing knowledge on the functional relevance of miRNAs during cardiac disease, the shortage of effective therapies, and the ability to potently and specifically regulate miRNAs in vivo has catalyzed efforts to explore pharmacological manipulation of miRNAs for treating heart disease. Many preclinical rodent studies have shown effective cardiac delivery and miRNA inhibition after subcutaneous delivery of anti-miRs and have indicated the potent effects of inhibition under disease conditions,4 of which a few relevant examples are outlined below.

An especially intriguing miRNA for the heart is the cardiac-specific miRNA, miR-208a.14,16 This miRNA is located within an intronic region of the gene encoding for α-myosin heavy chain, the major contractile protein of the heart. In response to cardiac stress, the adult heart changes from the α- to the β-myosin heavy chain isoform, which is thought to contribute to a diminution of cardiac contractility. Both genetic deletion or anti-miR—based inhibition of miR-208a in rodents prevents myosin switching and reduces pathological remodeling of the heart under disease conditions.14,17 Its cardiac specificity and its role in contractility and remodeling make miR-208a an attractive therapeutic candidate.

Adult cardiomyocytes have lost their ability to divide, which prevents the heart from being able to repair itself following injury. Identifying the key regulators of cardiomyocyte proliferation and therapeutic manipulation of this process represents one of the central challenges in cardiovascular medicine today. Recent studies showed that miRNAs can regulate the process of heart regeneration in mice. One miRNA, called miR-15, is upregulated in response to cardiac stress and myocardial infarction (MI), which causes death of cardiomyocytes and loss of pump function.18,19 Inhibition of miR-15 with an anti-miR protects the cardiomyocyte against ischemic damage and allows cardiomyocyte proliferation, which enhances the regeneration ability of the adult heart following injury.20,21 These findings can potentially provide a powerful new means of promoting heart repair by preventing myocyte loss through miRNA modulation.

For vascular indications, anti-miR strategies have been effective in targeting miRNAs that are preferentially expressed in endothelial and smooth muscle cells. miR-92a, a member of the miR-17-92 cluster, has been implicated in neoangiogenesis following ischemic injury.22 Intravenous administration of anti-miR-92a showed efficacious inhibition of the miRNA, which resulted in enhanced blood vessel growth as well as functional improvement in damaged tissue in models of hind limb ischemia and myocardial infarction, as a result of the derepression of multiple pro-angiogenic factors.23 Recent data in a porcine model of ischemic injury showed a potential advantage of localized delivery of anti-miR-92a to the heart, since
catheter-based delivery, but not systemic delivery, of anti-miR-92a showed a reduction in infarct size, which correlated with an improved ejection fraction and left ventricular end-diastolic pressure, indicating relevance of more directed cardiac delivery approaches.23

Although, so far, no miRNA-based therapeutics for cardiovascular disorders have reached human trials, the wealth of positive preclinical data in numerous animal models of diseases such as heart failure, cardiac hypertrophy, fibrosis, and hyperlipidemia suggest that human data will soon be forthcoming.

Looking to the future

Their prominent roles during disease, their known and conserved sequences, and their heightened functions under conditions of disease make miRNAs attractive candidates for therapeutic manipulation. The realization that miRNAs might be viable therapeutic targets for many serious health conditions triggered many companies to focus on translating these exciting scientific discoveries into real-world, commercial uses. However, while we are making important steps forward in developing anti-miR chemistries as a novel uses, we are making important steps forward in developing anti-miR chemistries as a novel therapy, numerous hurdles and questions remain in the path toward the development of miRNA-based therapeutics in general.

While some miRNAs have a very cell- or tissue-specific expression pattern, many miRNAs are broadly expressed and may have multiple effects in different tissues. Potential sources of toxicity after administration of a miRNA inhibitor can result not only from toxicities induced by the chemistry or unwanted gene changes, but can also arise from effects of the anti-miR on off-target, nondiseased tissues. For more chronic treatment regimens, localized delivery strategies, such as cardiac catheters or injectable hydrogel techniques, should be contemplated to prevent unwanted side effects.

Currently, there are still many unknowns surrounding the mode of action, cellular uptake, and distribution of the different anti-miRs. Their long half-life and activity (up to weeks or even months) indicates a high degree of stability, but also suggests that the cell has a reservoir of anti-miRs that, during the course of time, slowly releases the anti-miRs into the cytoplasm to inhibit the newly formed miRNA copies. More in-depth biochemistry will be required to gain the appropriate insights.

Despite the hurdles that still need to be overcome, the excitement surrounding miRNAs as a novel class of drugs is tremendous and the anticipated success of the early forerunners that are already in the clinic will likely trigger the search for additional therapeutic miRNA targets. Further optimization of the inhibitors and mimics as well as the search for techniques for efficient and safe delivery in vivo will trigger advancement of miRNA therapeutics. While we await more efficacy data in human subjects, the next few years promise to provide important insights into miRNA and anti-miR biology, and hopefully even further strengthen the enthusiasm for this new therapeutic approach.

Eva van Rooij is cofounder and SAB member of miragen Therapeutics.

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Targeting microRNAs to promote cardiac repair and cardiomyocyte proliferation as a potential regenerative therapeutic approach

Philipp Jakob, MD; Ulf Landmesser, MD
Department of Cardiology, University Hospital Zürich, Zürich, Switzerland; Center for Molecular Cardiology, University of Zürich, Switzerland

Correspondence: Ulf Landmesser, Vice Chairman, Department of Cardiology, University Hospital Zürich, Raemistrasse 100, 8091 Zürich, Switzerland
E-mail: Ulf.Landmesser@usz.ch

Abstract
MicroRNAs (miRNAs) have emerged as important modulators of cardiovascular biology, repair, and regeneration. miRNAs posttranscriptionally regulate the expression of a network of proteins and consequently, cardiovascular development. miRNA targeting has been shown to: (i) improve the cardiac repair capacity of patient-derived circulating or bone marrow-derived mononuclear cells (BMCs); (ii) induce pluripotent stem cells or direct cardiac reprogramming; and (iii) promote cardiomyocyte proliferation after cardiac injury. Several groups, including our group, have recently targeted miR-126 or miR-34a to improve the cardiac repair potential of circulating mononuclear cells or BMCs from patients with ischemic cardiomyopathy. Moreover, targeting of several miRNAs induced reprogramming of mouse and human cells to pluripotency, and initial experimental studies suggest that fibroblasts can be directly converted into cardiomyocytes in vivo by targeting miRNAs—a process termed direct reprogramming. Recent data suggest a marked early postnatal cardiomyocyte proliferation in response to myocardial injury. Cardiomyocyte proliferation still occurs in adults, although at a very low rate (0.5% to 1% per year). These observations have stimulated the interest in investigating therapeutic strategies to promote cardiomyocyte reentry into the cell cycle to compensate for cardiomyocyte loss after myocardial infarction. Experimental studies have shown that miR-1, miR-133a, miR-29a, and the miR-15 family repress cardiomyocyte proliferation, whereas miR-199a and miR-590 promote cardiomyocyte proliferation in injured murine hearts. Therapeutic targeting of miRNAs is being intensely examined to promote cardiac repair and regeneration, a development aided by high-throughput functional screening assays. Large animal data suggest that targeting miRNAs can improve cardiac function after injury, indicating a potential future applicability for this approach to enhance cardiac repair and regeneration in the clinical setting. ■ Heart Metab. 2014;65:9-14

Keywords: dyspnea; exercise; heart failure; skeletal musculature
Heart failure is a leading cause of mortality and morbidity. Interventional and surgical revascularization, pharmacological treatment, and devices, such as cardiac resynchronization therapies, are strategies to improve cardiac function and improve symptoms and outcomes. However, the prognosis of patients with heart failure remains poor, and the frequent underlying cause, myocardial cell loss, has not been successfully addressed. Although a very limited number of cardiac cells harbor a potential to reenter the cell cycle or stimulate cardiomyocytes toward a proliferative state, these mechanisms cannot compensate for the acute or slowly proceeding cardiomyocyte loss occurring after acute myocardial infarction or in chronic heart failure (CHF), a progressive disease.

Therefore, therapeutic interventions to enhance cardiac regeneration directly are being intensely investigated as a potential novel therapeutic strategy. MicroRNAs (miRNAs) regulate cardiac developmental processes, cardiomyocyte proliferation, and viability by posttranscriptionally modulating messenger RNA (mRNA) translation into proteins. As miRNAs can be targeted using anti-miRNAs or miRNA mimics, exogenous modification of miRNA levels emerges as an interesting novel therapeutic strategy to enhance cardiac repair and regeneration. This review will focus on miRNAs involved in cardiac repair, cardiac lineage commitment, and regulation of cardiomyocyte proliferation.

MicroRNA biogenesis

miRNAs are small (~19 to 22 nucleotides long), single-stranded, noncoding RNAs. The primary transcript is transcribed by RNA polymerase, which is then processed by Drosha. The precursor miRNA (pre-miRNA) is transported from the nucleus to the cytoplasm, where it is frequently cleaved by Dicer. One strand of the unwind, double-stranded miRNA helix is preferentially integrated in the RNA-induced silencing complex (RISC) associating with other RNA-regulating proteins (eg, the Argonaute family). Within the RISC, miRNAs repress protein expression by interacting with partially complementary mRNAs sequences (3' untranslated regions), which leads to degradation or translational repression of the targeted mRNA. In humans, more than 1500 miRNAs have been identified. miRNAs target several genes to hundreds of genes for potent regulation of biological processes, and in addition, several miRNAs can collectively target one mRNA. These combinatorial interactions allow for a complex fine-tuning of regulatory processes, including cardiac repair, lineage commitment, proliferation, and survival of cardiomyocytes.

Targeting microRNAs as a strategy to prime circulating or bone marrow–derived mononuclear cells to improve their cardiac repair capacity

Cell-based therapies, using different autologous patient-derived cell populations, are being intensely examined as a potential strategy to improve cardiac function after injury (Figure 1). However, in several clinical trials of cardiac cell–based therapies, the functional and clinical benefits have been modest, which is potentially related to the impaired cardiac repair capacity of adult patient–derived cell populations. In this context, several groups, including ours, have demonstrated a dysregulation of miRNA expression in early angiogenic outgrowth cells (EOCs) of patients with CHF. Notably, overexpression of miR-126, which is reduced in EOCs from patients with CHF, enhanced the in vivo cardiac repair capacity. Another approach is to prevent apoptosis of transplanted cells, a process thought to substantially decrease the cardiac repair capacity after cell transplantation due to low survival of transplanted cells. Expression of miR-34a, a proapoptotic miRNA, was increased in bone marrow mononuclear cells (BMCs) from patients with myocardial infarction. Treatment of BMCs with miR-34a inhibitors improved their capacity to restore cardiac function in a murine infarct model. Hu et al applied a cocktail consisting of miR-21, miR-24, and miR-221 to cardiac progenitor cells, and showed an increase in their survival after cardiac transplantation in an experimental myocardial infarct model, which resulted in a better preserved left ventricular function. Bim, an apoptosis inducer, was repressed by these three miRNAs, demonstrating that multiple miRNAs can synergistically repress one target.

Hence, miRNAs have the potential to improve the
impaired cardiac repair capacity of adult stem cells and progenitor cells. In addition, miRNA modulation of adult stem cells and progenitor cells may serve as a strategy to enhance cardiac repair processes in cell-based therapies.

**Targeting microRNAs to promote cardiomyocyte lineage commitment in pluripotent stem cells**

Stimulation of cardiomyocyte lineage commitment has been studied in embryonic stem cells (ESCs) and inducible pluripotent stem cells (iPSCs) as a potential strategy to promote regeneration of the myocardium (Figure 1). Human ESC-derived cardiomyocytes enhanced cardiac function in a rat myocardial infarction model. Recently, transplantation of human ESC-derived cardiomyocytes has been shown to integrate and survive after transplantation in nonhuman primates in an experimental myocardial infarct model, resulting in remuscularization of substantial amounts of the infarcted monkey heart, albeit with an occurrence of nonfatal ventricular arrhythmias. Dynamic regulation of miRNAs is involved in differentiation of ESCs toward a cardiomyocyte fate. In vitro studies have shown that miR-1, together with miR-499, are upregulated during the differentiation of human ESC (hESCs) and cardiac progenitor cells toward cardiomyocytes and overexpression of these miRNAs enhances differentiation toward a cardiomyocyte fate.  

miR-1 mediates developmental cardiomyocyte differentiation and its role in cardiomyocyte differentiation of hESCs in vivo was recently analyzed. Transplantation of hESCs treated with miR-1 mimics improved cardiac function and increased the number of donor-derived cardiomyocytes. Of interest, a decrease in cardiac apoptosis was detected in these...

**Fig. 1** Potential therapeutic approaches targeting miRNAs to enhance cardiac repair processes and regeneration. miRNA-based therapies have been shown to efficiently enhance mechanisms involved in cardiac repair and regeneration in experimental models. **A.** Isolated aged and adult progenitor cells frequently show an impaired cardiac repair potential, partly due to dysregulation of miRNAs. Priming of these cells by targeting miRNAs may result in an enhanced cardiac repair capacity. **B.** Direct inhibition of proteins involved in cell survival, apoptosis, and proliferation of cardiomyocytes and endothelial cells by local or systemic direct targeting of miRNAs may enhance cardiac repair and regenerative processes. **C.** Conversion of skin fibroblasts into iPSCs can be achieved by transfection with miRNAs. Thereafter, iPSCs can be differentiated toward a cardiomyocyte fate and used for cardiac regeneration after transplantation. **D.** miRNAs have the potential to directly convert cardiac fibroblasts into cardiomyocyte-like cells in vivo. The efficacy of such approaches in human cells needs to be further investigated.

**Abbreviations:** iPSCs, inducible pluripotent stem cells; miRNAs, microRNAs.
mice, suggesting that miR-1 not only facilitates cardiomyocyte differentiation, but also protects resident cardiomyocytes within the hostile milieu after cardiac injury. The role of miRNAs to enhance the cardiomyocyte lineage commitment of iPSCs has to be further explored in future studies.

Together, these studies reveal important roles of miRNAs for cardiac lineage commitment in stem cells. However, profiling of miRNAs involved in this differentiation process revealed complex spatiotemporal expression of different miRNAs.

Targeting microRNAs to stimulate heart repair by direct cardiac reprogramming

Apart from governing ESC/iPSCs toward cardiomyocytes, another potential option for regenerating the heart is transdifferentiation of resident cardiac host cells directly into cardiomyocytes (Figure 2). This process, termed direct reprogramming, circumvents the step of dedifferentiation into pluripotent stem cells and pursues reprogramming directly from endogenous non-cardiomyocytes (e.g., cardiac fibroblasts) toward functional cardiomyocytes. Qian et al reported the feasibility of direct reprogramming of mouse fibroblasts into cardiomyocytes. Transduction of fibroblasts derived from mouse hearts and skin with three cardiac transcription factors (Gata4, Mef2c, Tbx5) directly induced cardiomyocyte-like cells with expression of cardiomyocyte-specific structures and promoters. These findings were successfully translated into a murine myocardial infarction model by using viral transfection of cardiac transcription factors. Interestingly, Jayawardena et al 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 directed these cells toward cardiomyocytes with expression of cardiomyocyte markers and functions in vitro. Moreover, after an experimentally induced myocardial infarction, intramyocardial injection of this set of miRNAs converted cardiac fibroblasts into cardiomyocyte-like cells, which was shown by genetic tracing methods. Recently, Nam et al reported direct reprogramming of human fibroblasts, which are more resistant to reprogramming techniques, into cardiomyocyte-like cells. Use of cardiac-specific transcription factors (Gata4, Mef2c, Tbx5, and Hand2) alone were sufficient to reprogram mouse fibroblasts, but not human fibroblasts. Addition of myocardin (Myocd) effectively induced cardiac gene expression. Furthermore, the use of four transcription factors (Gata4, Hand2, Tbx5, and Myocd) together with miR-1 and miR-133 further improved reprogramming efficiency toward a cardiomyocyte phenotype.

Cardiac troponin T could be detected in 19% of transfected human fibroblasts. However, calcium transients and beating cells were rarely observed, indicating that together with the lack of upregulation of some cardiac genes, a full cardiomyocyte differentiation could not be achieved, yet.

Targeting microRNAs to stimulate cardiomyocyte proliferation in injured hearts

For many years, cardiomyocyte proliferation was thought to be a prerequisite in embryogenesis and for lower vertebrates. However, elegant studies have now shown that cardiomyocyte proliferation still occurs in adults, although at a low level (~1% turnover rate/year). Experimental studies have reported proliferation of cardiomyocytes after surgical injury in neonatal mice. These observations indicate postnatal regeneration of the heart and the possibility of adult cardiomyocytes to reenter the cell cycle, also in an unstimulated state. Spatiotemporal expression of miRNAs plays an important role in cardiac muscle development; therefore, it may be used as a therapeutic strategy to stimulate endogenous regenerative processes leading to duplication of resident cardiomyocytes. Cardiac deletion of enzymes required for the biogenesis of miRNAs resulted in dilated cardiomyopathy and premature lethality, indicating a pivotal role of miRNAs to modulate proliferative and apoptotic processes of cardiomyocytes.

Several miRNAs are involved in cardiac growth and function by either enhancing or decreasing mitotic pro-

![Fig. 2 miRNAs involved in the regulation of cardiomyocyte differentiation and proliferation. Abbreviation: miRNAs, microRNAs.](image-url)
cesses. miR-1 is a skeletal and cardiac muscle specific miRNA consisting of two miRNAs, miR-1-1 and miR-1-2, which are encoded by two genes. Mice lacking miR-1-2 die early due to ventricular septal defects. Mutant adult mice with normal cardiac function revealed hyperplasia, which is partly due to increased expression of proteins involved in cardiac morphogenesis and development, such as Hand2. In contrast, cardiac-specific overexpression of miR-1 leads to decreased ventricular cardiomyocyte proliferation.

miR-133a, which is cotranscribed with miR-1, is involved in cardiac development. Deletion of miR-133a-1 and miR-133a-2 causes lethal ventricular septal defects in embryonic and neonatal stages and dilated cardiomyopathy in surviving adult mice. In these double-knockout mice, disorganization of sarcomeres and increased proliferation and apoptosis of cardiomyocytes were detected. Consistently, cell cycle genes were upregulated in double-knockout mice. In contrast, diminished cardiomyocyte proliferation was observed in miR-133 transgenic mice. These observations indicate that miR-133a is pivotal for withdrawal from the cardiomyocyte cell cycle and full differentiation into cardiomyocytes.

The transient regenerative capacity in postnatal murine hearts was used to detect upregulated and downregulated miRNAs using a microarray approach. miR-195, a member of the miR-15 family, is highly upregulated in mouse hearts between days 1 and 10 after birth. Therapeutic inhibition of the miR-15 family with anti-miRNAs in neonatal mice increased cardiomyocyte proliferation by enhancement of cell cycle genes. Further investigations showed that miR-15 on cardiac regeneration after cardiac injury in postnatal mice. Myocardial infarction in pups at day 1 functionally and fully resolves through day 21. Cardiac-specific overexpression of miR-195 in these mice impaired the cardiac regenerative capacity with extensive fibrosis in the infarcted area and diminished proliferating cardiomyocytes, consistent with the antiproliferative effects of the miR-15 family. Furthermore, pretreatment of postnatal mice with anti-miR-15 improved left ventricular function after induction of myocardial infarction in adult mice.

A similar approach using a miRNA array was also conducted in cardiomyocytes of rats to decipher alterations in miRNA expression on postnatal days 2 and 28. miR-29a expression increased extensively from day 2 to day 28 and was investigated for its antiproliferative capacity. Indeed, treatment of neonatal cardiomyocytes with miR-29a inhibitors significantly increased cardiomyocyte proliferation. Direct targets include cyclin D2, a cell cycle regulator.

These studies investigated miRNAs with antiproliferative effects on cardiomyocytes. In contrast, an elegant study reported that miRNAs can also induce proliferation of cardiomyocytes. To detect miRNAs involved in cardiomyocyte proliferation, a functional high-throughput screening was performed. Neonatal cardiomyocytes were transfected with a miRNA library consisting of 875 miRNAs. 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 left ventricular function.

The miR-17-92 miRNA cluster is involved in proliferative processes. Cardiac specific deletion of miR-17-92 leads to decreased cardiomyocyte proliferation in postnatal hearts. Consistently, overexpression of miR-17-92 in embryonic and postnatal cardiomyocytes increased their proliferative capacity, culminating in a thickened myocardium due to hyperplasia. Intriguingly, induced cardiac expression of miR-17-92 in adult mice, where the proliferative capacity of cardiomyocytes is diminished, resulted in an increased cardiomyocyte proliferation. In addition, cardiac overexpression of miR-17-92 preserved cardiac function after myocardial infarction. These studies indicate that reentry of cardiomyocytes into the cell cycle can be induced by administration of pro-proliferative miRNAs.

Conclusion

miRNAs are powerful mediators of the cardiac repair capacity in adult cell populations, cardiomyocyte lineage commitment in pluripotent stem cells, and cardiomyocyte cell cycle regulation. Therefore, therapeutic miRNA targeting to enhance cardiac repair, either by pretreatment of patient-derived cells used for cell-based therapies or direct local or systemic application, is a highly interesting potential therapeutic option to promote cardiac regeneration. Efficient inhibition of
deleterious miRNAs in large animal models by using locked nucleic acid (LNA)-modified antisense miRNA has now been reported and a clinical phase 2 trial using LNA-modified antisense miRNA in patients with chronic hepatitis C successfully decreased viral load without toxic side effects. Whereas anti-miRNA treatment seems to be effective, stable overexpression using miRNA mimic constructs is more challenging and needs an optimized delivery system. Of note, miRNAs do not exclusively mediate their functions within the cell compartment, but are also actively or passively secreted. Therefore, pleiotropic effects may further enhance the therapeutic potential after miRNA delivery.

Moreover, a cell-specific delivery of miRNAs may be desirable because one miRNA can induce opposite effects in different cell types. The understanding of intercellular communication of miRNAs and their respective targets and functions in each cell type may contribute to an optimization of delivery strategies for miRNA-targeted therapies.

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MicroRNAs as potential biomarkers and therapies for human pathologies

The transcriptional modulation by microRNAs (miRNAs) plays a fundamental role in a variety of cardiac diseases, such as myocardial infarction, hypertrophy, and heart failure; peripheral artery disease; metabolic disorders, such as diabetes mellitus; as well as in the regulatory processes to maintain the cellular homeostasis of cell differentiation, proliferation, and apoptosis. Importantly, miRNAs cannot only be detected in the cytoplasm of involved cell types, but also in noncellular compartments such as plasma, sputum, or urine where they remain remarkably stable. Transportation of miRNAs occurs either freely, bound to proteins or lipids, or via incorporation into microvesicles or exosomes. Besides the various properties of these particles, it is now crucial to not only characterize miRNAs, but also their packaging, eg, in terms of morphology. Several techniques have been developed in order to reliably measure exosome size, shape, and dispersity. Among these, nanoparticle tracking analysis (NTA) is a newly developed light-scattering technique for the rapid sizing of exosomes in vivo.
and evaluation of extracellular vesicle concentration (Figure 1). In this technique, microvesicles or exosomes are illuminated through a passing laser beam, with each individual particle scattering the light into the field of view of a microscope and onto the image sensor of a video camera.

One of the basic principles in order to take advantage of the therapeutic potential of miRNAs is based on identifying distinctly dysregulated miRNAs in cardiac diseases; this is being extensively studied in cellular models, in various small and large animal models, and ultimately in humans. Current work includes identification of miRNA expression profiles in neonatal mouse hearts by deep sequencing, study of the cargo system of circulating miRNAs in the pathophysiology of cardiac hypertrophy, and reprogramming of cardiac fibroblasts toward a cardiomyocyte lineage via a single transient transfection of certain miRNAs. The latter idea evolved to functional screening of the whole human miRNome, which led to the identification of miR-25 as a suppressor for intracellular calcium handling in heart muscle. Hence, high-throughput functional screening of the whole human miRNome helps identify novel targets able to restore cardiac contractility.

Common to all techniques for miRNA evaluation is the development toward faster turnover of even smaller amounts of material, a higher dynamic range, and meeting excellent sensitivity and specificity criteria. Emphasis is laid on new imaging techniques enabling sensitive and nondestructive detection of miRNA expression and activity, combined with high dynamic and local resolution properties.

Assessment of microRNAs by molecular imaging

Despite their huge potential, miRNAs are still in the early translational process toward clinical application. A recent publication on the role of miRNA-30c showed that transgenic mice overexpressing cardiac miRNA-30c developed a severe form of dilated cardiomyopathy. This underlines that misbalanced expression of only a single miRNA can lead to a series of downstream molecular changes and severe organ dysfunction. Yet our knowledge of possible harmful and off-target effects of miRNAs remains very scarce. Hence, it is crucial to be able to monitor miRNAs efficiently and reliably to further comprehend their function in regulatory pathways.

Over the years, advances have led to sophisticated assays (Figure 2) characterized by their ability not only to capture a single biological response, but also to meet diagnostic-grade standards of sensitivity, versatility, and fidelity. As such, miRNA expression levels can be measured at the benchside by a host of methods, eg, quantitative reverse transcription polymerase chain reaction (qRT-PCR), microarray analysis, northern blots, and in situ hybridization. In addition, assays based on firefly luciferase, isolated from the North American firefly Photinus pyralis, rank among the

**Abbreviations**

miRNA: microRNA; NGS: next generation sequencing; NTA: nanoparticle tracking analysis; qRT-PCR: quantitative reverse transcription polymerase chain reaction

**Fig. 1** Exosome nanoparticle tracking (NTA). NTA is a light-scattering technique for rapid sizing and evaluation of extracellular vesicle concentrations, eg, of exosomes, which are visualized in Panel A. Distribution of the calculated vesicle size is shown in Panel B. Image and graph courtesy of Christian Harz, NanoSight Ltd.

**Abbreviations:** con/ml, concentration per mL; cum %, cumulative percentage.
more and more extensively used methods due to its wide dynamic range of measurable activity. Today, it represents only a small step from analysis of a single miRNA to quantification of whole miRNomes via microarrays. Finally, the revolution of next-generation sequencing (NGS) techniques made comprehensive profiling of miRNAs possible even in a clinical setting, due to excellent detection sensitivity, a large dynamic range of detection, and high accuracy in differential expression analysis. Furthermore, NGS allows sequence motif-independent detection of completely novel miRNAs or miRNA modifications. It was not until recently that NGS made it possible to investigate the human cardiac miRNome by quantification of miRNA expression in healthy and diseased human heart. Leptidis et al. discovered more than 800 miRNAs expressed in the human heart, with more than 250 differentially expressed in human dilated or hypertrophic cardiomyopathy, making them potential therapeutic targets.

**Fig. 2** Comparison of destructive and nondestructive imaging techniques for monitoring miRNAs. miRNA expression levels can be measured with a variety of destructive methods, which means that the tissue or cell has to be lysed. Shown are real-time polymerase chain reaction, microarray, NGS, and biosensors. Fluorescence- and bioluminescence-based imaging methods and molecular beacons are representatives for nondestructive assays. Bioluminescence-based imaging depends on enzymatic reactions between luciferase and its substrate, whereas a molecular beacon employs a single-stranded and stem-looped DNA oligonucleotide complementary to its target miRNA. Abbreviations: miRNA, microRNA; NGS, next-generation sequencing.
Among the major disadvantages of the aforementioned methods is their “invasiveness,” requiring the destruction of the target tissue, which hampers dynamic analysis and in vivo detection of miRNAs. Moreover, amplification of unidentified poor-quality RNA in the initial phase of the expression profiling may yield poor-quality data and can be costly, laborious, and time consuming. Thus, for example, in the field of oncology, molecular profiling of cancers has led to the development of second-generation assays based on custom-designed microarrays. Clinically applicable assays must be highly reproducible, sensitive, and specific, as well as having a comparably high dynamic range. The potential implementation of a miRNA signature as a biomarker for cardiovascular diseases may not only enable patients with a high cardiovascular mortality risk to be distinguished from those at low risk, but also helps to predict poor clinical outcomes in patients suffering, eg, from heart failure. However, current techniques using target amplification are relatively time consuming, which requires the development of assays with faster turnaround times of about 20 min, so that they are suited also for the diagnosis of acute coronary syndromes. An increasing number of studies concentrate on amplification-free, eg, biosensor-based techniques for miRNA assays, which combine a selective molecular probe with a highly sensitive transducer for rapid and extremely sensitive detection at attomolar to femtomolar levels. When compared with classic biomolecular approaches, biosensor-based techniques excel in terms of measurement reliability, reduced time-to-results, and sample preparation-free protocols, together with disadvantages due to target degradation, sequence similarities, as well as variable melting temperatures.

In order to overcome some of the obstacles, noninvasive methods of repetitive imaging are needed for proper in vivo assessment of miRNA expression patterns. These currently comprise fluorescent proteins, bioluminescent enzymes, molecular beacons, as well as various nanoparticles, enabling visualization of miRNA biological processes in living individuals (Figure 3).

Back in 1962, the discovery of green fluorescent protein (GFP) in the Aequorea victoria jellyfish paved the way for the development of a variety of fluorescent proteins with unique emission wavelengths suitable for visualizing specific molecules within cells. As such, the DNA fragment encoding the fluorescent protein is fused to repetitive miRNA seed-targeting sites complementary to the specific miRNA. In 2009, Ko et al were able to establish a miRNA reporter gene imaging system to monitor miR-124a during neurogenesis, comprising a reporter gene vector, cell transfection, in vitro luciferase assay, and in vivo bioluminescence imaging of miRNAs. The group around Kato created a two-color fluorescence imaging system using a proviral vector, which allowed the more precise evaluation of miR-133 activity during myogenesis. Such fluorescence-based imaging approaches allow easy and noncytotoxic expression. However, despite appealing in vitro results, there are several in vivo drawbacks, including high autofluorescence from the background as well as poor tissue penetration of the excitation light with subsequent low signal-to-noise ratios.

Bioluminescence imaging–based methods (BLI), based on enzymatic reactions between luciferase and its substrate, provide an alternative to fluorescence imaging. Besides increased sensitivity and cost effectiveness, BLI are able to overcome the much-dreaded background signal issue. Tu and coworkers engineered a dual-luciferase reporter system for quantitative imaging of miR-22 in isoproterenol-induced cardiac hypertrophy for repetitive and noninvasive monitoring of dynamic changes of miR-22 expression in a real-time manner. Moreover, the constructed miRNA reporter system showed that knockdown of miR-22 by antagomiR-22 is capable of silencing cardiac endogenous miR-22 expression, resulting in partial inhibition of isoproterenol-induced cardiac hypertrophy. Concerns from scientists regarding BLI methods involve decreased signal intensity in the presence of a functional miRNA as a result of the destabilized transcriptional activity of its target mRNA.
A different approach to direct in vivo imaging involves molecular beacons and nanoparticles. A molecular beacon consists of a single-stranded stem-loop DNA oligonucleotide complementary to its target miRNA, and represents a simple, fast, cost-effective, and specific method for monitoring intracellular miRNAs in living cells. For instance, Kang et al. were able to successfully image the biogenesis of miR-206 and miR-26a during myogenesis both in vitro and in vivo by means of a molecular beacon. Moreover, Baker et al. could demonstrate that molecular beacons are suitable for distinguishing mature from precursor miRNAs, and therefore capable of reliably quantifying miRNA expression. Limitations of molecular beacons and nanoparticles include their largely unknown toxicity, their inhomogeneous distribution in the body and tissues, and the possible requirement of a vectorization agent.

To date, the vast majority of miRNA imaging strategies based on fluorescent proteins, Bli, molecular beacons, or nanoparticles is still in its infancy. All the endeavors undertaken to image intracellular targets remain more burdensome than imaging cell surface targets.

Conclusion

As every single imaging modality struggles with its own shortcomings, it has become increasingly important to find ways to combine the results of different methods in a multimodality imaging approach or optimize assays for a distinct target miRNA application. Special emphasis has to be laid on developing efficient in vivo monitoring of proper miRNA function and controlling downstream physiological effects in order to fully exploit the diagnostic and therapeutic value of miRNAs.

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The role of microRNAs (miRNAs) in disease pathologies is ever increasing, and substantial evidence has accumulated for their role in cardiovascular diseases, raising possibilities for therapeutic intervention. miRNAs, 18-25 nucleotide single-stranded noncoding RNAs, are responsible for the negative epigenetic regulation of gene expression through complementary base pairing to their target messenger RNAs (mRNA). This miRNA-mRNA complementarity occurs in a 6-8 nucleotide “seed” region at the 5’ miRNA end, although evidence exists for recognition sites outside the seed region too. The function of miRNAs is to either prevent translation or promote transcript degradation. A single miRNA can target many effectors of a biological process, while cotargeting networks of miRNAs that regulate the expression of a specific component of a signaling pathway have also been reported, indicating miRNA redundancy and extremely high complexity of miRNA regulatory networks. Specific miRNAs have been implicated in a number of cardiovascular events that could lead to heart failure, including cardiac hypertrophy, myocardial infarction, cardiac fibrosis, and arrhythmias.

The potential use of microRNAs (miRNAs) to design novel therapeutic approaches in cardiovascular diseases (CVD) has emerged as a promising strategy. miRNAs are small, noncoding RNAs that regulate gene expression epigenetically. Increasing evidence suggests that miRNAs operate as regulatory networks that target functionally related genes in physiological and pathological conditions. This property offers a unique opportunity to devise functional interventions that can overcome any redundancy mechanisms in disease. Additionally, miRNAs typically exert mild effects under baseline conditions, but have a more pronounced response under stress suggesting that off-target effects on uninjured tissue will be limited. Attractive as it may seem, miRNA biology can also be a serious concern for the safety of the potential applications, as miRNAs tend to function in a context-dependent manner and participate in both positive and negative feedback loops. Besides their role as mediators of disease, the presence of miRNAs in biological fluids has now been well documented. These circulating miRNA signatures may offer a great diagnostic and prognostic tool as disease biomarkers. Here we will review the recent developments in miRNA research with a particular emphasis on secreted miRNAs as targets for novel therapeutic approaches to cardiovascular diseases.

**Abstract**

The potential use of microRNAs (miRNAs) to design novel therapeutic approaches in cardiovascular diseases (CVD) has emerged as a promising strategy. miRNAs are small, noncoding RNAs that regulate gene expression epigenetically. Increasing evidence suggests that miRNAs operate as regulatory networks that target functionally related genes in physiological and pathological conditions. This property offers a unique opportunity to devise functional interventions that can overcome any redundancy mechanisms in disease. Additionally, miRNAs typically exert mild effects under baseline conditions, but have a more pronounced response under stress suggesting that off-target effects on uninjured tissue will be limited. Attractive as it may seem, miRNA biology can also be a serious concern for the safety of the potential applications, as miRNAs tend to function in a context-dependent manner and participate in both positive and negative feedback loops. Besides their role as mediators of disease, the presence of miRNAs in biological fluids has now been well documented. These circulating miRNA signatures may offer a great diagnostic and prognostic tool as disease biomarkers. Here we will review the recent developments in miRNA research with a particular emphasis on secreted miRNAs as targets for novel therapeutic approaches to cardiovascular diseases.

**Keywords:** cardiovascular disease; microRNA; therapeutic application
In the vasculature, tissue remodeling in atherosclerosis and aortic aneurysms was shown to be orchestrated by miRNAs that tightly regulate different aspects of the disease. Here we will discuss the therapeutic potential of miRNAs in cardiovascular diseases.

MicroRNA targets in the heart

Since the first report of embryonic lethality in mice with cardiac deletion of Dicer, multiple studies have explored the role of miRNAs in heart development and function under physiological conditions and in disease. Several miRNAs highly expressed in the heart have been discovered, and remarkable findings for their mechanisms of function have been uncovered. miRNA-based therapeutic applications for the heart have provided promising results in mouse models. These studies are now slowly progressing to large animal models (Table I).

<table>
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<tr>
<th>Species</th>
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<th>Disease model</th>
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<td>Pigs</td>
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<tr>
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</tr>
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Table I: miRNA-based therapeutic applications in large animal models.

Abbreviations: anti-miR33, 2’-fluoro/3’-methoxyethyl (2’-F/3’-MOE)-modified, phosphorothioate-backbone-modified, antisense miR33; LNA, locked nucleic acid modified antisense; HDL, high-density lipoprotein; miRNA, microRNA; VLDL, very-low-density lipoprotein.
erated to elucidate potential effects on atherosclerosis lesion progression. Screening of the miRNA pool in human atherosclerotic plaques also demonstrated differential miRNA expression (miR-21, miR-34a, miR-92a, miR-146a/b, miR-210, miR-322-5p). The role of miRNAs in cholesterol homeostasis and its impact on atherosclerosis regression is one of the best studied mechanisms. miR-33 was shown to modulate expression of genes involved in cholesterol efflux. Silencing miR-33 in vivo in mice increased hepatic expression of ABCA1 (adenosine triphosphate–binding cassette transporter 1) and plasma high-density lipoprotein (HDL) levels. This reverse cholesterol transport led to atherosclerosis regression. More importantly, promising findings were obtained from inhibition of miR-33 in nonhuman primates, African green monkeys. In line with observations in mice, a sustained increase in plasma levels of HDL was detected while very-low-density lipoprotein (VLDL) levels were reduced without any evidence of adverse effects, suggesting that targeting miR-33 could be an attractive strategy to combat atherosclerosis.

Vascular remodeling in abdominal aortic aneurysms (AAA) is also mediated by miRNAs. miR-29b emerged as a key regulator of extracellular matrix deposition in several models of aortic dilatation and AAA in mice following systemic injection of anti-miRs. Interestingly, in mice, the increased miR-29b expression in the aged aorta renders it susceptible to aneurysm formation. In humans, differential expression of miR-29b was detected in the ascending aorta in Marfan syndrome, a connective tissue disorder that can lead to the development of aortic root aneurysms. Local instead of systemic administration of inhibitors for miR-29 will safeguard against off-target fibrotic effects in organs such as the liver and kidneys, which receive extremely high doses of the compounds.

Clinical studies

Despite the encouraging data from animal models of injury, more preclinical studies are required before miRNA therapeutics can enter any clinical trials. There are several issues that need to be addressed. Application of antisense oligonucleotides that act as inhibitors of a miRNA may be toxic for the liver and kidneys, which will clear most of these compounds from the circulation. These reagents will have to be chemically modified to increase their resistance to nucleases and thus their stability, enhance their cellular uptake, and reduce their renal clearance. Their specificity and route of delivery may be additional causes of concern. Nevertheless, miravirsen, an inhibitor against miR-122, was successfully used for phase 1 and 2 trials against hepatitis C virus (HCV). No significant renal toxicity was observed, the compound was biostable, and displayed extremely high target affinity. A prolonged dose-response reduction in the viral RNA was detected, indicating that miR-122 inhibition could be a safe and effective strategy to combat HCV infection. This is the most advanced miRNA therapeutic application so far. Of note, targeting miR-122 has benefited from its unique pattern of expression. miR-122 is highly expressed in the liver and therefore can be effectively targeted even when very low concentrations of the reagents are applied.

MicroRNAs as biomarkers of disease

Circulating miRNAs represent an attractive tool for the development of noninvasive diagnostic tests, and distinct signatures have been proposed to correlate with various cardiovascular diseases. Encouraging results were also obtained for their value as potential prognostic markers in CVD. A signature of three miRNAs (miR-126, miR-223, and miR-197) was reported to have a predictive value for incident myocardial infarction (MI) in a prospective population-based cohort (n=820), and addition of these miRNAs to the Framingham Risk Score for hard coronary heart disease led to a significant improvement in risk classification that actually exceeded the impact of alternative biomarkers such as C-reactive protein. Furthermore, in an independent cohort of patients who received percutaneous coronary intervention (PCI) and dual antiplatelet therapy (n=491), circulating miR-126 was significantly associated with major adverse cardiovascular events within 1 year. miRNA signatures with a potential value as early biomarkers of acute coronary syndrome (miR-1, miR-499, and miR-21, n=332) that added diagnostic value to high-sensitivity troponin T (hsTnT) have also been proposed. In a patient cohort of hypertrophic cardiomyopathy (HCM) patients and their controls (HCM, n=82), a circulating signature of three miRNAs (miR-27a, miR-29a, and miR-199a-5p) was reported to correlate with left ventricular mass. Importantly, miR-29a also associated with myocardial fibrosis, hence providing the first po-
The therapeutic potential of microRNAs in heart and vascular diseases

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The potential biomarker for myocardial remodeling in HCM. In the vasculature, circulating miR-126 correlated with subclinical and manifest peripheral artery disease in a prospective cohort (n=820), suggesting a direct link between miRNAs and vascular homeostasis. Interestingly, circulating miRNA signatures do not always have an additive value to known biomarkers of disease. In a cohort of acute coronary syndrome (n=444), cardiac-enriched miRNAs showed no independent association with the outcome once adjusted for TnT expression. In a similar manner, in a cohort of ST-segment–elevation myocardial infarction (STEMI, n=216) despite the association of circulating miR-133a with myocardial salvage, larger infarcts, and more pronounced reperfusion injury, no independent prognostic value for adverse cardiovascular events was detected. In a separate study, upregulation of miR-208b and miR-499 in the circulation of STEMI patients (n=397) correlated well with creatine kinase, but failed to provide a significant reclassification index following adjustment for hsTnT.

While some recently identified confounding factors in such studies will have to be taken into consideration and advanced statistical methods employed to address the high dimensional and collinear data obtain from profiling of the extracellular miRNA pools, large multicenter independent studies are urgently needed to clarify the robustness of the circulating miRNA signatures and their potential as biomarkers of cardiovascular diseases (Figure 1).

MicroRNAs as mediators of intercellular communication

The discovery that membrane vesicles contain functional miRNA pools sparked enthusiasm for their potential role in intercellular communication. These actively secreted vesicles may act as miRNA carriers and mediate paracrine effects. In the vasculature, regulation of vascular smooth muscle cell (SMC) contractility and proliferation by endothelial cell (EC)–secreted miRNAs was demonstrated. Vesicle-mediated transfer of miR-143/145 from ECs to SMCs was shown to confer atheroprotection. Shear stress stimulated EC secretion of vesicles enriched in miR-143/145 that could effectively target SMC gene expression and prevent neointima formation in vivo. Besides this local effect on the vessel wall, transfer of miRNAs from circulating cells to the vessel wall has also been explored. Monocyte-derived microvesicles enriched in miR-150 could enhance EC migration via a c-Myb–mediated mechanism both in vitro and in vivo. Likewise, miR-223–derived platelet microparticles induced apoptosis in the recipient ECs in response to glycation end products via targeting the expression of insulin like growth factor 1 receptor (IGF-1R). HDL complexes were recently proposed as carriers of miR-223 to ECs. Intriguingly, miR-223, which is not normally expressed in these cells, induced downregulation of intercellular adhesion molecule 1 (ICAM-1) expression, thus conferring an anti-inflammatory phenotype to the ECs. Although these studies are extremely interesting, our understanding of the molecular mechanisms involved is still poor. While the functionality of the miRNA pools in vesicles/HDL complexes has been clearly demonstrated, evidence for a specific role of the delivered miRNAs in the recipient cell is still sparse. Of note, identifying an exogenous miRNA that has the potential to perform a function does not necessarily imply that this function is indeed exerted. Delineating the mechanisms of delivery will be vital in exploiting microvesicles as a new delivery route for therapeutic agents.

Conclusions

miRNA-based therapeutics is still in its infancy. Evidence from the studies performed mostly in preclinical models is extremely promising, but further work is required to determine the safety and efficacy of such therapeutic approaches. Designing protocols for local administration rather than systemic delivery of miRNA
reagents would greatly facilitate a targeted manipulation and limit potential side effects. Nevertheless, miRNA therapeutics as a novel approach to combat cardiovascular diseases is worth exploring.

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Nonmetabolic effects of trimetazidine and ventricular remodeling: role in regulatory gene expression

Pericle Di Napoli, MD
Cardiovascular Section, SMDN, Center for Cardiovascular Medicine and Cerebrovascular Disease Prevention, Sulmona (AQ), Italy

Correspondence: Dr Pericle Di Napoli, Cardiovascular Section, SMDN - Center for Cardiovascular Medicine and Cerebrovascular Disease Prevention, Via Trento 41, 67039 Sulmona (AQ), Italy
E-mail: dinapoli@unich.it

Abstract
Although the management of ischemic heart disease and chronic heart failure (CHF) has made considerable progress over the past years, CHF is still a tremendous medical burden. The metabolic and therapeutic approach might play a significant role in reducing disease progression and mortality rate. The metabolic modulator trimetazidine, a partial inhibitor of long chain 3-ketoacyl CoA thiolase activity, the last enzyme involved in β-oxidation, seems to exert positive effects in the management of left ventricle remodeling in heart failure patients by reducing the progression of disease, and by improving quality of life and prognosis. Recent reports suggest that trimetazidine could also exert nonmetabolic effects useful in preventing left ventricle remodeling by modulating the expression of various regulatory genes (nitric oxide synthase, endothelin-1, tumor necrosis factors, atrial natriuretic peptide, glucose transporters) involved in endothelial and mitochondrial function, myocardial cell death, and tissue fibrosis. Heart Metab. 2014;65:26-30

Keywords: heart failure; mRNA; remodeling; trimetazidine

In ischemic heart disease (IHD) and chronic heart failure (CHF) therapeutic strategies have traditionally focused on the modification of hemodynamic alterations (blood pressure, heart rate, wall stress) occurring in the ischemic or failing heart. However, in addition to hemodynamic alterations, deep changes both in systemic and in cardiac metabolism were evidenced.

Trimetazidine (TMZ) has been reported to exert anti-ischemic properties without affecting myocardial oxygen consumption and blood supply. The beneficial effect of this agent has been attributed to the preservation of phosphocreatine and adenosine triphosphate (ATP) intracellular levels, and a reduction in cell acidosis, calcium overload, and free radical-induced injury caused by ischemia. TMZ affects myocardial substrate utilization by partially inhibiting oxidative phosphorylation and by shifting energy production from free fatty acids (FFAs) to glucose oxidation. This effect appears to be predominantly caused by a selective block of long chain 3-ketoacyl coenzyme A (CoA) thiolase activity, the last enzyme involved in β-oxidation. Various studies performed in patients with postischemic left ventricular dysfunction, have shown that TMZ may be beneficial in terms of improvement in left ventricular function, control of
Trimetazidine effects on mRNA

Symptoms, and recently, prognosis. This pharmacological approach could also be useful in the treatment of patients with heart failure of various etiologies, probably due to the reduction in whole body resting energy expenditure or, recently, to nonmetabolic effects.

Recent reports suggested that TMZ could exert nonmetabolic effects that are useful in preventing left ventricle remodeling by modulating the expression of various regulatory genes involved in endothelial and mitochondrial function, myocardial cell death, and tissue fibrosis, contributing to minimize gene regulations occurring in response to ischemia or wall stress changes.

Metabolic changes in chronic heart failure and ischemic heart disease

The classic mechanism of CHF includes neurohormone (adrenergic nervous system and renin-angiotensin system) and ventricular remodeling. The overexpression of various biologically active molecules contributes to the progression of the disease. More attention has recently been given to subcellular remodeling, including changes in biochemical composition and molecular structure of various subcellular organelles such as the extracellular matrix, sarcoplasmic reticulum, mitochondria, and energy metabolism. The changes in metabolic remodeling are the availability of the metabolic substrate and the decline in metabolic capability.

In the normal heart, the large amounts of ATP essential to maintain contractile function and metabolism are generated mainly by mitochondrial oxidative metabolism, with a small percentage derived from glycolysis. The heart can use many different energy substrates (FFA, glucose, lactate, ketones, amino acids), but mitochondrial ATP is primarily produced by the oxidation of FFAs and pyruvate (derived from either glycolysis or lactate). Approximately 10% to 40% of ATP is produced via pyruvate oxidation, whereas the remaining 60% to 90% is derived from the oxidation of FFA. An important enzyme at the crossing point between carbohydrate oxidation and FFA metabolism is pyruvate dehydrogenase (PDH), which decarboxylates pyruvate to acetyl-CoA. PDH activity is influenced not only by glycolysis, but also by an inhibitory effect exerted through FFA oxidation.

In situations where the circulating FFA concentrations are high, the oxidation of glucose and pyruvate and the activity of PDH are decreased. Pyruvate is re-directed toward lactate production and released from the heart. This produces protons, which the heart must also clear; a process that requires energy and results in redirecting ATP away from contractile function, thereby decreasing cardiac efficiency. On the other hand, decreasing plasma FFA concentrations or directly inhibiting FFA oxidation increases PDH activity and, hence, pyruvate oxidation and cardiac efficiency. A number of membrane transporters and enzymes are involved in transferring the substrates from the cytosol into the mitochondrial matrix. Particularly, the enzyme malonyl-CoA has an inhibitory effect on the enzyme carnitine palmitoyltransferase–1 (CPT1), a key physiological regulator of FFA oxidation in the heart, and acts to suppress FFA oxidation. Increases in malonyl-CoA decrease the rate of FFA oxidation and, conversely, reductions in malonyl-CoA activity will increase the rates of FFA uptake and oxidation.

In CHF, complex metabolic changes occur. Various studies document the reversion to the fetal metabolic phenotype, consisting of a shift from FFA metabolism to glucose metabolism, which is analogous to the metabolic behavior of the fetal heart. This reversion involves the expression of some fetal genes and it is useful to improve metabolic efficiency. The main mechanisms involved are: (i) depression of genes encoding the FFA metabolism and downregulation of their transcription factors, including the peroxisome proliferator-activated receptor (PPAR) family, PPARγ coactivator-1 protein family, retinoid X-receptor-α, and CPT1; and (ii) incline of glucose metabolism, including adenosine monophosphate-activated protein kinase, glucose transporters (GLUTs), and phosphofructokinase 2. Other nonfetal metabolic phenotypic changes include the hyperadrenergic states and insulin resistance, leading to an increase in FFA metabolism.

Abbreviations

ANP: atrial natriuretic peptide; ATP: adenosine triphosphate; BNP: brain natriuretic peptide; CHF: chronic heart failure; CoA: coenzyme A; CPT1: carnitine palmitoyltransferase–1; eNOS: endothelial nitric oxide synthase; FFA: free fatty acid; IHD: ischemic heart failure; NO: nitric oxide; PDH: pyruvate dehydrogenase; TMZ: trimetazidine; TNF: tumor necrosis factor
Several studies show a decrease in tissue ATP content, an increase in adenosine diphosphate, and a decrease in the phosphorylation potential, thus impairing the kinetics for the utilization of ATP for cell contraction. In addition, heart failure impairs the capacity for the creatine kinase system to transfer mitochondrial ATP to the myofibril, and decreases mitochondrial oxidative capacity, in part because of a decrease in electron transport chain activity. The electron transport chain is usually altered in heart failure. The impairment in the electron transport chain reduces the in vivo capacity for myocardial ATP generation and limits cardiac contractile function during high-level work, such as exercise or acute adrenergic stress. Studies in animals and humans suggest increased or normal FFA oxidation in early heart failure and impaired fatty acid oxidation in severe heart failure.\(^6\)

**Trimetazidine effects on the expression of regulatory genes involved in myocardial remodeling**

The main mechanism of action of TMZ can be attributed to the optimization of energy metabolism due to the inhibition of FFA oxidation. However, additional nonmetabolic effects are under investigation and remain to be elucidated.

The potential nonmetabolic TMZ effects on vasculature and endothelial function appear interesting (Table I). TMZ effects on endothelial function have been investigated in humans and animal models.\(^7\)-\(^9\) TMZ induces an increase in nitric oxide (NO) production, and blunts endothelin-1 (ET1) release in the ischemic rat heart. The beneficial effects of TMZ in the ischemic-reperfused rat heart are mediated by NO because TMZ treatment results in an increased coronary endothelial NO synthase (eNOS) expression at both mRNA and protein levels and in a preservation of coronary microvasculature (ultrastructural damage and microvascular permeability) after ischemia. The protective effects of TMZ against ischemia-reperfusion endothelium damage is strictly related to eNOS expression and NO bioavailability, and it is abolished by inhibiting NO synthase with L-NAME; this effect is due to posttranscriptional mechanisms since the effect is not affected by the transcriptional inhibitor actinomycin-D. In patients with IHD, Fragasso et al evidenced reduced plasma ET-1 levels without alterations in plasma NO.\(^8\) Recently, short-term TMZ therapy was shown to improve the parameters related to heart rate variability and angina, reduce ET-1, and increase NO levels in patients with IHD.\(^8\) TMZ also improved endothelin-dependent relaxation in patients with ischemic cardiomyopathy via antioxidant properties. In a model of renal ischemia-reperfusion, TMZ treatment significantly augmented glycogen synthase kinase 3-β phosphorylation and reduced levels of cytochrome c and voltage-dependent anion channel phosphorylation. It significantly improved the survival rate, attenuated cytolysis, oxidative stress, and improved renal function.\(^10\)

A pivotal step in the evolution of chronic heart disease is the remodeling process. The main factors involved are the progressive cell loss due to apoptotic or necrotic cell death and subsequent tissue architecture rearrangement with the substitution of contractile mass with noncontractile fibrous tissue.\(^1\) Inflammation plays an important role in the pathogenesis of ventricular remodeling. It has been demonstrated that inflammatory cytokines, including interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α), remarkably increase after myocardial infarction and are involved in the subsequent left ventricular remodeling. Studies revealed that pathophysiological relevant concentrations of TNF-α promote progressive left ventricular dysfunction and remodeling in rats. Gene expression and serum levels of inflammatory markers, including interleukins (IL) and TNF-α, are usually detected to evaluate the extent of systemic
inflammation. In a model of smoking-induced inflammation, TMZ significantly reduced gene expression and serum levels of IL-1β, IL-6, and TNF-α, which might be an important protective mechanism against left ventricular remodeling via attenuating oxidative stress, apoptosis, and inflammation.11

Atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) have important physiological roles in fluid homeostasis, vascular tone, and cardiac pathology, including myocardial ischemia and left ventricular dysfunction and remodeling. BNP and the N-terminal precursor fragment (NT-proBNP) have been regarded as the biomarkers of choice when obtaining diagnostic and prognostic information in patients with acute and chronic heart failure. These peptides are commonly involved in the remodeling process via antifibrotic actions, inhibition of aldosterone secretion, and cell proliferation (smooth muscle cell, mesangial cell, fibroblasts). Studies have also assessed a possible connection between natriuretic peptide concentrations and the risk of mortality in random populations, suggesting an association between plasma concentrations and mortality, independently of other risk factors.12,13

In a rat model of infarct-induced heart failure, short-term TMZ treatment significantly reduced ANP mRNA levels, but did not significantly affect BNP mRNA levels or genes involved in fatty acid metabolism.14 In long-term studies performed in patients with ischemic cardiomyopathy, TMZ’s beneficial effect on BNP plasma levels were also reported.15 These effects could have a relevant role in reducing ventricular remodeling and the decline of cardiac function in patients with IHD and heart failure.

Liu et al also reported that TMZ inhibits myocardial fibrosis through the signaling pathway involving NADPH oxidase, reactive oxygen species, and connective tissue growth factor.16,17 Ruixing et al reported that, in a rabbit model of ischemia-reperfusion, trimetazidine also reduced cardiomyocyte apoptosis and ischemia-reperfusion injury via antioxidant properties.18

TMZ also exerts a direct antiapoptotic effect by regulating microRNA (miRNA) expression. These are small, noncoding RNAs that regulate gene expression and exert an antiapoptotic role protecting muscle cells from wall stresses, hypoxia, and $H_2O_2$-induced apoptosis.19

In addition to antifibrotic and antiapoptotic effects, glucose metabolism modulation has been suggested to positively affect cardiac function in heart failure and IHD. Metabolic modulation is linked to the fact that glucose is a more energy-efficient fuel than FFAs. A shift from predominant long-chain FA utilization to glucose utilization will result in an increase in ATP production per unit of oxygen utilization. Impaired glucose metabolism and insulin resistance are commonly considered as maladaptive responses involved in the progression of ventricular function decline. Insulin resistance in heart muscle was recently shown to be related to reduced GLUT4 protein content.19 TMZ could also act, at a metabolic level, by promoting glucose oxidation, increasing insulin sensitivity and modulating GLUT4 expression. In diabetic rats after myocardial infarction, Zhang et al reported that TMZ improves left ventricular diastolic function and the remodeling process by increasing expression of GLUT4 mRNA and protein and inhibits myocardial fibrosis.20

Conclusions

Metabolic therapy could have a relevant role in IHD and CHF treatment. In these clinical conditions, trimetazidine exerts positive effects on ventricular function, quality of life, and prognosis. Although the main mechanism of action is the improvement in cardiac energetic efficiency, emerging reports suggest a potential role of its nonmetabolic effects, such as regulation of gene expression involved in the remodeling process. Future studies should be performed to understand these novel aspects and their clinical relevance.

REFERENCES


MicroRNA biology

MicroRNAs (miRNAs) are short, noncoding RNAs (=22 nucleotides) that modify gene expression by downregulating genes at the post-transcriptional level during various developmental or disease processes.1 These ≈22 nucleotide RNAs bind to their targets in the 3’ untranslated region (UTR) of messenger RNAs (mRNAs) to inhibit translation or evoke degradation of the mRNA. With miRNAs highly integrated into every cellular event, the potential impact on cardiac function is profound when considering the ramifications of misregulation of processes such as fibrosis,2,3 angiogenesis,4 cell growth,5-7 apoptosis,8 and electrophysiology.9 This convergence has led to the investigation of miRNAs as sources of pathophysiology and as biomarkers of heart failure (HF) in addition to therapeutic targets.

MicroRNA changes in heart failure

Although numerous miRNAs are expressed in the heart; miR-1, miR-133, miR-208, and miR-499 are among the most highly expressed. While miR-1 and miR-133 are also present in skeletal muscle, miR-208 and miR-499 appear to be more cardiac specific. Referred to as the myomiRs, miR-1, miR-133a/b, and miR-206 play a key role in myogenic differentiation including cardiomyocyte differentiation.10 Accordingly, as heart failure progresses, remodeling occurs, inducing an embryonic developmental miRNA genetic signature. A seminal 2007 study by Thum et al11 impressively demonstrated that adult hearts in HF were strikingly similar to fetal hearts with approximately 80% of miRNAs regulated analogously.1,12 One of the changes noted was increased expression of miR-21, which has now become one of the most consistent findings in HF (Table I). Inconsistencies between studies analyzing miRNA signatures in HF have complicated therapeutic development. The discrepancies are primarily the result of the type and stage of HF that patients or animal models are undergoing. Although these differences do not impede the use of miRNAs as therapeutics, it does warrant caution for selection.
MicroRNAs as biomarkers of heart failure

Another advantage of miRNAs is that, in addition to tissue specificity, many miRNAs can be found in the serum and are collectively known as circulating miRNAs (c-miRNAs). Easily detected in peripheral blood, c-miRNAs enable tracking of HF progression and can serve as biomarkers of efficacy in therapeutic trials (Table I). Serum levels of miR-208a/b, miR-499, miR-133a/b, miR1, and miR-423-5p have all been tested as biomarkers and compared with the current standard biomarker for HF, troponin.13,14 All showed good correlation with miR208a, demonstrating the highest level of sensitivity (≈90%).15 c-miRNA cardiac signatures were further validated by a study that assessed c-miRNA profiles before and after placement of ventricular assist devices. The study showed normalization of >70% of miRNAs tested, demonstrating feasibility for a noninvasive therapeutic assessment.12

Anti-microRNA oligonucleotides as therapeutics

There are primarily two mechanisms to regulate miRNAs therapeutically. For those miRNAs that are upregulated in HF, anti-miR oligonucleotides (AMOs) can be designed and synthesized to be complementary to mature miRNAs and inhibit binding to their 3’UTR targets. AMOs that are further modified to optimize uptake in vivo and minimize off-target effects are termed antagonirs. They include incorporation of a 2’-O-methyl group to resist nucleases and a phosphorothiate linkage (PS) to improve distribution in tissues. There are numerous examples of successful preclinical studies using antagonirs in vivo.12 An antagonir designed to sequester miR-133 was shown to reverse cardiac hypertrophy when delivered systemically in mice,16 while delivery of a miR-21 antagonir suppressed fibrosis and hypertrophy.17

Encouraging results in preclinical studies have led to the integration of miRNAs into clinical trials. The majority of this inclusion has focused on miRNA expression signatures as biomarkers for disease progression and surrogate markers for therapeutic efficacy following pharmacological therapies.18 Challenges for translation of antagonir therapy for HF include: (i) efficient delivery to the heart; (ii) repeated dosing; and (iii) potential off-target effects. One antagonir has reached clinical trial—miravirsen. Miravirsen, a potential treatment for hepatitis C virus (HCV) infection, is an antagonir of miR-122, a hepatic-specific miRNA that directly targets HCV.19 Dose-dependent efficacy was achieved in chronic HCV-infected patients with miravirsen without adverse events.19 This trial, along with impending trials using miRNA-based therapies for cancer,18 provide valuable safety data that can be applied to future trials for HF.

MicroRNA mimics

In disease states where miRNA expression has been downregulated, miRNA mimics can be delivered to normalize expression. The mimic must be recognized by the miRNA biogenesis machinery as a double-stranded oligonucleotide in order to be processed and expressed appropriately. Off-target effects pose even greater risks and thus encapsilation into viral vectors with specific tissue tropisms have come to the forefront as the preferred method for delivery. Adeno-associated virus (AAV) is the most widely used vector for miRNA delivery. It is a small virus (<4.7 kb) that does not cause disease and persists as an episome for years to decades in tissues. There

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Expression in HF</th>
<th>Therapeutic or biomarker</th>
<th>Expected outcome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-21</td>
<td>Upregulated</td>
<td>Therapeutic</td>
<td>Reverse hypertrophy/fibrosis</td>
<td>12,17</td>
</tr>
<tr>
<td>miR-208</td>
<td>Upregulated</td>
<td>Both</td>
<td>Normalization of contractility</td>
<td>13</td>
</tr>
<tr>
<td>miR-423-5p</td>
<td>Upregulated</td>
<td>Biomarker</td>
<td>Serum biomarker of heart failure</td>
<td>12</td>
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<tr>
<td>miR-499</td>
<td>Downregulated</td>
<td>Both</td>
<td>Cardioprotection</td>
<td>1,14</td>
</tr>
<tr>
<td>miR-1, miR-133</td>
<td>Downregulated</td>
<td>Therapeutic</td>
<td>Prevention/reversal of cardiomyopathy</td>
<td>6,7,12</td>
</tr>
<tr>
<td>miR-29</td>
<td>Downregulated</td>
<td>Both</td>
<td>Reversal of fibrosis</td>
<td>3</td>
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</table>

Table I MicroRNA (miRNA) therapeutic targets for heart failure.
are multiple serotypes, including AAV-8 and AAV-9, that lead to efficient transduction of cardiomyocytes.\(^3\) AAV-9 delivery of several miRs (miR-122, miR-29, and miR-21) in preclinical murine studies has demonstrated a strong proof-of-principle for this approach (Table I). Delivery of AAV vectors carrying miR mimics directly to the heart rather than through the systemic circulation provides additional benefits including dose reduction, minimizing off-target gene expression, and maximizing transduction levels in the heart. Challenges for direct delivery to the heart still pose some translational hurdles due to the protected internal location; however intracoronary delivery has now been shown to be safe in a gene therapy trial with patients with advanced heart failure.\(^2\) This phase 2 study tested safety and efficacy of intracoronary infusion of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) delivered using AAV-1. AAV-1–SERCA intracoronary delivery was shown to be safe and well tolerated in the 1-year study with a trend toward clinical significance in functional capacity; however intracoronary delivery has now been shown to be safe in a gene therapy trial with patients with advanced heart failure.\(^2\) This phase 2 study tested safety and efficacy of intracoronary infusion of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) delivered using AAV-1. AAV-1–SERCA intracoronary delivery was shown to be safe and well tolerated in the 1-year study with a trend toward clinical significance in functional capacity of patients in the high-dose group.\(^2\) This sentinel study has laid the foundation for treatment of HF using AAV-mediated intracoronary delivery.

**Conclusions**

The therapeutic potential of miRNA mimics and antagonors for HF is irrefutable. Preclinical studies have shown virtually complete reversal of HF in animal models and antagonors are now transitioning into the clinic for other diseases such as HCV. Caution is warranted, particularly with the use of miRNA mimics, as the long-term effects of overexpression are not known yet. miRNAs have evolved to tightly regulate gene expression in a highly dynamic and titrated manner. Upregulation of miRNAs may be beneficial for normalizing disease states; however, overexpression may tilt the balance in the wrong direction. As clinical safety and efficacy data accumulate in favor of a stepwise approach with targeted delivery of miRNA-based therapies, the risk of complications will be lowered. To summarize, miRNA therapy for heart failure is a very promising approach to treatment. As miRNA signatures become more defined, it will be feasible to identify early indicators of heart failure prior to clinical manifestations. Early intervention to normalize miRNA expression in the heart has the potential to revolutionize the way heart disease is managed in the next decade.

**REFERENCES**

What are microRNAs?

Thomas Thum, MD, PhD
Institute for Molecular and Translational Therapeutic Strategies (IMTTS), Hannover Medical School, Hannover, Germany; National Heart and Lung Institute, Imperial College, London, UK

Correspondence: Thomas Thum, MD, PhD, Institute for Molecular and Translational Therapeutic Strategies (IMTTS), Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany
E-mail: thum.thomas@mh-hannover.de

Abstract
MicroRNAs (miRNAs) are short regulatory noncoding endogenous RNA species that are involved in virtually all cellular processes. Intriguingly, they are highly conserved among species and very rarely harbor genetic variants, underlining their important role during the evolution of species. miRNAs fine-tune and regulate proteins involved in all main processes, including cell signaling pathways, as well as cellular functions and developmental steps. miRNAs are expressed in all organs and cells, and multiple functional aspects of miRNAs underscore their key role in physiology and pathophysiology. The development of novel molecular biology tools contributed to the recent successes in miRNA research. Moreover, miRNAs emerge as interesting biomarker candidates in cardiovascular disease, but also as therapeutic targets. Finally, so-called long noncoding RNAs regulate gene and protein expression and are also briefly discussed in this short review article. ■ Heart Metab. 2014;65:34-36

Keywords: cardiac remodeling; cardiovascular disease; heart failure; microRNA

Biogenesis and importance of microRNAs

MicroRNAs (miRNAs) are endogenous, small (~22 nucleotides) noncoding RNAs that are highly conserved among species and regulate expression of target mRNAs by binding to their 3′ untranslated region (UTR), followed by translational repression. In rare cases, miRNAs can also target 5′UTR or coding regions. miRNAs are indeed ubiquitously expressed, but may differ in expression patterns. In the cardiovascular system, miRNAs have an enormous gene regulatory potential, and play key roles in the physiology and pathophysiology of the entire cardiovascular system.

miRNAs originate from transcripts that are either coexpressed with the host gene transcript or located in intergenic regions of the genome. Initially, the early transcript, called “primary (pri)-miRNA,” is processed within the cellular nucleus by the ribonuclease III enzyme Drosha and the double-stranded RNA-binding protein DGC8 into a so-called precursor (pre)-miRNA (a short hairpin RNA molecule) and thereafter exported to the cytosol by exportin-5. Within the cytosol, there is a digestion of pre-miRNA into small mature RNA molecules by the endonuclease Dicer, followed by binding to the RNA-induced silencing complex (RISC) complex. During cardiovascular development and in disease, there are significant changes of miRNA expression profiles leading to derailed orchestration of cardiovascular genes.

MicroRNAs as therapeutic targets in cardiovascular disease

Cardiac miRNA is necessary for a balancing and buffer system to regulate protein expression, in order to maintain homeostasis in the heart. A disturbed balance of miRNA expression occurs in almost all cardiovascular pathologies. Major cardiovascular disorders with deregulated miRNA expression include...
MicroRNAs as biomarkers in cardiovascular disease

Surprisingly, it is possible to detect circulating miRNAs in serum/plasma, suggesting that they may serve as potential biomarkers for diseases and/or may have paracrine effector capacity. Circulating miRNAs are protected from RNase-dependent degradation by several mechanisms, including their inclusion in microvesicles, exosomes, and apoptotic bodies, and by formation of protein-miRNA complexes resistant to degradation. There is now ample evidence for the diagnostic use of circulating miRNAs as biomarkers, but here only a few studies can be highlighted. For instance, in patients with diabetes or coronary artery disease (CAD), reduced levels of miR-126 are observed, whereas cardiac muscle-enriched miRNAs (miR-133a, miR-208a) tended to be higher in patients with CAD. In patients with acute myocardial infarction (AMI), the muscle-enriched miRNA miR-1 was significantly upregulated in the circulation compared with non-AMI controls. Our group has also shown increased plasma miRNA post-AMI as well as some prognostic value of selected circulating miRNAs in terms of predicting future death (Widera et al21). During viral myocarditis, mild elevation of miR-208b and miR-499 was found. A recent study found circulating long noncoding RNAs to be of diagnostic and predictive value in post-MI and heart failure patients.

Outlook and conclusions

There are three major important points on the usefulness of miRNAs that I wish to explicitly highlight:

1. miRNA research has identified a new layer of mechanistic complexity in genome research, and has opened a new angle on how to identify new and potentially important mechanisms of cellular functions. Multiple novel pathways involving not only miRNAs, but also regulatory upstream mechanisms as well as downstream target networks, have only been identified because of miRNA research activities.

2. miRNAs emerged as drug-able targets, which, within a couple of years, made their way into clinical phase 1 and 2 trials, and have an enormous future therapeutic potential. Research has started using small and large animal models of diseases and, indeed, recently, the first miRNA therapeutic study in patients with hepatitis C has been published with many other ongoing clinical miRNA studies (www.clinicaltrials.org).

3. miRNAs have been shown to be stable in plasma and other body fluids and have been shown to be potentially interesting biomarkers of diseases, especially for diagnostic and prognostic evaluations. A potential future strength is the combination of miRNA panels as powerful predictors of outcome and/or therapy effectiveness in multiple diseases.

miRNA research is still relatively young, but has opened new avenues in the mechanistic understanding of physiology and pathophysiology, in drug
What are microRNAs?

therapy, and in diagnostic/prognostic patient evaluation. Following the improved development of miRNA-based diagnostic and therapeutic interventions, other RNA species such as long noncoding RNAs have emerged as interesting, either as targets and/or biomarkers.

The author has filed and licensed microRNA-related patents.

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MicroRNAs as therapeutics for heart disease

John R. Ussher, PhD
Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Canada

Correspondence: Dr John R. Ussher, Katz 2-020C, Faculty of Pharmacy and Pharmaceutical Sciences, Katz Centre for Pharmacy and Health Research, 11361 87 Avenue, University of Alberta, Edmonton, AB T6G 2E1, Canada
E-mail: jussher@ualberta.ca

Abstract

MicroRNAs (miRNAs) are small, noncoding RNA molecules that have been demonstrated to play critical roles in multiple aspects of whole body physiology by repressing downstream messenger RNA (mRNA) target transcription. With reference to the cardiovascular system, a number of miRNAs undergo deregulated expression in response to numerous stresses including ischemia, heart failure, and pulmonary hypertension, which led to the discovery of novel targets for treating these various cardiovascular diseases, including miRNA-214, miRNA-155, and miRNA-204. As our understanding of miRNA biology and the regulation of their downstream mRNA target genes improves, it should lead to the discovery of novel targets for the treatment of various types of cardiovascular disease. ■ Heart Metab. 2014;65:37-39

Keywords: cardiovascular disease; heart; heart failure; ischemic heart disease; microRNA; pulmonary arterial hypertension

Cardiovascular disease, consisting primarily of ischemic heart disease and heart failure, is a primary cause of death and disability in the world today.1,2 Fortunately, epidemiological studies and randomized clinical trials have provided compelling evidence that cardiovascular disease is highly manageable.3 Current treatment regimens, which are comprised of either percutaneous or surgical techniques to enhance myocardial blood supply, or pharmacotherapy to reduce myocardial oxygen demand, have greatly improved the overall prognosis of patients living with cardiovascular disease. Nevertheless, a large number of patients remain that prove to be either ineligible for, or refractory to, conventional treatment, and percutaneous or surgical revascularization is associated with a distinct set of risks. Thus, new approaches to treat such patients are necessary. One such potential novel therapy involves the targeting of microRNAs (miRNAs), which will be the focus of this article.

miRNAs are small, noncoding RNA molecules (~22 nucleotides in length) that have been demonstrated to play critical roles in multiple aspects of cardiac function via repression of target gene messenger RNA (mRNA) transcription. miRNAs are able to repress gene function by binding to sequences in the 3’ untranslated regions of their associated target mRNAs, which exhibit complementarity to nucleotides 2 to 8 of the associated miRNA, referred to as the seed region.4 miRNAs are divided primarily into 3 classes depending on the genomic location of their miRNA-encoding sequences; intergenic miRNAs are under the transcriptional control of distinct promoters, while both intronic and exonic miRNAs exhibit transcriptional control through their host-gene promoters.4 The generation of mature miRNAs and sub-
Abbreviations
LV: left ventricular; miRNA: microRNA; mRNA: messenger RNA; PAH: pulmonary arterial hypertension; TAC: transverse aortic constriction

sequent repression of gene expression first involves primary miRNAs (=hundreds to thousands of nucleotides in length), which fold imperfectly into hairpin-shaped precursor miRNAs (=70 to 100 nucleotides in length), and these precursor miRNAs are transported into the cytoplasm via exportin 5, and are further processed into a short, mature miRNA duplex via the endonuclease Dicer. This mature miRNA duplex is subsequently integrated into the RNA-induced silencing complex, resulting in translation inhibition, mRNA degradation, and the posttranscriptional repression of gene expression.

The remainder of this article will primarily illustrate novel findings supporting miRNA-targeted therapy as a novel approach for various forms of cardiovascular diseases including ischemia/reperfusion injury, cardiac hypertrophy, heart failure, and pulmonary arterial hypertension (Figure 1). For more detailed information on the complex regulation of miRNAs and their contribution to cardiac development and disease, please refer to the following in-depth reviews.4,5

In the setting of ischemic heart disease, miRNA expression is significantly altered and associated with the progression of disease pathogenesis, including perturbed angiogenesis, enhanced apoptosis, and contractile dysfunction.5-7 Elegant studies from Aurora et al demonstrated a significant role for miRNA-214 as an adaptive protective mechanism against ischemia/reperfusion injury via regulation of Ca2+ homeostasis and signaling.8 Indeed, they demonstrated a significant upregulation of miRNA-214 expression in wild type mice at both 24 hours and 7 days following a temporary left anterior descending (LAD) coronary artery occlusion. However, in whole heart lysates from mice deficient for miRNA-214 (miRNA-214-/-), there was a marked upregulation of the Na+/Ca2+ exchanger-1 protein (NCX1) at both 24 hours and 7 days following temporary LAD coronary artery occlusion, which contributes to ischemia/reperfusion injury via reverse mode Ca2+ exchange.9 Furthermore, other Ca2+-sensitive factors altered in miRNA-214-/- hearts at 7 days following temporary LAD coronary artery occlusion included calmodulin-dependent protein kinase Iκ (CamKIIκ) and cyclophilin D (CypD). Importantly, these alterations in NCX1/CamKIIκ/CypD were associated with an exacerbation of left ventricular (LV) remodeling and dysfunction at 7 days post-LAD coronary artery occlusion and were recapitulated in neonatal cardiomyocytes (NCMs) treated with anti-miRNA-214 (miRNA-214 antagonist) exposed to hypoxia/reoxygenation, which increased apoptosis.

During heart failure progression, Seok et al demonstrated that miRNA-155 expression is significantly reduced in heart extracts at 3 days, 2 weeks, and 4 weeks following transverse aortic constriction (TAC).9 Despite this reduction in miRNA-155 expression, the authors also observed, in mice deficient for miRNA-155, a marked improvement in LV function and adverse LV remodeling at 4 weeks post-TAC surgery, which was associated with a significant reduction in mRNA expression of two heart failure markers; brain natriuretic peptide and myosin heavy chain β.9 Similarly, genetic elimination of miRNA-155 in cardiac-specific calcineurin–overexpressing mice also improved LV function and adverse LV remodeling in this mouse model predisposed to early heart failure. Furthermore, inhibition of miRNA-155 in NCMs prevented phenylephrine-induced cellular hypertrophy.

miRNA-based targeting may also have a role in the setting of pulmonary arterial hypertension (PAH) because lung miRNA profiles from rats treated with monocrotaline (MCT) or subjected to chronic hypoxia demonstrate that the expression of a number of miRNAs are altered.10 Studies from Courboulin et al demonstrated that activation of signal trans-
ducer and activator of transcription 3 (STAT3) is responsible for miRNA-204 downregulation in primary cultured pulmonary arterial smooth muscle cells (PASMCs) from PAH patients, and that miRNA-204 expression negatively correlates with PAH severity in mice, rats, and humans. Moreover, treatment with a miRNA-204 mimic inhibited proliferation and increased apoptosis of PASMCs cultured from PAH patients, whereas nebulized delivery of this miRNA-204 mimic decreased mean pulmonary arterial pressure and pulmonary arterial wall thickness in rats with MCT-induced PAH. Analysis of these rats also demonstrated a significant reduction in proliferation and increased apoptosis in the distal pulmonary arteries of rats with MCT-induced PAH that were treated with the miRNA-204 mimic.

**Conclusions**

Taken together, these examples provide exciting evidence supporting miRNAs as novel targets for many facets of cardiovascular disease. At this stage, all miRNAs in development for cardiovascular disease are currently in preclinical stages, with the first phase 1 trial for a miRNA-based target, MRX34 (miRNA-34 mimic), currently in phase 1 trials to assess safety in the treatment of patients with primary liver cancer. Undoubtedly, the next decade of research in the field should provide valuable information regarding the true potential that miRNA-based therapies may have for the treatment of cardiovascular disease.

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**Adeno-associated virus**

Adeno-associated virus (AAV) is a nonpathogenic human virus that belongs to the Paroviridae family. Replication-defective AAV, which is devoid of all viral genes, is utilized as a gene transfer vector. AAV vectors do not integrate into the host genome. AAV vectors have several desirable properties for in vivo gene transfer, including low immunogenicity, lack of inflammatory response following in vivo administration, and the ability to deliver genes into postmitotic cells (e.g., cardiac myocytes) with high efficiency.

**Antagomir**

An antagomir is a chemically modified antisense oligonucleotide. Typically, a locked nucleic acid modification is utilized to restrict the ribose moiety of the nucleotide into a conformation ideal for complementary Watson-Crick binding. This modification confers long-lasting stability to the antagomir. Antagomirs bind and deplete microRNAs from cells, thereby preventing microRNA-induced messenger RNA degradation and translational inhibition.

**Dicer**

Dicer is a large multidomain protein possessing RNase III activity that is involved in processing larger RNA precursors into smaller RNA species. Dicer is responsible for cleaving pre-microRNAs into mature microRNAs, and double-stranded RNAs into small interfering RNAs.

**Episome**

Episome refers to an extrachromosomal genetic element (e.g., DNA) that is capable of replication when supplied with the necessary factors. Therefore, episomal expression vectors allow for gene transfer and subsequent transgene expression in the absence of chromosomal integration.

**Exosome**

Exosomes are small vesicles derived from late endosomes. Exosomes participate in secreting damaged proteins and RNA from the cell.

**Nanoparticle tracking analysis**

Nanoparticle tracking analysis allows for the visualization and analysis of particles in liquids by relating the rate of Brownian motion to particle size. This method is able to determine the size distribution of small particles in liquid suspension with a diameter of approximately 10 to 1000 nm.

**Pluripotency**

Pluripotency refers to a stem cell with the ability to differentiate into any of the three germ layers (endoderm, mesoderm, and ectoderm). Furthermore, pluripotent stem cells can be further divided into completely pluripotent (ability to form every cell of the embryo proper) or partially pluripotent (ability to form cells of all three germ layers, but does not exhibit all the characteristics of a completely pluripotent stem cell).

**RNA-induced silencing complex**

The RNA-induced silencing complex (RISC) is a multiprotein complex that incorporates one strand of small interfering RNA or microRNA and uses it as a template to recognize complementary messenger RNA. Upon recognition of the complementary strand, the RISC subsequently activates a protein within its multiprotein complex known as argonaute, which cleaves the RNA.

**Myocardin**

Myocardin is a serum response factor transcriptional coactivator found specifically in cardiac and smooth muscle, which belongs to a family of transcription factors and plays a key role in inducing smooth muscle differentiation.