Novel techniques and targets in cardiovascular microRNA research

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Abstract

MicroRNAs (miRNAs) are highly conserved, tiny (~22 nucleotides) non-coding RNAs that have emerged as potent regulators of mRNA translation. miRNAs exhibit fine-tuning of the control of proteins involved in cell signalling (AE) pathways and in vital cellular and developmental processes. miRNAs are expressed in cardiovascular tissues, and multiple functional aspects of miRNAs underscore their key role in cardiovascular (patho)physiology. The development and increasing use of novel molecular biology tools have contributed to the recent success in miRNA research. In the present review, we discuss current updates on important and novel miRNA techniques, including: (i) miRNA screening tools; (ii) bioanalytical target prediction tools; (iii) target validation tools; and (iv) manipulative miRNA expression tools. We also present an update about recently identified miRNA targets that play a key role in cardiovascular development and disorders.

Keywords

Cardiovascular disease • miRNA • miRNA target prediction • AntagomiR

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1. Introduction

MicroRNAs (miRNAs) are highly conserved, endogenous, small (~22 nucleotides) non-coding RNAs that regulate expression of target mRNAs.1 This control is executed mainly via miRNA binding to the 3′ untranslated region (UTR) of messenger RNA transcripts, followed by their translational repression.2 Most miRNAs differ in temporal or spatial expression patterns in the body, such as miR-1/miR133, which are specifically expressed in muscles.3 These tiny molecules show tremendous gene regulatory potential and play a key role in (patho)physiology of almost all organs, including the cardiovascular system.4

Despite intense research, cardiovascular disorders remain a leading cause of morbidity and mortality. Although the cardiovascular system is highly sensitive to micro-environmental changes at cellular and molecular levels, the aspect of miRNAs governing these changes is relatively new.5,6 Changes in miRNA expression profiles are noted during cardiogenesis as well as progression of heart diseases, indicating miRNA deregulation to be mechanistically involved in cardiovascular pathologies.4,7,8 Recent advances in molecular biology and biotechnology are having a strong impact on current miRNA research. In this review, therefore, we focus on important and novel miRNA techniques as well as recently published miRNA targets that are highly relevant to cardiovascular physiology and pathology.

2. Biogenesis and function of miRNAs

miRNAs originate from non-coding transcripts that are either co-expressed with the host gene transcript or located in intergenic regions of the genome;1,9 thus, miRNAs can be generated either as dependent transcripts utilizing promoters of the host protein-coding genes or stand-alone primary transcripts using their own promoters. This early transcript primary (pri)-miRNA is processed by the ribonuclease-III enzyme Drosha and the double-stranded RNA-binding protein DGCR-8 into so-called precursor (pre)-miRNA, a short-hairpin RNA molecule, and thereafter exported to the cytosol by exportin-5.1,10,11 In the cytosol, digestion of pre-miRNA into small RNA molecules by the endonuclease Dicer is followed by incorporation of one strand of duplex miRNA into Argonaute family protein that paves its assembly in RNA-induced silencing complex (RISC).12 miRNA-induced silencing complex (miRISC) renders protection and stability to the mature miRNA strand against RNase and guides miRNA to its target mRNA (see schematic summary in Figure 1).

miRNAs are not translated into a protein but modulate protein synthesis by binding to the 3′ UTR of protein-coding gene transcripts.
harbouring a sequence complementary to the miRNA seed sequence (2–8 nucleotides). miRNA–mRNA binding follows the Watson–Crick base pairing but does not always require a full complement homology. The complete homology initiates degradation of target miRNA, whereas the partial complementarity leads to the translational repression (see Figure 1). miRNA-mediated degradation, and therefore on/off switching of functional proteins, is more common in lower vertebrates than in mammals, where miRNAs mostly repress the gene translation and fine-tune the control over functional protein expression. This moderate control on gene expression by miRNA presumably suggests a wide scope of miRNA–mRNA interactions following either divergent (single miRNA with many targets) or convergent pathways (many miRNAs with single target) and a robust regulation of protein synthesis in living cells.

3. miRNA research techniques

miRNAs present a rapidly growing research area, with immense possibility to uncover novel and exciting mechanisms relevant to physiology and pathology. Scientists can now use sophisticated research tools to resolve complex findings of miRNA research. Here we summarize several categories of current miRNA research tools as follows: (i) miRNA screening tools; (ii) bioanalytical target prediction tools; (iii) target validation tools; and (iv) manipulative miRNA expression tools, which are briefly discussed in the following section. A scheme showing miRNA research tools useful for understanding the expression and function of miRNAs is given in Figure 1.

3.1 miRNA screening tools

Genome-wide screening of miRNA expression can be achieved in various physiological and pathophysiological conditions using powerful, high-throughput molecular biology tools, e.g. miRNA microarray and deep sequencing (see Figure 1). Such techniques utilize total RNA isolated from tissue, cells, or body fluids such as plasma, but often require additional purification and enrichment steps to increase sensitivity of the method because miRNAs represent only about 0.01% of total RNA. miRNA microarrays provide a qualitative estimation of the expression of hundreds of different miRNA transcripts in cultured cells, tissues, or serum/plasma samples in a single screen, but require further validation to quantify the expression more accurately. In comparison to the microarray techniques, deep sequencing is a more sensitive technique for small RNA sequences, which provides absolute abundance values of miRNAs. Deep sequencing generates huge data sets and thus requires high bioinformatic skills to interpret the acquired raw data. So far, this technique has been used only in a few recent cardiovascular studies, but successfully discovered novel miRNAs that remain undetected using the microarray technique. Deep sequencing has also revealed that a few, most abundant miRNAs of the mouse heart comprise about 40% of all miRNAs present in the heart.

Quantitative real-time PCR (qRT-PCR) techniques to quantify miRNAs represent a balance of cost, precision, and sample size and are often used to quantify individual miRNA expression following microarray studies. The challenge posed by reverse transcription...
of short-length miRNA transcripts has been tackled by using miRNA stem loop specific reverse transcriptase or universal reverse transcriptase with poly-A polymerase.\textsuperscript{16} The former modification reverse transcribes only particular miRNAs from a pool of miRNAs present in isolated total miRNAs, whereas the latter method utilizes polyadenyl elongation of all miRNAs by Escherichia coli poly-A polymerase followed by reverse transcription of poly-A miRNA using universal primers consisting an oligo(dT) sequence on its 5' end.\textsuperscript{16} The latter approach is more suitable for detecting miRNAs in samples with a very small amount of RNA, such as plasma. Quantification of circulating miRNAs in body fluids, such as plasma, requires a ‘spike in’ internal standard for array/qRT-PCR data normalization. The internal standards provided with RNA isolation kits are usually Caenorhabditis elegans specific miRNAs, absent in humans or rodents, which are added to each sample during the total RNA isolation procedure. While the careful selection of primers or internal standards is mandatory for optimal output, cross-reactivity among certain miRNA family members using RT-PCR techniques could still be unavoidable due to high sequence homology of closely related miRNAs, which often only differ by several bases in sequence; for example, miR-17 family members.\textsuperscript{16} Northern blotting and in situ hybridization (ISH) are widely used for visualizing individual pre-miRNA or mature miRNA expression in cells or tissues (see Figure 1).\textsuperscript{19,20} Northern blotting is a simple but relatively time- and sample-consuming technique. ISH in combination with immunohistochemistry is a very sensitive technique to visualize the spatial pattern of miRNA distribution in cells or tissues.\textsuperscript{21,22} Moreover, the use of modified locked nucleic acid (LNA) oligonucleotides and a non-radioactive detection system, such as fluorescent tags, has increased its sensitivity and usefulness.\textsuperscript{21} LNA is a new class of bicyclic high-affinity RNA analogues, in which the furanose ring of the ribose sugar is chemically locked in an RNA-mimicking conformation by an O\textasciiacute{}'\textasciiacute{}-methylene bridge,\textsuperscript{21} which results in extremely high hybridization affinity towards complementary DNA or RNA, thermal stability, and improved mismatch discrimination. LNA-modified oligonucleotides are also useful for single-nucleotide polymorphism (SNP) genotyping assays, antisense-based gene silencing, and gene expression profiling.\textsuperscript{21}ISH performed on whole-mount embryos is useful to detect miRNA involvement in cardiogenesis; for example, the differential expression of miR-1 and miR-126 has been investigated in embryonic mouse hearts and blood vessels, respectively.\textsuperscript{21,23} 

### 3.2 Bioanalytical target prediction tools

Several open-access computational databases have recently been developed to facilitate the analysis of miRNAs and their target prediction (Figure 1). Despite the fast growth of technology, prediction of miRNA–mRNA interactions still remains challenging, owing to the short length of miRNAs, requirement of only partial homology for binding, redundancy among members of miRNA family, and existence of multiple putative miRNA recognition sites. In fact, only a limited number of miRNA targets have been experimentally validated so far. The majority of the computational target prediction programs are based on several common features, such as complementarity between the 5'-seed of the miRNA and the 3' UTR of the target mRNA, thermodynamic stability of the miRNA–mRNA duplex, conservation among species, and the presence of several miRNA target sites. The properties of various in silico target prediction programs, such as TargetScan/TargetScanS, PicTar, MiRanda/mirSVR, miRBase/MicroCosm, DIANA MicroTest, PITA, and RNA22 have been reviewed in detail elsewhere.\textsuperscript{24} Some of the programs, such as TargetScan, PicTar, MiRanda/mirSVR, and miRBase/MicroCosm, are based on complementarity and conservation, whereas others (PITA and RNA22) consider target site accessibility and pattern recognition (an overview of miRNA target prediction programs is shown in Table 1). Although TargetScan offers an advantage of calculating the final score associated with protein down-regulation, it often neglects the miRNA binding sites showing partial complementarities with seed sequences.\textsuperscript{25} The PicTar algorithm incorporates co-expressed miRNAs and miRNAs with putative targets and considers a parallel cellular expression but does not consider non-conserved binding sites.\textsuperscript{26} The miRanda database provides miRNA binding sites with partial homology within the seed region that is advantageous for conservation analysis and determination in several mammalian tissues, but suffers from lower precision.\textsuperscript{24}

The novel Web-based databases GOmir\textsuperscript{27} and miRWalk\textsuperscript{28} have incorporated some new features, such as gene ontology. A duly updated miRWalk database (http://mirwalk.uni-hd.de/) considers all miRNAs, mitochondrial genes, and 10 kb upstream flanking regions for the calculation. It seeks complementary seed regions on the complete gene sequence and compares those with existing target prediction programs and also checks for published miRNA targets in PubMed. Additionally, it contains predicted miRNA binding sites on genes associated with hundreds of signalling pathways and OMIM (Online Mendelian Inheritance in Man) disorders in humans.

### Table 1 Overview of miRNA target databases

<table>
<thead>
<tr>
<th>Target prediction algorithm</th>
<th>Website address</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TargetScan</td>
<td><a href="http://www.targetscan.org/">http://www.targetscan.org/</a></td>
<td>Lewis et al.\textsuperscript{25}</td>
</tr>
<tr>
<td>TargetScanS</td>
<td><a href="http://genes.mit.edu/tscan/targetscanS2005.html">http://genes.mit.edu/tscan/targetscanS2005.html</a></td>
<td>Witkos et al.\textsuperscript{24}</td>
</tr>
<tr>
<td>PicTar</td>
<td><a href="http://pictar.mdc-berlin.de/">http://pictar.mdc-berlin.de/</a></td>
<td>Krek et al.\textsuperscript{26}</td>
</tr>
<tr>
<td>MiRanda/mirSVR</td>
<td><a href="http://www.microrna.org/">http://www.microrna.org/</a></td>
<td>Witkos et al.\textsuperscript{24}</td>
</tr>
<tr>
<td>MiRBase/MicroCosm</td>
<td><a href="http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/">http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/</a></td>
<td>Witkos et al.\textsuperscript{24}</td>
</tr>
<tr>
<td>DIANA-microT</td>
<td><a href="http://diana.pcbi.upenn.edu/cgi-bin/micro_t.cgi">http://diana.pcbi.upenn.edu/cgi-bin/micro_t.cgi</a></td>
<td>Witkos et al.\textsuperscript{24}</td>
</tr>
<tr>
<td>PITA</td>
<td><a href="http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html">http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html</a></td>
<td>Witkos et al.\textsuperscript{24}</td>
</tr>
<tr>
<td>RNA22</td>
<td><a href="http://bcscrw.watson.ibm.com/rna22.html">http://bcscrw.watson.ibm.com/rna22.html</a></td>
<td>Witkos et al.\textsuperscript{24}</td>
</tr>
<tr>
<td>GOmiR</td>
<td><a href="http://www.bioacademy.gr/bioinformatics/projects/GOmir/">http://www.bioacademy.gr/bioinformatics/projects/GOmir/</a></td>
<td>Roubelakis et al.\textsuperscript{27}</td>
</tr>
<tr>
<td>MiRWalk</td>
<td><a href="http://www.mra.uni-heidelberg.de/apps/zmf/mirwalk/micrnapredictedtarget.html">http://www.mra.uni-heidelberg.de/apps/zmf/mirwalk/micrnapredictedtarget.html</a></td>
<td>Dweep et al.\textsuperscript{28}</td>
</tr>
</tbody>
</table>
3.3 Target validation tools

To validate predicted miRNA–mRNA interactions, several experimental approaches can be used, such as biochemical methods, proteomic approaches, and RISCome analysis (quantification of miRNAs in the RNA-induced sequencing complex). Nowadays, novel high-throughput methods are used to observe the effect of miRNAs in cells by measuring protein expression changes in the presence or absence of an miRNA in the cell. Various approaches to validate targets of an individual miRNA or miRNA family are given in Table 2.

3.3.1 Biochemical methods

Apart from basic molecular biology techniques, qRT-PCR, western blotting, and reporter assays, novel strategies, such as hybrid PCR or pull-down PCR have been developed to validate miRNA targets (see Table 2).29,30 Huang et al. used an miRNA-specific primer containing the reverse and complementary sequence of the seed region of the known miRNA at the 3′-terminal end and identified 15 putative mRNA targets by hybrid PCR.29 The combination of bioinformatic prediction and the ratio of mRNA levels in the cytoplasm and the nucleus (C/N ratios) could identify potential target genes of hsa-miR-93 in cultured cells (Table 2).30 Experimental identification of miRNA targets is also done by using direct affinity purification of tagged miRNAs associated with their targets, such as biotin-tagged miRNAs, which are pulled down by streptavidin beads (Table 2).31 Additionally, a bacteriophage (MS2) affinity purification strategy has been used to elucidate that miR-133a regulates the cardiac transcription factor Hand2 in addition to miR-1.32 The Hand2 3′ UTR was fused to an MS2 binding site and cloned downstream from a green fluorescent protein reporter. MicroRNAs associated with the MS2-tagged Hand2 3′ UTR are pulled down by amylose beads, which bind to maltose-binding protein fused MS2 coat proteins on affinity columns.32 Accordingly, a labelled microRNA pull-down (LAMP) assay approach was used to search for target genes in C. elegans and zebrafish by labelling a precursor miRNA with digoxigenin.33

3.3.2 Proteomic/transcriptome analysis

Proteomic strategy is a promising tool to take account of altered protein levels as a direct global read out. Several groups used stable isotope labelling with amino acids in cell culture (SILAC) followed by quantitative mass spectrometry to identify miRNA–mRNA interactions.34 Yang et al. used the SILAC-based proteomic method to identify 94 putative targets of miR-143, which is known to be involved in tumourigenesis (Table 2).35 This outlines that in mammals miRNAs regulate most of their targets through translational inhibition. Fleissner et al. used proteomic analysis, such as difference in-gel electrophoresis followed by mass spectrometry, to identify miR-21 targets in human endothelial cells and circulating angiogenic cells after transfection with miR-21 precursors (Table 2).36

3.3.3 Other high-throughput miRNA target identification tools

The hitherto described methods focus on the miRNA–mRNA interaction at a global level. Novel approaches are needed to consider tissue types and pathological circumstances in order to understand the biological functions of miRNAs. A recent report applied a new strategy called ‘RNA-induced silencing complex (RISC) sequencing’ in combination with Argonaute-2 immunoprecipitation to identify targets of miR-133a and miR-499 in the heart (Table 2).37 This group performed comparative RNA sequencing of miRNAs that are recruited into the mouse cardiac RISComes and sequencing of the mouse cardiac transcriptome. Furthermore, cardiomyocyte-specific over-expression of miR-133a and miR-499 defined 209 targets of miR-133a and 81 targets of miR-499, which underscores its application in various cardiovascular diseases. The immunoprecipitation of RISC components is another promising approach to identify miRNAs targeted by miRNAs.38 Several new immunoprecipitation-based high-throughput assays have been designed recently, such as RIP-Chip (RNA-Binding Protein Immunoprecipitation-Microarray Profiling),39 PAR-CLIP (Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation),40 and HITS-CLIP (High-throughput Sequencing of RNAs isolated by cross-linking immunoprecipitation),11 and could be adapted to cardiovascular research in the future. Some of the novel approaches are listed in Table 2, but detailed description of these methods is beyond the focus of the present review.

3.4 Manipulative miRNA tools

miRNA manipulative tools provide a good opportunity to observe miRNA-mediated effects in vitro or in vivo by changing the expression or function of single or many miRNAs. Exogenously synthesized peptides that mimic (miRNA mimics or pre-miRNAs) or antagonize endogenous miRNAs (anti-miRs) can be transfected in living cells to modulate expression of the respective miRNA of interest.19 Sophisticated robotic devices are now available to transflect complete libraries of pre- or anti-miRs, which makes it possible to manipulate hundreds of miRNAs in a high-throughput manner and to compare their functions on selected end-points.42 Functional inhibition of miRNAs, without affecting their endogenous expression, can also be achieved by using other techniques, such as miRNAerasers or miRNA sponges, that prevent binding of endogenous miRNA to mRNA targets by utilizing over-expression of the miRNA target sequence.43

Table 2 List of miRNA research tools to identify miRNA targets

<table>
<thead>
<tr>
<th>Method</th>
<th>miRNA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>qRT-PCR, gene reporter</td>
<td>miR-21</td>
<td>Thum et al.19</td>
</tr>
<tr>
<td>assay, western blotting</td>
<td></td>
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</tr>
<tr>
<td>Hybrid PCR</td>
<td>miR-UL112-1</td>
<td>Huang et al.29</td>
</tr>
<tr>
<td>Cytoplasm/nucleus ratio</td>
<td>hsa-miR-93</td>
<td>Li et al.30</td>
</tr>
<tr>
<td>Biotin-tagged miRNAs</td>
<td>miR-bantam</td>
<td>Orom et al.31</td>
</tr>
<tr>
<td>Affinity purification</td>
<td>miR-133a</td>
<td>Vo et al.32</td>
</tr>
<tr>
<td>LAMP</td>
<td>lin-4, let-7, miR-1</td>
<td>Hsu et al.33</td>
</tr>
<tr>
<td>SILAC</td>
<td>miR-1, miR-124,</td>
<td>Baek et al.34</td>
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<tr>
<td></td>
<td>miR-181, miR-223,</td>
<td>Yang et al.35</td>
</tr>
<tr>
<td></td>
<td>miR-143</td>
<td></td>
</tr>
<tr>
<td>Proteomics</td>
<td>miR-21</td>
<td>Fleissner et al.36</td>
</tr>
<tr>
<td>RISC RNA sequencing</td>
<td>miR-133a, miR-499</td>
<td>Matkovich et al.37</td>
</tr>
<tr>
<td>RIP-CHIP</td>
<td>miR-17, miR-20,</td>
<td>Tan et al.38</td>
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<tr>
<td></td>
<td>miR-93, miR-106</td>
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<td></td>
<td>miR-107, miR-124,</td>
<td>Wang et al.39</td>
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<tr>
<td></td>
<td>miR-128, miR-320</td>
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<tr>
<td>PAR-CLIP</td>
<td>miR-7, miR-124</td>
<td>Hafner et al.40</td>
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<tr>
<td>HITS-CLIP</td>
<td>miR-124</td>
<td>Chi et al.41</td>
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</table>
Genetic manipulation to generate null or transgenic animals by global or tissue-specific deletion or over-expression of an miRNA is a valuable tool to validate the role of miRNAs, especially in developmental and physiological processes in vivo. Indeed, mice deficient in a central miRNA processing enzyme, Dicer, cannot synthesize new miRNAs, which results in embryonic lethality, whereas conditional deletion of Dicer in the mouse myocardium results in extensive cardiac remodelling. Likewise, targeted deletion of miR-1-2 suggested a role in cardiac conduction, morphogenesis, and cell cycle control, whereas knockdown of the endothelium-specific miRNA, miR-126, led to 40–50% embryonic lethality and haemorrhage (for an overview see review by Small and Olson).13

Genetic knockdown of miRNAs in zebrafish has revealed their potent roles during development (see embryonic development section of this review), whereas deletion of miRNAs, which has proved significant in vitro assays, does not always represent an overt phenotype in genetic mouse models. For example, miR-208b, miR-133a-1, miR-133a-2, or miR-499 knockout mice do not develop any (obvious) disease phenotype.46 Functional redundancy among miRNAs and evolutionary upgrading of regulatory buffers in mammals, perhaps to nullify the deleterious gene expression changes, could explain this phenomenon. Sometimes deletion of two related miRNAs is required to produce phenotypic changes rather than deletion of either miRNA alone. For instance, miR-133a-1 knockout or miR-133a-2 knockout animals develop normally, whereas lack of both miRNAs causes ventricular septal defects, leading to 50% embryonic or neonatal lethality and to dilated cardiomyopathy in mice.50 In mice, miR-1 is expressed at around embryonic day 8.5 and increases during cardiovascular development.53 Over-expression of miR-1 under control of β-myosin heavy chain (β-MHC) to express miR-1 at very early stages resulted in reduction of proliferating ventricular cardiomyocytes due to less cell division. In contrast, over-expression of miR-1 induced heart development in Xenopus.54 miR-1-2 null-mutant mice showed an increase in cardiomyocyte proliferation and an up-regulation of Hand2 along with cardiac hyperplasia, which is a known target of miR-133 as well.53,72 The common ventricular septal defects observed in miR-1-2 and miR-133 knockout mice indicate that the miR-1/133 cluster could control common signalling pathways involved in cardiomyocyte differentiation. Another study deciphered a role of the miRNAs miR-1 and miR-499 in proliferation of human cardiomyocyte progenitor cells and their differentiation into beating cardiomyocytes.56 The authors demonstrated that miR-1 and miR-499 repressed histone deacetylase-4 and Sox6, respectively, to enhance cardiomyocyte differentiation.

Morton et al. showed that miR-138 is involved in chamber-specific gene expression patterning in the zebrafish heart. The two-chambered heart of the zebrafish consists of a single atrium and ventricle, expressing unique myosin genes, separated by the atrioventricular canal, which expresses a different set of genes. Morton et al. demonstrated that the disruption of miR-138 in the ventricle resulted in an increase of atrioventricular gene expression and impaired ventricular cardiomyocyte morphology. The use of miRNA inhibitors (antagomiRs) against miR-138 revealed that miR-138 is required during a distinct temporal window (24–34 h post-fertilization) and targets several members of a common pathway. Another report, by Deacon et al., suggests a role of miRNA-143 in zebrafish cardiac chamber morphogenesis, demonstrating that miR-143, expressed during cardiogenesis, is essential for cardiac functions, such as development of the cardiac conduction system and sarcomere assembly.58 miR-143 directly repressed the cytoskeletal component adducin3, encoding F-actin capping protein. Loss of miR-143 or disruption of miR-143—adducin3 interaction blocks ventricular cardiomyocyte F-actin remodelling, leading to abnormal growth. Recently, Stankunas et al. demonstrated the involvement of miR-126 in heart valve development by controlling the vascular endothelial growth factor (VEGF) pathway. miR-126 null embryos show valve elongation defects and embryonic lethality. Another miRNA, miR-218, which affects VEGF signalling by targeting Robo-1 (Roundabout) receptor has also been reported to be involved in heart tube formation in zebrafish.60 These reports suggest that spatio-temporal expression of miRNAs during developmental processes governs the gene expression. Chinchilla et al. showed temporal expression of several miRNAs during mouse ventricular maturation using miRNA profiling assays,61 for instance, miR-27b is expressed during early stages of ventricular maturation and regulates the transcription factor MeF2c.

4. Novel miRNA targets in cardiovascular development and pathology

miRNAs influence key physiological processes, such as proliferation, migration, differentiation, secretion, excitation, conduction, cell cycle, and apoptosis, by altering protein expression of potential candidate targets. Changes in miRNA profiles in a cell-specific manner may therefore interfere with function and vital co-operation among different cell types, leading to developmental or functional failure in the cardiovascular system.19

4.1 Novel miRNA targets during cardiovascular development

Cardiovascular development is a well-co-ordinated process combining proliferation, migration, and differentiation of different cell types constituting the heart and vessels. The primary approach to understand the global impact of miRNAs in cardiovascular development is to study Dicer depletion in vitro and in vivo. Dicer depletion resulted in abnormal morphogenesis and development of heart failure in zebrafish, whereas in mice it resulted in embryonic death at embryonic day 7.5.48 Huang et al. demonstrated a role for miR-21 and miR-181a in development of severe ventricular septal defects and perinatal lethality manifested in neural crest cell-specific Dicer mutant mice.50 In addition, miR-21 promoted branching morphogenesis in cardiomyocytes by affecting matrix metalloproteinases and targeting Re1-silencing transcription factor and programmed cell death-4 (PDCD4).51,52

Several groups demonstrated the enrichment of the miR-1/133 cluster in cardiac muscles during pre- and post-natal development. miR-1 and miR-133 have antagonistic effects on muscle lineage differentiation, as miR-1 induces differentiation of mouse and human embryonic stem cells into the cardiac lineage, whereas miR-133 suppresses cell differentiation.53 In mice, miR-1 is expressed at around embryonic day 8.5 and increases during cardiovascular development.53 Over-expression of miR-1 under control of β-myosin heavy chain (β-MHC) to express miR-1 at very early stages resulted in reduction of proliferating ventricular cardiomyocytes due to less cell division. In contrast, over-expression of miR-1 induced heart development in Xenopus.54 miR-1-2 null-mutant mice showed an increase in cardiomyocyte proliferation and an up-regulation of Hand2 along with cardiac hyperplasia, which is a known target of miR-133 as well.53,72 The common ventricular septal defects observed in miR-1-2 and miR-133 knockout mice indicate that the miR-1/133 cluster could control common signalling pathways involved in cardiomyocyte differentiation. Another study deciphered a role of the miRNAs miR-1 and miR-499 in proliferation of human cardiomyocyte progenitor cells and their differentiation into beating cardiomyocytes.56 The authors demonstrated that miR-1 and miR-499 repressed histone deacetylase-4 and Sox6, respectively, to enhance cardiomyocyte differentiation.

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4.2 Novel miRNA targets in cardiovascular pathologies

The fine balance of cardiac miRNA and miRNA expression provides the basis of a stringent buffer system to regulate protein expression
to maintain homeostasis in the heart. A disturbed balance of the miRNA target gene expression is increasingly being appreciated in almost all sorts of cardiovascular pathologies. Rapid development and an enormous number of publications in this research area make it difficult to present an in-depth review on all miRNA targets within the limited frame of the present review. In the next paragraph, we therefore focus on certain novel miRNA targets in cardiovascular disorders, particularly on cardiac remodelling, and briefly on vascular disorders (see Figure 2). Details about the current knowledge concerning miRNA regulation in cardiovascular diseases have been recently reviewed elsewhere. 4.3 Novel miRNA targets in cardiac remodelling Cardiovascular remodelling is characterized by molecular, cellular, and interstitial changes and is manifested clinically as changes in size, shape, and function of the heart. Atherosclerosis of coronary arteries, altered haemodynamic load, inflammation, cardiac fibrosis, myocardial ischaemia, impaired post-ischaemic angiogenesis, and hypertrophy are key determinants of the clinical course of compensatory cardiac remodelling, which ultimately progresses into cardiac failure at later stages. Activation of various signalling effectors, second messengers, and transcription factors results in enhanced protein synthesis and hypertrophic growth of cardiomyocytes. These subtle changes during progression of cardiac remodelling are related to genome expression changes and are directly or indirectly governed by miRNA regulators, as summarized in Figure 2. Reports published in the last few years have increasingly investigated pro- or anti-hypertrophic roles of various miRNAs in cultured cells or in vivo animal models. Van Rooij et al. mentioned up-regulation of miR-21, miR-23, miR-24, miR-125b, miR-195, miR-199a, and miR-214 and down-regulation of miR-29c, miR-93, miR-150, and miR-181b in disease models. Likewise, miRNA expression changes could be seen in subjects with end-stage heart failure, and some of the up-regulated miRNAs correlated well in animal models. miRNAs not only exhibit direct repression of effector proteins but may also repress intermediate signalling molecules or transcription factors that modulate expression of a group of genes. A recent publication by da Costa Martins et al. suggests another miRNA to be involved in altered cellular signalling of the failing heart. They demonstrate that miR-199b, a direct target of calcineurin/NFAT signalling, is up-regulated during heart failure. Mice over-expressing miR-199b showed stress-induced cardiomegaly due to reduced Dyrk1a expression and NFAT hyperactivity, whereas in vivo inhibition of miR-199b by a specific antagonist normalized Dyrk1a expression,
reduced nuclear NFAT activity, and caused reversal of hypertrophy in mouse models of heart failure.\textsuperscript{65,66} The role of miRNAs in governing transcriptional changes, such as foetal gene reprogramming in hypertrophic end-stage heart failure, has been described and suggests their tremendous potential to regulate gene expression (Figure 2).\textsuperscript{67} Comparison of the miRNA transcriptome of the human foetal heart and adult failing heart showed a strong overlap.\textsuperscript{67} Furthermore, tamoxifen-induced deletion of Dicer showed cardiac hypertrophy and sudden death and reactivated the foetal cardiac gene programme.\textsuperscript{45}

Extracellular matrix remodelling induces a number of structural alterations, not only morphological changes of cardiomyocytes but also excessive accumulation of extracellular matrix proteins in perivascular and/or interstitial regions of myocardium, leading to cardiac hypertrophy and increased cardiac stiffness (fibrosis) that results in diastolic and later systolic dysfunction, and progression of the heart failure.\textsuperscript{68} Several microRNAs, including miR-21, miR-29, miR-30, miR-133, and miR-590, have been implicated in cardiac fibrosis (see Figure 2).

The role of cardiac fibroblasts enriched with miR-21 has been studied extensively. miR-21 is weakly expressed in the healthy heart but up-regulated markedly in fibroblast-rich infarct regions of the stressed heart in response to ischaemic injury or pressure overload in rodents and in subjects with end-stage heart failure.\textsuperscript{69} miR-21 targets an endogenous extracellular signal-regulated kinase/mitogen-activated protein kinase inhibitor, sprouty-1, which is primarily expressed in the fibroblast compartment of the heart.\textsuperscript{69} The miR-21 mediated down-regulation of sprouty-1 results in fibroblast growth factor secretion and survival signalling in fibroblasts, resulting in interstitial fibrosis and cardiac hypertrophy (see Figure 2).\textsuperscript{19} miR-21 also regulates matrix metalloproteinase-2 expression via targeting phosphatase and tensin homologue (PTEN) in fibroblasts, leading to fibrosis in pressure-overloaded mouse hearts.\textsuperscript{8} The miR-29 family, comprising miR-29a, miR-29b-1, miR-29b-2, and miR-29c, is down-regulated in near-infarct regions of rat hearts. miR-29 is a negative regulator of genes involved in the pathology of fibrosis, such as collagens, elastin, and fibrillins.\textsuperscript{65}

Other miRNAs, miR-30c and miR-133, target connective tissue growth factor, a key player in cardiac fibrosis. Up-regulation of connective tissue growth factor in hypertrophy and cardiac fibrosis coincides with down-regulation of these miRNAs, whereas over-expression of these miRNAs decreases connective tissue growth factor expression.\textsuperscript{70} By generating transgenic mouse models with cardiomyocyte-specific over-expression of miR-133, Matkovich et al. could prevent the down-regulation of miR-133 upon transaortic constriction, which improved myocardial fibrosis and diastolic dysfunction of the heart.\textsuperscript{71} Likewise, cardiac miR-133 and miR-590 are down-regulated in nicotine-induced atrial fibrosis and have been demonstrated to play a role via inversely regulating transforming growth factor-β and collagen production (Figure 2).\textsuperscript{72}

Cardiac remodelling is a compensatory mechanism to overcome the impaired efficiency of the heart to pump blood during progression of stress conditions, such as cardiac ischaemia, coronary artery disease, and chronic pressure overload. Oxidative stress is an important inducer of cardiac injury, which provokes apoptosis and necrosis in hypo-perfused myocardium. miR-21 was shown to prevent oxidative stress-mediated cardiomyocyte injury and apoptosis by targeting the PDCD4 gene and its downstream signalling molecule, activator protein-1.\textsuperscript{73} In contrast, miR-21 over-expression in endothelial or circulating angiogenic cells led to a down-regulation of superoxide dismutase-2, potentially leading to increased oxidative stress in endothelial cells (Figure 2).\textsuperscript{36} miR-499 has been reported to be protective against ischaemia/reperfusion-induced apoptosis and cardiac dysfunction by suppressing calcineurin-mediated dephosphorylation of dynamin-related protein-1, an activator of the mitochondrial fission programme, thereby inhibiting cardiomyocyte apoptosis.\textsuperscript{74} Another study, by Ren et al., demonstrated the crucial role of miR-320 in ischaemia/reperfusion-induced cardiac injury via regulation of heat-shock protein 20 (see Figure 2).\textsuperscript{75} In their study, over-expression of miR-320 in transgenic mice enhanced apoptosis and increased infarct size in the hearts, whereas administration of antagomiR reduced infarct size.

Myocardium of the remodelled heart exhibits disturbed cardiac conduction, partly due to altered expression of ion channel proteins in affected cardiomyocytes. Expression levels of cardiac miR-1, an abundant muscle-specific miRNA, are increased in patients suffering from coronary artery disease. miR-1 targets the inwardly rectifying potassium channel, resulting in reduced conduction velocity and ischaemic arrhythmia.\textsuperscript{76} miR-1 also regulates calcium channels. As its over-expression in cardiomyocytes exhibits spontaneous oscillations of intracellular calcium in the presence of isoproterenol by suppressing protein phosphatase 2, which in turn enhances activity of ryanodine receptor R2 and releases calcium from the sarcoplasmic reticulum.\textsuperscript{77} Modulation of pacemaker hyperpolarization-activated cyclic nucleotide potassium channels (HCN2/HCN4) by miR-1 and miR-133 has also been reported by Luo et al.\textsuperscript{78} (see Figure 2).

These channels are important in generating sinus rhythm and ectopic heartbeats in various pathological conditions that may lead to acute heart failure. Irreversible loss of terminally differentiated cardiomyocytes in the progressively remodelling heart can cause severe outcome such as heart failure. Owing to limited availability of donated hearts, development of cell-based regenerative therapy, for example, induced pluripotent stem cells, may provide some hope for patients suffering from end-stage heart failure. Pfaff et al. recently showed an important role of a novel miRNA family (miR-130/301/721) in stimulation of induced pluripotent stem cell generation from murine embryonic fibroblasts via repression of a homeobox transcription factor, Meox-2.\textsuperscript{79}

5. miRNA targets in vascular disorders

Several miRNAs are highly expressed in the vasculature and show altered expression during vascular pathologies, such as angiogenesis, atherosclerosis, arterial remodelling, vascular injury, and neointimal hyperplasia or restenosis (Figure 2).\textsuperscript{63} miR-21 influences migration of circulating angiogenic cells and impairs angiogenesis in patients suffering from coronary artery disease.\textsuperscript{36} Likewise, miR-24 enriched in cardiac endothelium regulates angiogenesis in ischaemic mouse hearts.\textsuperscript{22} In that study, blocking of endothelial miR-24 could limit myocardial infarct size in ischaemic mouse heart via preventing apoptosis and enhanced vascularization (see Figure 2). miR-126, which modifies endothelial cell functions, is suspected to influence atherosclerosis and inflammation by targeting vascular cell adhesion molecule 1.\textsuperscript{80} Migration and proliferation of smooth muscle cells are essential for tissue repair after vascular injury, but excessive smooth muscle cell migration and proliferation
may lead to vessel narrowing, neointima formation, and in-stent restenosis in pathological settings. miR-21 and miR-143/145 are deregulated after mechanical injury of large vessels, and normalizing their expression can prevent restenosis.\textsuperscript{81,82} miR-21 induces smooth muscle cell proliferation via modulating PTEN/Bcl-2, whereas miR-145 influences the phenotypical differentiation of smooth muscle cells by targeting Kruppel-like factors and myocardin (see Figure 2).

6. miRNAs as biomarkers

miRNAs secreted by cells accumulate in body fluids, such as plasma, and owing to their stability against RNase the circulating miRNAs could serve as sensitive and specific biomarkers for tissue injury or various pathologies. Recent pre-clinical and clinical studies have established the specificity of circulating miRNAs to various cardiovascular disorders. An increased plasma level of miR-423-5p is reported in heart failure patients, whereas elevated plasma concentrations of cardiac-specific miRNAs, such as miR-208 or miR-499, reflect myocardial ischaemia in rats and human subjects, respectively.\textsuperscript{83,84} In contrast, miR-126, miR-17, and miR-92a levels are suppressed in coronary artery disease.\textsuperscript{85} Certain circulating miRNAs also have a prognostic impact in patients with acute coronary syndrome.\textsuperscript{86} Whether circulating miRNAs are also able to target extracellular or intracellular targets is not known but currently under investigation.

7. Polymorphisms in binding sites of miRNA targets

Single-nucleotide polymorphisms are the most frequent variation in the human genome, occurring once every several hundred base pairs throughout the genome. SNPs have been studied extensively for defining the regions of disease candidate genes. SNPs in the 3′ UTR of some target miRNAs have been observed but often do not lead to a strong pathological phenotype, owing to redundancy in related or unrelated miRNAs as well as not so stringent miRNA–mRNA binding.\textsuperscript{87} In contrast, polymorphism in miRNA seed sequences is not common, perhaps due to divergent and vital regulatory roles of single miRNA among species. In a recent study, a SNP in the 3′ UTR of the angiotensin receptor-1 gene at the miR-155 binding region was suspected to have a role in the pathophysiology of hypertension.\textsuperscript{88} The report indicates that studying SNPs in miRNA binding sites of various genes associated with pathological phenotypes of the cardiovascular system could be important to relate miRNAs to cardiovascular pathologies.

8. Conclusions

The discovery of miRNAs has added a new dimension in our understanding of gene regulation involved in cardiovascular development and pathologies. The apparent role of miRNAs is not to control on–off switching but to fine-tune the protein expression that controls cardiovascular physiology and development. Regulatory networks of miRNAs offer exciting opportunities to modulate cardiac function therapeutically by manipulating protective or pathogenic miRNAs. Development of antagoniMS, which have been used successfully in small laboratory animals to prevent or treat the disorder state by normalizing the deregulated miRNAs, presents a big hope for future miRNA therapeutic strategies.\textsuperscript{89} However, translation of the laboratory knowledge into a practical therapeutic approach is challenging, as attempts to manipulate ubiquitously expressed miRNAs may result in off-target adverse effects. To avoid this, the whole spectrum of combinatorial and functional miRNA–mRNA interaction resulting in altered protein expression and disturbed signalling pathways should be intensively elucidated. Recently developed high-throughput techniques, described in this review article, will be helpful to investigate novel miRNA targets. Better understanding of miRNA-mediated deleterious gene regulation that underlies cardiovascular pathologies would be helpful to exploit these molecules in cardiovascular diagnostics and therapeutics.

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