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MicroRNAs as Possible Molecular Pacemakers

Emanuela Boštjančič and Damjan Glavač
University of Ljubljana, Faculty of Medicine, Institute of Pathology, Department of Molecular Genetics, Slovenia

1. Introduction

MicroRNAs (miRNAs) are endogenously expressed, small (approx. 22 nucleotides long) non-coding RNA molecules that regulate gene expression at the post-transcriptional level. They are encoded in almost all organisms, from viruses to humans (Soifer et al., 2007). Bioinformatic studies of the genomes of multiple organisms suggest that this short length maximizes target-gene specificity and minimizes non-specific effects. Generally, by targeting the 3'-untranslated region (UTR) of mRNAs in a sequence specific manner, they influence the translation (protein synthesis repression) or stability of the transcripts (mRNA degradation) (Ying et al., 2008). The role of endogenously expressed miRNA (the first miRNA to be discovered was *lin-4*) in down-regulating gene expression was first described by Victor Ambros and his colleagues in 1993 for *C. Elegans,* although the term microRNA was only introduced in 2001 (Lagos-Quintana et al., 2001; Lau, et al., 2001; Lee et al., 2001; Ruvkun, 2001). In humans, approx. 1700 mature miRNA have been cloned and sequenced (miRBase v17.0 database, release April 2011, http://www.mirbase.org). It is estimated that there could be as many as thousands of miRNAs in humans, thought to regulate approx. 30 % of genes within the human genome (Pillai et al., 2007).

1.1 MicroRNA biology

MicroRNAs are genome encoded, derived from the intergenic regions, exon sequences of non-coding transcription units or intronic sequences of either protein coding or non-coding transcription units. They are encoded as a single gene or gene clusters. It has been predicted that miRNAs constitute more than 3 % of human genes (Pillai, 2005). Intergenic miRNAs are transcribed as an independent transcription unit, as a monocistronic, bicistronic or polycistronic primary transcript (Bartel, 2004). Intronic miRNA are usually part of introns of pre-mRNA, preferentially transcribed in the same orientation as the mRNA, probably not transcribed from their own promoters but instead processed from introns, as are many snoRNA. Intronic miRNAs and their host transcripts are co-regulated and co-transcribed from the same promoter (Kim & Kim, 2007). Within the genome, there might be more than one copy of particular miRNAs. The suggestion has been made that some miRNAs are also encoded in antisense DNA, which is not transcribed to the mRNA (Bartel, 2004).

1.1.1 MicroRNA processing

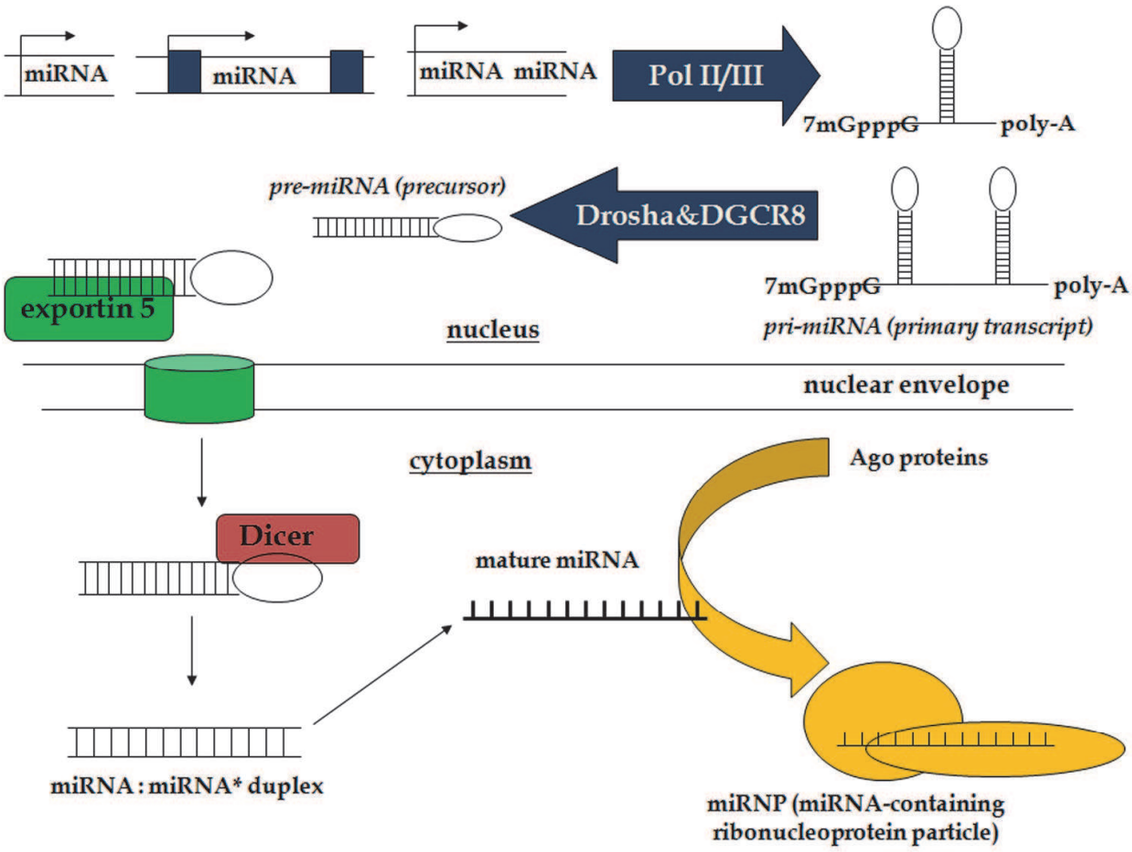
Regulation of miRNA expression depends on transcription factors and epigenetic mechanisms, such as DNA methylation and histone modification of the miRNA genomic

region. Over the course of their lifecycle, miRNAs must undergo extensive post-transcriptional modifications. Genes encoding miRNAs are transcribed with RNA-polymerase II or RNA-polymerase III (Pol II or Pol III) into a primary transcript, 200 nucleotides (nt) to several kilobases (kb) long, known as a *pri-miRNA*. Mature miRNA sequences are usually localized to regions of imperfect stem-loop. The resulting *pri-miRNA* (with poly-A tail and 7-methylguanosine cap) is processed by an RNase III enzyme called Drosha and a double-stranded RNA-binding protein, DGCR8 (DiGeorge syndrome critical region) in the cell nucleus, into a 70-nt stem-loop structure called a *pre-miRNA*. The resulting stem-loop structure, with a monophosphate at the 5' terminus and a 2-nt overhang with a hydroxyl group at the 3' terminus, is imported into the cytoplasm by a transporter protein, Exportin 5. After GTP hydrolysis, with consequent release of the *pre-miRNA*, the double-stranded RNA portion of *pre-miRNA* is bound and cleaved by Dicer (RNase III enzyme) together with co-factor TRBP (transactivating region binding protein). The action of these proteins removes the terminal loop and produces a miRNA:miRNA* duplex, which is a transient intermediate in miRNA biogenesis (20–25 nt), with a 2-nt overhanging its 3' UTR. One of the two strands of each fragment, known as the *guide strand* (miRNA), together with proteins argonaute (Ago 1-4), helicases, nucleases and RNA binding proteins, is incorporated into a complex called the miRNA-containing ribonucleoprotein complex (miRNP) or RNA-induced silencing complex (RISC). The resulting complex is responsible for base-pairing with complementary mRNA sequences. The other strand, miRNA* or *passenger strand*, is presumably degraded, although there are increasing prospects that either or both strands may be functional. It is believed that the *guide strand* is determined on the basis of the less energetically stable 5' end (Bartel, 2004; Pillai, 2005; Pillai et al., 2007; Ying et al., 2008). Intronic miRNAs bypass Drosha cleavage and rely on the action of the *pre-mRNA* splicing/debranching machinery to produce an approx. 60 nt precursor miRNA hairpin (*pre-miRNA*) (Kim & Kim, 2007). The miRNA processing is summarized in Figure 1.

1.1.2 MicroRNAs mechanism

The functional role of miRNA varies, depending on the organism, but the primary mechanism of miRNA action in mammals is to inhibit mRNA translation. The catalytic components of miRNP/RISC complex are Ago proteins. After base pairing between the miRNA and target mRNA, degradation of the target mRNA results when complementarity is perfect, or suppression of the translation occurs when base pairing between these two molecules is incomplete. Especially in animals, each miRNA can inhibit the translation of many different mRNAs (as many as 200 predicted target genes) without degrading the target mRNA. In addition, mRNA can be regulated by more than one miRNA. The cooperative action of multiple identical or different miRNP/RISCs appears to provide the most efficient translational inhibition. This explains the presence of multiple miRNA complementary sites in most genetically identified targets, and the cooperative action of miRNA:UTR interactions would provide an additional mechanism to increase the specificity of miRNAs. Proteins or mRNA secondary structures could restrict miRNP/RISC accessibility to the UTRs, or may facilitate recognition of the authentic mRNA targets (Bartel, 2004; Pillai, 2005; Pillai et al., 2007; Ying et al., 2008). It has been suggested that miRNAs may also be involved in regulation by binding to the 5' UTR of the target genes (Liu z. Et al, 2008). There is still the prospect that some miRNA might specify more than just post-transcriptional repression; some might in addition target DNA for transcriptional

silencing. Each of the examples (DNA methylation and silencing in plants, heterochromatin formation in fungi, DNA rearrangements in ciliates) suggests the existence of a nuclear RISC-like complex (Bartel, 2004).



Legend: Pol II/PolIII, RNA-polymerase II and III; poly-A, poly-A tail; 7mGpppG, 7-methylguanosine cap; Drosha, RNase III enzyme; DGCR8, double stranded RNA-binding protein; Exportin 5, transporter protein; Dicer, RNase III enzyme.

Fig. 1. Schematic overview of the miRNA biogenesis pathway

1.2 MicroRNA annotation in humans

After the small isolated RNAs are annotated as miRNAs, based on expression and biogenesis criteria, they need to be named (Ambros et al., 2003; Berezikov et al., 2006). Perhaps the best examples of naming annotated miRNA in this context are those of muscle-specific *hsa-miR-133a-1, hsa-miR-133a-2* and *hsa-miR-133b,* and heart associated *hsa-miR-199a-3p* and *hsa-miR-199a-5p.* The prefix *hsa* is designated for human miRNA (Homo sapiens), the term *miR* is designated for miRNA gene; the numbers *133* and *199* are unique identifying numbers that characterize the exact miRNA sequence; the letters *a* and *b* are used for paralogous miRNAs; numbers after the miRNA gene name, e.g., *hsa-miR-133a* numbers *1* and *2,* are used for one copy of genes encoded within the genome; *3p* or *5p*, in this case for *hsa-miR-199a,* is used when none of the miRNA duplex is degraded, or it has not yet been determined from which *pre-miRNA* arm the miRNA is degraded and from which *pre-miRNA* arm the miRNA is incorporated in the miRNP/RISC (Griffiths-Jones et al., 2008).

1.3 Target prediction and bioinformatics

MicroRNAs are generally conserved in evolution, some quite broadly, others only in more closely related species (Bartel, 2004). Many computational methods have recently been developed for identifying potential miRNA targets (Ioshikes et al., 2007). Most of these methods search for multiple conserved regions of miRNA complementarities within 3' UTR; the most important parameters are therefore evolutionary conservation with regard to the quality and stability of base pairing. The interaction between seven consecutive nucleotides in the target mRNAs 3' UTR and the 2-8 nt ("seed sequence") at the 5' miRNA end is believed to be important for base pairing. The majority of prediction programmes use pairing with the seed sequence as one of the major criteria. There are several available programs for predicting mRNA targets for specific miRNA or for predicting possible miRNA binding sites for specific mRNA, but none of these programs can be used as a means of independently validating the targets, and all predicted targets must be validated *in vitro* and *in vivo* (Kuhn et al., 2008). Further complicating target site prediction in mammals is the fact that not all 3' UTR sites with perfect complementarities to the miRNA seed nucleotides are functional. Moreover, mRNAs sites with imperfect seed complementarities can themselves be very good miRNA targets. In animals, there are far fewer mRNAs with near perfect complementarities to miRNAs. Bioinformatic analysis is therefore much noisier and more prone to false positives (Barnes et al., 2007). The most often used target prediction programs are perhaps TargetScan and PicTar, although others are often used, such as miRanda, microrna.org, miRBase etc. There is also a database available containing dysregulated miRNAs in different diseases or their profiling in various tissues (HMDD, Human MicroRNA Disease Database; Lu et al., 2008). Another useful database is Tarbase, in which all experimentally validated targets for all organisms and miRNAs are incorporated (Sethupathy et al., 2006).

2. MicroRNA in regulating physiological functions

Importance of miRNA processing pathway components. MicroRNAs and their associated proteins appear to be one of the more abundant ribonucleoprotein complexes within cells. Perhaps the best evidence of miRNAs being important for normal physiological functions is provided by experiments in which the components of the miRNA biogenesis pathway are depleted or over-expressed. Biochemical experiments in several eukaryotes have shown that DGCR8 is an essential co-factor of the RNAse II enzyme Drosha. In addition, the reduced enzymes Dicer and Drosha have been demonstrated in several diseases, as well as over-expression of Dicer, Ago 2 and exportin-5 (Soifer et al., 2007).

Outcomes of translational repression. By translational repression, miRNAs, in normal cell conditions can function in different ways. Firstly, for mRNAs that should not be expressed in a particular cell type, miRNAs reduce protein production to inconsequential levels (switch off the targets). Secondly, miRNAs can adjust protein output in a manner that allows for customized expression in different cell types but a more uniform level within each cell type (fine-tuning target expression). Thirdly, some miRNAs act as bystanders, for which down-regulation by miRNAs is tolerated or is negated by feedback processes (neutral target expression). MicroRNA functions have mainly been determined by *in vivo* experiments, by the phenotypic consequences of a mutated miRNA or an altered mRNA complementarity site, either of which can disrupt miRNA regulation. In some cases, function has been inferred from the effects of transgenic constructs that lead to ectopic expression of the miRNA (Bartel, 2004).

Physiological functions. Many miRNAs are expressed in a tissue-specific manner, e.g., *miR-208* is cardiac specific (van Rooij et al., 2007), *miR-122* is liver specific (Girard et al., 2008), and/or cell-type specific manner (e.g. *miR-223* is primarily expressed in granulocytes); they are important at distinct stages of development and have been found to regulate a variety of developmental and physiological processes (Williams, 2008). In terms of development, miRNAs are important in regulating morphogenesis and the maintenance of undifferentiated or incompletely differentiated cell types, such as stem cell differentiation, cardiac and skeletal muscle development, neurogenesis, hematopoiesis etc. Recently discovered miRNA functions include control of cell-fate decision, cell proliferation, cell death, neuronal patterning, modulation of hematopoietic lineage differentiation and controlling the timing of developmental transitions (Callis et al., 2007; Fazi & Nervi, 2008; Li & Gregory, 2008). In physiological conditions, miRNAs are involved in metabolism, regulation of insulin secretion, cholesterol metabolism, resistance to viral infection and oxidative stress, immune response etc (Lodish et al., 2007; Williams, 2008). With all different genes and expression patterns, it is reasonable to propose that every cell type at each developmental stage might have a distinct miRNA expression profile. MicroRNA biogenesis and activity is now regarded as a key regulatory mechanism in maintenance tissue identity during embryogenesis and adult life.

3. MicroRNAs and disease

Presence of SNPs. Disruption of miRNA target interaction in the form of single-nucleotide polymorphisms (SNPs), either in the miRNA gene or its target site (3′ UTR mRNA), can lead to complete gain or loss of the miRNA function and thus account for a diseased state (e.g. AT_1R and *miR-155*, Martin et al., 2007). In contrast to the miRNA target sites in mRNA transcripts, in which the potential of variation is huge, variants identified in miRNA precursor sequences tend to be extremely rare, usually restricted to one individual. The presence of SNPs in *pri-miRNA* or *pre-miRNA* can also affect the processing of miRNAs and their expression, which can also result in different disease outcomes (Barnes et al., 2007).
Aberrant expression of miRNAs. Recent advances in miRNA research have provided evidence of an miRNA association with various pathological conditions. These can be due to abnormal miRNA expression profiles, genomic rearrangements or epigenetic mechanisms activated in diseased human tissues. Aberrant miRNAs expression and processing is associated with genetic disorders, cancer, autoimmune and inflammatory diseases, and neurodegenerative and cardiovascular disorders (Perera & Ray, 2007). It is estimated that 50 % of miRNA genes are located at fragile chromosome sites and associated with the development of cancer. MicroRNAs can in addition act as tumour suppressors or proto-oncogens during the course of carcinogenesis (Cho, 2009).

4. MicroRNAs in heart physiology and disease

Cardiac specific Dicer deletion. One of the most important studies showing that miRNAs are important in heart physiology, as well as in heart disease, concerned the cardiac specific knockout of Dicer in prenatal mice. Dicer deletion resulted in rapidly progressive dilated cardiomyopathy, heart failure and postnatal mortality; early mortality was due to heart defects, such as pericardial edema and underdevelopment of the ventricular myocardium. Dicer mutant mice showed a severe decrease in heart contractile function, due to aberrant

expression and loss of cardiac contractile proteins, and profound sarcomere disarray, resulting in reduced heart rates. Decreased Dicer expression has consistently been detected in end-stage human cardiomyopathy and heart failure. In contrast, increased expression has been observed after left ventricular assist device support in humans, which is used to improve cardiac function (Chen et al., 2008). Furthermore, postnatal experiments of Dicer loss in cardiomyocytes of young mice resulted in sudden cardiac death, probably due to arrhythmias. Loss of Dicer in adult myocardium induces rapid and dramatic biventricular enlargement, accompanied by myocyte hypertrophy, myofiber disarray, ventricular fibrosis and strong induction of foetal gene transcripts (da Costa Martins et al, 2008). These results clearly demonstrated that components of miRNA processing are important for cardiac contractility, suggesting one of the crucial roles of miRNAs in normal and pathological functions of the heart. Changes in miRNA biogenesis affect both juvenile and adult myocardial morphology, suggesting a huge biological impact of miRNAs in the postnatal heart. It can therefore be concluded that Dicer down-regulation probably affects the expression of hundreds of miRNAs, which results in a severe disease outcome.

Heart disease. MicroRNA research in cardiovascular diseases has only just started. There is growing evidence to suggest that miRNAs are involved in the regulation of developmental, physiologic and pathologic conditions of the heart. Cardiac diseases, including those with progressive degeneration, might involve abnormal miRNA regulation leading to loss of renewal of the cardiac muscle cells. The majority of studies have been concerned with development, conduct and pathology, focusing on hypertrophy, end-stage heart failure, cardiomyopathy and myocardial infarction (Schipper et al., 2008; Thum et al., 2007; Xiao et al., 2008; Yin et al, 2008). Stress, associated with cardiac diseases, contributes to miRNA expression patterns in the heart, suggesting that miRNAs might function in stress-related factors affecting cardiac structure and function. Previous studies of cardiac disease have focused on miRNAs that are primarily expressed in cardiomyocytes; however, there is mounting evidence that other miRNAs expressed in the human heart have an impact on cardiovascular disease (Cordes et al., 2009; Rane et al., 2009; Roy et al., 2009; Song et al., 2010). In several studies, miRNA microarray analysis has been performed using cell lines and an animal model of hypertrophy (Cheng et al., 2007; Sayed et al., 2007; van Rooij et al., 2006; Tatsuguchi et al., 2007; Thum et al., 2008), human cardiomyopathies and aortic stenosis (Ikeda et al., 2007; Sucharov et al., 2008), end-stage heart failure (Matkovich et al., 2009; Thum et al., 2007), fibrosis (van Rooij et al., 2008), myocardial infarction (Roy et al., 2009) and development (Niu et al., 2008) and animal model of remodelling and reverse remodelling of the heart (Wang et al., 2009) and all other forms of myocardial ischemia. Among the genes activated by oxidative stress are the transcription factors that orchestrate the expression of a wide variety of responses affecting metabolism, angiogenesis, cell survival and oxygen delivery and, in addition, miRNA expression, thought to be critical for adaptation to low oxygen. In response to low oxygen, a number of miRNAs are up- or down-regulated, with several of these dependent on hypoxia-inducible-factor, a transcription factor that plays essential role in the homeostatic response to hypoxia (Kulshreshtha et al., 2007; Kulshreshtha et al., 2008).

Heart development. The heart is the first organ to form and to function during development. It has been established that miRNAs represent developmental expression patterns, important for timing developmental decisions and pattern formation (Morton et al., 2008). In addition, it has been shown that some miRNA patterns are similar in diseased and foetal hearts, supporting the concept of reactivation of the foetal gene program in cardiovascular diseases.

MicroRNA expression in failing hearts has an increased similarity to that of foetal cardiac tissue, suggesting that foetal gene expression in diseased hearts is a hallmark of cardiac stress (Thum et al., 2007).

4.1 Cardiac and muscle specific microRNAs in heart

MicroRNAs *miR-1*, *miR-133*, *miR-206* and *miR-208,* are considered to be muscle and/or cardiac specific because they are preferentially but not exclusively expressed in muscle and/or cardiac tissue. Among mammalian miRNAs identified so far, *miR-1* and *miR-133* are believed to have a muscle specific expression pattern, with an impact on the regulation of heart development. Cardiac expression of *miR-1* is controlled by *SRF* (serum response factor) and myocardin; similar to *miR-1*, *miR-133* expression in the heart is controlled by *SRF* (Niu et al., 2008). Currently, a number of miRNAs have been described as enriched or muscle specific, but, to the best of our knowledge, only *miR-208* has been described as cardiac specific.

4.1.1 Cardiac specific *miR-208*

As an identified cardiac specific miRNA, *miR-208* is believed to play an important role in response to stress, such as pressure overload, activated calcineurin or hypothyroidism. MicroRNA *miR-208* is encoded by an intron of *Myh6*, a gene encoding human and mouse α-cardiac muscle myosin heavy chain (*aMHC*). By targeting *THRAP1*, a co-factor of the thyroid hormone nuclear receptor, it mediates down-regulation of *aMHC* and up-regulation of β-cardiac muscle myosin heavy chain (*βMHC*) in mice, the primary contractile proteins of the heart. Changes in contractile proteins are accompanied by hypertrophy and fibrosis, resulting eventually in the diminution of contractility; these changes are also referred to as remodelling. Experimental models of *miR-208* null animals (mice), which failed to undergo stress-induced remodelling and hypertrophic growth in response to activated calcineurin signalling or pressure-overload-induced stress, and failed to induce *βMHC* up-regulation in response to hypothyroidism, support the suggested role of *miR-208* in remodelling (van Rooij et al, 2007). It was recently determined that this *miR-208* gene corresponds to *miR-208a* and that it is a member of a family that also includes *miR-208b*, which is encoded within an intron of *Myh7* (gene coding *ßMHC*). These two miRNAs (*miR-208a* and *miR-208b*) are differentially expressed in mice heart during development, paralleling the expression of their host genes (Callis et al., 2009).

4.1.2 Muscle specific *miR-1* and *miR-133*

Development. Muscle miRNAs are mainly controlled by myogenic transcription factors; through cardiac development they fine-tune regulatory protein levels in a spatiotemporal manner. MicroRNAs *miR-1* and *miR-133* are clustered on the same chromosome loci (*miR-1-1* and *miR-133a-2* on chromosome 20, and *miR-1-2* and *miR-133a-1* on chromosome 18) and are transcribed together in a tissue specific manner. Using cell culture and animal model experiments, it has recently been shown that *miR-1* and *miR-133* have opposite roles in muscle development, with *miR-1* promoting myoblast differentiation and *miR-133* promoting myoblast proliferation; both *miR-1* and *miR-133* target *SRF*, with *miR-1* also targeting transcription repressor, histone deacteylase *HDAC4* thus promoting myogenesis (Chen et al., 2006; Niu et al., 2008). Over-expression of *miR-1* in developing mouse hearts results in decreased cardiomyocyte proliferation and premature differentiation through

down-regulation of transcription factor *Hand2*. Target deletion of *miR-1* causes death *in utero* of the majority of offspring, due to defects in cardiac morphogenesis. The surviving ones die later due to conductivity problems. It is suggested that a precise dosage of *Hand2* is essential for normal cardiomyocyte development and morphogenesis (Zhao et al., 2005). Experiments using mouse models of an *miR-1-2* null animal suggest that *miR-1-2* has a non-redundant role with *miR-1-1* in the heart, despite their apparent overlapping expression patterns. Half of the *miR-1-2* null animals died, others suffered from incomplete ventricular septation, indicating abnormal cardiogenesis. It would be useful to know whether deletion of *miR-1-1* invokes a similar phenotype, and whether deletion of both copies causes a more severe phenotype (Zhao et al., 2007). Finally, it has been shown that, during development, *miR-133* regulates cardiogenesis by targeting nuclear factor *Nelf-A/Whsc2* (Care et al., 2007).

Apoptosis. Loss of cardiac muscle cells due to apoptotic cell death is a common process in heart development, as well as in myocardial ischemia, cardiac hypertrophy and heart failure. MicroRNAs are also implicated in cardiovascular disease as regulators of apoptosis. Opposite effects of *miR-1* and *miR-133* regulating cardiomyocyte apoptosis induced by oxidative stress have been described, with a pro-apoptotic role of *miR-1* (targeting *HSP60* and *HSP70,* heat-shock proteins) and anti-apoptotic role of *miR-133* (targeting caspase-9) (Xu et al., 2007).

Hypertrophy. Both *miR-1* and *miR-133* have been demonstrated to be dysregulated in hypertrophic and failing hearts and in myocardial infarction in both animals and humans. MicroRNA *miR-133* showed down-regulation in patients with hypertrophic cardiomyopathy and in mouse models of cardiac hypertrophy. The predicted targets for *miR-133* are *Rhoa*, a GDP-GTP exchange protein regulating cardiac hypertrophy, and *Cdc42*, a signal transduction kinase implicated in hypertrophy; both miRNAs are involved in cell growth, myofibrillar rearrangements and regulation of contractility. Another target was determined, *Nelf-A/Whsc2*, a nuclear factor involved in cardiogenesis but the role of *Nelf-A/Whsc2* in cardiac hypertrophy has not yet been defined (Care et al., 2007). Although it is also believed that *miR-1* expression is down-regulated during cardiac hypertrophy, results are somewhat controversial; additional genetic studies are therefore needed to demonstrate clearly a direct role of *miR-1* in the regulation of cardiac hypertrophy. However, *miR-1* targets in the context of cell growth, contractility and extracellular matrix have been determined, including *RasGAP*, *Cdk9*, *Rheb* and fibronectin (Care et al., 2007).

4.2 MicroRNAs controlling cardiac excitability

The electrical-conduction system, which maintains proper heart rhythmicity, has been shown to be regulated by miRNAs that regulate the expression of its components and therefore possess the potential to induce arrhythmia. Dysregulated miRNA expression might affect the expression of ion channel genes, leading to arrhythmogenesis; it has been postulated that miRNAs control cardiac excitability through this regulation. Using bioinformatics and experimental approaches, a number of miRNAs have recently been proposed as having the potential to regulate human ion channel genes. The matrix of miRNAs that are expressed in cardiac myocytes has been established, with the potential to regulate genes encoding cardiac ion channels and transporters. The author proposed that multiple miRNAs might be critically involved in the electrical/ionic remodelling process in heart disease through altering the expression of the genes in cardiac myocytes (Luo et al., 2010). MicroRNAs known up to date to target cardiac excitability are listet in Table 1 with corresponding target genes and their functions.

4.2.1 Cardiac specific *miR-208*

As previously reported, the expression of *miR-208a* and *miR-208b* is not just developmentally regulated in the heart, but also pathologically. In a recent study, it was postulated that *miR-208a* expression was sufficient to induce arrhythmias; furthermore, experiments on genetic deletion of *miR-208a* in mice revealed that *miR-208a* is required for proper cardiac conduction and expression of the transcription factor *GATA4* and gap junction protein connexin 40 (*Cx 40*). MicroRNA *miR-208* is therefore required for maintaining the expression of cardiac transcription factors known to be important for the development of the conduction system. Over-expression of *miR-208a* results in cardiac conduction abnormalities and suggests that *miR-208a* regulates cardiac conduction system components. Studies on mice lacking *miR-208a* suggest that these mice suffer atrial fibrillation. Furthermore, *Cx 40* expression is restricted to the atria, more precisely in the His bundle and Purkinje fibres. Consistent with the phenotype of mice lacking *miR-208a*, mice lacking *Cx 40* also suffer from first-degree AV block. It can be concluded, therefore, that *miR-208a* gain- and lost-of-function are associated with arrhythmias (Callis et al., 2009).

4.2.2. Muscle specific *miR-1* and *miR-133*

MicroRNAs miR-1 and miR-133. The slow delayed rectifier current I_{ks}, is constituted of channel complex, which is formed from *KCNQI* and *KCNEI*. Their expression is regionally heterogeneous; it is also changed by the pathological state of the heart. It has been experimentally established that the two genes, *KCNQI* and *KCNEI*, are targets for *miR-133* and *miR-1*, respectively. It was shown that expressions of *miR-1* and *miR-133* in the heart are spatially heterogeneous and that this may contribute to regional differences in the distribution of *KCNQI* and *KCNE1*. To confirm the hypothesis, it was shown that *KCNQI* has the opposite patterns of transmural and apical-basal gradients to those of *miR-133*, whereas the characteristic regional distribution of *miR-1* may be one of the causal factors for the converse transmural and apical-base gradients of *KCNEI*. Thus, in areas in which *miR-1* and *miR-133* are less abundant, I_{ks} are more densely expressed (Luo et al., 2007). In one of the first studies of miRNA influence on the cardiac conduction system, it was suggested that down-regulation of *miR-1* and *miR-133* contributes to arrhythmogenesis in hypertrophic and failing hearts, and that *miR-1* and *miR-133* play an important role in determining cardiac automaticity, possibly by re-expression of the pacemaker channel genes *HCN2* and *HCN4.* Both miRNAs are involved in hypertrophy, with *miR-133* believed to be a negative regulator of hypertrophic growth of heart muscle. By undergoing a remodelling process and hypertrophic growth, the heart adapts to impaired cardiac function. The remodelling process in the heart also includes electrical remodelling, which increases the risk of arrhythmogenesis by re-expression of the pacemaker channel *HCN* genes (hyperpolarization–activated cyclic-nucleotide-gated channels). The *HCN2* gene has been shown to be a target of *miR-133.* In addition, pacemaker channels *HCN2* and *HCN4* have both been shown to be targets for *miR-1*, so its down-regulation may also lead to re-expression of these pacemaker channels in a diseased heart. To date, these two miRNAs have a postulated role in regulating automaticity in the functioning of the I_f current. This is a mixed Na^+K^+ inward current, which is activated by hyperpolarization and is the main current of pacemaker activity in the sinoatrial node. I_f may therefore be controlled by *miR-1* and *miR-133* through the regulation of *HCN* density (Luo et al., 2008).

MicroRNA miR-1. Up-regulation of *miR-1* has been reported in patients with coronary artery disease and in animal models in the border zone of myocardial infarction. Over-expression

of *miR-1* in the hearts of adult rats leads to widened QRS complex, indicative of intraventricule conduction delay and the development of severe cardiac arrhythmia. Target mRNAs for *miR-1* have been predicted as ion channel genes *GJA1,* which encodes gap-junction protein connexin 43, and *KCNJ2,* which encodes the K^+ channel subunit Kir2.1. Knockdown of endogenous *miR-1* can inhibit ischemic arrhythmias. Therefore, *miR-1* might contribute to re-entry through decreased intracellular coupling via the repression of *GJA1* (Yang et al., 2007). In contrast to this research, it was shown that *miR-1* levels are greatly reduced in the left atrium of patients with persistent atrial fibrillation (AF), possibly resulting in up-regulation of Kir2.1 subunits, which leads to increased I_{k1}. Up-regulation of this inward-rectifier current is important for AF maintenance (Girmatsion et al., 2009). A further *miR-1* target involved in regulation of cardiac conductance is *Irx5,* a transcription factor that regulates cardiac repolarization by repressing *Kcnd2.* This gene encodes for potassium channel Kv4.2, which is responsible for transient outward K^+ current. Normal expression of all three components, *miR-1, Irx5* and *Kcnd2*, is required for maintaining the ventricular repolarisation gradient. Evidence was provided by deletion of the *miR-1-2* gene, which resulted in the death of half of the mice at birth, and those surviving to adulthood showed an aberrant heart rate and repolarization (Zhao et al., 2007). Calcium channels account for excitation-contraction coupling and also contribute to pacemaker activities. The effect of increased expression of *miR-1* on excitation-contraction coupling and Ca^{2+} cycling has been investigated in rat ventricular myocytes. It was shown that *miR-1* over-expression increased phosphorylation of the ryanodine receptor (*RyR2*) by selective decrease in expression of protein phosphatase *PP2A*. *RyR* channels on the sarcoplasmic reticulum (SR) are essential for activation of contractile filaments during myocardial contraction; *RyR2* is regulated by kinases and phosphathases, its activity depends on the phosphorylation state. Through translational inhibition, *miR-1* causes hyperphosphorylation of *RyR2* thus enhancing *RyR2* activity and promoting arhythmogenic SR Ca^{2+} release. The author concluded that *miR-1* enhances cardiac excitation-contraction coupling by selectively increasing phosphorylation of the L-type and *RyR2* channels via disrupting localization of *PP2A* activity to these channels (Terenteyev et al., 2009).

MicroRNA miR-133. In vitro studies have identified ERG (ether-a-go-go related gene) as an *miR-133* target, K^+ channel (I_{Kr}), in cardiomyocytes; its repression may contribute to depression and subsequent QT prolongation in diabetic hearts. ERG protein level was decreased in the ventricle of diabetic hearts; in contrast, increased expression of *miR-133* and SRF was detected in the same diabetic subjects. Down-regulation of *ERG* is responsible for arrhythmias in diabetic hearts caused by QT prolongation (Xiao et al., 2006).

4.2.3 Other miRNAs regulating cardiac excitability

Another possible mechanism of miRNA regulating the L-type Ca^{2+} current, reduction of which is associated with atrial electrical remodelling and atrial fibrillation, has recently been suggested. In particular, *miR-328* level was approx. 4-fold up-regulated, targeting the L-type Ca^{2+} channel genes, *CACNA1C* and *CACNB1*. The author therefore concluded that *miR-328* contributes to adverse atrial electric remodelling in AF and was postulated as a novel molecular mechanism for AF (Lu et al., 2010). Furthermore, it has been shown that nicotine-induced atrial remodelling, which represents an increased risk for atrial fibrillation, results in significant up-regulation of *TGF-ß1* and *TGF-ßRII* and, remarkably, a decrease in the levels of *miR-133* and *miR-590*, which at least partly accounts for *TGF-ß1* and *TGF-ßRII* up-regulation. It is suggested that the antifibrotic effect of both *miR-133* and *miR-590* are implicated in AF (Shan et al., 2009).

miRNA	Target gene	Target gene description	Regulating process	Reference
miR-1	*Hand2*	transcription factor, heart- and neural crest derivatives-expressed protein 2	developing ventricular chambers, cardiac morphogenesis	Zhao et al., 2005
	Irx5	iroquois homeobox 5	pattern formation of vertebrate embryos, heart development	Zhao et al., 2007
	GJA1	gap junction protein, alpha 1, connexin 43	conduction between cells and within a cell	Yang et al., 2007
	KCNJ2	potassium inwardly-rectifying channel, subfamily J, member 2		
	KCNEI	potassium voltage-gated channel, I_{ks}-related family, member 1	regional heterogeneity of expression	Luo et al., 2007
	HCN2, HCN4	hyperpolarization activated cyclic nucleotide-gated potassium channel	pacemaker channels	Luo et al., 2007
	PP2A	protein phosphatase 2A	regulating RyR2 phosphorylation	Terenteyev et al., 2009
miR-133	*ERG*	ether-a-go-go-like gene	long QT sindrom, diabetes mellitus	Xiao et al., 2007
	KCNQI	potassium voltage-gated channel, KQT-like subfamily, member 1	regional heterogeneity of expression	Luo et al., 2007
	HCN2	hyperpolarization activated cyclic nucleotide-gated potassium channel 2	pacemaker channels	Luo et al., 2007
	TGFB1, TGFBRII	transforming growth factor, beta 1 and its receptor	nicotin-induced atrial remodelling	Shan et al., 2009
miR-208	*GATA4, Cx 40*	GATA binding protein 4 and connexin 40	heart conduction and arrhythmia	Callis et al., 2009
miR-590	*TGFB1, TGFBRII*	transforming growth factor, beta 1 and its receptor	nicotin-induced atrial remodelling	Shan et al., 2009
miR-328	*CACNA1/ CACNB1*	voltage-dependent calcium channel	calcium transport	Lu et al., 2010

Table 1. MicroRNAs regulating cardiac excitation, its target genes and function.

5. Therapeutic potentials of microRNAs

MicroRNA expression patterns are dynamically regulated during various diseases, thus providing an opportunity to use them as biomarkers or diagnosis indicators and for prognosis. MicroRNAs are small molecules, making their *in vivo* delivery feasible. The use of chemically modified oligonucleotides either to target a specific miRNA or disrupt miRNA-mRNA binding may lead to inactivation of pathological miRNA. MicroRNAs may therefore serve as therapeutic targets in the future (Liu Z. et al, 2008; Soifer et al., 2008).

5.1 Replenishing small RNAs

For an miRNA that is under-expressed, re-introduction of the mature miRNA into the affected tissue would restore regulation of the target gene. For this purpose, artificial miRNA have been developed (miRNA mimic) to enhance the expression of beneficial miRNAs or the introduction of short hairpin duplex, similar to *pre-miRNA*, into the cell. These findings raise the hope that re-introduction of certain miRNAs (e.g., *miR-1, miR-133* or *miR-208),* depending on the disease outcome, might reduce life threatening arrhythmias, a frequent cause of death in patients with cardiovascular disease, or heart remodelling associated with prolonged ischemia, which often results in end-stage heart failure and poor prognosis (Liu Z. et al, 2008; Soifer et al., 2008). *In vivo* over-expression of *miR-133* protected animals from agonist induced cardiac hypertrophy, whereas reduction in wild-type mice (anti-miRNA antisense antagomir molecules secreted by implanted osmotic pumps) caused an increase in hypertrophic markers. This suggests that individual miRNAs are potential therapeutic agents, provided that their expression or delivery can be targeted to appropriate tissue. However, care should be taken, since over-production of *miR-133* induces arrhythmias (Care et al., 2007). Most of the developed protocols have used local administration in easily accessible tissue; systemic delivery has also some promising results; the major challenge remains tissue and cell-type specific targeting (Liu Z. et al, 2008; Soifer et al., 2008).

5.2 Inhibiting small RNAs

ASOs are short and single-stranded antisense oligonucleotides and, in the context of miRNA inhibition, are called AMOs, anti-miRNA oligonucleotides. Over-expressed miRNA can be down-regulated by reducing the mature miRNA level through direct targeting (mature miRNA, *pri-miRNA* or *pre-miRNA*) or by reducing the components of miRNA biogenesis. Chemically engineered oligonucleotides, termed "antagomirs", have been developed and proven to be efficient and specific silencers of endogenous miRNAs in mice. Chemical modifications and cholesterol conjugations stabilize and facilitate intravenous delivery of antagomirs. They interact with miRNAs in the cytoplasm and lead to specific miRNA down-regulation when injected systemically or locally (Liu Z. et al, 2008; Soifer et al., 2008). Direct injection of lipid-complexed antagomir oligonucleotides against *miR-1* into rat hearts protected the animals from induced arrhythmias, suggesting that transient down-regulation of *miR-1* could provide therapeutic benefits to those suffering from acute myocardial infarctions (Yang et al., 2007). In another approach, miRNA sponges have been developed to inhibit several miRNAs; miRNA sponges possess multiple binding sites and could be useful for sequestering an miRNA family. Furthermore, miR-masks and miR-erasers have also been developed; an miR-mask has been designed for masking the miRNA binding site on target mRNA, whereas an miR-eraser is similar to miR-sponges, except that the miR-eraser

uses only two copies of the antisense sequence. Gene-specific miRNA mimic and miRNA-masking antisense approaches have been used to test the possibility of using miRNAs and their corresponding targets as therapeutic targets. The expression of the cardiac pacemaker channel genes, *HCN2* and *HCN4*, has been manipulated via the mentioned approaches. MicroRNA mimics repressed protein levels, whereas miRNA masking markedly enhanced *HCN2/HCN4* expression and function (Xiao et al., 2007). In a recent study, the authors proposed that arhhythomgenesis after intracardiac skeletal myoblast (SKM) transplantation, a promising therapy for myocardial infarct repair, may be related to the differentiation state of (SKM). It was shown that *miR-181a* plays an important role in myobast differentiation, so using lentivirus mediated oligonucleotides against *miR-181a,* the authors demonstrated reduced arrhythmias post SKM transplantation (Li et al., 2009).
We need more knowledge concerning which miRNAs to target, how to produce and stabilize them, how to direct them to the heart and not systemically. The specificity of drug-like oligonucleotides is important, because of the off-target effect. The off-target effect is also a significant challenge, especially considering that miRNA-mediated repression often requires a homology of only six to seven nucleotides in the seed region of the miRNA and mRNA target site. Toxicity due to chemical modifications, which is used to facilitate cellular uptake and prevent degradation, should be take into account (Liu Z. et al, 2008; Soifer et al., 2008).

6. Conclusions

The field of miRNA is largely undiscovered territory, a young, emerging field of research, and we are just beginning to understand the role of miRNAs in the cardiovascular context. Recent advances in the research of miRNAs suggest that miRNAs modulate a wide variety of cardiac functions with developmental, (patho)physiological and clinical implications. The miRNA level in the myocardium must be kept within a proper concentration range to maintain normal cardiac conduction; excessive either decrease or increase in the level of some miRNAs can induce arrhythmia, which supports the central role of some miRNAs in fine-tuning the regulation of cardiac electrophysiology in pathological and normal conditions. The role of miRNAs in the pathogenesis of the heart and vessels points to the possibility of miRNA targets for the treatment of cardiovascular disease in previously unconsidered medical therapies.

7. References

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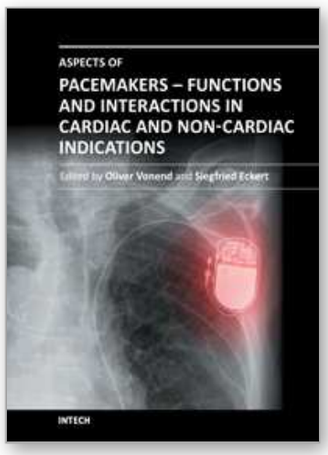

Aspects of Pacemakers - Functions and Interactions in Cardiac and Non-Cardiac Indications

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Outstanding steps forward were made in the last decades in terms of identification of endogenous pacemakers and the exploration of their controllability. New â€œartificialâ€ devices were developed and are now able to do much more than solely pacemaking of the heart. In this book different aspects of pacemaker â€“ functions and interactions, in various organ systems were examined. In addition, various areas of application and the potential side effects and complications of the devices were discussed.

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InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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