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MicroRNAs Telltale Effects on Signaling Networks in Cardiomyopathy

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1. Introduction

MicroRNAs (miRNAs) are single-stranded, highly conserved, short non-coding RNAs (~ 22 nucleotides) regulating target gene expression by base pairing with specific sequences of target mRNAs (Ambros, 2004). miRNAs negatively regulate gene expression post-transcriptionally by suppressing translation and/or inducing mRNA degradation. Bioinfomatically, it is estimated that human genome may contain approximately 1000 miRNAs (Bartel, 2004; Berezikov et al., 2005; Griffiths-Jones et al., 2008) and consistently, additional miRNAs are continually being identified (Griffiths-Jones et al., 2006). miRNAs modulate the expression of target proteins in a non-canonical manner by binding to specific sequences regulating functional networks. Consequentially, a single miRNA might target hundreds of distinct genes or alternatively expression of a single coding gene can be regulated by many different miRNAs (Lewis et al., 2005; Miranda et al., 2006). Recent studies show the important role of miRNAs in the regulation of a variety of physiological functions ranging from stem cell differentiation to cardiac muscle development and stress (Krichevsky et al. 2006; Chen et al., 2006; Zhao et al., 2005; Pedersen et al., 2007; Kloosterman et al., 2007; Felli et al., 2005; Tay et al., 2008). Furthermore, aberrant expression of miRNAs has been found in various diseases including cancer, diabetes and cardiac hypertrophy/failure.

The binding specificity of miRNAs depend on complementary base pairing of ~ 7 nucleotide seed sequence region at the 5′ end of the miRNA with the corresponding mRNA target. Another caveat that needs to be considered in the miRNA regulation is the miRNA sequences outside the 7 nucleotide seed region which pairs with the mRNA that may also play a role in determining the strength/efficacy of regulating the target mRNA. The binding of miRNAs to their cognate target mRNAs commonly results in decreased expression of target genes through translational repression or mRNA degradation (Fig. 1). Conversely, decreased expression of miRNAs will lead to increased target gene expression (Gregory et al., 2008).

This realm of knowledge has allowed for studies on miRNAs on their tissue specificity and disease specificity but critically little information is available with regards to temporal or

spatial expression profiles of miRNAs in the heart. By and large studies have used micro-array analysis to identify altered miRNAs to define signature of altered miRNAs in a specific cardiac phenotype. As miRNAs target multiple proteins, these signatures have been used to predict the array of molecules altered. Over time sophisticated computational approaches have been developed that has lead to identification of previously unrecognized targets within disease pathways of interest (Ivanovska and Cleary, 2008; Gusev et al., 2007). Among the computational tools the most commonly used target prediction algorithms include DIANA-microT (Kiriakidou et al., 2004), miRanda (Griffiths-Jones et al., 2006), TargetScan (Lewis et al., 2003), TargetScanS (Lewis et al., 2005), PicTar (Krek et al., 2005) and PITA (Kertesz et al., 2007). These algorithms rely on criteria like conservation among species, seed complementarity, thermo-stability of miRNA-mRNA hybrids, delta G of target mRNA binding site, and multiple miRNA binding sites in the 3′UTR (cooperativity) to predict targets (Bartel, 2009; Cacchiarelli et al., 2008; Ivanovska and Cleary, 2008; Gusev et al., 2007). Thus, use of these algorithms provide hundreds of targets indicating that miRNA alteration in expression could have wide ranging effects on molecules belonging to multiple signaling pathways. It is important to note that target prediction with these algorithms remains challenging but these are the tools currently available in field to provide a window into understanding the role of miRNAs. These predicted targets can then be used as a platform for identifying signaling pathways and networks that are altered manifesting in the phenotype. Critically these bioinformatic tools are evolving with the field and are pivotal to understanding the global role of miRNAs in cardiomyopathy. Although miRNA regulation adds another layer of complexity to the already complex etiology, understanding the regulation could provide novel therapeutic strategies due to miRNAs ability to target multiple molecules. In this regard, the focus of our article is to provide an overview of altered miRNAs in cardiac stress and the available tools that could be used to understand their global implications.

2. miRNA generation

2.1 Genome distribution, miRNA processing and nuclear export

miRNAs are encoded by their own genes which are an integral part of cell's genetic make up and are evolutionarily conserved (Ambros, 2004; Bartel, 2004). miRNAs can be transcribed as polycistronic primary transcripts or as individual transcripts from intergenic regions, exon sequences of non-coding strand or intronic sequences (Kim and Nam, 2006; Altuvia, et al., 2005) (Fig. 1). Intronic miRNAs are generally transcribed coincidentally with the gene and excised by the splicing machinery from the larger gene transcript in which they are embedded (Rodriguez et al., 2004). Indeed, intronic miRNAs may represent a simple way for a protein-coding gene to regulate other protein-coding genes in a non-canonical manner. miRNAs are transcribed by the RNA polymerase II as a primary transcript several kilobases long characterized by stem-loop hairpin structures called pri-miRNAs that are 5′ capped and a poly (A) to stabilize these pre-miRNAs similar to that of the traditional mRNAs (Lee et al., 2004). The generated pre-miRNA is processed in the nucleus and exported out through a regulated process. The stem-loop structures of pre-miRNAs are recognized by Drosha (a double-stranded specific RNase III) and its partner DGCR8 (a double stranded RNA binding protein) that cleave at the hair-pin base to release ~ 70-90 nucleotide stem-loop pri-miRNA precursor (Lee et al., 2003, 2004). In addition to this classical pathway, recent studies have identified alternate pathway wherein intronic pre-miRNA precursors "mirtrons" uses the cellular

splicing machinery to bypass Drosha mediated processing (Ruby et al., 2007; Okamura et al., 2007) . The cleaved stem-loop pre-miRNA hairpins are exported into the cytoplasm by the exportin-5 (a Ran-GTP-dependent nuclear transport receptor) (Yi et al., 2003). The interaction of exportin-5 with the pre-miRNA 'minihelix motif' (~14 nucleotide stem and a short 30 nucleotide overhang) is thought to stabilize the pre-miRNAs (Yi et al., 2003; Filipowicz, 2005) manifesting in efficient transport.

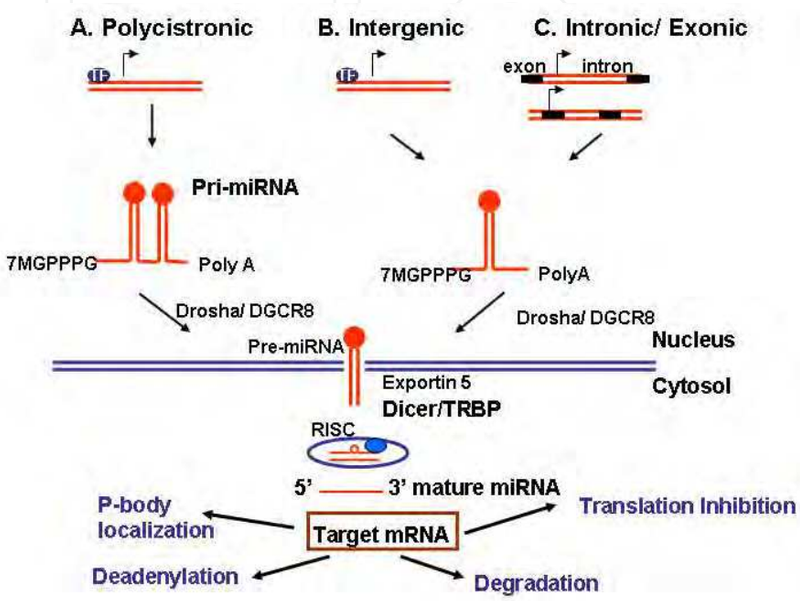

Fig. 1. MicroRNA (miRNA) genomic organization, biogenesis and function
Genomic distribution of miRNA genes. TF: transcription factor. (A) Clusters throughout the genome transcribed as polycistronic primary transcripts and subsequently cleaved into multiple miRNAs; (B) intergenic regions transcribed as independent transcriptional units; (C) intronic sequences of protein-coding or -non-coding transcription units or exonic sequences (black cylinders) of non-coding genes. Primary miRNAs(pri-miRNAs) transiently have a 7- methylguanosine (7mGpppG) cap and a poly(A) tail. The pri-miRNA is processed into a precursor miRNA (pre-miRNA) stem-loop of 60 nucleotides (nt) in length by the nuclear RNase III enzyme Drosha and its partner DiGeorge syndrome critical region gene 8 (DGCR8). Exportin-5 actively transports pre-miRNA into the cytosol, where it is processed by the Dicer RNaseIII enzyme, together with its partner TAR (HIV) RNA binding protein (TRBP), into mature, 22 nt-long double strand miRNAs. The RNA strand (in red) is recruited as a single-stranded molecule into the RNA-induced silencing (RISC) effector complex and assembled through processes that are dependent on Dicer and other double strand RNA binding domain proteins, as well as on members of the Argonaute family. Mature miRNAs then guide the RISC complex to the 3' untranslated regions (3'-UTR) of the complementary messenger RNA (mRNA) targets and repress their expression by several mechanisms: repression of mRNA translation, destabilization of mRNA transcripts through cleavage, de-adenylation, and localization in the processing body (P-body), where the miRNA-targeted mRNA can be sequestered from the translational machinery and degraded or stored for subsequent use. Nuclear localization of mature miRNAs has also been described and is a novel mechanism of action for miRNAs.

2.2 Generation of mature miRNA, activation and target recognition

The pre-miRNA is processed into a mature miRNA of ~22 nucleotides long by another double stranded RNase III called the Dicer (Hutvagner et al., 2001). Single stranded RNA is assembled into a RNA-inducing silencing complex (RISC) with the help of Dicer, TAR (HIV) RNA binding protein (TRBP), and dsRNA-binding proteins of the argonaute (AGO) family (Chapman and Carrington, 2007; Filipowicz, 2005; Schwarz et al., 2003; MacRae et al., 2008; Okamura et al., 2004). Additional factors have also been isolated and implicated (Chapman and Carrington, 2007; Filipowicz, 2005; Schwarz et al., 2003; MacRae et al., 2008; Okamura et al., 2004) to be a part of RISC complex bringing about miRNA-mediated silencing of gene expression that could be either a translational repression or degradation of mRNA. miRNAs recognize their target mRNAs through specific interaction of the 5′ end 'seed' region (2–8 nt from the 5′ end) and the complementary sequences of conserved target mRNAs (Bartel, 2004). Since only a few miRNAs have perfect complementarity to the target mRNAs leading to degradation, majority of the miRNAs have imperfect match resulting in translational repression (Nilsen, 2007). Another caveat in the miRNA silencing dynamics is the ability of multiple miRNAs to bind to the same mRNA initiating translational repression with different potencies. Repressed mRNAs are sequestered from translational machinery, degraded or stored for subsequent use in large macroscopic cytoplasmic foci, named processing bodies (P-bodies) upon silencing by miRNAs. The P-bodies contain a wide range of enzymes involved in RNA turnover, including de-capping enzymes, de-adenylases and exonucleases (Eulalio et al., 2007). In addition to their cytoplasmic role, miRNAs with nuclear localization sequence have been identified demonstrating their role in transcriptional control of gene expression (Chapman and Carrington, 2007; Volpe et al., 2002; Zilberman, et al., 2003; Aravin et al., 2007; Yu et al., 2008; Hwang et al., 2007; Calin et al., 2007).

3. miRNAs and cardiac development

3.1 miRNA expression in cardiac development

Studies have shown that miRNA mediated fine tuning leads to critical cell lineage commitment and embryonic tissue development (Latronico, et al., 2007; Farh et al., 2005; Ivey et al., 2008). Consistent with the role of miRNA in development, deletion of Dicer leads to embryonic lethality resulting from defects in cardiogenesis due to deficiencies in miRNAs biogenesis (Giraldez et al., 2006; Ebert et al., 2007). In tune with complex process of cardiogenesis many miRNAs are shown to be involved and the selection of miRNAs enriched during the differentiation of mouse embryonic stem cells to cardiomyocytes are detailed in Table 1 (Lakshmipathy et al., 2007; Thum et al., 2007). Significant increase in miRNA expression with development shows that miRNAs play an important role in early embryonic patterning and orchestrating organogenesis (Ivey et al., 2008). Expression pattern of miRNA-1 and -133 show that these two miRNAs play a key role in skeletal muscle proliferation and differentiation (Chen et al., 2006). Specifically, miRNA-1 promotes myogenesis by targeting histone deacetylase (HDAC4), a transcriptional repressor while miRNA-133 enhances myoblast proliferation by repressing serum response factor (SRF) (Zhao et al., 2005, 2007; Kwon et al., 2005; Niu et al., 2007). In this context, loss of function of miRNA-1 in Drosophila results in embryonic/larval lethality due to altered sarcomeric gene expression and increased number of undifferentiated muscle progenitors (Kwon et al., 2005). Where as miRNA-1 gain of function results in embryonic lethality due to insufficient

numbers of cardioblasts indicating that cardiogenesis and differentiation is a spatio-temporal process tightly regulated by miRNA-1 mediated by cardiac transcription factor Hand2 (Zhao et al., 2005; Srivastava et al., 1997). Another important component of miRNA expression is the Dicer mediated generation of miRNA during development. Dicer expression during development determines miRNA expression and its regulation. Indeed, Dicer-deficient animals fail to synthesize new miRNAs resulting in embryonic lethality in zebrafish (Giraldez et al., 2005) and mice (Bernstein et al., 2003). More importantly cardiac specific deletion of Dicer results in aberrant cardiac contractile protein expression and severe sarcomere disarray, leading to progressive dilated cardiomyopathy (DCM), failure and postnatal lethality (Chen et al., 2008).

Downregulated miRs		Upregulated miRs		Species
Development	Disease	Development	Disease	
1, 7d*, 10a/b, 26a/b, 29a/b/c, 30a-3p/a-5p/b/ c/d/e/e*, 30e, 93, 126-5p, 133a/b, 139, 149, 150, 151, 155, 181b, 185, 187, 194, 218, 292-5p, 373, 378, 451, 466, 486		1, 18, 20, 23b, 24, 26a, 30c, 133, 43, 182, 183, 200a/b, 292-3p, 293, 295, 335	10b, 15b, 17-5p, 18b, 19a/b, 20b, 21, 23a/b, 24, 25, 27a/b, 29a, 31, 103, 106a, 107, 125b, 126, 127, 140/*, 142-3p, 146, 153, 154, 195, 199a/a*/b, 200a, 208, 210, 211, 214, 217, 218, 221, 222, 330, 341, 351, let-7b/c, 424	Mouse
16, 17-5p, 19b, 22, 23b, 24, 27a, 30a-5p/b/c/e-5p, 107, 126, 130b, 135a, 136, 148a, 150, 182, 186, 192, 199a*, 218, 299-5p, 302b*, 302c*, 325, 339, 342, 452/*, 494, 495, 497, 499, 507, 512-5p, 515-5p, 520d*/h, 520, 523, 526b/b*, 378,7		1, 20, 21, 26a, 92, 127, 129, 130a, 199b, 200a, 335, 424	1, 7a/b/c/d/e/f, 10b, 17-3p, 21, 23, 24, 26a, 28, 29a/b/c, 32, 34b, 98, 106b, 125a/b, 126*, 129/-3p, 130a, 132, 195, 196a, 199a/b, 200c, 204, 205, 208, 210, 211, 212, 213, 214, 215, 292-3p, 294, 295, 296, 297, 300, 302a, 320, 322, 330, 331, 333, 340, 341, 343, 365, 367, 372, 373, 377, 381, 382, 423, 424, 429, 432, 500, 520c, 525*,	Human

Upregulated (enriched) miRNA during mouse cardiac development as shown by Srivastava and colleagues. Data about altered miRNAs in heart failure and animal models of heart disease originate from different results published earlier. Note that in some cases results were not consistent between the different laboratories.

Table 1. Summary of regulated microRNAs (miRNAs) in cardiac development and disease.

3.2 Regulation of miRNA transcription

Understanding transcriptional regulation of miRNAs is critical as expression of miRNAs is a major determinant of miRNA dependent regulatory mechanisms. As miRNAs are transcribed like other genes, they are regulated by transcription factors and expression of transcription factors determines the miRNA expression. SRF is a cardiac enriched transcription factor that regulates sarcomere organization in the heart and SRF expression follows a restrictive pattern during development (Olson and Schneider, 2003; Niu et al., 2007; Barron et al., 2005). SRF expression is very important as multiple SRF binding sites have been identified in promoters of genes regulating contractility, cell movement, and growth signaling (Sun et al., 2006; Zhang et al., 2005). Consistent with the role of SRF in cardiac development, several miRNAs have been identified to contain SRF binding sites in their promoter including miRNA-1-1, -1-2, -21, -206, -214, -133 and others (Niu et al., 2007). In addition, studies have unequivocally have shown that miRNA-1-1, -1-2 and -133 are

regulated by SRF transcription factor alone or in conjunction with co-factors like GATA5, MyoD, Nkx 2.5 or MEF2 (Fig. 2) (Zhao et al., 2005; Chen et al., 2006; Niu et al., 2007; Rao et al., 2006; Xiao et al., 2007). It is important to note that the outlined co-factors of SRF by themselves can act as transcriptional regulators in their own right increasing complexity of regulation. For example, MEF2 along with MyoD is known to regulate miRNA-1-2/133a-1 in myotomes during embryogenesis and all skeletal fibers in adulthood (Liu et al., 2007).

3.3 miRNA expression patterns

miRNA expression is greatly enriched in a tissue/cell-specific manner indicating unique signature patterns for each type. This enrichment and signature pattern suggests that miRNAs play a critical role in regulating and maintaining the specific cellular phenotype which is of essence in an organ with diverse cell/tissue types contributing to effective functioning. In this regard, heart as an organ contains many "non-cardiomyocyte" cell types like endothelial cells, smooth muscle cells and fibroblasts and each of which have distinct function in the heart. Consistently, differential enrichment of miRNAs are observed in cardiomyocyte versus cardiac fibroblasts indicating important role in cellular specificity (Landgraf et al., 2007; Kuehbacher et al., 2007; Harris et al., 2008). Although specific miRNA enrichments are being found in different cell types (Kuehbacher et al., 2007; Harris et al., 2008), lot more work needs to be done to determine contribution of miRNAs towards regulation of global networking pathways that defines specific fingerprints for each cell type. The current studies have all been focused on one or two miRNAs (Chen et al., 2006; Gregory et al., 2008; Harris et al., 2008) which by themselves may not be sufficient to determine a cellular phenotype indicating requirement of more comprehensive studies on specific miRNAs signature for cellular phenotype.

4. miRNAs and cardiac disease

The heart is responsive to physiological stimuli or pathological stress and accordingly undergoes remodeling to meet the demand (Catalucci et al., 2008). Following stress, the heart undergoes extensive remodeling in the form of physiological or pathological hypertrophy defined as an augmentation of ventricular mass due to increased cardiomyocyte size. Cardiac hypertrophy is characterized by initial compensatory mechanisms that adapt the heart towards sustaining the cardiac output. However, this process is only an initial 'adaptive' response and chronic exposure to stress eventually leads to impaired function that, in many cases, progresses to failure. This maladaptive change is accompanied by alterations in the underlying molecular map including a switch in the gene expression program leading to reexpression of fetal genes (Catalucci et al., 2008; Thum et al., 2007). The involvement of miRNAs in this pathological process has been recognized and is thought to be integral 'switch' in the gene expression program. Intense efforts have been put into identifying miRNAs altered in pathology and evolving signature of deregulated miRNAs identified cardiac disease is detailed in Table 2.

4.1 Myocardial hypertrophy, remodeling, and heart failure

Cardiac remodeling is characterized by structural alterations of myocardial tissue, modification of the extracellular matrix, and reshaping of left ventricle geometry and performance (Catalucci et al., 2008; Dash et al., 2001). The presence of chronic stress results in deleterious remodeling.

microRNA	Experiment	Phenotype
miRNA-1-2	Mouse knockout	Cardiac septal defects, hyperplasia and delay between atrial an ventricular repolarizations (PR interval) was shortened (Zhao et al.,2007)
miRNA-1	Neonatal cardiomyocyte	Inhibits FBS/endothelin/isoproterenol overexpression mediated hypertrophy (Ikeda et al., 2009)
miRNA-21	TAC and isoproterenol induced hypertrophy	Upregulated in compensatory hypertrophy and reduced in decompensation (Sayed et al., 2008)
	Neonatal cardiomyocyte overexpression	Outgrowths in the cardiomyocytes accompanied by connections via gap junctions (Sayed et al., 2008)
	Transgenic cardiomyocyte-specific expression	No specific phenotype indicating minimal role for miRNA21 in cardiomyocytes (Thum et al., 2008)
	Cardiac fibroblast overexpression	Anti-apoptotic (Thum et al., 2008)
miRNA-23a	Antagomir infusion using minipumps	Isoproterenol-induced cardiac hypertrophy is attenuated with miRNA23a antagomirs (Lin et al., 2009)
	Neonatal cardiomyocyte overexpression	Induces hypertrophy (van Rooij et al., 2006;, Lin et al., 200955)
miRNA-23b	Neonatal cardiomyocyte overexpression	Induces hypertrophy (van Rooij et al., 2006)
miRNA-24	Antagomir infusion using minipumps	Isoproterenol-induced cardiac hypertrophy is not altered (Lin et al , 2009)
	Neonatal cardiomyocyte overexpression	Induces hypertrophy (van Rooij et al., 2006)
miRNA-27	Antagomir infusion using minipumps	Isoproterenol-induced cardiac hypertrophy is not attenuated (Lin et al., 2009)
miRNA-92	miRNA inhibitor treatment of neonatal cardiomyocytes	Minimal effect on fetal gene expression (Sucharov et al., 2008)
miRNA-100	miRNA mimic treatment of neonatal cardiomyocytes	Results in re-expression of fetal genes (Sucharov et al., 2008)
miRNA-129	Neonatal cardiomyocyte transfection	Induces hypertrophy (Thum et al., 2007)
miRNA-133	Neonatal cardiomyocyte transfection	Inhibited hypertrophy (Care et al., 2007)
	Antagomir infusion using minipumps	Induces cardiac hypertrophy (Care et al., 2007)
	Double knockout of miRNA-133-a/b	Embryonic myocyte proliferation, septal defects, and surviving adults have severe dilated cardiomyopathy (Liu et al., 2008)
	Transgenic cardiomyocyte specific expression	Inhibitor of cardiomyocyte proliferation (Liu et al., 2008)
	Transgenic cardiomyocyte-specific expression subjected to TAC	Cardiac hypertrophy is not inhibited, but decreases myocardial fibrosis and cardiomyocyte apoptosis (Matkovich et al., 2010)
miRNA-195	Neonatal cardiomyocyte overexpression	Induces hypertrophy (van Rooij et al., 2006)
	Transgenic cardiomyocyte specific expression	Induces cardiac hypertrophy and dilated cardiomyopathy (van Rooij et al., 2006)
miRNA-199a	Neonatal cardiomyocyte overexpression	Cardiomyocyte enlargement (van Rooij et al., 2006)]
miRNA-208	Knockout mice subjected to TAC or bred to mouse model of hypertrophy	No hypertrophic response in both the cases [van Rooij et al., 2007)
miRNA-214	Neonatal cardiomyocyte overexpression	Cardiomyocyte hypertrophy (van Rooij et al., 2006)
	Transgenic cardiomyocyte specific expression	No phenotype (van Rooij et al., 2006)

Table 2. miRNAs experimentally determined to play a role in cardiac hypertrophy/ cardiomyopathy

Multiple studies have been carried out to reveal important roles of miRNAs in cardiac hypertrophy and heart failure. Studies have found that a unique set of miRNAs are upregulated, downregulated or unaltered during the adaptive response of the heart to stress stimuli (Latronico et al., 2007). Furthermore, unique subset of miRNAs are known to be altered within the various etiologies of heart failure indicating significant role of miRNAs in these disease states (Sucharo et al., 2008). Consistent with the reexpression of fetal gene program, a high degree of similarity has been found between the miRNA expression pattern occurring in failing human hearts and those observed in the 12- 14 week-old hearts (Thum et al., 2007). Approximately, 80% of the analyzed miRNAs are similarly altered in failing adult and fetal human hearts compared to non-failing hearts. Multiple miRNAs have been implicated in cardiomyocyte hypertrophy and studies have consistently found upregulation of miRNA-21, -23a, -23b, -24, -195, -199a and miR-214 and downregulation of miRNA-1, -7, -133 and 378 (Naga Prasad and Karnik, 2010). Many of these miRNAs have been tested for hypertrophic response in neonatal cardiomyocytes. Concordant data from human and mice samples indicate that miRNAs may be involved in common pathway mediating hypertrophic response (Thum et al., 2007; van Rooij et al., 2006; Chen et al., 2008; Ikeda et al., 2007; Tatsuguchi et al., 2007; Sayed et al., 2007; Cheng et al., 2007) .

Among the miRNAs altered in various cardiac etiologies, some of them have been studied indepth and these include miRNA-1, -21, -133 and -208. It is well known that miRNA-1 is downregulated with a week of transverse aortic banding and its expression is inversely

correlated with cardiac hypertrophy (Table 2) (Sayed et al., 2007; Catalucci et al., 2008; Ikeda et al., 2009, Naga Prasad et al., 2009 after al., 2009)). Similarly, Care et al., observed impaired expression of both miR-1 and miR-133 in patients with hypertrophic cardiomyopathy and atrial dilatation as well as in 3 different murine models of cardiac hypertrophy (Catalucci et al., 2008). In vitro cellular overexpression of miRNA-133 resulted in suppression of protein synthesis and block in hypertrophic response. Contrastingly, utilization of a decoy for miRNA-133 resulted in cellular hypertrophy and in vivo administration resulted in significant myocardial hypertrophy associated with reexpression of the fetal gene program (Catalucci et al., 2008). Some of targets of miRNA-133 have been validated and many are still being validated to provide evidence of miRNA-133 targeting multiple molecules to bring about hypertrophic response. miRNA-133 is encoded by 133a-1 and –2 and deletion of individual miRNA have no obvious cardiac abnormalities but combined deletion results in severe cardiac malformations with embryonic and post-natal lethality (Care et al., 2007). In contrast, overexpression of miRNA-133a results in embryonic lethality (E 15.5) caused by ventricular septal defected and impaired cardiomyocyte proliferation resulting in thinning of ventricular walls unable to meet hemodynamic needs (Care et al., 2007). These studies reveal that miRNA-133 plays a key role in myocardial development, hypertrophy and function. miRNA-208 is unique as it is a cardiac-specific miRNA encoded within the intron of α-myosin heavy chain (α-MHC) gene. miR-208 knockout mice are viable and do not show any obvious cardiac phenotype, but they fail to undergo stress-induced cardiac remodeling, hypertrophic growth, and α-MHC upregulation following transverse aortic constriction (Table 2) (van Rooij et al., 2007). It is believed that miRNA-208 regulation of this process involves α-MHC alterations balancing α-MHC.

While miRNA-208 mediates cardiac function by cardiomyocyte specific expression, miRNA-21 regulates cardiac function by its expression in both myocytes as well as cardiac fibroblasts. Recent studies (Thum et al., 2008) have shown progressive upregulation of miR-21 during late stages of heart failure, with an expression profile restricted exclusively to cardiac fibroblasts (Table 2). Upregulation of miR-21 was shown to be responsible for increased extracellular signal-regulated kinase (ERK) signaling through inhibition of its target, spry1 (sprouty 1), an inhibitor of the ERK/extracellular signal-regulated kinase pathway. These studies suggest that miRNA-21 expression results in increased fibroblast survival and reduced interstitial fibrosis independent of cardiomyocyte loss that may provide protective effects (Thum et al., 2008). Likewise, it has been (van Rooij et al., 2008) recently demonstrated that downregulation of the fibroblast-enriched miRNA-29 family in fibrotic areas surrounding a cardiac infarct is responsible for the regulation of mRNAs that encode a multitude of proteins involved in fibrosis such as collagens, fibrillins, and elastins. In addition to these miRNAs, we have recently shown that 8 miRNAs are differentially expressed in human dilated cardiomyopathy (DCM) (Naga Prasad et al., 2009). The miRNA-1, -29b, -7, and -378 were significantly down-regulated in the DCM samples compared with non-failing controls. In contrast, miRNA-214, -342, -125b and -181b were significantly upregulated in DCM compared with non-failing controls. These studies identified miRNA-7 and -378 as novel miRNAs which are significantly downregulated during end stage cardiac dysfunction whose role in cardiac pathology remains to be determined.

4.2 Arrhythmia

One of the well known contributing factors for heart failure are the changes in ion channel function and expression leading to electrophysiological remodeling in both atria and

ventricles. Although the role of miRNAs with regard to arrhythmia is not yet well established, recent evidence supports their role in the induction of arrhythmia. Expression of miRNA-1 by viral transduction following myocardial infarction in rat resulted in significant enlargement of the QRS complex, prolongation of the QT interval, and an increased incidence of arrhythmias (Yang et al., 2007). Conversely, a low incidence of fatal arrhythmias was obtained when antisense for miRNA-1 was used. These studies further identified that miRNA-1 targets GJA1 (connexin 43) and KCNJ2 a critical K^+ channel subunit both of which are required for maintenance of membrane potential. Consistently, miRNA-1 and -2 double knockout mice that survived until birth had high incidence of electrophysiological abnormalities resulting in sudden death (Zhao et al., 2007). In addition to miRNA-1, miRNA-133 has been implicated in contributing towards cardiac disease by altering electrophysiological remodeling. In particular, downregulation of miRNA-133 in hypertrophic hearts has been associated with an increase in ion channels HCN2/HCN4 which when upregulated, enhance automaticity and the development of arrhythmia (Luo et al., 2008). Moreover, in a model of diabetic cardiomyopathy, overexpression of miR-133 has been shown to downregulate the ERG (ether a-go-go-related gene) with consequent QT prolongation responsible for arrhythmias (Xiao et al., 2007). Although only two miRNAs have been extensively studied with regards to arrhythmia, it is only matter of time that more miRNAs will be found to play a critical contributing role in complex electrophysiological remodeling that may cause heart failure and sudden death.

5. Cardiac microRNA targets

5.1 Identification of microRNA targets

To comprehensively understand the miRNA function and potential therapeutic use in heart disease, identification and validation of miRNA targets is of fundamental importance. A large number of bioinformatic methods have been developed to predict miRNA targets based on the assumption that the 5′-nucleotides of miRNAs are most critical for target recognition (Lai, 2002; Lewis et al., 2003). Such methods easily result in the prediction of hundreds of potential miRNA targets which are difficult to validate using conventional means. Target accessibility is an important factor for miRNA target repression as nearly all the miRNA binding sites reside in the 3′-UTRs of target mRNA that is located in the unstable regions of mRNA structure calculated on the basis of free energy predictions and RNA structure (Zhao et al., 2005; Lee et al., 2002). Although various target prediction algorithms use the sequence complimentarity as a major determinant, newer tools are being developed as our understanding of the miRNA biology improves. In addition to the previous tools a novel miRNA target identification algorithms are being developed that also include target accessibility by evaluation of energy states of sequences flanking the miRNA target (Lewis et al., 2003; Lai, 2002). Such a tool has become a necessity as previous prediction algorithms seem to have higher levels of false positives. In this context, however it remains to be determined whether this stringent approach may identify less false-positive targets without missing others (Bruneau, 2005). A potential relationship between altered miRNA expression and changes in messenger RNA expression profiles in failing human left ventricles has recently been explored (Thum et al., 2007). Computational prediction identified multiple potential target genes with at least one binding site for highly upregulated miRNAs during heart failure. In contrast, transcriptome analysis conducted in parallel showed that theoretically predicted target genes were upregulated, demonstrating

no obvious preponderance of gene repression (Thum et al., 2007). This obvious disconnect could be because the analysis was carried out at transcriptome level and not at a proteome level. It is potentially possible that the target proteins are significantly altered in response to miRNA alterations. The observed increase in miRNA transcripts could be due to feed back mechanism of reduced protein levels of the respective target proteins. These observations bring to focus our incomplete understanding of mRNA targeting by miRNA and we still have lot more to learn with regards to determining the underlying mechanisms regulating these processes. However, simultaneous use of current prediction algorithms and proteomic analysis should be able to provide a realistic idea on the target proteins. An important caveat that needs consideration is the sensitivity of proteome analysis which may still miss out on proteins altered at lower potency by miRNAs.

5.2 MicroRNA targets in cardiac disease

Despite the shortcomings of the tools available to accurately predict the targets, various studies have used traditional and non-traditional tools to verify and validate the targets of miRNAs. In many cases the targets have been identified in the knock-out or overexpression system which provides validity to the targets and it is further strengthened by the function of the target protein. We quote some of the examples below that provide a view point the way studies are currently being carried to unequivocally show that a specific protein is a miRNA target. Targeted deletion of miR-1-2 in mice causes 50% lethality mainly because of ventricular wall defects (Zhao et al., 2007) along with arrhythmias leading to sudden death. This has been linked to upregulation of Irx5, which is a target of miR-1 (Zhao et al., 2007). Conversely, it is also known that miRNA-1 expression resulted in repression of target KCNJ2 and GJA1 channels that code for the main potassium channel subunit Kir2.1 and connexin (Yang et al., 2007). This to a certain degree explains higher degree of arrhythmias found in patients with coronary artery disease where miRNA-1 expression is elevated and similar elevation is observed in mice following myocardial infarction. In contrast to these findings our studies in end-stage dilated cardiomyopathy and studies by others on aortic stenosis have found reduction in miRNA-1 expression (Naga Prasad et al., 2009). A preview of these studies show varied expression pattern of miRNA-1 based on the variations of diseases, biopsy locations, technical differences, or altered cellular composition of the biopsies. This indicates a need for appreciation of the differences so that in future a much more representative pattern develops for each of the altered miRNAs. In this context, miRNA-1 targets have been very well summarized that includes Ras GTPase-activating protein (RasGAP), cyclin-dependent kinase 9 (Cdk9), Ras homolog enriched in brain (Rheb), and fibronectin **(**Latronico et al., 2007).

On a similar note, miRNA-133 targets have also been well studied and they have been identified using in vitro and in vivo techniques (Care et al., 2007). They include Cdc42 (implicated in cytoskeletal modifications during cardiac remodelling), Rho-A (a GTP–GDP-binding molecule, also critical for hypertrophy), and NELF-A/WHSC2 (a nuclear factor involved in heart genesis). While Rho-A and Cdc42 have already been established as fundamental factors for cell growth, cytoskeletal reorganization, and regulation of contractility in cardiomyocytes, (Brown et al., 2006; Ke et al., 2004) the role of NELF-A/WHSC2 in cardiac hypertrophy is yet to be defined. Transduction of cardiomyocytes both in vitro and in vivo with an adenoviral vector containing a Whsc2 transgene resulted in protein synthesis inhibition, but induced the fetal gene program and upregulation of Rho-A, (Care et al., 2007) supporting the postulation that WHSC2 could play a selective role in hypertrophy. In addition

to targeting molecules modulating cardiac hypertrophy, miRNA-133 also targets molecules regulating cardiac conductance. In the diabetic heart, upregulation of miR-133 expression results in downregulation of protein expression of the ether-a-go-go-related gene (ERG), encoding the rapid delayed rectifier potassium channel (Xiao et al., 2007) .

The studies on miRNA-133 targets are interesting suggesting a lot needs to be understood in terms of alteration of these miRNAs in stress results in a unique pathological phenotype. Such a view is further supported by studies on miRNA-208 which is expressed specifically in the cardiomyocytes (van Rooij et al., 2007). A major target of miRNA-208 is thrap1 [thyroid hormone receptor (THR)-associated protein 1] and reduction in the expression of miRNA-208 results in loss of negative regulation on THRAP-1 (van Rooij et al., 2007). The resulting increase in THRAP 1 protein affects the THR-regulated expression of α-MHC and β-MHC, which are inversely regulated through a positive and negative thyroid hormone response element on their promoters. This shift in expression is thought to be the underlying factor for the blunted response to pressure overload in miR-208 knockout mice. In this context, our studies have shown that miRNA-7 is significantly down-regulated in end-stage human heart failure and upon TAC in mice and consistently its targets ERBB2 (epidermal growth factor receptor 2) and COL1A (Collagen 1) are upregulated (Naga Prasad et al., 2009). The above discussed studies are only a representative window on plethora of studies identifying targets for various miRNAs altered in conditions of cardiac stress. These studies have been discussed with an aim to provides a bird eye view of the complexity in regulation of target protein expression by miRNAs and to appreciate the diverse effects miRNA alteration can have in a pathology accounting for the phenotype.

6. Specific molecules and network pathways altered in cardiac disease

In this section we will specifically discuss the study initiated by our group to uncover the specific set of molecules and pathways altered during end stage human heart failure. Our published study comprehensively showed alterations in eight miRNAs which are significantly altered in heart failure out of which two new miRNAs that are yet to be implicated in cardiac pathophysiology. We have built signaling pathway networks using predicted targets for the miRNAs and identified nodal molecules that control these networks. Genome-wide profiling of miRNAs was performed using custom-designed miRNA microarray followed by validation on an independent set of samples. To gain an unbiased global perspective on regulation by altered miRNAs, predicted targets of eight miRNAs were analyzed using the Ingenuity Pathways Analysis network algorithm to build signaling networks and identify nodal molecules. The majority of nodal molecules identified in our analysis were targets of altered miRNAs and well known regulators of cardiovascular signaling. A heart failure gene expression data base was used to analyze changes in the expression patterns for these target nodal molecules (Naga Prasad et al., 2009). Indeed, expression of nodal molecules was altered in heart failure and inversely correlated to miRNA changes validating our analysis. Importantly, using network analysis we were successful in identification of a limited number of key functional targets that may regulate expression of the myriad proteins in heart failure and could be potential therapeutic targets. Furthermore, we have been able to independently see these 2 new miRNAs mir-7 and -378 in TAC mice hearts (Naga Prasad et al., 2009). We have shown miRNA-7 and -378 to be downregulated in the end stage human heart failure and targets of miRNA-7, ERBB2 and Col1A to be upregulated. miRNA-7-1 is encoded by the intron-1 of the HNRNPk gene. Similarly, we found miRNA-378 target SLC2A to be upregulated. Out of 1785 predicated

targets, 1716 could be mapped to signaling networks in the IPATM, and 995 predicated targets were found to be network-eligible. The 995 network-eligible candidates mapped to 43 networks that are predicted to be involved in the cross-talk with the peripheral molecules bridging different networks. A representative network with NFkB, a known mediator in cardiac dysfunction (Naga Prasad et al., 2009) as a central node, is shown in Fig. 2 wherein the members that network with NFkB are targets for the miRNAs 1, 29b, 125b, 181b 214, 342, and 378. As individual miRNA acts on each target, the net effect on the node would be the collective influence of all the members connected to the central node, NFkB (Fig. 2) Based on this consideration, we predicted that the complete NFkB regulatory signaling network (Fig. 2) would be significantly down-regulated in DCM since molecules in this network are predicted targets for up-regulated miRNAs 125b, 181b, 214, and 243.

To directly evaluate whether nodal molecules are potential targets for altered miRNAs, immunoblotting studies in the end-stage human heart failure samples revealed an inverse co-relation with the level of respective miRNAs (Fig. 3). Immunoblotting showed ERBB2, HDAC4, COL1, MMP2, and TIMP2 were significantly up-regulated in human DCM (Fig. 3) and were inversely correlated to the down-regulation of their respective miRNAs. In contrast, STAT3 and E2F3 are down-regulated, consistent with the observation of up-regulation of their respective miRNAs suggesting that expression of these molecules may be regulated by miRNAs. Interestingly, we did not observe changes in expression levels of RB1 or EZH2 (Fig. 4) despite being predicted targets for miRNAs consistent with our data.

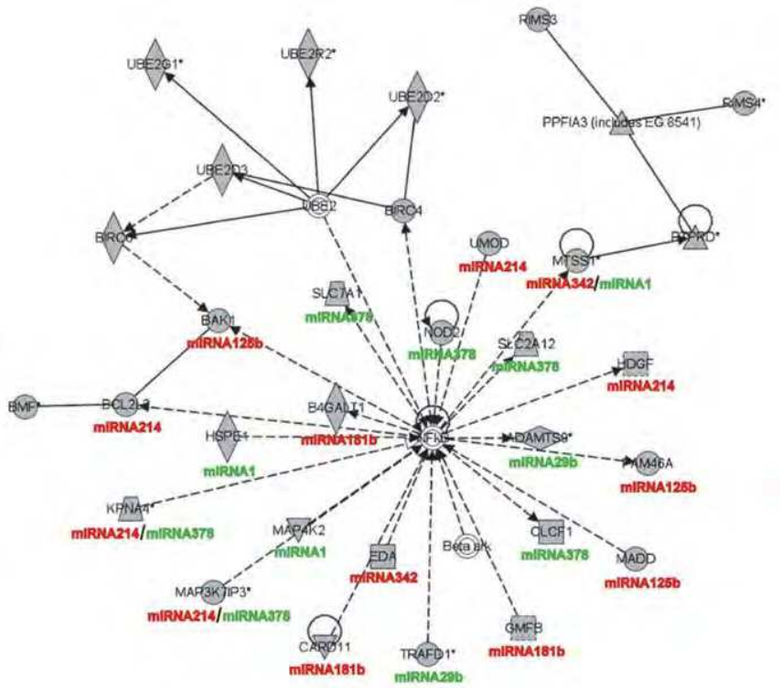

Fig. 2. A representative network showing NF-κB as high connectivity node: The hub is the center of the web of signaling connections and NF-κB is connected to nearly all the molecules in the network. Altered miRNAs in end-stage heart failure are overlaid with their respective predicted targets. miRNA represented in green are downregulated and in red are upregulated in end-stage human dilated cardiomyopathy. Importantly, NF-κB is not a predicted target to any of the altered miRNAs in DCM, yet it could be regulated by alterations in miRNA targets.

7. miRNA databases and computational tools

In recent years, many miRNA database systems have been developed and each of the databases has unique capabilities as they distinguish themselves by the types of data collected, the organization principles, and sources of the data contents. Therefore, it is important for researchers to use all of them to make an informed decision regarding execution of their experimental plan. These databases provide valuable resources to the research community towards understanding the functions of miRNAs in gene regulation. Critically, these databases contain miRNA sequences, annotations and nomenclature, miRNA targets and their relationships, as well as in some cases miRNA expression profiles in different cell types and tissues.

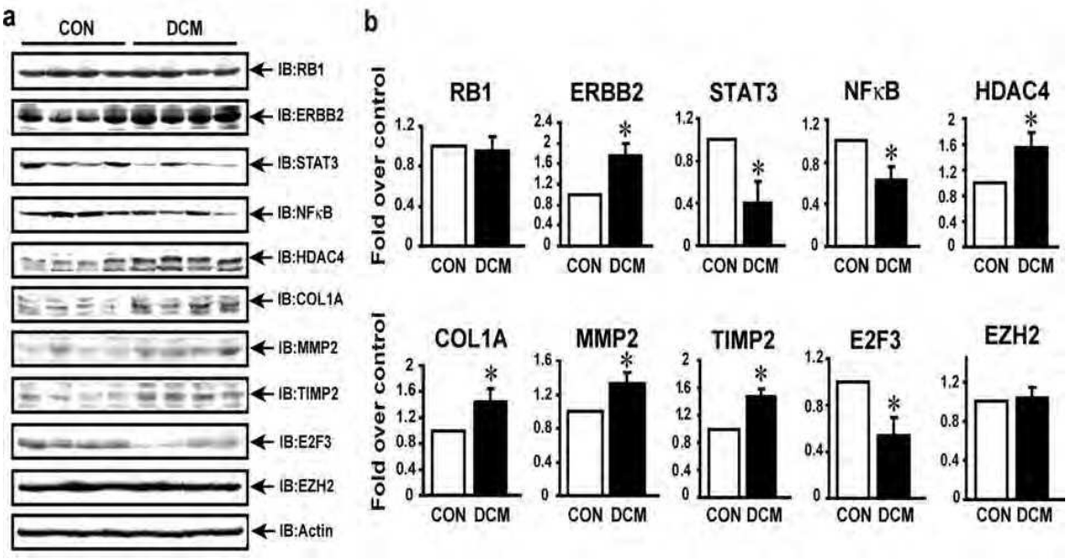

Fig. 3. Immunoblotting in end stage human heart failure *a,* Western immunoblotting analysis was carried out on nonfailinghuman hearts (*CON*, controls; *n*=8), and hearts from patients diagnosed with DCM (*n*=8). 170 microgram of myocardial lysate was resolved with SDS-polyacrylamide gel and immunoblotted (*IB*) with respective antibodies. The blots were stripped and re-probed multiple times with various antibodies, including beta-actin antibody that was used to ensure equal loading. *b,* densitometric analysis in the DCM samples is represented as fold over nonfailing controls.*, $p < 0.001$ control *versus* DCM.

miRBase (Griffiths-Jones et al., 2008) and Rfam (Gardner et al.,) are two major databases containing miRNA sequences and their annotations. miRBase database is an online repository for miRNA sequences and annotations that provides naming service for new miRNA genes isolated by researchers prior to publication. As of April 2011, miRBase contains 16772 mRNA entries from 153 species. Each entry represents a predicted hairpin portion of a miRNA transcript with information on the location and sequence of the mature miRNA as well as functional information/references. In this regard, Rfam database contains information about non-coding RNA families and annotations for family of RNA genes. As of June 2011, Rfam contains 1973 RNA families annotating over 2,756,313 regions in 1,723 unique species. Each family in Rfam is represented by multiple sequence alignments, its consensus secondary structures, and the associated probabilistic covariance models. In

addition, Rfam coordinates a community annotation system providing access through Wikipedia allowing researchers to update entries and create families in the database.

An essential aspect of the functional analysis of miRNAs is the annotation of their targets. Increasing number of miRNA target genes are being identified and confirmed experimentally and simultaneously numerous target prediction algorithms are being developed to enhance the certainty of prediction. miRNA target prediction data base TargetScan (Lewis et al., 2003) predicts miRNA targets in mammals by searching for the presence of conserved 8-mer and 7-mer sites that match the seed region of the miRNA. The criteria for prediction and ranking include stringent seed sequence base pairing, untranslated region (UTR) context, the degree of target sequence conservation across the range of species and finally the thermodynamic stability of the predicted pairings. In addition to TargetScan, there are also several other miRNA target prediction algorithms like PicTar (Lall et al., 2006), MiRanda (John et al., 2004) , EMBL (Stark et al., 2005). They all adopt similar criteria (of rigorous seed pairing, site number, site type and context, likelihood of preferential conservation, and predicted site accessibility) for the target prediction. Historically, during the early phases of target prediction algorithm development, the predicted targets would vary remarkably with many non-overlapping predictions. But with time, utilization of similar criteria has significantly reduced the discrepancies with many overlaps of predicted targets. Such cross-references across the data bases for the miRNA targets is provided by miRecords. This computational tool provides an integrated view of experimentally validated miRNA targets and displays predicted targets generated by 11 established miRNA target prediction programs.

Since targets for many miRNAs are being identified, there is an ongoing simultaneous effort to develop databases that exclusively provides information on validated miRNA targets like TarBase (Sethupathy et al., 2006) and miRTarBase. TarBase database collects manually curated and experimentally supported miRNA targets in animal species, plants and viruses. It includes more than 1300 targets and each target is described by the miRNA it binds, the experiments that tested this relationship and the link to citation. Database miRTarBase (Hsu et al.,) curates 3576 experimentally validated microRNA-target interactions between 657 microRNAs and 2297 target genes among 17 species. In order to provide a disease perspective on the role of miRNA and their targets in pathology, a unique database miR2Disease [miR2Disease] has been generated. miR2Disease (Jiang et al., 2009) documents 1939 curated miRNA-disease relationships between 299 human miRNAs and 94 human diseases. Each entry in the database contains information on the miRNA ID, the disease name, validated targets, expression patterns of the miRNA, a brief description of the miRNA-disease relationship and citation.

Another important issue the readers need to appreciate is that despite the knowledge that there may be 1000 potential miRNA genes in the human genome, all of them have not yet been experimentally validated. These miRNA encoding gene predictions come from the sequence based curation of the genome to determine whether a specific DNA sequence characteristically fits the requirement for encoding a miRNA. RepTar (Elefant et al.,) is one such database that curates genome-wide predicted miRNAs of human and mouse. Furthermore, it can also predict cellular targets of human and mouse viral miRNAs. In addition to the miRNA sequence and target databases, a growing number of entries have been recorded at gene expression databases such as Gene Expression Omnibus (GEO) (Barrett et al.,) at NCBI and ArrayExpress (Brazma et al., 2006) Archive at EBI.

Although we are a long way away from validating all the predicted targets of altered miRNAs in heart failure, the predicted targets provide us a window to assess the global signaling pathways that could potentially be altered by the miRNAs in the heart failure. We have used this idea to build signaling networks of predicted targets of miRNAs altered in human heart failure which involves the role of miRNA target interaction with the biological pathways which ultimately generate the phenotype. A well known web-based tool is the Ingenuity Pathway Analysis (IPA) which is commonly used to model, analyze, and understand biological data derived from mRNA/miRNA gene expression arrays, SNP microarrays, proteomics, as well as small scale experiments. The core of IPA is its knowledge database on genes, proteins, chemicals, and molecular relationships. The database contains highly structured information about molecular interactions and functional annotations as well as contextual details of the biological interactions. IPA provides modules that can be used to integrate data at multiple levels to obtain insight into the molecular interactions, cellular phenotypes and disease processes of the biological system like heart failure. The main analysis modules include 1) IPA Core Analysis for identifying signaling and metabolic pathways, molecular networks, and biological processes that are related to the biological data; 2) IPA-Metabolomics for extracting biological insight into cell physiology and metabolism; 3) IPA-Tox for analyzing toxicity of candidate compounds; and 4) IPA-Biomarker for identifying the most promising and relevant biomarker candidates within experimental datasets. In addition, IPA's Path Designer provides researchers with help for transforming customized networks/pathways into pathway graphics for easy representation. It is well known historically that as the field evolves, so also the tools and miRNA target filter in the IPA analysis is one such evolution. miRNA Target Filter was introduced in the recent version of IPA (IPA 9.0) that allows researcher to examine both predicted and experimentally confirmed miRNA targets. Furthermore, it prioritizes targets based on related biological context and allows visualization of molecular interactions between miRNAs, their targets and other related molecules. IPA uses TargetScan database for predicted targets and TarBase for experimentally confirmed targets.

8. miRNAs as therapeutic targets

The current evidence from multiple studies show that miRNAs are altered with cardiac stress and genetic manipulation shows that miRNAs may actively contribute towards the deleterious cardiac phenotype. The regulation of cardiac phenotype by miRNAs, indicates that miRNAs can be used in therapeutic strategies to ameliorate deleterious outcomes. Since miRNAs are RNAs, they can be manipulated using the existing antisense and gene therapy approaches in vivo. Modified antisense oligonucleotides targeting the mature miRNA sequence, antimiRs, can reduce the levels of pathogenic or aberrantly expressed miRNAs (Krutzfeldt et al., 2005). Conversely, miRNA mimics can elevate the levels of miRNAs with beneficial outcomes (Xiao et al., 2007). Since miRNAs typically act as inhibitors of gene expression, the effect of adding specific miRNA mimics to a system is to decrease the expression of the mRNAs controlled by the miRNA. Conversely, the effect of inhibitors of specific miRNAs is to relieve the inhibition of the genes normally targeted by the miRNA. Thus, the primary effect of a miRNA inhibitor is activation of gene expression and a miRNA mimic is suppression of gene expression.

8.1 Antisense miRNA oligonucleotides and miRNA mimics

Disease condition is contributed by upregulation or downregulation of miRNAs. In conditions of upregulation specific reduction of the miRNA would be therapeutically desirable. One of the efficient ways to inhibit miRNAs would be the use of chemically modified single-stranded reverse complement oligonucleotides. The synthetic reverse complement oligonucleotide approach affects miRNA levels by (1) binding the mature miRNA within the RISC and acting as a competitive inhibitor; (2) binding to the pre-miRNA and preventing its processing or entry into the RISC; (3) interfering with the processing or export of the pre- or pri-miRNA from the nucleus. In any case, the net result is a reduction in the concentration of a specific miRNA-programmed RISC. This approach is similar in concept to traditional antisense targeting of mRNAs, except the number of targeting sites for a miRNA is very limited. Although conceptually comparable, only a handful of modifications have been achieved for inhibition of miRNAs. Such a technique has been effectively used to knock down let-7 in Drosophila (Hutvagner, Simard et al. 2004) while miRNA-122 was the first mammalian miRNA to be targeted for liver (Krutzfeldt et al., 2005). In this context, miRNA-133 (Altuvia et al., 2005) and miRNA-29 (van Rooij et al., 2008) have been effectively used to alter cardiac phenotype suggesting that this specific technique would be a viable option for therapeutic strategy. More recently, the technique of modifying the oligonucleotides with 2'O-methoxyethyl phosphorothioate is being extensively used as it seems to provide long term stability for the administered oligos thus extending the beneficial effects in vivo.

Anti-sense oligos can be used in conditions of targeting upregulated miRNAs and in conditions where reduction in miRNA level causes a disease state, beneficial therapeutic approach would be to increase its concentration. Instead of delivering the single-stranded oligonucleotide equivalent of the mature miRNA, an increase in the effective concentration of a reduced miRNA can be achieved through the use of synthetic RNA duplexes in which 1 strand is identical to the native miRNA. In this case, short double stranded oligonucleotides are designed in which 1 strand is the mature miRNA sequence (guide strand) and a complimentary or partially complementary stand is complexed with the mature miRNA sequence (passenger strand). The double stranded structure is required for recognition and loading into the RISC (Martinez et al., 2002). The only caveat in this kind of the design is to make sure that the passenger strand is eliminated and does not act as a new miRNA that may complicate the interpretation. Alternatively, approaches similar to that undertaken with siRNA using bioinformatic and chemical modification can be used and provides attractive means to elevate miRNA levels.

8.2 Therapeutic targeting of miRNA and challenges

Despite the ability to manipulate miRNAs in vivo to provide unique opportunities therapeutically, miRNA-based therapeutics pose challenges that are different from those associated with classic drugs. One of the major stumbling blocks for miRNA targeting is the issue of specificity. While specificity for a single cellular target is vital in classic drugs, miRNAs have numerous molecular targets raising the possibility that targeting of a miRNA may perturb multiple cellular functions both deleterious as well as beneficial. Since miRNAs are new set of molecules that are being targeted, better understanding of pharmacokinetics, biodistribution, and cell penetration is required to develop these as therapeutics. It is known that native nucleic acids are rapidly degraded by a variety of nucleases and

phosphodiesterases in blood. Furthermore, biological environments and requirement of chemical modifications on the synthetic nucleotide derivatives may alter miRNA biophysical properties reducing the efficiency of therapeutic function. In this regards, several modifications that increase the stability of the oligonucleotides, including phosphorothioate, 2′-O-methyl, and 2′-fluoro substitutions can be effectively put to use in developing therapeutic strategies (Soutschek et al., 2004).

8.3 Methods of Delivery

In addition to efforts on developing miRNAs that are stable by modification, intense efforts are also ongoing to identify agents capable of targeted delivery of nucleic acids to tissues and cells. Delivery approaches can be broadly divided into 2 categories, conjugation and formulation. Conjugation strategies include direct attachment of targeting and cell-penetrating peptides, antibodies, and other bioactive molecules to the oligonucleotide. Formulation approaches vary broadly and include complex lipid emulsions from natural sources, synthetic liposomes, polyplexes, polymers and nanoparticles. To enter mammalian cells, the reverse-complement oligonucleotide needs to cross the lipid bilayer of the cell membrane and can be achieved by packaging the oligonucleotide into liposomes or nanoparticles that facilitates endocytosis. Alternatively, the oligonucleotide can be linked to a lipophilic moiety or receptor ligand, such as cholesterol that seems to greatly enhance cellular uptake (Soutschek et al., 2004). Despite significant advances in systemic delivery technology, most nucleic acid delivery agents developed to date have demonstrated efficacy in delivery to the liver. Therefore, effective delivery approaches especially to the heart would be a great stepping stone in the direction towards use of synthetic nucleic acids as therapeutics for cardiovascular disease. Interestingly, heart failure affords a unique opportunity to expand the potential for local delivery through the use of catheters providing additional level of sophistication.

9. Conclusions

It is remarkable to consider that miRNAs were first shown to function in mammals less than a decade ago, and the concept of miRNA manipulation in vivo to regulate disease-related processes is already becoming a feasible future therapeutic approach. Moreover, the rapidly expanding number of miRNAs makes it likely that the relatively few miRNAs studied to date represent only a subset of the miRNAs of interest in human disease. Given the established involvement of miRNAs in many facets of heart disease, it becomes pertinent to understand the underlying basis for its contributing role before taking on miRNA based human trials. Understanding the role of miRNAs in regulating various targets is the weakest link in this chain of fast moving area of miRNA that needs effort and resources. We believe the efforts are needed simultaneously in the direction of indepth contemporary proteomics along with assessment of miRNAs providing platform for linking miRNA to target protein expression which are the ultimate determinants of the phenotype. Therefore, identifying and validating miRNA targets are of paramount importance and establishment of the miRNA targets will provide a sound foundation for development of global signaling networks. Understanding the global regulation of networks by a miRNA rather than a specific target would be a more feasible approach to understand the overall function of miRNAs in development and disease conditions as a single miRNA could target both

synergistic as well as antagonistic pathways. Appreciation of this unique regulation by miRNAs in physiology as well as pathology is the incentive to develop tools and technology to better understand the role of miRNAs in effecting global change rather than specific molecules in a given pathway.

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11. References

Altuvia, Y., P. Landgraf, et al. (2005). "Clustering and conservation patterns of human microRNAs." *Nucleic Acids Res* 33(8): 2697-706.

Ambros, V. (2004). "The functions of animal microRNAs." *Nature* 431(7006): 350-5.

Aravin, A. A., G. J. Hannon, et al. (2007). "The Piwi-piRNA pathway provides an adaptive defense in the transposon arms race." *Science* 318(5851): 761-4.

Barrett, T., D. B. Troup, et al. "NCBI GEO: archive for functional genomics data sets--10 years on." *Nucleic Acids Res* 39(Database issue): D1005-10.

Barron, M. R., N. S. Belaguli, et al. (2005). "Serum response factor, an enriched cardiac mesoderm obligatory factor, is a downstream gene target for Tbx genes." *J Biol Chem* 280(12): 11816-28.

Bartel, D. P. (2004). "MicroRNAs: genomics, biogenesis, mechanism, and function." *Cell* 116(2): 281-97.

Bartel, D. P. (2009). "MicroRNAs: target recognition and regulatory functions." *Cell* 136(2): 215-33.

Berezikov, E., V. Guryev, et al. (2005). "Phylogenetic shadowing and computational identification of human microRNA genes." *Cell* 120(1): 21-4.

Bernstein, E., S. Y. Kim, et al. (2003). "Dicer is essential for mouse development." *Nat Genet* 35(3): 215-7.

Brazma, A., M. Kapushesky, et al. (2006). "Data storage and analysis in ArrayExpress." *Methods Enzymol* 411: 370-86.

Brown, J. H., D. P. Del Re, et al. (2006). "The Rac and Rho hall of fame: a decade of hypertrophic signaling hits." *Circ Res* 98(6): 730-42.

Bruneau, B. G. (2005). "Developmental biology: tiny brakes for a growing heart." *Nature* 436(7048): 181-2.

Cacchiarelli, D., D. Santoni, et al. (2008). "MicroRNAs as prime players in a combinatorial view of evolution." *RNA Biol* 5(3): 120-122.

Calin, G. A., C. G. Liu, et al. (2007). "Ultraconserved regions encoding ncRNAs are altered in human leukemias and carcinomas." *Cancer Cell* 12(3): 215-29.

Care, A., D. Catalucci, et al. (2007). "MicroRNA-133 controls cardiac hypertrophy." *Nat Med* 13(5): 613-8.

Catalucci, D., M. V. Latronico, et al. (2008). "Physiological myocardial hypertrophy: how and why?" *Front Biosci* 13: 312-24.

Chapman, E. J. and J. C. Carrington (2007). "Specialization and evolution of endogenous small RNA pathways." *Nat Rev Genet* 8(11): 884-96.

Chen, J. F., E. M. Mandel, et al. (2006). "The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation." *Nat Genet* 38(2): 228-33.

Chen, J. F., E. P. Murchison, et al. (2008). "Targeted deletion of Dicer in the heart leads to dilated cardiomyopathy and heart failure." *Proc Natl Acad Sci U S A* 105(6): 2111-6.

Cheng, Y., R. Ji, et al. (2007). "MicroRNAs are aberrantly expressed in hypertrophic heart: do they play a role in cardiac hypertrophy?" *Am J Pathol* 170(6): 1831-40.

Dash, R., V. Kadambi, et al. (2001). "Interactions between phospholamban and beta-adrenergic drive may lead to cardiomyopathy and early mortality." *Circulation* 103(6): 889-96.

Ebert, M. S., J. R. Neilson, et al. (2007). "MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells." *Nat Methods* 4(9): 721-6.

Elefant, N., A. Berger, et al. "RepTar: a database of predicted cellular targets of host and viral miRNAs." *Nucleic Acids Res* 39(Database issue): D188-94.

Eulalio, A., I. Behm-Ansmant, et al. (2007). "P-body formation is a consequence, not the cause, of RNA-mediated gene silencing." *Mol Cell Biol* 27(11): 3970-81.

Farh, K. K., A. Grimson, et al. (2005). "The widespread impact of mammalian MicroRNAs on mRNA repression and evolution." *Science* 310(5755): 1817-21.

Felli, N., L. Fontana, et al. (2005). "MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation." *Proc Natl Acad Sci U S A* 102(50): 18081-6.

Filipowicz, W. (2005). "RNAi: the nuts and bolts of the RISC machine." *Cell* 122(1): 17-20.

Gardner, P. P., J. Daub, et al. "Rfam: Wikipedia, clans and the "decimal" release." *Nucleic Acids Res* 39(Database issue): D141-5.

Giraldez, A. J., R. M. Cinalli, et al. (2005). "MicroRNAs regulate brain morphogenesis in zebrafish." *Science* 308(5723): 833-8.

Giraldez, A. J., Y. Mishima, et al. (2006). "Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs." *Science* 312(5770): 75-9.

Gregory, P. A., A. G. Bert, et al. (2008). "The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1." *Nat Cell Biol* 10(5): 593-601.

Griffiths-Jones, S., R. J. Grocock, et al. (2006). "miRBase: microRNA sequences, targets and gene nomenclature." *Nucleic Acids Res* 34(Database issue): D140-4.

Griffiths-Jones, S., H. K. Saini, et al. (2008). "miRBase: tools for microRNA genomics." *Nucleic Acids Res* 36(Database issue): D154-8.

Gusev, Y., T. D. Schmittgen, et al. (2007). "Computational analysis of biological functions and pathways collectively targeted by co-expressed microRNAs in cancer." *BMC Bioinformatics* 8 Suppl 7: S16.

Harris, T. A., M. Yamakuchi, et al. (2008). "MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1." *Proc Natl Acad Sci U S A* 105(5): 1516-21.

Hsu, S. D., F. M. Lin, et al. "miRTarBase: a database curates experimentally validated microRNA-target interactions." *Nucleic Acids Res* 39(Database issue): D163-9.

Hutvagner, G., J. McLachlan, et al. (2001). "A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA." *Science* 293(5531): 834-8.

Hutvagner, G., M. J. Simard, et al. (2004). "Sequence-specific inhibition of small RNA function." *PLoS Biol* 2(4): E98.

Hwang, H. W., E. A. Wentzel, et al. (2007). "A hexanucleotide element directs microRNA nuclear import." *Science* 315(5808): 97-100.

Ikeda, S., A. He, et al. (2009). "MicroRNA-1 negatively regulates expression of the hypertrophy-associated calmodulin and Mef2a genes." *Mol Cell Biol* 29(8): 2193-204.

Ikeda, S., S. W. Kong, et al. (2007). "Altered microRNA expression in human heart disease." *Physiol Genomics* 31(3): 367-73.

Ivanovska, I. and M. A. Cleary (2008). "Combinatorial microRNAs: working together to make a difference." *Cell Cycle* 7(20): 3137-42.

Ivey, K. N., A. Muth, et al. (2008). "MicroRNA regulation of cell lineages in mouse and human embryonic stem cells." *Cell Stem Cell* 2(3): 219-29.

Jiang, Q., Y. Wang, et al. (2009). "miR2Disease: a manually curated database for microRNA deregulation in human disease." *Nucleic Acids Res* 37(Database issue): D98-104.

John, B., A. J. Enright, et al. (2004). "Human MicroRNA targets." *PLoS Biol* 2(11): e363.

Ke, Y., L. Wang, et al. (2004). "Intracellular localization and functional effects of P21-activated kinase-1 (Pak1) in cardiac myocytes." *Circ Res* 94(2): 194-200.

Kertesz, M., N. Iovino, et al. (2007). "The role of site accessibility in microRNA target recognition." *Nat Genet* 39(10): 1278-84.

Kim, V. N. and J. W. Nam (2006). "Genomics of microRNA." *Trends Genet* 22(3): 165-73.

Kiriakidou, M., P. T. Nelson, et al. (2004). "A combined computational-experimental approach predicts human microRNA targets." *Genes Dev* 18(10): 1165-78.

Kloosterman, W. P., A. K. Lagendijk, et al. (2007). "Targeted inhibition of miRNA maturation with morpholinos reveals a role for miR-375 in pancreatic islet development." *PLoS Biol* 5(8): e203.

Krek, A., D. Grun, et al. (2005). "Combinatorial microRNA target predictions." *Nat Genet* 37(5): 495-500.

Krichevsky, A. M., K. C. Sonntag, et al. (2006). "Specific microRNAs modulate embryonic stem cell-derived neurogenesis." *Stem Cells* 24(4): 857-64.

Krutzfeldt, J., N. Rajewsky, et al. (2005). "Silencing of microRNAs in vivo with 'antagomirs'." *Nature* 438(7068): 685-9.

Kuehbacher, A., C. Urbich, et al. (2007). "Role of Dicer and Drosha for endothelial microRNA expression and angiogenesis." *Circ Res* 101(1): 59-68.

Kwon, C., Z. Han, et al. (2005). "MicroRNA1 influences cardiac differentiation in Drosophila and regulates Notch signaling." *Proc Natl Acad Sci U S A* 102(52): 18986-91.

Lai, E. C. (2002). "Micro RNAs are complementary to 3' UTR sequence motifs that mediate negative post-transcriptional regulation." *Nat Genet* 30(4): 363-4.

Lakshmipathy, U., B. Love, et al. (2007). "MicroRNA expression pattern of undifferentiated and differentiated human embryonic stem cells." *Stem Cells Dev* 16(6): 1003-16.

Lall, S., D. Grun, et al. (2006). "A genome-wide map of conserved microRNA targets in C. elegans." *Curr Biol* 16(5): 460-71.

Landgraf, P., M. Rusu, et al. (2007). "A mammalian microRNA expression atlas based on small RNA library sequencing." *Cell* 129(7): 1401-14.

Latronico, M. V., D. Catalucci, et al. (2007). "Emerging role of microRNAs in cardiovascular biology." *Circ Res* 101(12): 1225-36.

Lee, N. S., T. Dohjima, et al. (2002). "Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells." *Nat Biotechnol* 20(5): 500-5.

Lee, Y., C. Ahn, et al. (2003). "The nuclear RNase III Drosha initiates microRNA processing." *Nature* 425(6956): 415-9.

Lee, Y., M. Kim, et al. (2004). "MicroRNA genes are transcribed by RNA polymerase II." *EMBO J* 23(20): 4051-60.

Lewis, B. P., C. B. Burge, et al. (2005). "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets." *Cell* 120(1): 15-20.

Lewis, B. P., I. H. Shih, et al. (2003). "Prediction of mammalian microRNA targets." *Cell* 115(7): 787-98.

Liu, N., A. H. Williams, et al. (2007). "An intragenic MEF2-dependent enhancer directs muscle-specific expression of microRNAs 1 and 133." *Proc Natl Acad Sci U S A* 104(52): 20844-9.

Luo, X., H. Lin, et al. (2008). "Down-regulation of miR-1/miR-133 contributes to re-expression of pacemaker channel genes HCN2 and HCN4 in hypertrophic heart." *J Biol Chem* 283(29): 20045-52.

MacRae, I. J., E. Ma, et al. (2008). "In vitro reconstitution of the human RISC-loading complex." *Proc Natl Acad Sci U S A* 105(2): 512-7.

Martinez, J., A. Patkaniowska, et al. (2002). "Single-stranded antisense siRNAs guide target RNA cleavage in RNAi." *Cell* 110(5): 563-74.

Miranda, K. C., T. Huynh, et al. (2006). "A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes." *Cell* 126(6): 1203-17.

Naga Prasad, S. V., Z. H. Duan, et al. (2009). "Unique microRNA profile in end-stage heart failure indicates alterations in specific cardiovascular signaling networks." *J Biol Chem* 284(40): 27487-99.

Naga Prasad and Karnik SS (2010). ''MicroRNAs--regulators of signaling networks in dilated cardiomyopathy.''*J Cardiovasc Transl Res*. 3(3):225-34.

Nilsen, T. W. (2007). "Mechanisms of microRNA-mediated gene regulation in animal cells." *Trends Genet* 23(5): 243-9.

Niu, Z., A. Li, et al. (2007). "Serum response factor micromanaging cardiogenesis." *Curr Opin Cell Biol* 19(6): 618-27.

Okamura, K., J. W. Hagen, et al. (2007). "The mirtron pathway generates microRNA-class regulatory RNAs in Drosophila." *Cell* 130(1): 89-100.

Okamura, K., A. Ishizuka, et al. (2004). "Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways." *Genes Dev* 18(14): 1655-66.

Olson, E. N. and M. D. Schneider (2003). "Sizing up the heart: development redux in disease." *Genes Dev* 17(16): 1937-56.

Pedersen, I. M., G. Cheng, et al. (2007). "Interferon modulation of cellular microRNAs as an antiviral mechanism." *Nature* 449(7164): 919-22.

Rao, P. K., R. M. Kumar, et al. (2006). "Myogenic factors that regulate expression of muscle-specific microRNAs." *Proc Natl Acad Sci U S A* 103(23): 8721-6.

Rodriguez, A., S. Griffiths-Jones, et al. (2004). "Identification of mammalian microRNA host genes and transcription units." *Genome Res* 14(10A): 1902-10.

Ruby, J. G., C. H. Jan, et al. (2007). "Intronic microRNA precursors that bypass Drosha processing." *Nature* 448(7149): 83-6.

Sayed, D., C. Hong, et al. (2007). "MicroRNAs play an essential role in the development of cardiac hypertrophy." *Circ Res* 100(3): 416-24.

Schwarz, D. S., G. Hutvagner, et al. (2003). "Asymmetry in the assembly of the RNAi enzyme complex." *Cell* 115(2): 199-208.

Sethupathy, P., B. Corda, et al. (2006). "TarBase: A comprehensive database of experimentally supported animal microRNA targets." *RNA* 12(2): 192-7.

Soutschek, J., A. Akinc, et al. (2004). "Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs." *Nature* 432(7014): 173-8.

Srivastava, D., T. Thomas, et al. (1997). "Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND." *Nat Genet* 16(2): 154-60.

Stark, A., J. Brennecke, et al. (2005). "Animal MicroRNAs confer robustness to gene expression and have a significant impact on 3'UTR evolution." *Cell* 123(6): 1133-46.

Sucharov, C., M. R. Bristow, et al. (2008). "miRNA expression in the failing human heart: functional correlates." *J Mol Cell Cardiol* 45(2): 185-92.

Sun, Q., G. Chen, et al. (2006). "Defining the mammalian CArGome." *Genome Res* 16(2): 197-207.

Tatsuguchi, M., H. Y. Seok, et al. (2007). "Expression of microRNAs is dynamically regulated during cardiomyocyte hypertrophy." *J Mol Cell Cardiol* 42(6): 1137-41.

Tay, Y. M., W. L. Tam, et al. (2008). "MicroRNA-134 modulates the differentiation of mouse embryonic stem cells, where it causes post-transcriptional attenuation of Nanog and LRH1." *Stem Cells* 26(1): 17-29.

Thum, T., P. Galuppo, et al. (2007). "MicroRNAs in the human heart: a clue to fetal gene reprogramming in heart failure." *Circulation* 116(3): 258-67.

Thum, T., C. Gross, et al. (2008). "MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts." *Nature* 456(7224): 980-4.

van Rooij, E., L. B. Sutherland, et al. (2006). "A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure." *Proc Natl Acad Sci U S A* 103(48): 18255-60.

van Rooij, E., L. B. Sutherland, et al. (2007). "Control of stress-dependent cardiac growth and gene expression by a microRNA." *Science* 316(5824): 575-9.

van Rooij, E., L. B. Sutherland, et al. (2008). "Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis." *Proc Natl Acad Sci U S A* 105(35): 13027-32.

Volpe, T. A., C. Kidner, et al. (2002). "Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi." *Science* 297(5588): 1833-7.

Xiao, J., X. Luo, et al. (2007). "MicroRNA miR-133 represses HERG K+ channel expression contributing to QT prolongation in diabetic hearts." *J Biol Chem* 282(17): 12363-7.

Yang, B., H. Lin, et al. (2007). "The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2." *Nat Med* 13(4): 486-91.

Yi, R., Y. Qin, et al. (2003). "Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs." *Genes Dev* 17(24): 3011-6.

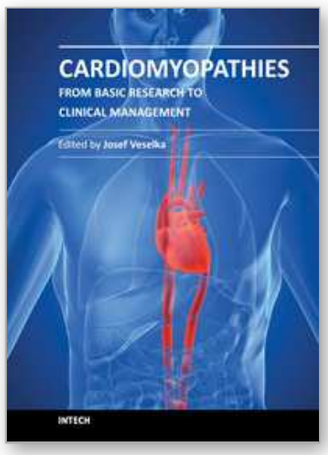
Yu, W., D. Gius, et al. (2008). "Epigenetic silencing of tumour suppressor gene p15 by its antisense RNA." *Nature* 451(7175): 202-6.

Zhang, S. X., E. Garcia-Gras, et al. (2005). "Identification of direct serum-response factor gene targets during Me2SO-induced P19 cardiac cell differentiation." *J Biol Chem* 280(19): 19115-26.

Zhao, Y., J. F. Ransom, et al. (2007). "Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2." *Cell* 129(2): 303-17.

Zhao, Y., E. Samal, et al. (2005). "Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis." *Nature* 436(7048): 214-20.

Zilberman, D., X. Cao, et al. (2003). "ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation." *Science* 299(5607): 716-9.



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Cardiomyopathy means "heart (cardio) muscle (myo) disease (pathy)". Currently, cardiomyopathies are defined as myocardial disorders in which the heart muscle is structurally and/or functionally abnormal in the absence of a coronary artery disease, hypertension, valvular heart disease or congenital heart disease sufficient to cause the observed myocardial abnormalities. This book provides a comprehensive, state-of-the-art review of the current knowledge of cardiomyopathies. Instead of following the classic interdisciplinary division, the entire cardiovascular system is presented as a functional unity, and the contributors explore pathophysiological mechanisms from different perspectives, including genetics, molecular biology, electrophysiology, invasive and non-invasive cardiology, imaging methods and surgery. In order to provide a balanced medical view, this book was edited by a clinical cardiologist.

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