HOSPITAL CHRONICLES 2012, 7(1): 9–15

EXPEDITED REVIEW

Micro-RNAs: New Biomarkers and Novel Applications for Heart Disease Prevention and Treatment

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KEY WORDS: *microRNA; cardiovascular disease; genomics; molecular cardiology; cardiac hypertrophy; cardiomyopathy; heart failure*

ABBREVIATIONS

cTnI = cardiac troponin I
DNA = deoxyribonucleic acid
DYRK = dual-specificity tyrosine phosphorylation-regulated kinase
GATA = (transcription factor binding to the consensus DNA sequence) guanine/adenine/ thymine/adenine
HSP = heat shock protein
LNA = locked-nucleic acid
MEF2A = myocyte-specific enhancer factor 2A
miRNA = micro RNA
mRNA = messenger RNA
MuRF1 = muscle ring finger1
NFATc3 = nuclear factor of activated T cells 3
PPARγ = peroxisome proliferator-activated receptor γ
PTGS = post transcriptional gene silencing
RISC = RNA-induced silencing complex, also known as a microRNA ribonucleoprotein complex (miRNP)
RNA = ribonucleic acid
Thrap1 = thyroid hormone receptor-associated protein 1

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Manuscript received January 17, 2012; Revised manuscript received January 30, 2012; Accepted January 31, 2012

ABSTRACT

MicroRNAs (MiRNAs) have emerged as pivotal modulators of mammalian cardiovascular development and disease over the past few years. They represent a biological mechanism for regulating gene expression and consequently a broad range of physiological and pathological molecular processes. Importantly, multiple miRNAs have been implicated in heart disease onset and progression, whereas in the case of cardiac hypertrophy and heart failure, recent data are changing our perception of disease pathogenesis and therapeutics. This review presents the concept of miRNAs, mechanisms of generation, and representative examples of associations with cardiovascular disease pathogenesis and therapeutic potential.

INTRODUCTION

The 21st century is considered by many the era of Genomic Medicine. Indeed fascinating advances have been achieved both in terms of high-throughput technologies and biological insight, leading to a deeper understanding of human disease, improved diagnostic and prognostic tools, and better therapeutic approaches. In Cardiology, extensive work over decades led to the identification of genetic and environmental factors contributing to disease pathogenesis. However, genetics alone does not suffice to explain the full pathological spectrum and variable prognosis of these complex multifactorial heart diseases. Furthermore, available therapies are far from ideal. The latest advances in genomics are now opening the way to a new era for Molecular Cardiology, with novel concepts and molecules meriting in depth exploration. One of the most rapidly evolving cases is this of microRNAs (miRNAs).

Micro-RNAs (miRNAs) have emerged as pivotal modulators of mammalian cardiovascular development and disease over the past few years. They represent another biological mechanism for regulating gene expression and consequently a broad range of physiological and pathological molecular processes. MiRNAs are a group of small (18-25 nucleotide-long), single stranded, evolutionary conserved, non-coding regulatory RNA molecules. They are transcribed from DNA (intergenic, intronic or polycistronic) but are not translated into proteins. Instead, they bind messenger RNA (mRNA) molecules and interfere with their stability and consequantly their translation

The authors confirm that the work is original and has not been previously published elsewhere. There are no conflicts of interest to declare

to proteins. The primary transcripts of miRNAs, pri- miRNAs, are hairpin-shaped molecules bearing a 5'cap and a poly (A) adenosine tail on the 3'edge.[1,2] After a first processing step inside the nucleus by the Microprocessor complex (Dorsha nuclease and Pasha protein) 70 nucleotide-long pre-miRNAs are generated. In turn, they are transported to the cytoplasm by exportin 5, and cleaved by the Dicer protein to form mature double stranded miRNA molecules (Fig. 1).[3-5] These double-stranded molecules are cut into two single-stranded miRNAs, with the "active" one being chosen by the Argonaut protein and embodied in a protein complex to form the active miRNP complex (miRNA-containing ribonucleoprotein particle) and the remaining one being decomposed.[6-9]

The mature miRNA can direct miRNP complexes to the appropriate mRNA targets using their base-pair complementarity, and thus regulate mRNA expression levels. As reported for most animal miRNAs, they can bind multiple, partially complementary sites in the 3'-UTR regions of target mRNAs, and in the case of perfect base-pairing the target mRNA is destroyed.[10] Alternatively, multiple miRNAs may bind partially to an mRNA, resulting in reduced levels of the corresponding protein with minimum effect on the mRNA levels.[11] The overall outcome is post transcriptional gene silencing (PTGS).

MicroRNAs are implicated in numerous physiological functions, including cell- and tissue- specific differentiation and development. Closing up to the cardiovascular system, a role for miRNAs has been proposed in cardiac development and function, as well as in heart disease. Multiple miRNAs

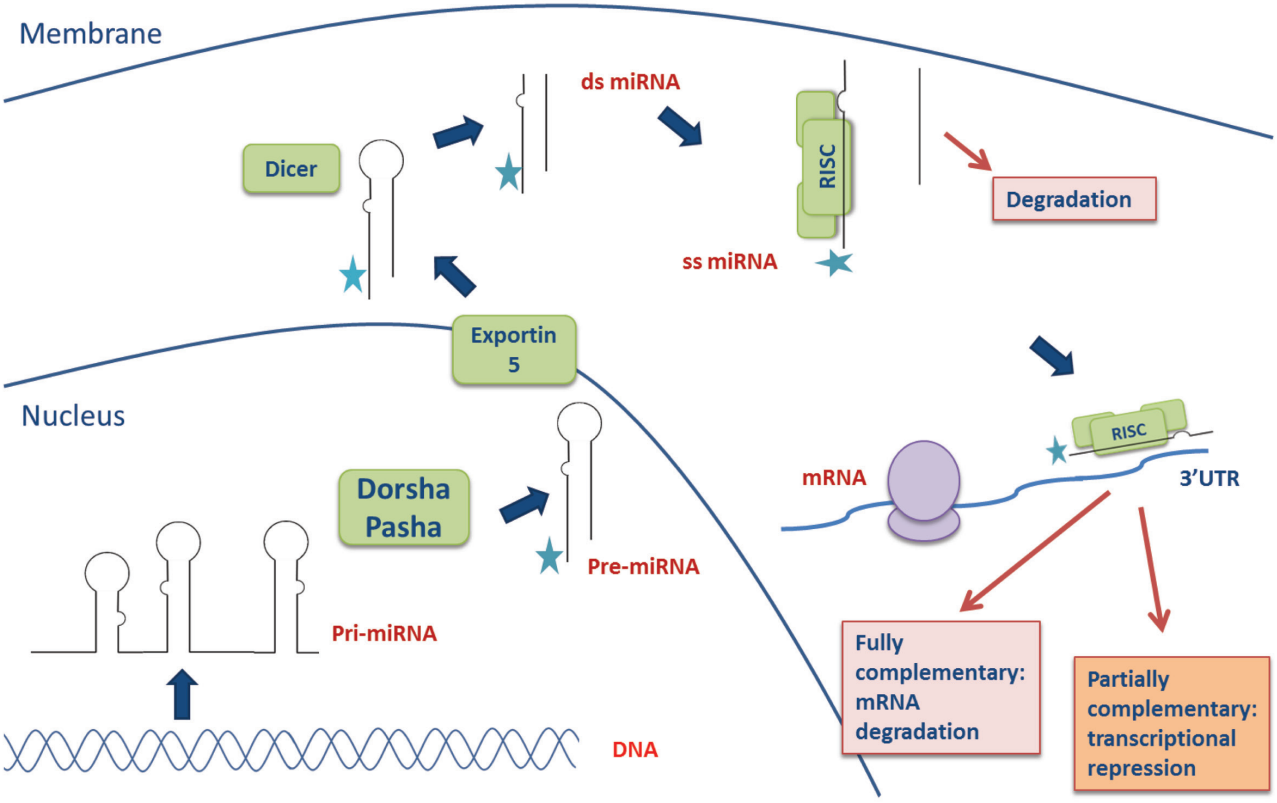

FIGURE 1. Schematic representation of microRNAs' formation and course of action. MicroRNAs (miRNAs) are transcribed from intergenic, intronic or polycistronic DNA, in the first instance as hairpin-shaped molecules (primary transcript or pri- miRNAs), which bear a 5'cap and a poly (A) adenosine tail on the 3'edge. Inside the nucleus, these primary transcripts are modified by the Microprocessor complex (Dorsha nuclease and Pasha protein) to form 70 nucleotide-long pre-miRNAs. Exportin 5 facilitate pre-miRNAs translocation to the cytoplasm, where they are cleaved by the Dicer protein to form mature double stranded 18-25 nucleotide-long miRNA molecules. After being cut into two single stranded miRNAs, the "active" one is selected by the Argonaut protein and embodied in a protein complex to form the active miRNP. The remaining single stranded miRNA is decomposed. The miRNP complexes are directed to the appropriate mRNA targets and bind multiple, partially complementary sites in their 3'-UTR regions. In the case of perfect base-pairing the target mRNA is destroyed, whereas partial binding multiple miRNAs to an mRNA results in reduced levels of the corresponding protein with minimum effect on the mRNA levels. 3'UTR = three prime untranslated regions; DNA = deoxyribonucleic acid; ds = double-stranded; mRNA = messenger RNA; RNA = ribonucleic acid; RISC = RNA-induced silencing complex, also known as a microRNA ribonucleoprotein complex (miRNP); ss = single-stranded.

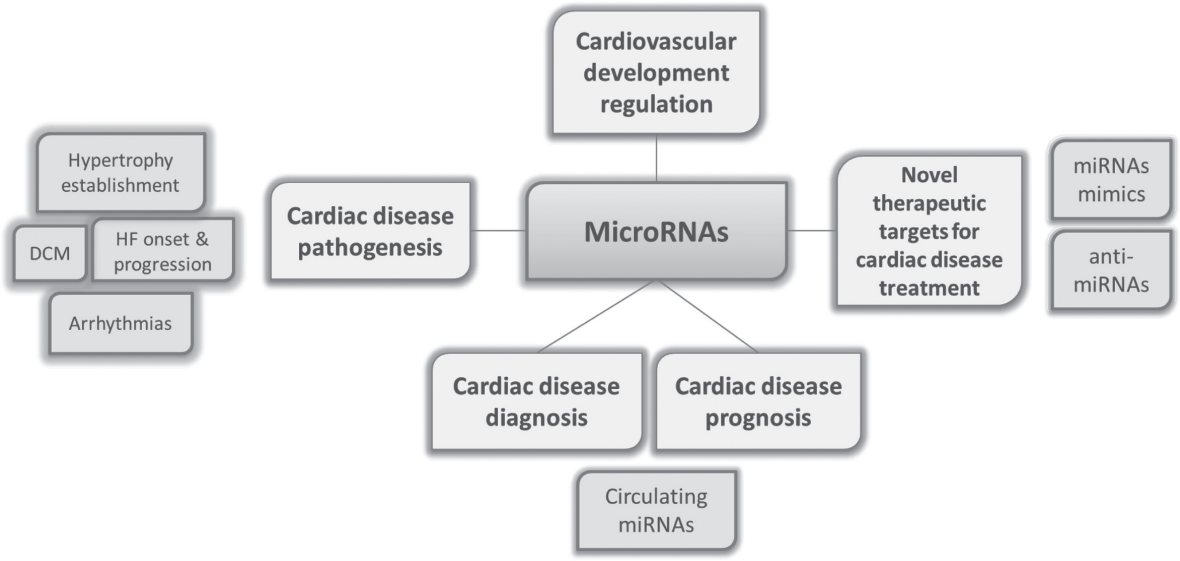

FIGURE 2. MicroRNAs in health and disease. MicroRNAs are implicated in cardiac physiology and pathology. These roles render them valuable biomarkers for diagnostic and prognostic purposes, as well as promising therapeutic targets. DCM = dilated cardiomyopathy; HF = heart failure.

TABLE 1. Examples of MicroRNA Involvement in Cardiac Pathophysiology.

MicroRNAs	Pathological effect/outcome
miR-195	*in vivo* hypertrophy, dilated cardiomyopathy
miR-23a	*in vivo* hypertrophy
miR-23b	*in vivo* hypertrophy
miR-24	*in vivo* hypertrophy
miR-214	*in vitro* hypertrophy
miR-21 miR-129 miR-212	*in vivo* hypertrophy
miR-199a	*in vivo* hypertrophy
miR-199b	*in vivo* hypertrophy, heart failure
miR-27b	*in vivo* hypertrophy, heart failure
miR-208a miR-499	*in vivo* hypertrophy, arrhythmias
miR-1 miR-133	*in vitro* inhibition of hypertrophy

have been implicated in heart disease onset and progression, whereas in the case of cardiac hypertrophy and heart failure, recent data are changing our perception of disease pathogenesis and therapeutics.

MICRORNAs RELATED TO CARDIOVASCULAR PATHOGENESIS

A representative study from this field involved global miRNA expression profiling in the left ventricles of heart failure patients. It showed significant alterations in many miRNAs, indicating that they hold a key role in disease pathogenesis and progression. The reported changes varied based on the primary disease (e.g. dilated or ischemic cardiomyopathy or aortic stenosis) and they were sufficiently distinct to allow accurate patient classification.[12] Of note, 87% of the over-expressed miRNAs along with 84% of the under-expressed miRNAs in those tissues were similar to the miRNA expression profiles of fetal heart tissue, thus implicating the specific miRNAs in the activation of the "fetal gene expression program", a hallmark of the hypertrophic and failing myocardium.[13]

PRO-HYPERTROPHIC MiRNAs

Other studies in hypertrophic hearts from humans and mouse models identified significantly elevated miR-195 levels. *In vitro* and *in vivo* analysis of miR-195 proved that its overexpression is sufficient for the development of cardiac hypertrophy over the course of a few weeks, and development of dilated cardiomyopathy and heart failure in the long-term.[14] MiR-23a, miR-23b, miR-24 and miR-214 were also found overexpressed in cardiac hypertrophy. In order to assess their contribution to disease pathogenesis, they were overexpressed in cardiomyocytes *in vitro,* and as hypothesized, they led to hypertrophy. In contrast to miR-195, miR-214 overexpression

alone does not seem to lead to a pathological phenotype *in vivo*. An explanation for this finding may be provided by another study, reporting that only the combined overexpression of miRNAs (specifically miR-21, miR-129 and miR-212), as opposed to their separate overexpression, could lead to hypertrophy.[13] These findings accentuate miRNAs' critical role in cardiac function, the differences between them but also their synergistic action.

Further elucidating the role of miR-23a in cardiac hypertrophy, it was shown that its expression was directly activated by the nuclear factor of activated T cells 3 (NFATc3), and in turn it targeted and suppressed the negative regulator muscle ring finger1 (MuRF1). Treatment with anti-miRNA oligonucleotides (antagomirs – described in section "MicroRNAs as therapeutic targets") led to MuRF1 upregulation and inhibition of isoproterenol-induced cardiac hypertrophy in mouse hearts.[15] Of note, mice with MuRF1 ablation present with pressure-induced hypertrophy.[15,16] These findings together support that miR-23a acts as a mediator of the calcineurin/NFATc3 pathway, facilitating its prohypertrophic action, which is consistent with observations of miR-23a upregulation in pressure overload- induced[14,17-19] and isoproterenol-induced cardiac hypertrophy.[15]

The calcineurin/NFAT pathway seems to play a pivotal role in hypertrophy regulation by miRNAs, with more than miR-23a being implicated in the process. MiR-199b upregulation, recorded in patients and mouse models with heart failure, acts through the calcineurin/NFAT in hypertrophy as well. In addition, NFAT activates miR-199b expression, thus allowing miR-199b to suppress NFAT's negative regulator dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1a (Dyrk1a), participating in an auto-amplification loop. In accordance, severe pressure-overload induced cardiac hypertrophy develops via Dyrk1a downregulation in transgenic mice overexpressing miR-199b or being *Dyrk1a* haploinsufficient. Consistently, when miR199b levels were normalized by antagomirs, hypertrophy and heart failurre were inhibited and the phenotype was reversed.[20] MiR-23a and miR199b serve as mediators of the calcineurin/NFAT pathway in hypertrophy and could have a therapeutic potential. Meantime, a possible synergistic action of these two miRs towards hypertrophy remains to be investigated.

Another miRNA likely implicated in the pathogenesis of cardiac hypertrophy and heart failure is miR-27b. In mouse models of cardiac hypertrophy induced by cardiac-specific Smad4 ablation, miR-27b upregulated, while its cardiac-specific overexpression in transgenic mice suffices to induce hypertrophy and dysfunction, and its suppression could inhibit phenylephrine-induced hypertrophy *in vitro*.[21] Ttransforming growth factor-1 (TGF-1) was shown to inhibit miR-27b expression and peroxisome proliferator-activated receptor γ (PPARγ) was verified as a direct target. These findings were further confirmed in a pressure overload-induced heart failure mouse model treated with 27b antagomirs.[21]

MiR-208, being exclusively expressed in the heart, is another promising miRNA implicated in hypertrophy. Although it does not affect cardiac development, its absence inhibits the development of hypertrophy. Specifically, miR-208 ablation in genetically modified mice is not associated with any developmental abnormality or pathological heart condition during the first months of life. Only a slight decrease in cardiac function is noted after the first two months, which seemed to deteriorate with age. Strikingly however, these mice do not develop hypertrophy upon exposure to pro-hypertrophic stimuli including activation of calcineurin pathway, pressure overload or hypothyroidism.[22] Since miR-208 loss leads to elevated thyroid hormone receptor-associated protein 1 (Thrap1) levels, it is possible for miR-208 to act via Thrap1. These findings establish a connection between miRNAs and hormone-regulated cardiac muscle physiology, and could further improve our understanding of cardiac function and its regulation.

Complementary studies demonstrated that miR-208 functions together with miR499 to regulate the "myosin switch", part of the fetal gene program activated during hypertrophy. MiR-208 has two isoforms, miR208a and miR-208b, both of which are sources of mature miR-208. MiR-208a, located in the Myh 6 (αMyh) intronic region, regulates the expression of βMyh myosin genes Myh7 and Myh7b, and their respective intronic miRNAs: miR-208b and miR-499, in the adult heart.[23] Developmentally normal neonatal transgenic mice lacking miR-208a had the expected levels of αMyh and βMyh, whilst adult transgenic mice exhibited decreased miR499 levels, did not present with the expected βMyh upregulation and did not develop hypertrophy in response to hypothyroidism.[22] Consistently, overexpression of miR499 in miR-208a deficient mice restored the βMyh levels and the normal hypertrophic response to thyroid hormone inhibition, indicating that miR499 acts downstream of miR-208a.[23] Meanwhile, overexpression of miR-208a in transgenic mice induced hypertrophic growth likely through Thrap1 and myostatin suppression, without however, upregulation of atrial natriuretic factor or βMyh, or significant alterations in hypertrophy-related miRNAs' expression levels. Moreover, the transgenic mice presented with arrhythmias, indicating that miR-208a is essential for normal cardiac conduction, as well.[24]

A pro-hypertrophic role has also been attributed to mir-199a. Specifically, its knockdown resulted in cell size decrease and attenuation of phenylephrine-induced hypertrophy, whereas its overexpression *in vitro* led to increased cell size and decreased αMyh.[25]

ANTI-HYPERTROPHIC MiRNAs

Apart from overexpression, downregulation of specific miRNAs can also contribute to cardiac hypertrophy indicating an antihyperyrophic role. For example, miR-1 and miR-133 downregulation suffices to trigger cardiac hypertrophy in

overall healthy animals, whereas their overexpression in *in vitro* hypertrophy models inhibits the hypertrophic phenotype.[17,26] This is likely achieved through modulation of multiple molecular mechanisms. One study demonstrated that these two miRNAs are involved in cardiomyocyte apoptosis, via heat shock protein 60 (HSP60), HSP70 and caspase 9,[27] and another implicated miR-1 in calmodulin downregulation and NFAT/calcineurin mediated reduction of calcium-calmodulin signaling.[28] Furthermore the calcium dependent transcription factors MEF2A and GATA4 were downregulation. Since NFAT and MEF2A cooperate with the transcription factor GATA4 to activate hypertrophic gene expression in cardiomyocytes, miR-1 exerts its antihypertrophic role, at least in part, through regulation of an essential subset of cardiac genes.[29]

MICRORNAs' POTENTIAL IN THE DIAGNOSTIC AND PROGNOSTIC SETTING

Although deciphering disease pathogenesis is the first step towards combating it, the identification of diagnostic and prognostic markers remains another crucial aspect in improving quality of life, healthcare and treatment selection. MicroRNAs are promising to make a significant contribution towards this direction as well. Although miRNA expression in cardiac tissue has been studied in the research setting, a number of steps are still needed in terms of validation of the research findings to the clinical setting, as well as development of suitable (accurate, easy and economical) molecular tests. Towards this direction, circulating miRNAs could serve a valuable role in the cardiology clinic. For example, in a rat model of isoproterenol-induced myocardial injury the levels of circulating miR-208 were significantly ($P < 0.0001$) increased, along with cardiac troponin I (cTnI), which is a widely used biomarker of myocardial injury.[30] Similarly, in humans, upregulation of miR-1,[31,32] miR-208a,[33] miR-208b,[34] miR-133, miR328,[35] and miR-499,[34,36] has been reported in acute myocardial infarction patients in comparison with controls, while increased levels of the circulating miR-423-5p and miR-499 have been associated with heart failure. In parallel to upregulation, miRNA downregulation can also serve as a disease marker as exemplified by the coronary artery disease related underexpression of miR-126, miR-17 and miR-92a.[37]

MICRORNAs AS THERAPEUTIC TARGETS

The emerging critical role of miRNAs in cardiac function and disease pathogenesis renders them promising therapeutic targets. The inactivation of miRNA targets could be achieved either by directly targeting selected miRNA molecules or by inhibiting miRNA binding to their mRNA targets. A variety of molecular tools are already available to enable the targeted modulation of miRNA pathways. The two main categories include 1) anti-miRNAs (antagomirs), modified antisense oligonucleotides that bind selected mature microRNAs and reduce their intracellular levels; 2) miRNA mimics, that mimic the action of selected miRNAs.[38]

Specifically, *anti-miRNAs (antagomirs)* can act at multiple levels including i) binding to mature miRNA within the RNA-induced silencing complex (RISC) and serving as a competitive inhibitor, ii) binding to pre-miRNA and inhibiting their processing and incorporation to the RISC complex, and iii) inhibiting the processing or the exit of pre-miRNA or pri-miRNA from the nucleus. In all cases the end result is the increase in the levels of the selected mRNA. For example, administration of antimiR-21 to a mouse pressure-overload-induced disease model inhibited interstitial fibrosis and attenuated cardiac dysfunction.[39] Similarly, treatment of Dahl hypertensive rats with hypertension-induced heart failure, with antimiR-208a resulted in prevention of pathological "myosin switching" and cardiac remodeling, whereas cardiac function, overall health and survival were markedly improved.[40]

On the other hand, *miRNA mimics* aim at decreasing the levels of selected mRNAs. For example, miR-9, a suppressor of myocardin, is downregulated in cardiac hypertrophy and has been considered a potential therapeutic target. A double-stranded RNA miRNA mimic for miR-9 was recently assessed *in vivo*, in an isoproterenol-induced cardiac hypertrophy rat model, and successfully reduced myocardin levels, leading to attenuation of cardiac hypertrophy and improvement of cardiac function.[41]

Other emerging miRNA-related therapeutic approaches include the miRNA sponges, the miRNA erasers and the locked-nucleic acid (LNA) chemistry, which was only recently successfully employed in primates.[42]

Although miRNA modulation stands as a highly promising tool in the therapeutic setting, significant efforts are still required in order to understand their full spectrum of functions, their exact pharmacodynamic and pharmacokinetic characteristics, as well as their potential adverse reactions or side effects. Moreover, direct comparisons of the specificity and efficacy of the different miRNA antagonism and mimicking chemistries will need to be made and the most effective route and means of administration need to be determined.

FUTURE DIRECTIONS / PERSPECTIVES

The developments in the miRNA field are rapid, with thousands of publications in PubMed over the course of a decade. The emerging findings are not only promoting our understanding of different biological systems, such as the pathogenetic mechanisms of cardiovascular disease, but importantly, they are promising to soon transition to the Clinic.

It is anticipated that in the near future monitoring of miRNA expression will serve as routine diagnostic and/or prognostic testing. Large scale, multi-ethnic studies will first need to validate the accuracy of different miRNA markers and the sensitivity of different analytical approaches. Specific and detailed guidelines will then need to be established by international regulatory authorities and accreditation mechanisms will have to be set in place for miRNA diagnostic laboratories. Once this framework is available, miRNA diagnostic testing could be performed using either a biopsy of the affected organ / tissue or withdrawal of body fluids (e.g. plasma, saliva, semen). The cutting-edge technologies such as microarrays and next-generation sequencing are transforming the field through ultra rapid analysis of the miRNome, while for targeted evaluation of specific miRNA-markers, more classical molecular biology approaches can be applied. Interpretation of the results will require the continuous education of clinicians and their close collaboration of biomedical scientists. The test results would ultimately ensure more accurate, potentially faster diagnosis/ prognosis, and are anticipated to contribute towards the selection of targeted, and therefore, more effective therapies.

CONCLUSION

Despite our only recent encounter with miRNAs, extensive research has revealed their significant role in a broad range of physiological and pathological mechanisms. In the context of heart failure, miRNAs have been directly implicated in its onset and progression, while the latest findings are now changing our perception of disease pathogenesis. Importantly, miRNAs have opened the way to a new line of therapeutic approaches that could play a significant role in the near future.

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