

# MicroRNA Targeting in Heart: A Theoretical Analysis

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## 1. Introduction

Cardiovascular disease remains the major cause of morbidity and mortality; according to statistics, heart failure, the syndrome consequential to many diseases of the cardiovascular system, is estimated to have a prevalence of 1-2% and an annual incidence of 5-10 per 1,000 in the developed countries and is the leading cause of hospitalization in the population over 50 years of age. Worse so, there is a clear tendency of increasing prevalence of cardiovascular disease in this planet particularly in the developing nations. This problem casts enormous health concern and costly socioeconomic burden worldwide.

We have entered post-genome era after the human genome project had been completed years ago. Only <2% of all transcribed bases of the entire human genome constitutes the genetic sequence encoding proteins and the rest of 98% accounting for ~70% of all genes carry the sequences for RNAs not encoding a polypeptide chain that was used to be considered for many years "junk DNA" of no physiologic function; proteins were generally assumed the sole biopolymer capable of regulatory function. Intriguingly, the proportion of transcribed non-protein-coding sequences increases with developmental complexity and is a better indicator of phylogenetic level than the number of protein-coding genes of an organism. It is now known that "junk DNA" encodes non-protein-coding RNAs (ncRNAs) that are involved in determining the expression of protein-coding genes by regulating the activity of that 2% of the genome. These ncRNAs include microRNAs (miRNAs), once ignored completely or overlooked as cellular detritus, which were discovered over a decade ago have recently taken many by surprise because of their widespread expression and diverse functions.

miRNAs are endogenous small mRNAs of ~22 nucleotide in length which act primarily to repress gene expression at the post-transcriptional level. To date, ~6400 vertebrates mature miRNAs have been registered in miRBase, an online repository for miRNA, among which ~5100 miRNAs are found in mammals which include >850 human miRNAs. These miRNAs are predicted to regulates >60% of protein-coding genes. The discovery of miRNA challenges the central dogma of molecular biology that has been hold since the latter half of the 20th century. With the recent rapid evolution of miRNA research, researchers have begun to appreciate the roles of these small non-protein-coding mRNAs in the cardiovascular system. Based on the published studies, it is now clear that miRNAs are involved in nearly all aspects of cardiac function and pathogenesis (Wang, 2010; Wang et al., 2008; Yang et al., 2008).

It is known that an individual miRNA has the potential to target multiple protein-coding genes and *vice versa* a single protein-coding gene may be regulated by multiple miRNAs, implying that the action of miRNAs is not gene-specific. This fact creates an obstacle to our thorough understanding of miRNA functions and the mechanism underlying these functions. For example, we have recently found that *miR-125-5p* target GJA1 (encoding gap junction channel protein connexin43) and GJC1 (encoding another gap junction channel protein connexin40) to cause slowing of both ventricular and atrial conduction promoting arrhythmogenesis in failing heart where its expression is upregulated (Wang, 2010). However, based upon computational prediction it can also target SCN5A (encoding Nav1.5 Na<sup>+</sup> channel  $\alpha$ -subunit). An immediate question is whether the potential repression of SCN5A also plays a role in *miR-125-5p*-induced conduction slowing. On the other hand, GJA1, GJC1 and SCN5A are predicted to be regulated by other miRNAs in addition to *miR-125-5p* (such as *miR-101*, *miR-125*, *miR-130*, *miR-19*, *miR-23*, *miR-26* and *miR-30*); whether these miRNAs are also involved in the deregulation of these genes in heart failure remained unknown. This same uncertainty may exist in the interactions between literally all miRNAs and protein-coding genes. Proper experimental approaches are ultimately required to clarify these issues. However, at present it is not feasible to have thorough elucidation of the complete set of target genes of a given miRNA or of the complete array of mRNAs that regulate a given protein-coding gene; computational prediction remains the best alternative for rapid identification of miRNA target genes.

This chapter aims to shed light on how to take rational uses of bioinformatics analysis to identify the miRNAs from the currently available miRNA databases which have the potential to regulate human genes related to cardiac function and pathology and to validate the analysis taking several pathological settings associated with the deregulated miRNAs in the heart. The pathological conditions to be discussed include arrhythmogenesis, apoptosis and fibrogenesis that are known to be critical to the adverse electrical, cellular and structural remodelling processes in various diseased states of heart such as myocardial infarction, cardiac hypertrophy and heart failure.

## 2. Ion channel genes as targets for miRNAs

Cardiac cells are excitable cells that can generate and propagate excitations; At the cellular level, excitability is reflected by cardiac action potential, which is orchestrated by transmembrane proteins like ion channels and transporters and intracellular proteins for Ca<sup>2+</sup> handling. (Wang, 2010; Wang et al., 2008; Yang et al., 2008). Deregulation of miRNA expression has been implicated in a variety of diseased conditions of the heart and aberrant expression of miRNAs can render expression deregulation of ion channel genes resulting in channelopathies—arrhythmogenesis leading to sudden cardiac death. Indeed, we and others have shown the critical involvement of miRNAs, particularly the muscle-specific miRNAs *miR-1* and *miR-133*, in regulating every aspects of cardiac excitability to affect arrhythmogenesis under various pathological conditions including myocardial infarction, cardiac hypertrophy, diabetic cardiomyopathy, etc. This property of miRNAs is conferred by their ability to target ion channels and intracellular Ca<sup>2+</sup> handling proteins at the post-transcriptional level, as already revealed by numerous studies. Even though, our current knowledge about miRNA regulation of cardiac ion channels is still rather preliminary with limited experimental data available in the literature. This is largely due to the limitations of

our technologies and approaches to conduct thorough characterization of miRNA targeting. As a surrogate to these limitations, we have conducted a rationally designed bioinformatics analysis in conjunction with experimental approaches to identify the miRNAs which have the potential to regulate human cardiac ion channel genes and to validate the analysis with several pathological settings associated with the deregulated miRNAs and ion channel genes in the heart (Luo et al., 2010). In this way, we have been able to identify an array of miRNAs that are expressed in cardiac cells and have the potential to regulate the genes encoding cardiac ion channels, transporters and intracellular  $\text{Ca}^{2+}$  handling proteins. Our data well explain the ionic remodelling processes occurring in hypertrophy/heart failure, myocardial ischemia, or atrial fibrillation at the level of miRNA; the changes of miRNAs appear to have anti-correlation with the changes of many of the genes encoding cardiac ion channels under these pathological conditions.

### 2.1 *In Silico* analysis of miRNA targets

We used the miRecords miRNA database and target-prediction website for our initial analysis. The miRecords is resource for animal miRNA-target interactions developed at the University of Minnesota (Xiao et al., 2009). The miRecords consists of two separate databases. The Validated Targets database contains the experimentally validated miRNA targets being updated from meticulous literature curation. The Predicted Targets database of miRecords is an integration of predicted miRNA targets produced by 11 established miRNA target prediction programs. These algorithms include DIANA-microT, MicroInspector, miRanda, MirTarget2, miTarget, NBmiRTar, PicTar, PITA, RNA22, RNAhybrid, and TargetScan/TargetScanS.

As an initial "screening" process, we performed miRNA target prediction through the miRecords database (Xiao et al., 2009). This miRNA database integrates miRNA target predictions by 11 algorithms. Four of the 11 algorithms (microInspector, miTarget, NBmiRTar, and RNA22) were removed from our data analysis because they failed to predict; these websites require manual input of 3'UTR sequences of the genes. Thus, our data analysis was based upon the prediction from seven algorithms (TargetScan, DIANA-miT3.0, miRanda, PicTar, PITA, RNAHybrid, and miRTarget2) (Enright et al., 2003; Kertesz et al., 2007; Kiriakidou et al., 2004; Krek et al., 2005; Lewis et al., 2003 & 2005; Rehmsmeier et al., 2004; Wang & El Naqa., 2008). These prediction techniques are based on algorithms with different parameters (such as miRNA seed:mRNA 3'UTR complementarity, thermodynamic stability of base-pairing (assessed by free energy), evolutionary conservation across orthologous 3'UTRs in multiple species, structural accessibility of the binding sites, nucleotide composition beyond the seed sequence, number of binding sites in 3'UTR, and anti-correlation between miRNAs and their target mRNAs). A then-updated set of RefSeq genes and their annotations was used to define a set of human 3' UTRs. Orthologous UTRs (based on whole-genome alignments) were obtained for 22 other species from UCSC Genome Bioinformatics. Conservation of each miRNA site was evaluated using phylogenetic branch lengths of all species containing the site based on the methods by Friedman et al (2009). For all highly conserved miRNAs, the probability of preferentially conserved targeting for each site was estimated as described (Friedman et al., 2009). Predicted consequential pairing to 3' end of miRNAs was included only if the raw 3' pairing score (Grimson et al., 2007) is at least 3.0.

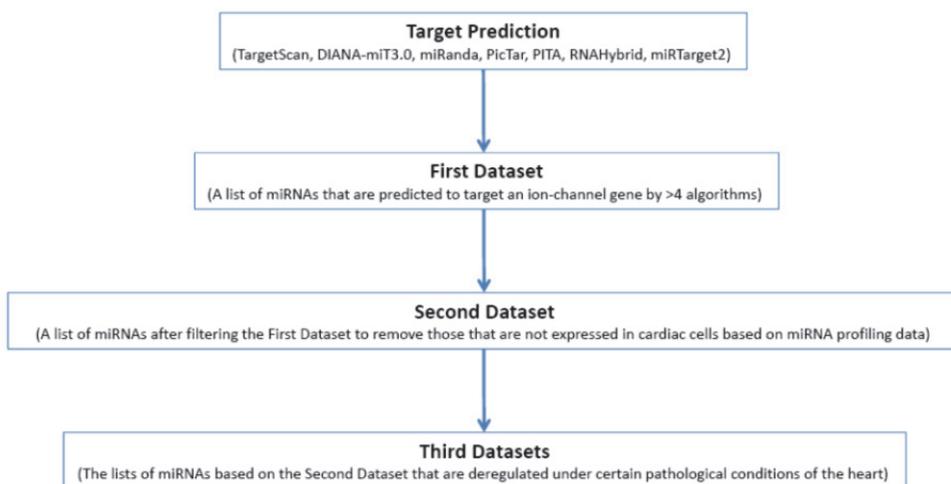


Fig. 1. Flow chart illustrating the procedures of our analysis. The First Dataset include all miRNAs in the then-updated database that have the potential to target at least an ion-channel-coding gene as predicted by at least 4 of the 7 algorithms. The Second Dataset limits the miRNAs from the First Dataset to only those that are expressed in cardiac cells. These miRNAs represent those that most likely play a role in regulating ion channel expression in the heart. The Third Datasets are the lists of miRNAs that have been shown to be deregulated in the pathological conditions under analysis, e.g. myocardial infarction, heart failure, etc. These miRNAs represent those that likely play an important role in defining ion channel expression under a given diseased state.

Each of the seven algorithms provides a unique dataset. Some of the algorithms have higher sensitivity of prediction but lower accuracy and the others weight on the accuracy in the face of reduced sensitivity. We collected all miRNAs predicted by at least four of the seven algorithms to have the potential to target any one of the selected cardiac ion channel and ion transporter genes. Meanwhile, we also collected all ion channel and ion transporter genes that contain the potential target site(s) (the binding site(s) with favourable free energy profiles) for at least one of then-registered 718 human miRNAs in the miRNA database (miRBase).

Expression of miRNAs is tightly controlled by the genetic programme to ensure certain spatial (depending on cell-, tissue-, or organ-type) and temporal (depending on developmental stage) patterns. The expression profile under a defined condition is considered the miRNA expression signature or miRNA transcriptome of a particular tissue. One way to minimize the possibility of false positive predictions and to narrow down the list of putative miRNA targets would be to compare the *in silico* target predictions to the miRNA transcriptome signatures in the biological system of interest. We therefore conducted miRNA microarray analysis of miRNAs including all 718 human miRNAs for their expression in left ventricular tissues of five healthy human individuals. Using this set of cardiac miRNA expression profiling data in conjunction with published data obtained by real-time RT-PCR by Liang et al (2007), we refined the miRNA–target prediction by filtering out the miRNAs that are not expressed in the heart and focusing on the top 20 abundant

miRNAs in human heart (*miR-1*, *miR-133a/b*, *miR-16*, *miR-100*, *miR-125a/b*, *miR-126*, *miR-145*, *miR-195*, *miR-199\**, *miR-20a/b*, *miR-21*, *miR-26a/b*, *miR-24*, *miR-23*, *miR-29a/b*, *miR-27a/b*, *miR-30a/b/c*, *miR-92a/b*, *miR-99*, and *let-7a/c/f/g*). In this way, we generated the modified datasets for subsequent analyses and obtained an overall picture of control of expression of ion channel genes by miRNAs in heart under normal conditions.

Next, we intended to apply the theoretical prediction to explaining some established observations of the electrical remodeling related to deregulation of both miRNAs and the genes for ion channels and transporters. Three pathological conditions, cardiac hypertrophy/heart failure, ischemic myocardial injuries, and atrial fibrillation, were studied based on the expression profiles and participations of miRNAs in these conditions as previously reported. The results are presented in the section following the next.

## 2.2 Control of expression of ion channel genes by miRNAs under normal conditions

The above analyses allow us to reach the following notes.

1. One hundred ninety-three out of 718 human miRNAs or out of 222 miRNAs expressed in the heart have the potential to target the genes encoding human cardiac ion channels and transporters.
2. Only two genes *CLCN2* and *KCNE2* were predicted not to contain the target site for miRNAs expressed in the heart.
3. It appears that the most fundamental and critical ion channels governing cardiac excitability have the largest numbers of miRNAs as their regulators. These include *SCN5A* for  $I_{Na}$  (responsible for the upstroke of the cardiac action potential thereby the conduction of excitations), *CACNA1C/CACNB2* for  $I_{Ca,L}$  (accounting for the characteristic long plateau of the cardiac action potential and excitation-contraction coupling), *KCNJ2* for  $I_{K1}$  (sets and maintains the cardiac membrane potential), *SLC8A1* for *NCX1* (an antiporter membrane protein which removes  $Ca^{2+}$  from cells), *GJA1/GJC1* (gap junction channel responsible for intercellular conduction of excitation), and *ATP1B1* for  $Na^+/K^+$  pump (establishing and maintaining the normal electrochemical gradients of  $Na^+$  and  $K^+$  across the plasma membrane). Each of these genes is theoretically regulated by >30 miRNAs.
4. The atrium-specific ion channels, including *Kir3.4* for  $I_{KACh}$ , *Kv1.5* for  $I_{Kur}$ , and *CACNA1G* for  $I_{Ca,T}$ , seem to be the rare targets for miRNAs (<5 miRNAs).
5. All four genes for  $K^+$  channel auxiliary  $\beta$ -subunits *KCNE1*, *KCNE2*, *KChIP*, and *KCNAB2* were also found to have less number of regulator miRNAs (<10).
6. Intriguingly, 16 of these top 20 miRNAs are included in the list of the predicted miRNA-target dataset; the other four cardiac-abundant miRNAs *miR-21*, *miR-99*, *miR-100* and *miR-126* are predicted unable to regulate the genes for human cardiac ion channels and transporters.
7. There is a rough correlation between the number of predicted targets and the abundance of miRNAs in the heart. It appears that the miRNAs within top 8 separate from the rest 12 less abundant miRNAs in their number of target genes. The muscle-specific miRNA *miR-1* was predicted to have the largest number of target genes (9 genes) among all miRNAs most abundantly expressed in the heart, followed by *miR-30a/b/c*, *miR-24* and *miR-125a/b* that have 6 target genes each. The muscle-specific miRNA *miR-133* has four target genes and three of them (*KCNH2*, *KCNQ1* and *HCN2*) have been experimentally verified (Luo et al., 2008; J Xiao et al., 2007; L Xiao et al., 2008).

8. Comparison of the target genes of the three muscle-specific miRNAs miR-1, miR-133 and miR-208 revealed that they might play different role in regulating cardiac excitability. It appears that miR-1 may be involved in all different aspects of cardiac excitability: cardiac conduction by targeting GJA1 and KCNJ2, cardiac automaticity by targeting HCN2 and HCN4, cardiac repolarization by targeting KCNA5, KCND2 and KCNE1, and Ca<sup>2+</sup> handling by targeting SLC8A1. By comparison, miR-133a/b mainly controls cardiac repolarization through targeting KCNH2 (encoding HERG/*I<sub>Kr</sub>*) and KCNQ1 (encoding KvLQT1/*I<sub>Ks</sub>*), the two major repolarizing K<sup>+</sup> channels in the heart. miR-208 was predicted to target only KCNJ2 (encoding Kir2.1 for *I<sub>K1</sub>*). The non-muscle-specific let-7 seed family members seem to regulate mainly cardiac conduction by targeting SCN5A (Nav1.5 for intracellular conduction) and GJC1 (Cx45 for intercellular conduction). miR-30a/b/c and miR-26a/b, miR-125a/b, miR-16, and miR-27a/b were predicted to be L-type Ca<sup>2+</sup> channel “blockers” through repressing  $\alpha$ 1c- and/or  $\beta$ 1/ $\beta$ 2-subunits.

### 2.3 Application of our bioinformatics analysis to heart failure

The mechanisms for arrhythmogenesis in failing heart involve (Nattel et al., 2007): (1) Abnormalities in spontaneous pacemaking function (enhanced cardiac automaticity) as a result of increases in atrial and ventricular If due to increased expression of HCN4 channel may contribute to ectopic beat formation in CHF; (2) Slowing of cardiac repolarization thereby prolongation of APD due to reductions of repolarizing K<sup>+</sup> currents (including *I<sub>K1</sub>*, *I<sub>Ks</sub>*, and *I<sub>tot</sub>*) provides the condition for occurrence of early afterdepolarizations (EADs) leading to triggered activities; (3) Delayed afterdepolarizations (DADs) due to enhanced Na<sup>+</sup>-Ca<sup>2+</sup> exchanger (NCX1) activity in cardiac hypertrophy/CHF is a consistent finding by numerous studies. Upregulation of NCX1 expression is the major cause for the enhancement; (4) Reentrant activity due to slowing of cardiac conduction velocity.

To date, there have been seven published studies on role of miRNAs and cardiac hypertrophy (Carè et al., 2007; Cheng et al., 2007; Sayed et al., 2008; Tatsuguch et al., 2007; Thum et al., 2007; van Rooij et al., 2006, 2007). The common finding of these studies is that an array of miRNAs is significantly altered in their expression, either up- or down-regulated, and that single miRNAs can critically determine the generation and progression of cardiac hypertrophy. The most consistent changes reported by these studies are up-regulation of miR-21 (6 of 6 studies), miR-23a (4 of 6), miR-125b (5 of 6), miR-214 (4 of 6), miR-24 (3 of 6), miR-29 (3 of 6) and miR-195 (3 of 6), and down-regulation of miR-1, miR-133, miR-150 (5 of 6 studies) and miR-30 (5 of 6). These miRNAs were therefore included in our analysis of target genes encoding ion channel and transporter proteins. Our analyses suggest the following.

1. It is known that cardiac myocytes are characterized with re-expression of the funny current (or pacemaker current) If that may underlie the increased risk of arrhythmogenesis in hypertrophic and failing heart (Luo et al., 2008), which is carried by HCN2 channel in cardiac muscles. We have previously verified that downregulation of miR-1 and miR-133 caused upregulation of HCN2 in cardiac hypertrophy (Luo et al., 2008). This may contribute to the enhanced abnormal cardiac automaticity and the associated arrhythmias in CHF.
2. The NCX1 is upregulated in cardiac hypertrophy, ischemia, and failure. This upregulation can have an effect on Ca<sup>2+</sup> transients and possibly contribute to diastolic

- dysfunction and an increased risk of arrhythmias (Flesch et al., 1996; Nattel et al., 2007; Pogwizd & Bers, 2002). Our target prediction indicates that SLC8A1, the gene encoding NCX1 protein, is a potential target for both miR-1 and miR-30a/b/c. The downregulation of miR-1 and miR-30a/b/c in hypertrophy/failure is deemed to relieve the repression of SLC8A1/NCX1 since a strong tonic repression miR-1 and miR-30a/b/c is anticipated considering the high abundance of these miRNAs. On the other hand, upregulation of miR-214 tends to repress NCX1, but the expression level of miR-214 is of no comparison with those of miR-1 and miR-30a/b/c; its offsetting effect should be minimal. Our prediction thus provides a plausible explanation for the upregulation of NCX1 through the miRNA mechanism.
3. A variety of Na<sup>+</sup> channel abnormalities have been demonstrated in heart failure. Several studies suggest that peak  $I_{Na}$  is reduced which can cause slowing of cardiac conduction and promote re-entrant arrhythmias (Zicha et al., 2004). It has been speculated that post-transcriptional reduction of the cardiac  $I_{Na}$   $\alpha$ -subunit protein Nav1.5 may account for the reduction of peak  $I_{Na}$ . In this study, we found that the only miRNA that can target Nav1.5 and is upregulated in cardiac hypertrophy/CHF is miR-125a/b. As an abundantly expressed miRNA, upregulation of miR-125a/b could well result in repression of SCN5A/Nav1.5.
  4. The gap junction channel proteins connexin43, connexin45 and connexin40 are important for cell-to-cell propagation of excitations. Downregulation of connexin43 expression is associated with an increased likelihood of ventricular tachyarrhythmias in heart failure (Kitamura et al., 2002). Other connexins, including connexin45 (Yamada et al., 2003) and connexin40 (Dupont et al., 2001), are upregulated in failing hearts, possibly as a compensation for connexin43 downregulation. Our analysis indicates that the upregulation of miR-125a/b and miR-23a/b should produce repression of connexin43 and connexin45 and the down regulation of miR-1, miR-30a/b/c and miR-150 should do the opposite. These two opposing effects may cancel out each other.
  5. Prolongation of ventricular APD is typical of heart failure to enable the improvement of contraction strength, thereby supporting the weakened heart. However, APD prolongation consequent to decreases in several repolarizing K<sup>+</sup> current ( $I_{to1}$ ,  $I_{Ks}$ , and  $I_{K1}$ ) in failing heart often results in occurrence of early afterdepolarizations (EADs) (Beuckelmann et al., 1993; Tsuji et al., 2000). Our prediction failed to provide any explanation at the miRNA level: None of the upregulated miRNAs may regulate the genes encoding repolarizing K<sup>+</sup> channels. On the contrary, downregulation of miR-1 and miR-133 predict upregulation of KCNE1/minK and KCNQ1/KvLQT1, respectively.
  6. A majority of published studies showed a decrease in  $I_{K1}$  in ventricular myocytes of failing hearts (Beuckelmann et al., 1993; Rose et al., 2005). But whether KCNJ2/Kir2.1, the major subunit underlying  $I_{K1}$ , is downregulated remained controversial in previous studies and the mechanisms remained obscured. One study noted decreased KCNJ2 mRNA expression but unaltered Kir2.1 protein level (Rose et al., 2005). With our prediction, the upregulated miRNAs (miR-125, miR-214, miR-24, miR-29, and miR-195) predict reduction of inward rectifier K<sup>+</sup> channel subunits including KCNJ2/Kir2.1, KCNJ12/Kir2.2, KCNJ14/Kir2.4, and KCNK1/TWIK1, whereas the downregulated miRNAs (miR-1 and miR-30a/b/c) predict increase in KCNJ2/Kir2.1.
- In summary, our analysis of target genes for deregulated miRNAs in hypertrophy/CHF may explain at least partly the enhanced cardiac automaticity (relief of HCN2 repression

and increased NCX1 expression) and reduced cardiac conduction (repression of Nav1.5). But the data suggest that miRNAs are hardly involved in the abnormality of cardiac repolarization in cardiac hypertrophy and heart failure since the genes for the repolarizing K<sup>+</sup> channels were not predicted as targets for the upregulated miRNAs. The prediction of NCX1 upregulation as a result of derepression from miRNAs may be of particular importance aberrantly enhanced NXC1 activity has also been noticed in atrial fibrillation occurring in CHF.

#### 2.4 Application of our bioinformatics analysis to myocardial infarction (MI)

MI is manifested as cascades of electrical abnormalities and even lethal arrhythmias as a result of deleterious alterations of gene expression outweighing adaptive changes (Carmeliet, 1999). Ischemic myocardium demonstrates characteristic sequential alterations in electrophysiology with an initial shortening of APD and QT interval during the early phase (<15min) of acute ischemia and subsequent lengthening of APD/QT after a prolonged ischemic period and chronic myocardial ischemia (Carmeliet, 1999; Nattel et al., 2007). To exploit if miRNAs could be involved in the remodelling process, several original studies have been published. We first identified upregulation of *miR-1* in acute myocardial infarction and the ischemic arrhythmias caused by this deregulation of *miR-1* expression (Yang et al., 2007). Subsequently, miRNA expression profiles in the setting of myocardial ischemia/reperfusion injuries were reported by four groups (Dong et al., 2009; Luo et al., 2010; Ren et al., 2009; Roy et al., 2009).

Based on these published data, we made an analysis to exclude that miRNAs that were found deregulated by a study but not by others and that were found deregulated in rat heart but was not expressed in human heart. In this way, we identified an array of miRNAs that are likely deregulated in the setting of myocardial ischemia. The MI-upregulated miRNAs include *miR-1*, *miR-23*, *miR-29*, *miR-20*, *miR-30*, *miR-146b-5p*, *miR-193*, *miR-378*, *miR-181*, *miR-491-3p*, *miR-106*, *miR-199b-5p*, and *let-7f*; and the downregulated miRNAs include *miR-320*, *miR-185*, *miR-324-3p*, and *miR-214*. Interesting to note is that some of the miRNAs demonstrated the opposite directions of changes in their expression between ischemic myocardium and hypertrophic hearts. For example, *miR-1*, *let-7*, *miR-181b*, *miR-29a* and *miR-30a/e* are upregulated in ischemic myocardium, but downregulated in hypertrophy. Similarly, *miR-214*, *miR-320* and *miR-351* are down-regulated in ischemic myocardium, but up-regulated in hypertrophy. This fact further reinforces the notion that different pathological conditions are associated with different expression profiles: miRNA signatures. Our analysis yielded the following notions.

1. Six upregulated miRNAs (*miR-1*, *miR-29*, *miR-20*, *miR-30*, *miR-193* and *miR-181*) were predicted to target several Kir subunits (KCNJ2, KCNJ12, KCNJ, and KCNK1), but none of the downregulated miRNAs can target these genes (Fig. 4). This is in line with the previous finding that  $I_{K1}$  is reduced and membrane is depolarized in ischemic myocardium (Carmeliet, 1999; Nattel et al., 2007; Yang et al., 2007).
2. The cardiac slow delayed rectifier K<sup>+</sup> current ( $I_{Ks}$ ) is carried by co-assembly of an  $\alpha$ -subunit KvLQT1 (encoded by KCNQ1) and a  $\beta$ -subunit minK (encoded by KCNE1). Loss-of-function mutation of either KCNQ1 or KCNE1 can cause long QT syndromes, indicating the importance of  $I_{Ks}$  in cardiac repolarization. In ischemic myocardium, persistent decreases in minK with normalized KvLQT1 protein expression have been observed which may underlie unusual delayed rectifier currents with very rapid

- activation (Sanguinetti et al., 1996; Dun & Boyden, 2005), resembling currents produced by the expression of KvLQT1 in the absence of minK. We have experimentally established KCNE1 as a target for *miR-1* repression [Luo et al., 2007], which was also predicted in the present analysis. Moreover, no other miRNAs were predicted to target KCNQ1. This finding is coincident with the observations on the diminishment of minK alone without changes of KvLQT1 in ischemic myocardium.
3. It has been observed that cells in the surviving peri-infarct zone have discontinuous propagation due to abnormal cell-to-cell coupling (Gardner et al., 1985; Peters, 1995; Spear et al., 1983). This is largely due to decreased expression and redistribution of gap junction protein connexins (Cxs). In this study, seven out of 12 upregulated miRNAs were predicted to target Cxs including GJA1/Cx43, GJC1/Cx45, and GJA5/Cx40, but only one downregulated miRNA *miR-185* may regulate GJA5/Cx40. This result clearly points to the role of miRNAs in damaging cardiac conduction in ischemic myocardium. Indeed, repression of GJA1/Cx43 to slow conduction and induce arrhythmias in acute myocardial infarction has been experimentally verified by our previous study (Yang et al., 2007).
  4. In ischemic myocardium, fast or peak sodium current ( $I_{Na}$ ) density is reduced, which may also account partly for the conduction slowing and the associated re-entrant arrhythmias (Friedman et al., 1975; Pu & Boyden, 1997; Spear et al., 1983). Our analysis showed that *let-7f* and *miR-378* may target SCN5A/Nav1.5 and upregulation of these miRNAs is anticipated to cause reduction of  $I_{Na}$  via downregulating SCN5A/Nav1.5 in myocardial infarction. By comparison, none of the downregulated miRNAs may repress SCN5A/Nav1.5 based on our target prediction.
  5. Transient outward  $K^+$  current ( $I_{to1}$ ) is reduced in myocardial ischemia and in rats,  $I_{to1}$  decreases correlate most closely with downregulation of KCND2-encoded Kv4.2 subunits. *miR-1* is predicted to repress KCND2/Kv4.2, and *miR-29* may target KCHIP2 that is known to be critical in the formation of  $I_{to1}$ .
  6. L-type  $Ca^{2+}$  current ( $I_{Ca,L}$ ) is diminished in border-zone cells of dogs. *miR-30* family has the potential to target CACNA1C/Cav1.2 and CACNB2/Cav $\beta$ 2, and *miR-124*, *miR-181*, *miR-320* and *miR-204* to target CACNB2. Upregulation of *miR-30*, *miR-124* and *miR-181* therefore would decrease CACNA1C/Cav1.2 and CACNB2/Cav $\beta$ 2 expression, but downregulation of *miR-320* and *miR-204* tends to increase the expression of these genes. Considering the relative abundance of these miRNAs, it seems that the decreasing force overweighs the increasing force with a balance towards a net inhibition of  $I_{Ca,L}$ .
  7.  $Na^+/K^+$  ATPase is a sarcolemmal ATP-dependent enzyme transporter that transports three intracellular  $Na^+$  ions to the extracellular compartment and moves two extracellular  $K^+$  ions into the cell to maintain the physiological  $Na^+$  and  $K^+$  concentration gradients for generating the rapid upstroke of the action potential but also for driving a number of ion-exchange and transport processes crucial for normal cellular function, ion homeostasis and the control of cell volume. It is electrogenic, producing a small outward current  $I_p$ . We noticed that the ischemia-induced upregulation of *miR-29* and *miR181* expression might render inhibition of  $Na^+/K^+$  ATPase activity as they possibly target the ATP1B1  $\beta$ -subunit of the enzyme. This may contribute to the electrical and contractile dysfunction in the ischemic/reperfused myocardium due to the ischemia-induced inhibition of the  $Na^+/K^+$  ATPase and the failure of intracellular  $Na^+$  to recover completely on reperfusion [Fuller et al., 2003].
- In a whole, it appears that the expression signature of miRNAs in the setting of myocardial ischemia and the predicted gene targeting of these miRNAs coincide with the ionic remodelling process under this pathological condition. The miRNAs seem to be involved in

all aspects of the abnormalities of cardiac excitability during ischemia, as manifested by the slowing of cardiac conduction due to reduced  $I_{Na}$  and  $Cx43$ , the depolarized membrane potential to adversely affect cardiac conduction due to reduced  $I_{K1}$ , the impaired excitation-contraction coupling and contractile function due to reduced  $I_{Ca,L}$  and  $Na^+/K^+$  ATPase, and the delayed cardiac repolarization due to reduced  $I_{Ks}$  and  $I_{to1}$ .

### 3. Apoptosis-related genes as targets for miRNAs

It has been nearly 40 years since Kerr named the novel death process “apoptosis,” from the Greek word meaning “falling of the leaves”, an active process that leads to cell death (Kerr et al., 1972). The human body destroys  $\sim 60 \times 10^9$  cells/day through an apoptotic process in response to various stresses such as physiological, pathogenic, or cytotoxic stimuli (Reed, 2002). Unlike necrosis, apoptosis is a complex endogenous gene-controlled event that requires an exogenous signal—stimulated or inhibited by a variety of regulatory factors, such as formation of oxygen free radicals, ischemia, hypoxia, reduced intracellular  $K^+$  concentration, and generation of nitric oxide. Progressive cell loss due to apoptosis is a pathological hallmark implicated in a wide spectrum of degenerative diseases such as heart disease, atherosclerotic arteries and hypertensive vessels, Alzheimer’s disease, etc (Jaffe et al., 1997; Palojoki et al., 2001; Sabbah et al., 1998). Apoptosis as an early and predominant form of cell death has been detected in human acute myocardial infarcts and it was shown to increase in reperfused myocardium. Apoptosis is also believed to account for the loss of cell mass in failing heart. Evidence for the role of miRNAs in cardiomyocyte apoptosis has been rapidly accumulating. My group documented the first of such evidence; the muscle-specific miRNAs miR-1 and miR-133 produce opposing actions on cardiomyocyte apoptosis with the former being proapoptotic while miR-133 being antiapoptotic (Xu et al., 2007). miR-21 is also an antiapoptotic miRNA. It has been shown to produce beneficial effects against  $H_2O_2$ -induced injury on cardiac myocytes and ischemia/reperfusion injury via antiapoptosis through its target Programmed Cell Death 4 (PDCD4). Based on our computational prediction, many other miRNAs, such as the miR-17~92 cluster and its two paralogs miR-106a~363 and miR-106b~25 clusters, also have the potential to regulate cardiomyocyte apoptosis by targeting the related genes in the signalling pathways (unpublished observations).

Following similar procedures we used to predict ion channel genes as targets for miRNAs described in section 2.1, we analyzed the genes known to be crucial for cell survival and death for miRNA regulation.

#### 3.1 Control of cardiomyocyte apoptosis by miRNAs under normal conditions

Our analyses allowed us to have an overall picture on how the cardiomyocyte homeostasis may be maintained by miRNAs and to divide miRNAs roughly into two groups: pro-apoptotic miRNAs and anti-apoptotic miRNAs, though there is no clear-cut distinction as each miRNA may simultaneously target both survival and apoptotic genes. This property indicates that cardiomyocyte survival and death is tightly controlled and delicately balanced. Any changes of expression of miRNAs can shift the balance leading to alterations of cell fate.

1. Among the top 20 most abundant miRNAs in the heart, only miR-99 and miR-100 have no predicted target genes relevant to apoptosis and others have 1 to 27 targets.
2. The let-7 family, miR-16, miR-20a/b, miR-125a/b, and miR-29a/b were predicted to some major survival genes including BCL2, BCL2L2, AKT2, AKT3, STAT3, IGF0-1 and MCL1. Thus, they are more likely to be pro-apoptotic miRNAs. Indeed, miR-29 has

- been experimentally proven to be pro-apoptotic (Ye et al., 2010). And our unpublished observations indicate a strong promotion of cardiomyocyte apoptosis by miR-20a/b. The studies conducted in cancer cells support our notion that miR-16 induces apoptosis (Cimmino et al., 2005; Tsang & Kwok, 2010), though its effects on cardiac cells have not yet determined.
3. The miR-30 family, miR-24, miR-23a/b, miR-26a/b, miR-27a/b, miR-145, miR-92a/b, and miR-199a/b may be anti-apoptotic miRNAs as they were predicted to target many important cell death genes, such as CASP3 (encoding caspase 3), CASP7, BCL2L11, BAK1, BAX, FOS, etc. Among these miRNAs, miR-199 and miR-24 have been shown to produce cardioprotective effects against apoptosis (Qian et al., 2011). miR-145 is known to mediate inhibition of proliferation and induction of apoptosis of cancer cells (Ostenfeld et al., 2010; Sachdeva & Mo, 2010).
  4. Several miRNAs were predicted to target both survival and apoptotic genes; these include the muscle-specific miRNAs miR-1, miR-133, miR-21, miR-195. In theory, these miRNAs are neutral without affecting cell death or can produce either pro-apoptotic or anti-apoptotic effect depending on particular cellular context: expression of particular target genes for a particular miRNA. Indeed, miR-1 and miR-133 do not affect cardiomyocyte apoptosis under normal conditions, but when many survival and death genes are increased in their expression in response to oxidative stress, miR-1 promotes cardiomyocyte apoptosis by targeting heat shock protein 60 whereas miR-133 protects against apoptosis by repressing caspase 9 (Xu et al., 2007). miR-21 has been commonly believed to elicit cardioprotective effects in myocardial ischemia and ischemia/reperfusion injuries (). But in tumor cells it has been reported to be pro-apoptotic, anti-apoptotic or neutral. For example, knockdown of miR-21 in cultured glioblastoma cells resulted in a significant drop in cell number. This reduction was accompanied by increases in enzyme activity of caspases 3 and 7, as well as terminal deoxyribonucleotidyl transferase-mediated dUTP - digoxigenin nick end-labelling (TUNEL) staining (Chan et al., 2005; Corsten et al., 2007). In MCF-7 human breast cancer cells, miR-21 elicits anti-apoptotic effects (Si et al., 2007; Zhu et al., 2007). However, in neuroblastoma cells, miR-Lat was reported to protect against apoptotic cell death (Gupta et al., 2006). This property of miR-21 in cancer cells is in line with our prediction.
  5. The cardiac-specific miRNAs miR-208a/b and miR-499 do not seem to have significant role in regulating apoptosis since they were predicted to target only a small number of genes involved apoptosis signalling: CDKN1A and E2F6 whose expression levels are low in heart. Moreover, the expression levels of these cardiac-specific miRNAs are also in the low range.

### 3.2 Control of cardiomyocyte apoptosis by miRNAs in ischemic myocardium

Myocardial infarction (MI), a typical situation of metabolic stress, is presented as cascades of cellular abnormalities as a result of deleterious alterations of gene expression outweighing adaptive changes. MI can cause severe cardiac injuries and the consequences are contraction failure, electrical abnormalities and even lethal arrhythmias, and eventual death of the cell. Apoptosis is an important mechanism for the cell death occurring in ischemic myocardium. Previous work on miRNAs and apoptosis has been mostly limited to the context of cancer, while studies on apoptosis regulation by miRNAs in non-cancer cells have been sparse. The first evidence for the role of miRNAs in cardiomyocyte apoptosis was obtained in 2007 from my laboratory demonstrating the proapoptotic effect of miR-1 and anti-apoptotic effect of

miR-133 in response to oxidative stress (Xu et al., 2007), with miR-1 causing proapoptotic effects confirmed by other groups (Yu et al., 2008; Tang et al., 2010). Subsequent studies in 2009 and 2010 revealed the involvement of other miRNAs such as miR-21, miR-24, miR-29, miR-199a, and miR-320 in ischemic myocardial injury (Cheng et al., 2010; Qian et al., 2011; Rane et al., 2009; Ren et al., 2009; Ye et al., 2010; Yin et al., 2008).

Extracting of the overlapping results from different laboratories and filtering with the cardiac expression profile verified by real-time RT-PCR in human hearts allowed us to identify an array of miRNAs that are likely deregulated in the setting of myocardial ischemia. The upregulated miRNAs include miR-1, miR-23, miR-29, miR-20, miR-30, miR-146b-5p, miR-193, miR-378, miR-181, miR-491-3p, miR-106, miR-199b-5p, and let-7f; the downregulated miRNAs include miR-320, miR-185, miR-324-3p, and miR-214. We then applied our procedures to these miRNAs and our analyses yielded the following notions.

1. Among the upregulated miRNAs, only miR-99 and miR-100 have no predicted target genes relevant to apoptosis and others have 1 to 27 targets related to cell survival and death. Notably, a majority of these miRNAs are predicted to be pro-apoptotic: let-7f, miR-1, miR-20, miR-29, miR-106, miR-181, miR-193, miR-378 and miR-491-3p, leaving the other four (miR-23, miR-30, miR-146b-5p and miR-199b-5p) being anti-apoptotic.
2. Among the downregulated miRNAs, except for miR-185 which is expressed with extremely low abundance, miR-214, miR-320 and miR-324 are supposed to be neutral as they were predicted to target both survival (AKT3, STAT3 and MCL1) and apoptotic (CASP3, BAX, CDK6, etc) genes. Their downregulation therefore may not cause significant impact on cell death in MI. However, it has been reported that overexpression of miR-320 in cultured adult rat cardiomyocytes enhanced apoptotic cell death, whereas knockdown produced cytoprotective effect against apoptosis, on simulated ischemia/reperfusion injuries, through targeting HSP20 (Ren et al., 2009), which is not within the list of our present theoretical prediction.
3. Taken together, it appears that the pro-apoptotic force is enhanced more than the anti-apoptotic force, being in agreement with the fact that apoptosis is increased in the setting of MI.

#### **4. Fibrosis-related genes as targets for miRNAs**

In tissues composed of post-mitotic cells, like heart, new cells cannot be regenerated; instead, fibroblasts proliferate to fill the gaps created due to removal of dead cells. In the normal heart, two thirds of the cell population is composed of nonmuscle cells, the majority of which are fibroblasts (Maisch, 1995; Manabe et al., 2002). Cardiac fibroblasts, along with cardiomyocytes, play an essential role in the progression of cardiac remodelling. Damaging insults evoke multiple signalling pathways that lead to coordinate and sequential gene regulation; the initial events lead to the activation of cardiac fibroblasts. Cardiac fibrosis is the result of both an increase in fibroblast proliferation and extracellular matrix (ECM) deposition. Cardiac myocytes are normally surrounded by a fine network of collagen fibres. Myocardial fibrosis is an established morphological feature of the structural myocardial remodelling that is a characteristic of all forms of cardiac pathology (Berk et al., 2007; Khan & Sheppard, 2006). A growing body of evidence indicates that, along with cardiomyocytes hypertrophy, diffuse interstitial fibrosis is a key pathologic feature of myocardial remodelling in a number of cardiac diseases of different (e.g. ischemic, hypertensive, valvular, genetic, and metabolic) origin. Acute myocardial infarction due to coronary artery occlusion represents a

major cause of morbidity and mortality in humans (Fox et al., 2007). The loss of blood flow to the left ventricular free wall of the heart after MI results in death of cardiomyocytes and impaired cardiac contractility. Scar formation at the site of the infarct and interstitial fibrosis of adjacent myocardium prevent myocardial repair, diminish coronary reserve and contribute to loss of pump function, and predisposes individuals to ventricular dysfunction and arrhythmias, which, in turn, confer an increased risk of adverse cardiovascular events (Swynghedauw, 1999). Elucidation of the precise mechanisms responsible for the actions of these factors could forge new frontiers in both risk identification and prevention of fibrosis-derived clinical complications in patients with cardiac disease.

A subset of miRNAs is enriched in cardiac fibroblasts compared to cardiomyocytes. A number of studies have demonstrated the involvement of miRNAs in regulating myocardial fibrosis in the settings of myocardial ischemia or mechanical overload. In this conceptual framework, the investigation of miRNAs might offer a new opportunity to advance our knowledge of the pathogenesis of fibrosis. Characterization of individual miRNAs or miRNA expression profiles that are specifically associated with myocardial fibrosis might allow us to develop diagnostic tools and innovative therapies for fibrogenic cardiac diseases. The identification of miRNAs as potential regulators of myocardial fibrosis has clinical implications; the search for a miRNA expression pattern specific to fibrosis might provide a novel diagnostic approach. Yet, the molecular mechanisms that lead to a fibrogenic cardiac phenotype are still being elucidated.

#### **4.1 Control of cardiac fibrogenesis by miRNAs under normal conditions**

Our analysis revealed that 19 of the 20 most abundant miRNAs in the heart have the potential to repress multiple genes known to be involved in fibrogenesis including various types of collagens (COL), CTGF (connective tissue growth factor), FBN1/2/3 (fibrillin1/2/3), ASPN (asporin), MMP2 (matrix metalloproteinase 2), FN1 (fibronectin 1), and various types of TRP channels (transient receptor potential). miR-126 is the only one among the 20 most abundant miRNAs that was not predicted to regulate any fibrosis-relevant genes. The cardiac-specific miRNAs miR-208a and miR-208b seem to have minimal effects on fibrosis since they have only two target genes *OMG* (oligodendrocyte myelin glycoprotein) and *TTN* (titin). Another cardiac-specific miRNA miR-499 is likely an anti-fibrotic miRNA as it was predicted to target 12 profibrotic factors including collagens, *LAMA1* (laminin 1), *FBN2*, *FN1*, *OMG*, *SLN* (sarcolipin), *TTN*, etc. It should be noted that all these target genes encode profibrotic proteins. Our data therefore indicate that the heart is evolved with a super-strong epigenetic program to prevent fibrogenesis or to suppress fibrosis under normal conditions.

Experimentally, several miRNAs including miR-29, miR-30, miR-133 and miR-590 were all found to produce anti-fibrotic effects (Duisters et al., 2009; Shan et al., 2009; van Rooij et al., 2008), whereas evidence exists for miR-208 (van Rooij et al., 2007) and miR-21 as pro-fibrotic miRNAs (Roy et al., 2009; Thum et al., 2008; van Rooij et al., 2008;). The former can be explained based on our prediction that miR-29, miR-30, miR-133 and miR-590 all have the potential to target profibrotic genes. The latter, however, seems not quite straightforward. Surprisingly, a murine genetic miR-21 knockout model failed to show an antifibrotic phenotype after cardiac stress suggesting differences in pharmacological and genetic miR-21 knockdown (Patrick et al., 2010). Indeed, the various miR-21 inhibitor chemistries have different effects on cardiac fibrosis (Thum et al., 2011). It is likely that other genes not included in our analysis may be the targets for miR-208 and miR-21 to produce pro-fibrotic actions.

## 4.2 Control of atrial fibrogenesis by miRNAs during atrial fibrillation

Atrial fibrillation (AF) is the most commonly encountered clinical arrhythmia that causes tremendous health problems by increasing the risk of stroke and exacerbating heart failure. It is characterized by a process termed atrial structural remodelling with increased atrial fibrosis. Indeed, atrial fibrosis has been strongly associated with the presence of heart diseases/arrhythmias, including congestive heart failure (CHF) and AF (Pellman et al., 2010; Tan & Zimetbaum, 2010).

To determine if miRNAs are involved in atrial structural remodelling, we first conducted expression profiling to identify deregulated miRNAs in the atrial tissues of a canine model of tachypacing-induced chronic AF, using miRNA microarray analysis comparing the differential expressions of miRNAs between control and AF dogs. Four miRNAs miR-223, miR-328, miR-664 and miR-517 were found increased by >2 folds, and six were decreased by at least 50% including miR-101, miR-133, miR-145, miR-320, miR-373 and miR-499. Real-time quantitative RT-PCR (qRT-PCR) analysis confirmed the significant upregulation of miR-223, miR-328 and miR-664 (miR-517 was undetectable), and the significant downregulation of miR-101, miR-320, and miR-499. Intriguingly, none of these deregulated miRNAs are within the list of top 20 most abundant miRNAs. But miR-223 and miR-328 are among the cardiac-enriched miRNAs. This notion would suggest that altered miRNA expression in this AF model tends to favour fibrogenesis; however, miRNAs are definitely not the major determinant for atrial structural remodelling associated with fibrosis.

In a recent study reported by Chen's group (Xiao et al., 2011), it was found that miRNA expression undergoes tremendous alterations in atrial tissues from AF patients with mitral stenosis. Intriguingly, out of 20 most abundant miRNAs in the heart, only let-7b/i and miR-30d were found significantly upregulated but 9 of 20 including miR-29, miR-133, miR-24, miR-26, miR-126, miR-125, miR-99, miR-20, and miR-23 were downregulated. Based on our computational prediction, these changes are expected to result in reduction of the anti-fibrotic force to promote atrial fibrogenesis.

## 5. Conclusion

The theoretical analyses in conjunction with experimental demonstration of miRNA expression profiles under various conditions performed presented here allowed us to establish a matrix of miRNAs that are expressed in cardiac cells and have the potential to regulate the genes encoding cardiac ion channels and transporters, proteins responsible for cell survival and death, and proteins involved in fibrogenesis in heart. These miRNAs likely play an important role in controlling cardiac excitability, cardiomyocyte homeostasis and cardiac fibrosis of the heart. In other words, the genes determining these processes may normally be under the post-transcriptional regulation of a group of miRNAs. Indeed, some of the predicted targets have already been demonstrated experimentally. Also we were able to link a particular remodeling process in hypertrophy/heart failure, myocardial ischemia, or atrial fibrillation to the corresponding deregulated miRNAs under that pathological condition; the changes of miRNAs appear to have anti-correlation with the changes of many of the genes responsible for cardiac electrophysiology, cardiomyocyte apoptosis and cardiac fibrosis under these situations. The present study should aid us to pinpoint the individual miRNAs that can most likely take part in the electrical and structural remodelling processes through targeting particular genes.

It should be noted, however, that the present computational study is in no way to replace experimental approaches for understanding the role of miRNAs in regulating expression of

genes; rather it merely presents a prediction of the odds of miRNA:mRNA interactions under normal situation and in the context of electrical/ionic remodeling under the selected circumstances of the heart. This theoretical analysis like all other computational studies needs to be eventually verified with the bench-top work and should not be considered original results. Nonetheless, with sparse experimental data published to date and the anticipated difficulties to acquire complete experimental data using the currently available techniques, the analytical procedures described here can well serve as first-hand information, providing a framework and guideline for future experimental studies.

The second limitation of the study is the possibility of underestimating the number of miRNAs that could regulate ion channels, apoptosis and fibrosis due to the stringent criterion for inclusion of miRNAs with positive prediction of targets by at least four out of seven algorithms; in the past, we had been able to experimentally verified nearly all the target genes predicted by only one algorithm miRanda for our pre-experiment analysis. However, the fact that our prediction includes all 20 most abundant miRNAs and other highly expressed miRNAs in the myocardium suggests that this limitation might not have significant negative impact on the accuracy of our analysis and inclusion of more miRNAs by more permissive criteria does not guarantee their physiological function if they are scarcely expressed in the heart. Yet it should be noted that the miRNA expression profiles were obtained from myocardium that also includes fibroblasts and caution needs to be taken when interpreting the expression data.

Another important notion is that despite that our prediction of miRNA targeting coincides with the changes of expression of relevant genes under the pathological conditions, it does not imply that miRNAs are necessarily the important or even the only determinant of the electrical remodeling processes. Our data to the most indicate the potential contribution of miRNAs to such conditions; other molecules like transcription factors must also be involved in the regulation of expression of ion channel genes under these conditions.

Finally, it is also difficult to predict the net outcome when two miRNAs target a same gene but alter in their expression in the opposite directions. Yet, with deepened and broadened understanding of miRNA targeting and action, these possible limitations should eventually be worked out.

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