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Regulation of Human Cardiac Ion Channel Genes by MicroRNAs: Theoretical Perspective and Pathophysiological Implications

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Key Words

miRNA • Ion channel • Gene expression • Heart • Myocardial infarction • Heart failure • Atrial fibrillation

Abstract

Excitability is a fundamental characteristic of cardiac cells, which is delicately determined by ion channel activities modulated by many factors. MicroRNA (miRNA) expression is dynamically regulated and altered miRNA expression can render expression deregulation of ion channel genes leading to channelopathies-arrhythmogenesis. Indeed, evidence has emerged indicating the crucial role of miRNAs in controlling cardiac excitability by regulating expression of ion channel genes at the post-transcriptional level. However, the very limited experimental data in the literature hinder our understanding of the role of miRNAs and the often one-to-one interaction between miRNA and ion-channel gene in the published studies also casts a doubt about fullness of our view. Unfortunately, currently available techniques do not permit thorough characterization of miRNA targeting; computational prediction programs remain the only source for rapid identification of a putative miRNA target *in silico*. We conducted a rationally designed bioinformatics analysis in conjunction with experimental approaches to identify the miRNAs from the currently available miRNA databases 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. We established a matrix of miRNAs that are expressed in cardiac cells and have the potential to regulate the genes encoding cardiac ion channels and transporters. We were able to explain a particular ionic remodeling process in hypertrophy/heart failure, myocardial ischemia, or atrial fibrillation with 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 encoding cardiac ion channels under these situations. These results indicate that multiple miRNAs might be critically involved in the electrical/ionic remodeling processes of cardiac diseases through altering their expression in cardiac cells, which has not been uncovered by previous experimental studies.

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Introduction

Cardiac cells are excitable cells that can generate and propagate excitations; excitability is a fundamental characteristic of cardiac cells. Cardiac excitability is conferred by three basic elements: automaticity, cardiac conduction, and membrane repolarization. Automaticity is a measure of the ease of cells to generate excitations or spontaneous membrane depolarization. Conduction refers to the propagation of excitation within a cell and between cells, and cardiac conduction velocity is determined by the rate of membrane depolarization and the intercellular conductance. The rate of membrane repolarization determines the length of action potential duration (APD) and effective refractory period (ERP) thereby the timeframe of availability for generation of a next excitation in a cardiac cell. These three intrinsic properties are reflected by electrical activities in cardiac cells. The electrical activities of the heart are orchestrated by a matrix of ion channels and transporters, the transmembrane proteins that control the movement of ions across the cytoplasmic membrane of cardiomyocytes. Sodium (Na^+) channels determine the rate of membrane depolarization and connexins (Cxs) are critical for gap junction communication, being responsible for excitation generation and inter-cell conductance of excitations, respectively. Calcium channels (mainly L-type Ca^{2+} channels) account for the characteristic long plateau phase of cardiac action potentials and excitation-contraction coupling, and also contribute to pacemaker activities. Potassium (K^+) channels govern the membrane potential and rate of membrane repolarization. Pacemaker channels, which carry the non-selective cation currents, are essential in generating sinus rhythm and ectopic heart beats as well. Intricate interplays of all these ion channels maintain the normal heart rhythm thereby contraction. Channelopathies, diseases caused by dysfunction of the ion channels, which may result from either genetic alterations in ion channel genes or aberrant expression of these genes, can render electrical disturbances predisposing to cardiac arrhythmias [1].

Evidence has emerged indicating the crucial role of microRNAs (miRNAs) in regulating expression of ion channel genes at the post-transcriptional level. The muscle-specific miRNA *miR-1* was shown to produce cardiac conduction disturbance in myocardial infarction [2] and in genetic knockout animal [3]; these studies opened up the new opportunity for studying the pathogenesis of miRNAs in the heart [4, 5]. While the role of miRNAs in oncogenesis and cardiac development has been well appreciated over the past few years, the involvement of miRNAs in the pathological process of cardiovascular system has only been recognized very recently. It is now clear that in addition to their role in cardiac development [6-12], miRNAs are also critically involved in the pathological processes of adult hearts, including cardiac hypertrophy [13-19], heart failure [14, 19], cardiomyopathy [20], angiogenesis [21] and arrhythmogenesis [2, 22-26]. In addition to myocardial infarction, we have also demonstrated the participation of miRNAs in other pathological settings. *miR-133*, another muscle-specific miRNA, was shown to regulate pacemaker channel HCN2 and HCN4 and contributes to the re-expression of these channels in hypertrophy heart [23]. This miRNA had also been shown to repress HERG K^+ channel gene KCNH2 contributing to the abnormal QT prolongation in an animal model of diabetes mellitus [24]. Both *miR-1* and *miR-133* may be involved in the spatial patterns of tissue distribution of ion channels [25].

An important message brought about by previous studies is that miRNA expression is dynamically regulated and altered miRNA expression can render expression deregulation of ion channel genes leading to channelopathies. Functional or mature miRNAs are around 22-nucleotides in length. In order for a miRNA to elicit functional consequences, its 5'-end 7 to 8 nts must have exact or nearly perfect complementarity to the target mRNA, the so-called 'seed' region, and partial complementarity with rest of its sequence [27-31]. A miRNA can either inhibit translation or induce degradation of its target mRNA or both, depending upon at least the following factors: (1) the overall degree of complementarity of the binding site, (2) the number of binding sites, and (3) the accessibility of the bindings sites (as determined by free energy states). The greater the complementarity of the accessible binding sites, the more likely a miRNA degrades its targeted mRNA, and those miRNAs that display imperfect sequence complementarities with target mRNAs primarily result in translational inhibition [27-31]. With better complementarity to the accessible binding sites, a miRNA could more likely degrades its targeted mRNA, and those miRNAs that display imperfect sequence complementarities with target mRNAs primarily result in translational inhibition. Greater actions may be elicited by a miRNA if it has more than one accessible binding site in its targeted miRNA, owing to the potential cooperative miRNA-mRNA interactions

from different sites.

miRNAs are abundant non-coding mRNAs in terms of the species of miRNAs existing in a cell: to date, ~6400 vertebrates mature miRNAs have been registered in miRBase, an online repository for miRNA [32], among which ~5100 miRNAs are found in mammals which include 718 human miRNAs. These miRNAs are predicted to regulates ~30% of protein-coding genes [33, 34]. One common concern that somewhat subsides researchers' inner confidence on the published experimental data on miRNA-target interactions with high-level skeptics and thus hinders our understanding of the function of miRNAs is the possibility that a single protein-coding gene may be regulated by multiple miRNAs and *vice versa* an individual miRNA has the potential to target multiple protein-coding genes. For instance, in our previous study, *miR-1* was shown to target GJA1 (encoding gap junction channel protein connexin43) and KCNJ2 (encoding the Kir2.1 K^+ channel subunit) to cause slowing of cardiac conduction leading to ischemic arrhythmogenesis [2]. However, it is conceivable that GJA1 and KCNJ2 are not the only ion channel targets for *miR-1*; it is also able to repress other genes such as SCN5A, CACNA1C, KCND2, KCNA5 and KCNE1 [4] and whether the repression of these genes other than GJA1 and KCNJ2 also contributes to the ischemic arrhythmogenesis remained unclear. On the other hand, GJA1 is predicted to be regulated by other miRNAs in addition to *miR-1* (including *miR-101, miR-125, miR-130, miR-19, miR-23*, and *miR-30*); whether these miRNAs are also involved in the deregulation of GJA1 in myocardial infarction remained unknown either. This same uncertainty or confusion expectedly exists in the interactions between literally all miRNAs and protein-coding genes. The only way to tackle this problem is the proper experimental approaches.

However, given the laborious nature of experimental validation of targets and the limited available experimentally validated data, computational prediction programs remain the only source for rapid identification of a putative miRNA target *in silico*. While currently available experimental approaches do not allow for 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 genes, appropriate theoretical analyses might aid to resolve this intricate problem. The present study aims to shed light on the issue by performing a rationally designed bioinformatics analysis in conjunction with experimental approaches to identify the miRNAs from the currently available miRNA databases 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.

Materials and Methods

Canine model of atrial fibrillation (AF)

Mongrel dogs (22 to 28 kg) of either sex were randomly divided into two groups: sham control (Ctl, n=6) and atrial tachypacing (n=7) groups. For animals in the A-TP group, dogs were sedated and anesthetized with morphine (2 mg/kg SC) and α-chloralose (120 mg/kg IV load, 29.25 mg/kg/h infusion), for electrode implantation via the jugular veins and atrioventricular (AV) block was created with radiofrequency ablation. A programmable pacemaker was inserted in a subcutaneous pocket with sterile techniques, and a tined atrial pacing lead was positioned in the right atrial appendage under fluoroscopic guidance. The dogs were subjected to continuous right atrial pacing at 400 bpm for 56 days (8 weeks) before experimental studies. The control dogs were sham-operated in the same way as atrial tachypaced dogs but without tachypacing. On study days, dogs were anaesthetized with morphine and α-chloralose and ventilated to maintain physiological arterial blood gases. Body temperature was maintained at 37°C. A median sternotomy was performed, and bipolar, Teflon-coated, stainless steel electrodes were hooked into the right and left atrial appendages for recording and stimulation. A programmable stimulator was used to deliver 2-ms pulses at twice-threshold current. The surface ECG and direct atrial activation electrograms were recorded.

AF vulnerability was tested at a basic cycle length (S1-S1 interval) of 300 ms, with single premature S2 extrastimuli delivered at each site by setting the coupling interval initially to 200 ms and decreasing by 10 ms decrements until AF was induced or failure to capture occurs. For the purpose of measuring AF duration, AF was induced by burst atrial pacing with 4x threshold 4-ms pulses at 20 Hz at a basic cycle length (BCL) of 300 ms. AF was considered sustained if it required electrical cardioversion for termination (cardioversion was performed after 30 min AF). To estimate the mean duration of AF, AF was induced 10 times if AF duration was <5 min, 5 times for AF between 5 and 20 min and 3 times for AF >20 min.

Rat model of myocardial infarction (MI)

Male Wistar rats (220-250 g) were randomly divided into control and MI groups. MI was established as previously described [2]. The rats were anesthetized with diethyl ether and placed in the supine position with the upper limbs taped to the table. A 1-1.5 cm incision was made along the left side of the sternum. The muscle layers of the chest wall were bluntly dissected to avoid bleeding. The thorax was cut open at the point of the most pronounced cardiac pulsation and the right side of the chest was pressed to push the heart out of the thoracic cavity. The left anterior descending (LAD) coronary artery was occluded and then the chest was closed back.

All surgical procedures were performed under sterile conditions. Twelve hours after occlusion, the heart was removed for Langendorff perfusion experiments or the tissues within ischemic zone (IZ), boarder zone (BZ) and non-ischemic zone (NIZ) distal to the ischemic zone were dissected for measurement of miRNA levels. Control animals underwent open-chest procedures without coronary artery occlusion.

Microarray analysis

The hearts were then removed from the dogs or rats and total RNA samples were extracted with Ambion's *mir*Vana miRNA Isolation Kit for miRNA expression analysis. The RNA samples were also isolated from left ventricular walls of healthy human hearts. miRNA expression profiles were analyzed using the miRNA microarray technology miRCURY™ LNA Array (Exiqon Company, Denmark). miRCURY™ LNA Array, including 718 mature human miRNAs plus 650 mature rodent miRNAs, incorporates Locked Nucleic Acid into an oligonucleotide probe, which greatly increases the affinity and specificity of that oligonucleotide for its complementary DNA or RNA target. Slides were scanned by the Genepix 4000B at 635 nm and the expression level was analyzed by Genepix Pro 6.0. The array output was received in Excel spreadsheets as lists of raw data and also as "simple detectable" data, which were the average of 4 signal values for each miRNA on the array. Differentially regulated miRNAs were defined as those with either <0.5- or >2-fold changes in expression for both arrays compared with the baseline expression levels from sham-operated dogs.

Quantitative real-time RT-PCR analysis

The *mir*Vana™ qRT-PCR miRNA Detection Kit (Ambion) was used in conjunction with real-time PCR with TaqMan for quantification of miRNAs in our study, as previously described in detail [2, 22, 23]. qRT-PCR was performed on a thermocycler ABI Prism® 7500 fast (Applied Biosystems) for 40 cycles. Fold variations in expression of an mRNA between RNA samples were calculated. The threshold cycle (C_T) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. To estimate copy numbers of transcript in a cardiac cells, a standard curve was generated by using a series of concentrations of synthetic *miR-1* and converting TaqMan C_T values into absolute copy numbers using the standard curve assuming 30 pg of total RNA in each cell [35, 36].

Computational prediction of miRNA target

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 [37]. 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.

Results and Discussion

Initial analysis of miRNAs with the potential to regulate cardiac ion channel genes

Our study was focused on the genes encoding cardiac cytoplasmic ion channels and electrogenic ion transporters (Table 1). The list includes Na^+ channel, Ca^{2+} channel, inward rectifier K^+ channel subunits, voltage-gated K^+ channel pore-forming α-subunits, ACh-activated K^+ channel α-subunits, ATP-sensitive K^+ channel α-subunit and receptor subunit, pacemaker hyperpolarization-activated cyclic-nucleotide gated cation channels, gap junction channel proteins, transient receptor potential channel subunits, chloride channel subunits, K^+ channel β-subunits, Na^+/Ca^{2+} exchanger NCX1, and Na^+/K^+-ATPase. These cytoplasmic ion channels and electrogenic ion transporters play the fundamental roles in generating, maintaining and shaping cardiac electrical activities (Table 1). Dysfunction of these proteins has been associated with a variety of pathological conditions of the heart.

As an initial "screening" process, we performed miRNA target prediction through the miRecords database [37]. This miRNA database integrates miRNA target predictions by 11 algorithms, as detailed in Methods section. 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) [38-44]. 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) and each of them are expected to provide a unique dataset. Some of them have higher sensitivity of prediction but low accuracy and the other 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 favorable free energy profiles) for at least one of the 718

Table 1. The genes encoding cardiac cytoplasmic ion channel proteins and electrogenic ion transporters selected for this study.

Gene Name	IUPHAR Name	Gene ID	Description
SCN5A	Nav1.5	ENSG00000183873	Pore-forming α-subunit of voltage-dependent Na^+ channel carrying TTX-insensitive Na^+ current (I_{Na}) responsible for phase 0 membrane depolarization of a cardiac action potential thereby cardiac conduction velocity and for type 3 familial long QT syndrome (LQT3) when mutated
SCN4B	Navβ4	ENSG00000105711	β-subunit of cardiac Na^+ channel able to enhance α-subunit conductance and is responsible for type 10 familial long QT syndrome (LQT10) when mutated
CACNA1C	Cav1.2	ENSG00000151067	Pore-forming α-subunit of voltage-dependent dihyropyridine-insensitive Ca^{2+} channel carrying L-type Ca^{2+} current ($I_{Ca,L}$) responsible for phase 2 plateau of a cardiac action potential and excitation-contraction coupling and for type 8 familial long QT syndrome leading to Timothy's syndrome when mutated
CACNB1	Cavβ1	ENSG00000067191	β1-subunit of Ca^{2+} channel able to enhance α-subunit conductance
CACNB2	Cavβ2	ENSG00000165995	β2-subunit of Ca^{2+} channel able to enhance α-subunit conductance
CACNA1G	Cav3.1c	ENSG00000006283	Pore-forming α-subunit of voltage-dependent Ca^{2+} channel carrying T-type Ca^{2+} current ($I_{Ca,T}$) contributing to pacemaker activity of sino-atrial nodal cells
CACNA1I	Cav3.3	ENSG00000100346	Pore-forming α-subunit of voltage-dependent Ca^{2+} channel carrying T-type Ca^{2+} current ($I_{Ca,T}$) contributing to pacemaker activity of sino-atrial nodal cells
KCNJ2	Kir2.1	ENSG00000123700	Pore-forming α-subunit and the major molecular component of inward rectifier K^+ channel carrying inward rectifier K^+ current (I_{K1}) responsible for setting the membrane potential and late phase repolarization of cardiac cells. Mutation of KCNJ2 leads to type 7 familial long QT syndrome (LQT7) and Andersen-Tawil syndrome.
KCNJ12	Kir2.2	ENSG00000184185	Pore-forming α-subunit of inward rectifier K^+ channel carrying inward rectifier K^+ current (I_{K1})
KCNJ4	Kir2.3	ENSG00000168135	Pore-forming α-subunit of inward rectifier K^+ channel carrying inward rectifier K^+ current (I_{K1})
KCNJ14	Kir2.4	ENSG00000182324	Pore-forming α-subunit of inward rectifier K^+ channel carrying inward rectifier K^+ current (I_{K1})
KCNJ3	Kir3.1	ENSG00000162989	Pore-forming α-subunit of acetylcholine-activated inward rectifier K^+ channel carrying ACh-sensitive K^+ current (I_{KACh})
KCNJ5	Kir3.4	ENSG00000120457	Pore-forming α-subunit of acetylcholine-activated inward rectifier K^+ channel carrying ACh-activated K^+ current (I_{KACh}), mainly expressed in atrial cells. It is an atrium-specific ion current
KCNJ8	Kir6.1	ENSG00000121361	Pore-forming α-subunit of ATP-sensitive K^+ channel carrying ATP-sensitive, inward rectifier K^+ current (I_{KATP})
ABCC9	SUR2	ENSG00000069431	Sulfonylurea receptor β-subunit of ATP-sensitive K^+ channel
KCNK1	TWIK1	ENSG00000135750	Pore-forming α-subunit of two-pore inward rectifier K^+ channel
KCNA5	Kv1.5	ENSG00000130037	Pore-forming α-subunit of voltage-gated K^+ channel carrying ultrarapid delayed rectifier K^+ current (I_{Kur}), mainly expressed in human heart. It is an atrium-specific ion current
KCNA4	Kv1.4	ENSG00000182255	Pore-forming α-subunit of voltage-gated K^+ channel carrying transient outward K^+ current (I_{to1}), mainly expressed in rabbit heart
KCND2	Kv4.2	ENSG00000184408	Pore-forming α-subunit of voltage-gated K^+ channel carrying transient outward K^+ current (I_{to1}), mainly expressed in rodent hearts
KCND3	Kv4.3	ENSG00000171385	Pore-forming α-subunit of voltage-gated K^+ channel carrying transient outward K^+ current (I_{to1}), mainly expressed in human heart. Being responsible for phase 1 rapid repolarization and the "spike and dome" morphology of cardiac action potentials
KCNH2	HERG	ENSG00000055118	Pore-forming α-subunit of voltage-gated, *ether-a-go-go*-related K^+ channel carrying rapid delayed rectifier K^+ current (I_{Kr}) responsible for phase 3 repolarization and drug-induced long QT syndrome and type 2 familial long QT syndrome (LQT2) when mutated
KCNQ1	KvLQT1	ENSG00000053918	Pore-forming α-subunit of voltage-gated K^+ channel carrying slow delayed rectifier K^+ current (I_{Ks}) responsible for phase 3 repolarization and drug-induced long QT syndrome and type 1 familial long QT syndrome (LQT1) when mutated
KCHIP2	KChIP2	ENSG00000120049	β-subunit of voltage-gated K^+ channel able to interact with Kv4.2 and Kv4.3 α-subunits to modulate transient outward K^+ current (I_{to1})
KCNE1	minK	ENSG00000180509	β-subunit of voltage-gated K^+ channel carrying slow delayed rectifier K^+ current (I_{Ks}) responsible for phase 3 repolarization and drug-induced long QT syndrome and type 5 familial long QT syndrome (LQT5) when mutated
KCNE2	MIRP1	ENSG00000159197	β-subunit of voltage-gated, *ether-a-go-go*-related K^+ channel carrying rapid delayed rectifier K^+ current (I_{Kr}) responsible for phase 3 repolarization and drug-induced long QT syndrome and type 6 familial long QT syndrome (LQT6) when mutated
KCNAB2	Kvβ2	ENSG00000069424	β-subunit of voltage-gated K^+ channel able to co-assemble with Kv1.4 α-subunit carrying transient outward K^+ current (I_{to1})
CLCN2	ClC2	ENSG00000114859	A molecular component of Cl^- channel thought to play a role in sinus nodal cells
CLCN3	ClC3	ENSG00000109572	A molecular component of volume-sensitive outwardly rectifying Cl^- channel
CLCN6	ClC6	ENSG00000011021	A molecular component of volume-sensitive outwardly rectifying Cl^- channel

(continued next page)

Gene Name	IUPHAR Name	Gene ID	Description
GJA1	Cx43	ENSG00000143140	Connexin43 gap junction channel protein mainly expressed in ventricular cells, responsible for intercellular conduction of excitation and the coordinated depolarization of cardiac muscle
GJA5	Cx40	ENSG00000152661	Connexin40 gap junction channel protein mainly expressed in atrial cells, responsible for intercellular conduction of excitation and the coordinated depolarization of cardiac muscle
GJC1	Cx45	ENSG00000182963	Connexin45 gap junction channel protein mainly expressed in atrio- ventricular nodal cells, responsible for intercellular conduction of excitation
HCN2	HCN2	ENSG00000099822	K^+/Na^+ hyperpolarization- activated cyclic n ucleotide-gated channel 2, the dominant isoform for ventricular funny current (I_f), responsible for abnormal automaticity of the heart
HCN4	HCN4	ENSG00000138622	K^+/Na^+ hyperpolarization -activated cyclic nucleotide -gated channel 4, the major molecular component for sinus funny current (I_f), responsible for phase 4 diastolic spontaneous membrane depolarization and pacemaker activity
TRPC4	TRPC4	ENSG00000133107	Member of the class C (canonical) transient receptor potential channels, activated by phospholipase C stimulation. Its expression is downregulated in cardiac hypertrophy
TRPM4	TRPM4	ENSG00000130529	Member of the class M (melastatin) transient receptor potential channels, activated by intracellular calcium. Its expression is downregulated in cardiac hypertrophy
SLC8A1	NCX1	ENSG00000183023	Na^+/Ca^{2+} exchanger, an antiporter membrane protein which removes Ca^{2+} from cells. The NCX removes a single Ca^{2+} in exchange for the import of three Na^+, generating a net inward current believed to account for delayed after depolarization
ATP1A3	Na/K ATPase α3	ENSG00000105409	The large catalytic α-subunit of the Na^+/K^+ pump, pumping three sodium ions out of the cell for every two potassium ions pumped in after each single action potential for establishing and maintaining the normal electrochemical gradients of Na^+ and K^+ across the plasma membrane
ATP1B1	Na/K ATPase β1	ENSG00000143153	The smaller glycoprotein β-subunit of the Na^+/K^+ pump

human miRNAs. From the above two datasets, we noticed two points. First, out of 718 mature human miRNAs registered in miRBase, 429 miRNAs find their potential target site(s) in the 3'UTR(s) of at least one of the genes encoding cardiac ion channels and ion transporters. Second, all of the genes encoding cardiac ion channels and ion transporters selected for analysis, except for CLCN2, are the potential targets for miRNA regulation.

miRNA expression profiling in human cardiac tissue

Expression of miRNAs in mammalian species under normal conditions is genetically programmed with certain spatial (depending on cell-, tissue-, or organ-type) and temporal (depending on developmental stage) patterns. This property generates the so-called expression signature of a particular tissue. One approach to decrease the incidence of false positive predictions and to narrow down the list of putative miRNA targets would be to compare these *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. We found 220 out of 718 human miRNAs being expressed in the cardiac tissue (http://www.mirna-tech.com/CPB/suppl).

According to the results reported by Liang *et al* for human heart [35], the top 20 abundant miRNAs in human heart are *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* (Fig. 1A). We verified the expression abundance of several selected miRNAs (*miR-1, miR-133a/b, miR-125a/b, miR-30a/b/c*, *miR-26a/b, miR-24, miR-27a/b, miR-23, miR-29a/b, miR-101, miR-21, miR-150* and *miR-328*) using RNA samples isolated from left ventricular tissues of healthy human subjects . A recent study by Rao et al [45] reported a similar array of abundant miRNAs in mouse heart. But differences between the two species exist: e.g. *miR-1* constitutes ~40% of total miRNA content in mouse, but in human, it is ranked the 2nd most abundant miRNA around 1/3 of the *miR-133* level; *miR-208* was found to be one of the top 20 abundant miRNA in mouse but not in human; and *miR-22, miR-143*, *miR-499* and *miR-451* were considered the most abundant miRNAs in mouse heart but not in human heart. Our analysis was focused on the miRNA transcriptome in human heart.

We considered the miRNAs with the same seed sequence as one single miRNA for these miRNAs expectedly have the same set of target genes. This consideration might change the relative abundance of

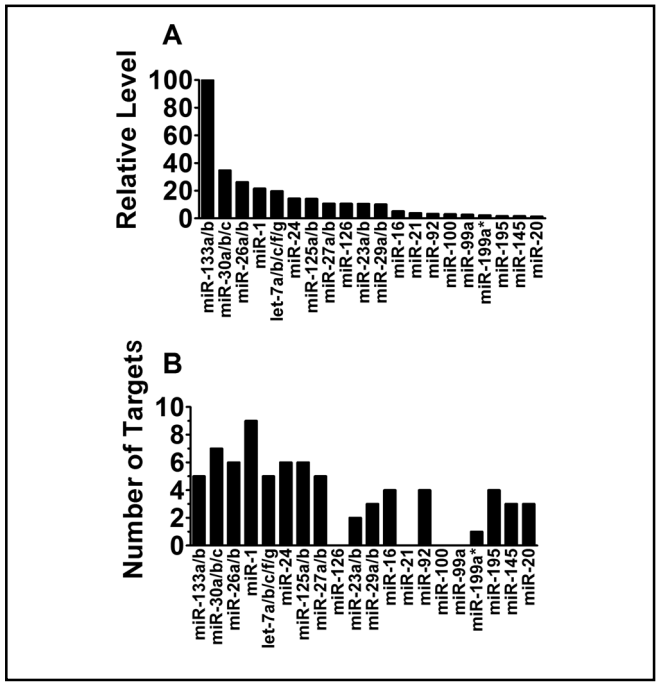

Fig. 1. miRNA expression signature in the heart. (A) Relative levels of the top 20 most abundantly expressed miRNAs in myocardium. (B) Number of predicted target genes of the top 20 most abundantly expressed miRNAs in myocardium. Note that *miR-126, miR-21, miR-99* and *miR-100* are not predicted to target any genes encoding cardiac ion channels and transporters.

miRNAs. For instance, *miR-1* was found more abundant than each of the *miR-30* or *miR-26* isoforms; but was considered less abundant than these latter two miRNAs when the seed family was taken as one miRNA, ranked top 4 after *miR-30a/b/c* (top 2) and *miR-26a/b* (top 3).

Detailed analysis of the miRNAs with the potential to regulate cardiac ion channel genes

Using this cardiac miRNA expression profiling data in conjunction with published data obtained by real-time RT-PCR by Liang et al [35], we refined the miRNA-target prediction by filtering out the miRNAs that are not expressed in the heart. In this way, we generated the modified datasets for subsequent analyses (http://www.mirna-tech.com/CPB/suppl). Detailed analysis of these two datasets revealed the following notes.

(1) One hundred ninety-three out of 718 registered 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 for 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 β-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 (Fig. 1B). 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 [22-25].

(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^{2+} handling by targeting SLC8A1. By comparison, *miR-133a/b* mainly controls cardiac

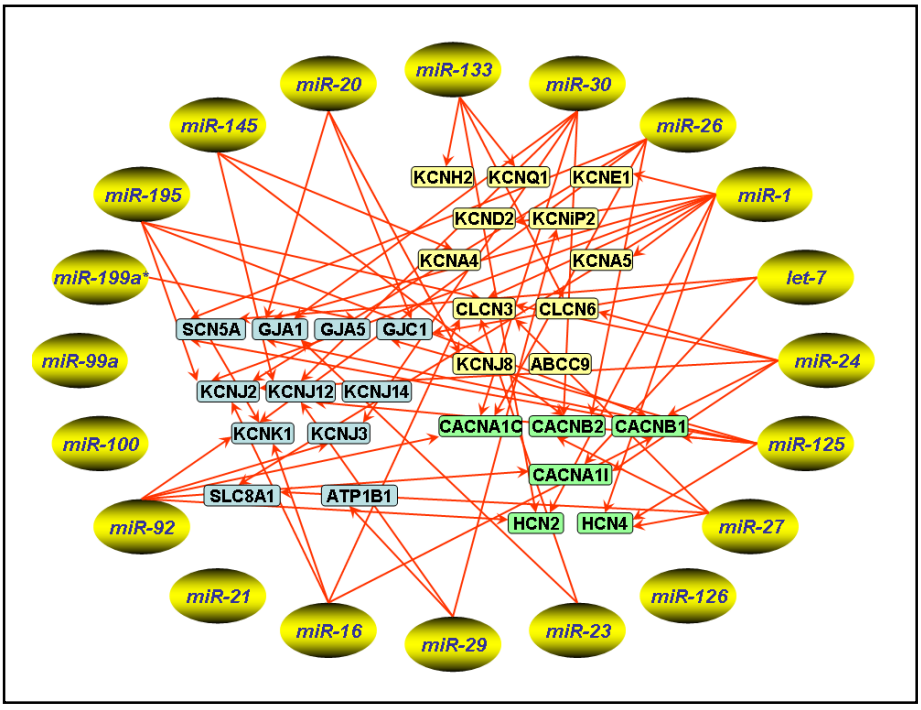

Fig. 2. Predicted gene targeting of the top 20 most abundantly expressed miRNAs in myocardium. The arrows indicate repression of the genes by the connected miRNAs. The target genes are roughly divided into groups in three different colors: the genes for cardiac conduction in blue, the genes for cardiac

repolarization through targeting KCNH2 (encoding HERG/I_{Kr}) and KCNQ1 (encoding KvLQT1/I_{Ks}), the two major repolarizing K^+ channels in the heart. *miR-208* was predicted to target only KCNJ2 (encoding Kir2.1 for I_{K1}). 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^{2+} channel "blockers" through repressing α1c- and/or β1/β2-subunits (Fig. 2).

Application of the theoretical analysis to explaining the electrical remodeling processes of cardiac diseases

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 because the participation of miRNAs in these conditions has previously been investigated.

Cardiac hypertrophy and heart failure

The adult heart is susceptible to stress (such as hemodynamic alterations associated with myocardial infarction, hypertension, aortic stenosis, valvular dysfunction, etc) by undergoing remodeling process, including electrical/ionic remodeling. The remodeling process may originally be adaptive in nature, but is in the face of increased risk of arrhythmogenesis. The mechanisms for arrhythmogenesis in failing heart involve [46]: (1) Abnormalities in spontaneous pacemaking function (enhanced cardiac automaticity) as a result of increases in atrial and ventricular I_f 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^+ currents (including I_{K1}, I_{Ks}, and I_{to1}) provides the condition for occurrence of early afterdepolarizations (EADs) leading to triggered activities; (3) Delayed afterdepolarizations (DADs) due to enhanced Na^+-Ca^{2+} 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 [13-19]. 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-*

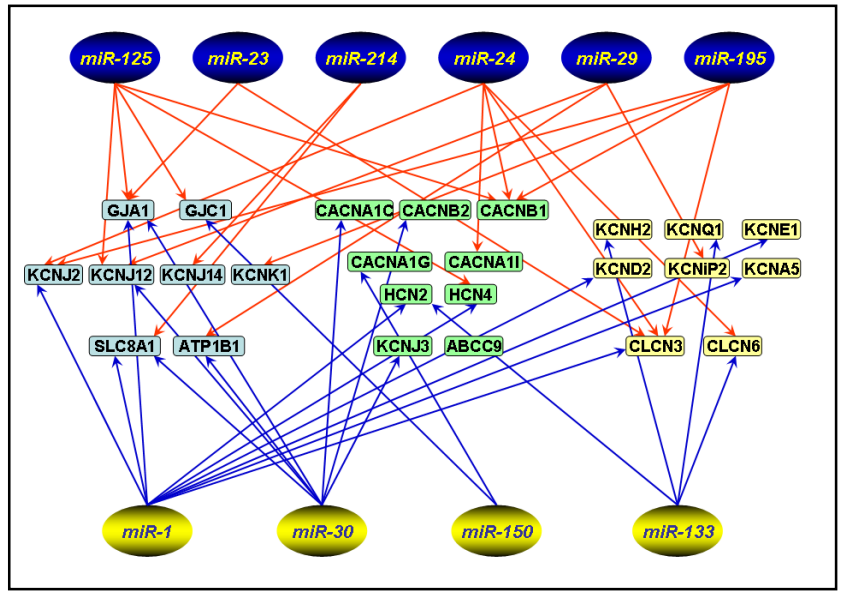

Fig. 3. Predicted gene targeting of the miRNAs deregulated in their expression in cardiac hypertrophy and congestive heart failure (CHF). The arrows in red indicate repression of the genes by the upregulated miRNAs (top row in blue) and those in blue indicate derepression of the genes by the downregulated miRNAs (bottom row in yellow). The target genes are roughly divided into groups in three different colors: the genes for cardiac conduction in blue, the genes for cardiac automaticity in green, and the genes for cardiac repolarization in yellow.

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, as shown in Fig. 3. Our analyses suggest the following.

It is known that cardiac myocytes are characterized with re-expression of the funny current (or pacemaker current) I_f that may underlie the increased risk of arrhythmogenesis in hypertrophic and failing heart [23], 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 [23]. This may contribute to the enhanced abnormal cardiac automaticity and the associated arrhythmias in CHF.

The NCX1 is upregulated in cardiac hypertrophy, ischemia, and failure. This upregulation can have an effect on Ca^{2+} transients and possibly contribute to diastolic dysfunction and an increased risk of arrhythmias [46, 47-51]. Our target prediction indicates that SLC8A1, the gene encoding NXC1 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.

A variety of Na^+ 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 [52-55]. It has been speculated that post-transcriptional reduction of the cardiac I_{Na} α-subunit protein Nav1.5 may account for the reduction of peak I_{Na} [55]. 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.

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 [56]. Other connexins, including connexin45 [57] and connexin40 [58], 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.

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^+ current (I_{to1}, I_{Ks}, and I_{K1}) in failing heart often results in occurrence of early afterdepolarizations (EADs) [59-64]. Our prediction failed to provide any explanation at the miRNA level: None of the upregulated miRNAs may regulate the genes encoding repolarizing K^+ channels. On the contrary, downregulation of *miR-1*

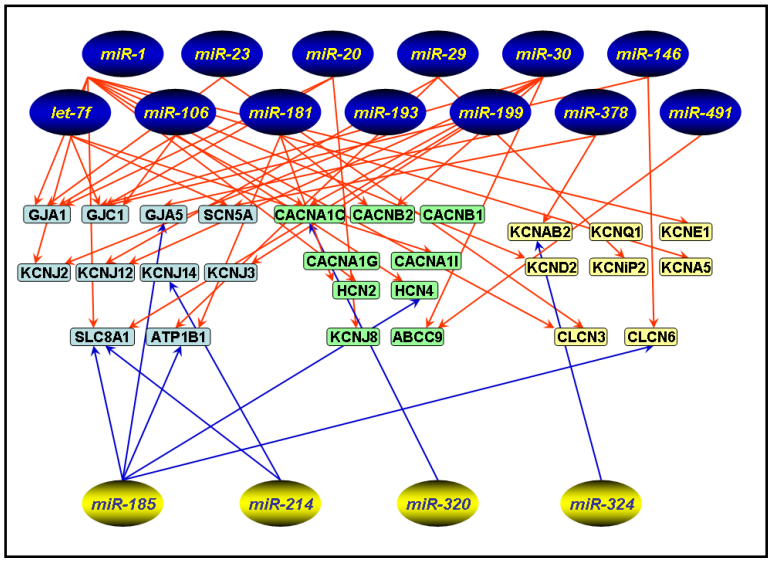

Fig. 4. Predicted gene targeting of the miRNAs deregulated in their expression in ischemic myocardial injuries. The arrows in red indicate repression of the genes by the upregulated miRNAs (top rows in blue) and those in blue indicate derepression of the genes by the downregulated miRNAs (bottom row in yellow). The target genes are roughly divided into groups in three different colors: the genes for cardiac conduction in blue, the genes for cardiac automaticity in green, and the genes for cardiac repolarization in yellow.

and *miR-133* predict upregulation of KCNE1/minK and KCNQ1/KvLQT1, respectively.

A majority of published studies showed a decrease in I_{K1} in ventricular myocytes of failing hearts [46, 61-64]. 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 [64]. With our prediction, the upregulated miRNAs (*miR-125, miR-214, miR-24, miR-29*, and *miR-195*) predict reduction of inward rectifier K^+ 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^+ 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.

Myocardial infarction (MI)

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 [65, 66]. MI can cause severe cardiac injuries and the consequences are contraction failure, electrical abnormalities and even lethal arrhythmias, and eventual death of the cell. 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 [46, 65, 66]. While these alterations may be adaptive to the altered metabolic status, they occur at the cost of arrhythmogenesis consequent to ischemic ionic remodelling. 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 [2]. Similar ischemic *miR-1* upregulation was reproduced by another two groups [67, 68]. Subsequently, miRNA expression profiles in the setting of myocardial ischemia/reperfusion injuries were reported by three groups [69-71].

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* (Fig. 4). This analysis excluded some 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.

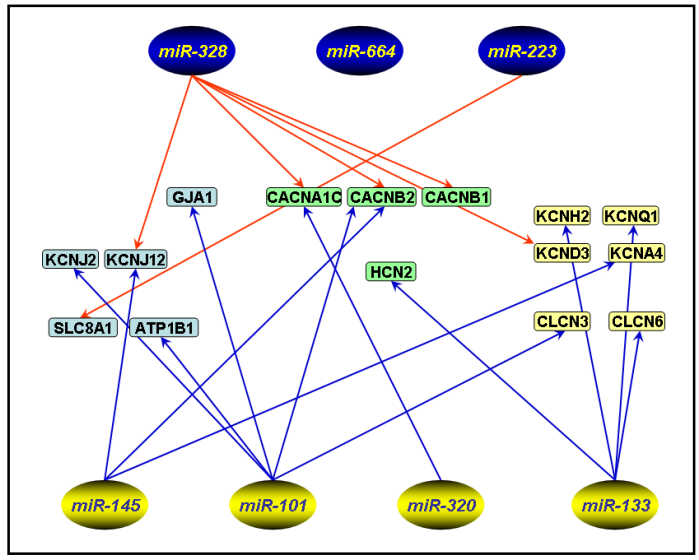

Fig. 5. Predicted gene targeting of the miRNAs deregulated in their expression in experimental atrial fibrillation. The arrows in red indicate repression of the genes by the upregulated miRNAs (top row in blue) and those in blue indicate derepression of the genes by the downregulated miRNAs (bottom row in yellow). The target genes are roughly divided into groups in three different colors: the genes for cardiac conduction in blue, the genes for cardiac automaticity in green, and the genes for cardiac repolarization in yellow

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 (Fig. 3). This fact further reinforces the notion that different pathological conditions have different expression profiles. Our analysis yielded the following notions.

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 [2, 65, 66].

The cardiac slow delayed rectifier K^+ current (I_{Ks}) is carried by co-assembly of an α-subunit KvLQT1 (encoded by KCNQ1) and a β-subunit mink (encoded by KCNE1) [72, 73]. 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 [73-75], resembling currents produced by the expression of KvLQT1 in the absence of minK [72, 73]. We have experimentally established KCNE1 as a target for *miR-1* repression [25], 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.

It has been observed that cells in the surviving peri-infarct zone have discontinuous propagation due to abnormal cell-to-cell coupling [76-78]. 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 (Fig. 5). 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 [2].

In ischemic myocardium, fast or peak I_{Na} density is reduced, which may also account partly for the conduction slowing and the associated re-entrant arrhythmias [79-81]. 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.

I_{to1} is reduced in myocardial ischemia and in rats, I_{to1} decreases correlate most closely with downregulation of KCND2-encoded Kv4.2 subunits [82, 83]. *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}.

$I_{Ca,L}$ is diminished in border-zone cells of dogs [46, 84, 85]. *miR-30* family has the potential to target CACNA1C/Cav1.2 and CACNB2/Cavβ2, 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/Cavβ2 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 $I_{Ca,L}$.

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 [86]. 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 β-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 [87].

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 (Fig. 4). 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}.

Atrial fibrillation (AF)

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 electrical remodeling: the rapid atrial activation rate during AF can remodel the atrial electrophysiology to promote the recurrence and maintenance of AF [46, 88]. A prominent finding in atrial electrical remodeling is shortening of atrial effective refractory period (ERP) favoring re-entrant arrhythmias, primarily because of the shortening of atrial APD as a result of two critical changes. The first change is the reduction of L-type Ca^{2+} current (I_{CaL}) that serves to shorten the plateau duration. And the second change is the increase in inward rectifier K^+ current I_{K1}, which underlies the shortening of the terminal phase [89-91]. Whereas it is known that the expression of the genes for these channels is deregulated during AF, the precise molecular mechanisms remained unclear. Additionally, expression of other ion channels such as KCND3 (encoding Kv4.3 for I_{to1}) and KCNA5 (encoding Kv1.5 for I_{Kur}) has been consistently found downregulated in AF, though the role of the changes in AF is yet to be elucidated [89-91]. The present analysis, however, aids us to get some insight into the issue.

We first conducted expression profiling to identify deregulated miRNAs in the atrial tissues of a canine model of tachypacing-induce 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* (Fig. 5). Our subsequent analysis was therefore based on the deregulated miRNAs verified by qPCR.

Our prediction indicates that three miRNA *miR-328, miR-145* and *miR-320* have the potential to repress both the α1c- and β2-subunits of cardiac L-type Ca^{2+} channel genes, CACNA1C and CACNB2, respectively. While increased miR-328 level should upregulate L-type Ca^{2+} channel expression, decreased *miR-145* and *miR-320* levels should downregulate it. In reality, these two opposing actions may offset each other.

Among the deregulated miRNAs, the only miRNA that may target KCND3 is *miR-328*. Hence, upregulation of *miR-328* predicts downregulation of Kv4.3 thereby reduction of I_{to1} in AF.

Increase in I_{K1} is a hallmark of atrial electrical remodeling in AF. *miR-101* was predicted to target KCNJ2/Kir2.1 and downregulation of this miRNA should upregulate KCNJ2/Kir2.1 due to a relief of repression. Repression of KCNJ12/Kir2.2 due to *miR-328* upregulation may be canceled out by a derepression upon

miR-145 downregulation.

Impulse initiation by automaticity and triggered activity as well as impulse initiation resulting from reentry in AF has been suggested [92]. The hyperpolarization activated cation current or funny current I_f is a candidate for contributing to abnormal automaticity [93]. The downregulation of *miR-133* in AF predicts enhancement of I_f through derepression of HCN2 to induce abnormal cardiac automaticity.

Our data did not predict involvement of miRNAs in the alterations of the genes for I_{Kur}, I_{Kr} and I_{Ks}.

Taken together, it appears that the miRNA expression signature identified in a canine model of tachypacing-induced AF is related to the atrial ionic remodelling process. Specifically, downregulation of *miR-101* and *miR-133* may contribute to enhanced I_{K1} and I_f, respectively, and upregulation of *miR-328* may underlie the reduction of I_{to1}, in AF. Whether this upregulation also contributes to the reduction of $I_{Ca,L}$ need to be examined experimentally. The characteristic decrease of I_{Kur} in AF is unlikely related to miRNA deregulation.

Cardiovascular diseases remain the major cause of mortality and morbidity in developed countries. Most of the cardiac deaths are sudden, occurring secondary to ventricular arrhythmias, the electrical disturbances that can result in irregular cardiac contraction. Abnormally altered cardiac excitability suggests that for arrhythmias to arise, the normal matrix of ion channels and transporters must be perturbed by arrhythmogenic substrates to produce a proarrhythmic conditions to permit rhythmic disturbances caused by impaired excitation conduction/propagation, enhanced automaticity, or abnormal repolarization. In some cases, abnormalities of these ion channels, channelopathies, can be attributed to mutations in the genes encoding the channel proteins, which can predispose to arrhythmias. In other cases, malfunction of ion channels can also be ascribed to abnormally altered expression. The present study aims to acquire an overall picture about the potential expression regulation of ion channel and transporter genes by miRNAs and the possible implications of this regulatory mechanism. The theoretical analysis in conjunction with experimental demonstration of miRNA expression profiles under various conditions performed in this study 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. These miRNAs likely play an important role in controlling cardiac excitability and keeping the normal electrical activities of the heart. In other words, the ion channel genes may normally be under the post-transcriptional regulation of a group of miRNAs in addition to the muscle-specific miRNAs *miR-1* and *miR-133* as already demonstrated experimentally. Also were we able to link a particular ionic 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 encoding cardiac ion channels under these situations. Intriguingly, the miRNA targeting under three different conditions clearly demonstrated three different patterns with that in hypertrophy/CHF showing balanced repression and derepression, in MI showing repression overweighing derepression, and in AF showing the opposite: derepression overweighing repression. Another important notion revealed by this study is that though we have elucidated role of *miR-1* and *miR-133* in controlling cardiac excitability and the associated arrhythmogenesis in the above-mentioned three pathological conditions, it is now clearly that other miRNAs that are deregulated are also likely involved in these processes. In reality, it is conceivable that the electrical/ionic remodeling processes under various conditions are caused by many miRNAs in addition to other regulatory molecules. The present study should aid us to pinpoint the individual miRNAs that can most likely take part in the remodeling 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 for cardiac ion channels and transporters; 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, this study 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 ion channel-regulator miRNAs because of the stringent criterion for inclusion of miRNAs with positive prediction of targets by at least four out of 11 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 ion channel expression 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 above limitations should eventually be worked out.

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