MICRORNA-135A REGULATION OF SODIUM CALCIUM EXCHANGER
GENE EXPRESSION IN CARDIOMYOCYTES

by

ERIC DUONG

Department of Pharmacology and Therapeutics
Faculty of Medicine
McGill University
McIntyre Medical Building
3655 Sir William Osler Promenade
Montreal, Quebec, Canada H3G 1Y6

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MICRORNA-135A REGULATION OF SODIUM CALCIUM EXCHANGER GENE EXPRESSION IN CARDIOMYOCYTES

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ERIC DUONG

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Masters of Science

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This thesis is dedicated to my parents and the countless sacrifices they made to afford me endless possibility; no tears, just dreams.
MicroRNAs (miRNAs) are key regulators of cardiac electrical and structural remodeling and are implicated in pathologies such as arrhythmias, ischemic heart disease and hypertrophy. miRNA expression has yet to be studied in the context of complete atrioventricular block (CAVB) and the subsequent bradycardic arrhythmogenic remodeling. A time course microarray and RT-qPCR analysis of left ventricular miRNA expression in a mouse CAVB model revealed a strong, early and sustained reduction in miR-135a over 4 weeks, a miRNA unexplored in the cardiovascular field. Several other miRNAs were differentially expressed by 4 weeks post-CAVB as well.

To better understand the role of miR-135a in the cardiomyocyte, we identified relevant target genes using literature searches and prediction algorithms, followed by biological confirmation using miR-135a overexpression with luciferase assays, real time qPCR and Western blot. Na\(^+\)-Ca\(^{2+}\) exchanger 1 (NCX1), encoded by the gene SLC8A1, was identified as a regulated gene; miR-135a overexpression reduced NCX1 3’UTR luciferase reporter activity (-42%) and NCX1 mRNA (-47%) and protein (-35%) expression.

Given the significance of NCX1 activity in automaticity and Ca\(^{2+}\) handling in neonatal cardiomyocytes, we sought to investigate the functional effects of miR-135a overexpression in spontaneously beating primary neonatal rat ventricular myocytes (NRVMs). 72-hour overexpression of miR-135a reduced spontaneous beating frequency at 33°C from 147±8 bpm (negative control) to 55±6 bpm, and caused beat-to-beat interval irregularities. Ca\(^{2+}\) transient measurements (fura-2 AM) demonstrated no miR-135a associated alterations when NRVMs were electrically stimulated at 2 Hz; however, rapid application of 20-mM caffeine revealed an increase in decay time constant (+54%) in miR-135a NRVMs. Additionally, the Ca\(^{2+}\) overloading toxic effects of 100-µM ouabain, a cardiac glycoside reliant on NCX1 activity, was
eliminated by miR-135a overexpression, further reducing potentially arrhythmogenic spontaneous Ca\(^{2+}\) release events (SCREs).

The findings of this study illustrate that miR-135a overexpression negatively regulates NCX1 expression, leading to alterations of NRVM automaticity, Ca\(^{2+}\) extrusion rate, arrhythmogenic Ca\(^{2+}\) loading and SCREs. miR-135a may contribute to pro-arrhythmic remodeling following CAVB.
RÉSUMÉ

Les microARNs (miARNs) sont des régulateurs clés des remodelages électrique et structurel cardiaques et sont impliqués dans des pathologies telles que les arythmies, les cardiopathies ischémiques et l'hypertrophie. L'expression des miARNs n'a pas encore été étudiée dans le contexte d’un bloc auriculo-ventriculaire complet (BAVC) et du remodelage arythmogénique bradycardique subséquent. Une puce d’expression à miARNs et l'analyse individuelle par RT-qPCR de l'expression de miARNs du ventricule gauche au cours du remodelage post-BAVC chez la souris ont révélé une forte réduction précoce et soutenue de miR-135a sur 4 semaines, un miARN non étudié dans le domaine cardiovasculaire. Un certain nombre d'autres miARNs sont également différemment exprimés 4 semaines après le BAVC.

Pour mieux comprendre le rôle de miR-135a dans le cardiomyocyte, nous avons identifié des gènes cibles pertinents en se basant sur des recherches bibliographiques et sur des algorithmes de prédiction. Nous avons confirmé ces études par des validations biologiques en étudiant les effets d’une surexpression de miR-135a avec des tests luciférase, de la qPCR en temps réel et de l’immunobuvardage de type Western. L’échangeur Na⁺-Ca²⁺ 1 (NCX1), codé par le gène SLC8A1, a été identifié comme un gène potentiellement régulé par miR-135a. La surexpression de miR-135a est parvenue à réduire l'activité du gène rapporteur Luciférase couplé à la séquence3’UTR de NCX1 (-42%) et réduit également l'expression de l’ARNm de NCX1 (-47%) et de la protéine (-35%).

Étant donné l'importance de l'activité de NCX1 dans l'automaticité et l’homéostasie calcique dans les cardiomyocytes néonataux, nous avons cherché à étudier les effets fonctionnels de la surexpression de miR-135a dans des cardiomyocytes ventriculaires néonataux de rat (NRVM) ayant des battements spontanés. La surexpression de miR-135a durant 72 heures a
permis de réduire la fréquence de battement spontané par minute (bpm) - enregistrée à 33°C - de 147±8 bpm (pour le contrôle négatif) à 55±6 bpm et a également provoqué des irrégularités de l’intervalle mesuré entre chaque battement. Les mesures des transitoires calciques (par la sonde fura-2 AM) n’ont pas montré d’altération associée à la surexpression de miR-135a lorsque les NRVM étaient stimulés électriquement à 2 Hz. Cependant, l’application rapide de 20 mM de caféine a révélé une augmentation de la constante de temps de décroissance des transitoires calciques (+ 54%) dans les NRVM surexprimant miR-135a. En outre, les effets toxiques d’une surcharge calcique par l’ajout de 100 uM d’ouabaïne, un glycoside cardiaque dépendant de l’activité de NCX1, ont été éliminés par une surexpression de miR-135a, réduisant davantage les événements de libération spontanés de Ca²⁺ (SCREs) potentiellement arythmogènes.

Les résultats de cette étude montrent que la surexpression de miR-135a régule négativement l'expression de NCX1, entraînant des altérations de l'automatique des NRVM, du taux d'extrusion du Ca²⁺ et de la charge calcique arythmogène, ainsi que des SCREs. miR-135a pourrait ainsi contribuer au remodelage pro-arythmique suite à un BAVC.
ACKNOWLEDGEMENTS

In retrospect, my tenure in Montreal encompassed some of the most transformative years of my adult life. For the academic milestone I was able to achieve during this time, I have several individuals I would like to acknowledge.

First, I would like to extend my sincere gratitude to Dr. Stanley Nattel, for the opportunity and honor to work under his supervision. His expertise, passion, mentorship and patience were instrumental in the development of my own personal and professional competencies. I only wish I got to better know the man behind the name.

I would like to thank Dr. Paul Clarke, my departmental advisor, for his guidance and brutal honesty, which made McGill feel a little more like home.

To all my colleagues, past and present: Mona Aflaki, Martin Aguilar, Vincent Algalarrondo, Doa’a Al-u’datt, Kuiau Chen, Kirstin Dawson, Roddy Hiram, Hai Huang, Jeremy Liu, Tao Liu, Jonathan Melka, Xiaoyan Qi, Hua Qiang, Ali Saeid, Nazlee Sharmin, Athiththan Sivapiragasam, Yoshihiro Sobue, Yiguo Sun, Artavazd Tadevosyan, Kazuko Tajiri, Patrick Vigneault, Jiening Xiao, Feng Xiong; thanks for the support, guidance and friendship. Do not be strangers!

To Chantal St-Cyr, Natalie L’Heureux, Audrey Bernard and Jennifer Bacchi, thanks for getting me through each and every day with the technical support and organization.

To Patrice Naud, thanks for being a great neighbor and translator.

To my surrogate sisters Yolanda Chen and Sirirat Surikaew, thanks for looking after and putting up with me.

To my lovely neighbors Youri Laplante-El Haili, Xiaoyan Luo, Olivia de Montgolfier, Cecile Martel, Albert Nguyen, Adeline Raignault, Carol Yu; thanks for the running buffer.
To my uncle Quang Trinh Nguyen, the words of wisdom you offered me will never be forgotten.

To my aunt Tu Kinh Duong, the loving care you provided is not overlooked.

To my grandfather Kinh Toan Duong and Lucky, may you both rest in peace.

To my parents and siblings, I am forever grateful for the support and space you have always given me to pursue my passions.

I would finally like to acknowledge the Canadian Institutes of Health Research and the Department of Pharmacology and Therapeutics of McGill University for providing the financial and academic tools necessary to make this work possible.
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LIST OF ABBREVIATIONS

[ ] – concentration

AAV – adeno-associated virus

AP – action potential

APD – AP duration

ATP – adenosine 5’-triphosphate

AVB – atrioventricular block

AVN – atrioventricular node

BPM – beats per minute

Ca^{2+} – calcium ion

CAVB – complete AVB

CICR – Ca^{2+}-induced Ca^{2+}-release

CO – cardiac output

DAD – delayed afterdepolarization

DNA – deoxyribonucleic acid

EAD – early afterdepolarization

ECC – excitation contraction coupling

HF – heart failure

I – current

I/R – ischemia/reperfusion

K^{+} – potassium ion

LTCC – L-type Ca^{2+} channel

LV – left ventricle
MCU – mitochondrial Ca\textsuperscript{2+} uniporter
MI – myocardial infarction
miRISC – microRNA induced silencing complex
miRNA or miR – microRNA
MRE – microRNA response element
Na\textsuperscript{+} – sodium ion
NCX – Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger
NFAT – nuclear factor of activated T cells
NRVM – neonatal rat ventricular myocyte
PLB – phospholamban
PMCA – plasma membrane Ca\textsuperscript{2+} ATPase
pri-miRNA – primary microRNA
RMP – resting membrane potential
RNA – ribonucleic acid
RT-qPCR – real time – quantitative polymerase chain reaction
RV – right ventricle
RYR – ryanodine receptor
SAN – sinoatrial node
SCRE – spontaneous Ca\textsuperscript{2+} release event
SERCA – sarco/endoplasmic reticulum Ca\textsuperscript{2+} ATPase
SR – sarcoplasmic reticulum
TAC – transverse aortic constriction
TdP – torsades de pointes
TMD – transmembrane domain

UTR – untranslated region

VGNC – voltage gated Na⁺ channel
PREFACE – CONTRIBUTION OF AUTHORS

This thesis is written in manuscript form based on the thesis preparation and submission guidelines of the Faculty of Graduate Studies and Research, McGill University.

MicroRNA-135a Regulates Sodium-Calcium Exchanger Gene Expression and Spontaneous Activity

Eric Duong, Jiening Xiao, Xiao Yan Qi, Stanley Nattel

The candidate, Eric Duong, performed cell isolation, culture and transfection for the biological experiments described in this study. The candidate also conducted and obtained experimental data from luciferase reporter assays, real time qPCR, Western blot, cell shortening measurements and Ca\(^{2+}\) fluorescence microscopy. Statistical data analyses and manuscript writing were also completed by the candidate.

Xiao was responsible for the design and cloning of 3’UTR reporter constructs for the luciferase assays, while Qi consulted with the design of the Ca\(^{2+}\) fluorescence microscopy experiments. Nattel assisted with project development and supervision, and provided intellectual and editorial input.
CHAPTER 1 – LITERATURE REVIEW
1.1 Overview

The heart is an electromechanical pump that drives the circulatory system, the main function of which is to supply peripheral organs with nutrients and oxygen, while simultaneously removing metabolic waste. The rhythmic contractions of the heart are the product of the electrical and biomechanical properties of the cardiomyocyte, the predominant and tissue defining cell type that makes up the myocardium and the electrical conduction system of the heart.

Irregularities of the normal rate and/or rhythm of the heart are known as arrhythmias. A bradycardia can be caused by atrioventricular block, where the slow ventricular escape rhythm is independent from the atria. The resulting increased volume load on the ventricles induces homeostatic adaptations in cardiomyocyte gene expression to improve contractility; however, this electrical and structural remodeling is arrhythmogenic and predisposes for a potentially lethal ventricular tachyarrhythmia associated with sudden cardiac death.\textsuperscript{1} The contributory ion channel and structural protein expression changes in this context have been well documented and differs between species.\textsuperscript{2-4}

MicroRNAs are epigenetic, negative regulators of gene expression that have emerged as key players in cardiac physiology and pathology,\textsuperscript{5} but their role in bradycardic arrhythmogenic remodeling is currently unknown.
1.2 Cardiac Structure and Function

1.2.1 Cardiac Structure

The heart is the muscular pump at the center of the circulatory system. Its pump action forces blood through the body’s circulatory network to deliver essential substances such as oxygen and nutrients to all tissues and cells, while synchronously removing metabolic waste products. The atria of the heart receive blood as it enters the heart, while the ventricles pump blood out of the heart (Figure 1). These structures are named right or left, based on their relative position. The heart is contained within a fibrous sac called the pericardium, which lines the epicardium of the heart. The cavities of the atria and ventricles are lined with endocardium, while the muscle mass of the chambers is the myocardium. The myocardium, most evidently in the thicker left ventricle (LV), can be separated into subendocardial, midmyocardial and subepicardial transmural layers.

1.2.2 Cardiac Function

The pulmonary and systemic circulations are maintained by the right and left sides of the heart, respectively. Pulmonary circulation routes deoxygenated blood from systemic circulation to the respiratory system for re-oxygenation and removal of carbon dioxide, a metabolic waste generated by tissues undergoing aerobic respiration. This replenished blood returns to the heart and is directed toward systemic circulation to resupply the tissues. Movement of blood is the product of coordinated muscular systoles (contractions to empty chamber) and diastoles (relaxations to fill chamber) of the four chambers. For each heartbeat, atrial emptying precedes ventricular contraction; this and the overall spontaneous rhythm of the heart is the product of an electrical conduction system that spans the heart. As a unified structure, the volume of blood pumped by the heart within a minute defines its cardiac output (CO), which is calculated as heart
rate multiplied by stroke volume. Changes in CO can be physiological, such as an increase during exercise, or it can be diminished by cardiac pathologies.

**Figure 1.** The four chambers of the heart are made up of several cell types that contribute to the structural, biochemical, mechanical and electrical properties of the functional heart. The electrical conduction system consists of specialized cardiomyocytes that generate and conduct electrical impulses. Resulting contractions force blood throw the chambers, where unidirectional flow is ensured by the presence of one-way valves. From Xin M, Olson EN, and Bassel-Duby R, Nat Rev Mol Cell Biol, 2013.

The electrical conduction system that traverses the heart can be broken into several regions (Figure 1). Pacemaker impulses are typically generated at the sinoatrial node (SAN), a group of specialized cells in the right atrium. This impulse forms an electrical wave that travels through and activates the right then left atrium. The wave collects at the atrioventricular node (AVN), which slows conduction so that the atria can empty before ventricular contraction begins. The electrical wave is then rapidly conducted through the AV bundle consisting of specialized Purkinje cells. The bundle separates into right and left bundle branches, followed by
left anterior and posterior fascicles, that innervate the extensive ventricular tissue. This network, known as the His-Purkinje system, consists of rapidly conducting cells that synchronize ventricular activation for a unified contraction.

1.3 Mechanisms of Cardiac Function

1.3.1 Electrophysiology

Cardiomyocytes, the defining cardiac cell type, possess an excitation-contraction coupling (ECC) system that gives rise to the heart’s electrical and mechanical properties. The electrical function is based on the dynamic movement of ions, including Na\(^+\), Ca\(^{2+}\) and K\(^+\) (Table 1), across the sarcolemma that create either inward (net positive charge enters the cell) or outward (net positive charge leaves the cell) currents, which contributes to a pattern of flux in the membrane potential over a period of time, known as an action potential (AP).\(^6,7\) The classical cardiac AP contains four phases which can be characterized by specific currents and changes in membrane potential (Figure 2).

![](image1)

<table>
<thead>
<tr>
<th>Ion</th>
<th>Intracellular Concentration</th>
<th>Extracellular Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>5 - 34</td>
<td>140</td>
</tr>
<tr>
<td>Potassium</td>
<td>104 - 180</td>
<td>5.4</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.0001 – 0.001</td>
<td>3</td>
</tr>
</tbody>
</table>

*Table 1. Ion concentrations (mM) inside and outside mammalian myocytes. Adapted from Katz AM, Physiology of the Heart. 5th ed. 2011; Fearnley CJ, Roderick HL and Bootman MD, Cold Spring Harb Perspect Biol, 2011.*

The negative resting membrane potential (RMP) in phase 4 is primarily determined by the electrochemical gradient of K\(^+\) as it flows through background K\(^+\) channels, since the membrane is relatively impermeable to other ions. The cardiomyocyte RMP hovers around the K\(^+\) equilibrium potential (-85mV), and is influenced by the inwardly rectifying K\(^+\) current I\(_{K1}\) (inward current) and the Na\(^+\)/K\(^+\) ATPase (outward current). In phase 0, if the cardiomyocyte is depolarized past the threshold potential, voltage gated Na\(^+\) channels (VGNCs) rapidly activate
and inactivate. The influx of Na\(^+\) along its electrochemical gradient further depolarizes the cardiomyocyte to >0mV, which activates several voltage gated currents along the way that contribute to phases 1, 2 and 3. In phase 1, the transient outward K\(^+\) currents \(I_{to,slow}\) and \(I_{to,fast}\) begin a brief early repolarization back to the RMP. In phase 2, while still at more positive potentials, L-type Ca\(^{2+}\) channels (LTCCs) activate to create an inward Ca\(^{2+}\) current \(I_{Ca,L}\); this is counterbalanced by the activation of several outward K\(^+\) currents, such that a plateau in the membrane potential results. In phase 3, the Ca\(^{2+}\) channels close while the delayed rectifier K\(^+\) currents \((I_{Kr}, I_{Ks}, I_{Kur}, I_{K,Ca})\) remain open, restoring the membrane potential to the resting state. By phase 4, these K\(^+\) channels close, while \(I_{K1}\) maintains the RMP and prevents depolarization.

The AP mechanism described above is most representative of the human ventricular cardiomyocyte, and is represented in Figure 2A. While the phase 4 and 0 are similar, the phase 2 plateau is significantly shorter in murine cardiomyocytes because repolarization must be more rapid to allow for a faster resting heart rate. In fact, AP duration (APD) is approximately ten times shorter in mice. Furthermore, in the human, \(I_{Ks}\) and \(I_{Kr}\) are the predominant repolarizing currents, whereas in mice, \(I_{to1}, I_{K,slow1}\) and \(I_{K,slow2}\) and \(I_{ss}\) are the prevailing K\(^+\) currents; this indicates there are species specific ion channel subunit expression profiles. On the other hand, atrioventricular differences in ion channel expression also exist. Human atrial cardiomyocytes have less \(I_{K1}\) than their ventricular counterparts, leading to a more depolarized RMP. They also have an atrial specific ultra-rapid \(I_{Kur}\) current that contributes to earlier repolarization and less Ca\(^{2+}\) influx, indicative of a shorter plateau. Further differences exist between ventricular cardiomyocytes and the spontaneously firing cardiomyocytes in the conducting system, as indicated by their different AP waveforms (Figure 2B). Ultimately, ion channel expression dictates the AP waveform and the function of the particular cardiomyocyte.
All cardiomyocytes express intercalated discs. Fascia adherens and desmosomes keep adjacent cardiomyocytes tightly linked together, while gap junctions formed by connexion channels allow for nonselective and low resistance flow of ions and small molecules. These structures allow all cardiomyocytes to be interconnected, permitting rapid propagation of electrical impulses throughout the myocardium and enhancement of overall organ integrity.

Figure 2. Generation of cardiac electrical activity. A) The various phases of the cardiac action potential and the corresponding electrical ionic currents. B) The direction of electrical impulse propagation from the sinoatrial node and the typical action potentials elicited at different regions of the heart. From Nattel S et al., Physiol Rev, 2007.

1.3.2 Calcium and Contractility

The Ca$^{2+}$ influx during the AP is the essential second messenger for producing contractile activity (Figure 3). Adult cardiomyocytes have specialized transverse tubules, which are invaginations of the sarcolemma that bring clusters of LTCCs into close proximity with the intracellular sarcoplasmic reticulum (SR). The SR is the major contributor to cytosolic Ca$^{2+}$ homeostasis; it expresses sarco/endoplasmic reticulum Ca$^{2+}$ ATPase (SERCA) and ryanodine
receptor channel (RYR) at the membrane, which allow for SR Ca\textsuperscript{2+} influx and efflux, respectively.\textsuperscript{6} Small amounts of Ca\textsuperscript{2+} entering the cardiomyocyte via LTCCs can bind and activate RYRs, which then empties the SR of its Ca\textsuperscript{2+} stores and drastically elevates the systolic [Ca\textsuperscript{2+}].\textsuperscript{17} This amplification of the Ca\textsuperscript{2+} signal is termed Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}-release (CICR), which all for greater activation of the contractile apparatus. Relaxation of the sarcomere and diastole of the myocardium are products of Ca\textsuperscript{2+} uptake into the SR by SERCA and/or extrusion into the extracellular space following ECC by Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX), plasma membrane Ca\textsuperscript{2+} ATPase (PMCA) and mitochondrial Ca\textsuperscript{2+} uniporter (MCU).\textsuperscript{16} Quantitatively, SERCA and NCX play the most significant roles overall.\textsuperscript{18} Their relative expression and contribution to Ca\textsuperscript{2+} removal vary between species and during development; NCX activity is more evident in larger mammals\textsuperscript{19} and earlier in development when the SR is maturing,\textsuperscript{20, 21} but generally SERCA is the dominant mechanism for diastolic removal of Ca\textsuperscript{2+} from the cytosol.

The flux in cytosolic [Ca\textsuperscript{2+}] versus time relationship, from AP onset to sarcomere relaxation, is described as a Ca\textsuperscript{2+} transient. There are several defining features that represent specific movements into and out of the cytoplasm. The basal level from which [Ca\textsuperscript{2+}] rises then falls is termed the diastolic baseline. Here, the Ca\textsuperscript{2+} that activated the myofilaments has been moved into the SR or out of the cell, and the Ca\textsuperscript{2+} that remains is heavily buffered by troponin, calmodulin and membrane binding.\textsuperscript{22} The rise in the transient is the product of CICR, the rate and amplitude of which are regulated by RYR and SR Ca\textsuperscript{2+} content.\textsuperscript{23} SR content can be controlled by SERCA and its interaction with the inhibitory accessory protein phospholamban (PLB). When phosphorylated, PLB relieves its inhibition of SERCA and stimulates the rate of Ca\textsuperscript{2+} uptake into the SR.\textsuperscript{24} SR content is also autoregulated by the cytosolic [Ca\textsuperscript{2+}],\textsuperscript{25} such that if influx increases or efflux decreases, such as with higher stimulation frequency and prolonged
APD, the SR compensates to allow for greater storage. The expression and open probability of RYR are significant factors for determining the fraction of SR content to be released for a given $I_{Ca}$ trigger, termed ECC gain. Open probability can also be increased by phosphorylation, stabilized by FK binding proteins or limited by acidification and reduced cytosolic [ATP].

High SR [Ca$^{2+}$] also stimulates RYR open probability, such that a state of cellular Ca$^{2+}$ overload will promote spontaneous SR Ca$^{2+}$ release; conversely, low SR [Ca$^{2+}$] will typically attenuate CICR in order to allow the Ca$^{2+}$ stores to re-establish themselves. Typically, the SR load and amplitude of the Ca$^{2+}$ transient will correspond with the strength of contraction.

The rate of decay is the product of SERCA, NCX, PMCA and MCU activities that bring the [Ca$^{2+}$] back down to diastolic baseline. During a typical transient, total Ca$^{2+}$ entry via $I_{Ca}$ has an equal sarcolemmal extrusion and total Ca$^{2+}$ released from the SR has an equal reuptake, thus effectively maintaining Ca$^{2+}$ homeostasis within the cardiomyocyte.

The study of Ca$^{2+}$ dynamics has advanced significantly with the use of fluorescent Ca$^{2+}$ sensitive indicators, such as fura-2, that allow for accurate intracellular [Ca$^{2+}$] measurements. Additionally, acute 10-20mM caffeine administration has proven to be a useful tool for studying transient parameters in isolated cardiomyocytes. Caffeine binds the RYR to induce sustained unloading of the SR Ca$^{2+}$ store, thereby inhibiting reuptake by the SR. This enables identification of total SR Ca$^{2+}$ load and the extent of Ca$^{2+}$ decay mediated mainly by NCX extrusion mechanics.
Figure 3. Components and time course of excitation-contraction coupling in the cardiomyocyte. Red arrows indicate paths of Ca$^{2+}$ entry into the cytosol through LTCC and RYR that facilitate the rise of the transient, while green shows removal via SERCA, NCX, PMCA and MCU and corresponds with transient decay. From Bers DM, Nature, 2002.

1.3.3 Automaticity and Rhythmicity

The coupled membrane voltage and intracellular Ca$^{2+}$ clock mechanism has been proposed to explain the spontaneous diastolic depolarization that results in the automaticity of SAN and AVN cardiomyocytes. These cells express several electrogenic membrane proteins that contribute to the surface membrane voltage clock (M clock), that include and are not exclusive to: L-type Ca$^{2+}$ channels (I_{Ca,L}), T-type Ca$^{2+}$ channels (I_{Ca,T}), hyperpolarization-activated cyclic nucleotide-gated channels (“funny” current, I_{f}), delayed outward rectifying K$^+$ currents (I_{K}), NCX (I_{NCX}) and the Na$^+/K^+$ ATPase (I_{Na,K}). Diastolic depolarization can be divided into early and late phases, and M clock involvement begins early. The I_{K} that drives repolarization
conducts less as the membrane becomes more negative, enabling $I_r$ to initiate depolarization and transition toward $Ca^{2+}$ clock involvement.\textsuperscript{35} The $Ca^{2+}$ clock involves all the $Ca^{2+}$ handling machinery concerned in ECC. As the membrane potential returns to its resting state at the end of an AP, the SR $Ca^{2+}$ load is restored by SERCA mediated reuptake. Subsequent rhythmic SR $Ca^{2+}$ release through RYRs activates a depolarizing $I_{NCX}$ that accelerates the late diastolic depolarization phase of the M clock.\textsuperscript{36} The depolarization generated culminates with $I_{Ca,T}$ then $I_{Ca,L}$ activation, initiating an AP and CICR. The M and $Ca^{2+}$ clock rely on each other to function properly; the $Ca^{2+}$ clock requires an AP to replenish $Ca^{2+}$ stores, while the M clock requires intracellular $Ca^{2+}$ increases to drive $I_{NCX}$.\textsuperscript{35} The rate of the pacemaker is therefore dependent on the rates at which $Ca^{2+}$ stores are replenished and the threshold at which spontaneous $Ca^{2+}$ release from the SR begins.

Purkinje fibres exhibit the classic ventricular AP waveform, but possess automaticity that is driven by the clock system.\textsuperscript{37} They have a much slower intrinsic rate, ranging from 25-40bpm in man. The AVN has a firing rate of 40-60bpm, while the SAN sets the physiological heartrate at 60-100bpm.\textsuperscript{6} Embryonic and neonatal cardiomyocytes also rely on spontaneous intracellular $Ca^{2+}$ release to drive automaticity and contractility, where $Ca^{2+}$ flux through RYRs and inositol-1,4,5-triphosphate receptors drive NCX activity.\textsuperscript{38}
1.3.4 Sodium Calcium Exchange

NCX activity, as has been noted, is a critical component of ECC. NCX is carried out by the NCX1 protein expressed in cardiomyocytes. The cardiac isoform is NCX1.1, which is encoded by the SLC8A1 gene. NCX1 is organized into ten transmembrane domains (TMDs) with a large cytoplasmic loop between TMDs5-6. NCX1 is organized into ten transmembrane domains (TMDs) with a large cytoplasmic loop between TMDs5-6. The regions of TMDs2-3 and TMDs7-8 are responsible for ion transport; in the complete structure (Figure 5), they are in close proximity and together they bind three Na$^+$ and one Ca$^{2+}$. The cytoplasmic loop contains two Ca$^{2+}$ binding domains, such that intracellular Ca$^{2+}$ binding causes positive allosteric modulation of NCX activity.
NCX consists of the counter-transport of three Na\(^+\) for one Ca\(^{2+}\), creating a net positive charge difference across the membrane and a detectable ionic current. NCX operates in two directions; the direction of exchange depends on the electrochemical gradients of Na\(^+\) and Ca\(^{2+}\), as well as the membrane potential. It has an equilibrium potential of \(~-40\text{mV}\).\(^{18}\) In forward mode, Ca\(^{2+}\) is extruded with Na\(^+\) influx, moving net positive charge inwardly; in reverse mode, there is an influx of Ca\(^{2+}\) with Na\(^+\) extrusion, creating an outward movement of positive charge.

![Figure 5](image)

**Figure 5.** Topological model of cardiac NCX1. A) Illustration of the transmembrane domains and positions of the regulatory and splicing sites. N and C indicate the N- and C-termini, respectively. B) A model of helix packing of the domains that form the pore. From Shigekawa M, and Iwamoto T, Circ Res, 2001.

NCX activity influences the AP, ECC and Ca\(^{2+}\) cycling in working cardiomyocytes. NCX1 is widely distributed in the sarcolemma, but populations localize in T-tubules in close proximity to VGNCs, LTCCs and RYRs.\(^{42}\) During the Na\(^+\) influx in phase 0, a local rise in intracellular [Na\(^+\)] can induce NCX reverse mode, bringing Ca\(^{2+}\) in and priming ECC by enhancing LTCC and RYR Ca\(^{2+}\) transport.\(^{43}\) At high local [Na\(^+\)] and with the positive membrane potential following phase 0 of the AP, NCX maintains reverse mode activity and elicits an outward current that contributes to repolarization and APD;\(^{44}\) however, as intracellular [Ca\(^{2+}\)] increases with CICR, Ca\(^{2+}\) entry via reverse mode NCX becomes limited. As the membrane
begins to repolarize, NCX acts in forward move to extrude Ca\(^{2+}\). This generates depolarizing currents, similar to those contributing to diastolic depolarization and automaticity.

To maintain Ca\(^{2+}\) homeostasis, NCX expression/function displays an inverse relationship with SR Ca\(^{2+}\) load.\(^ {45}\) Reduced NCX expression with no change in I\(_{Ca}\) allows SERCA to contribute more to Ca\(^{2+}\) removal, thereby enhancing SR content. The opposite is also true. This relationship, however, is affected by resting intracellular [Na\(^{+}\)].\(^ {46}\) A higher [Na\(^{+}\)] will limit Ca\(^{2+}\) efflux via forward mode NCX, leading to higher resting intracellular [Ca\(^{2+}\)] that may further increase coupling gain. This is evident in mice and rats, who have higher cytosolic [Na\(^{+}\)] compared to rabbits, guinea pigs and humans.\(^ {45}\) This is also the case with cardiac glycosides (Figure 6), a family of medicines used to treat heart failure (HF) by improving contractility. They inhibit the Na\(^{+}/K\(^{+}\) ATPase and induce an increase in intracellular [Na\(^{+}\)].\(^ {47}\) Ca\(^{2+}\) efflux via NCX forward mode following CICR becomes severely limited and SERCA must compensate, thereby enhancing the SR Ca\(^{2+}\) load and producing an inotropic effect.\(^ {48}\) The increased intracellular [Na\(^{+}\)] also forces Ca\(^{2+}\) influx via NCX reverse mode, further contributing to the inotropy and Ca\(^{2+}\) overload.\(^ {49}\)

Global knockout of NCX1 in mice leads to cell apoptosis, heartbeat defects and abnormal myofibrillary organization that results in embryonic lethality.\(^ {50}\) Conversely, mice with cardiac-specific loss of NCX1 survive to adulthood, where cardiac function is largely preserved and there are no compensatory changes in expression of Ca\(^{2+}\) handling proteins.\(^ {50}\) These cardiomyocytes have an obvious decline in Ca\(^{2+}\) extrusion capacity, yet have similar Ca\(^{2+}\) transients compared to the wild type. The adaptations that allow this include reduced sarcolemmal Ca\(^{+}\) influx and increased coupling gain, probably due to an increase in [Ca\(^{2+}\)] near
LTCCs and RYRs.\textsuperscript{51} NCX knockout in atrial cells abolishes SAN spontaneous depolarization,\textsuperscript{52} while SAN-specific knockout disrupts rhythmicity by inducing bursts of activity between periods of quiescence,\textsuperscript{53} both studies point to the crucial role of NCX in the coupled clock mechanism.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure6.png}
\caption{The effects of ouabain, a cardiac glycoside, of Ca\textsuperscript{2+} transients are dependent on NCX expression. NCX knockout does not significantly affect the Ca\textsuperscript{2+} transient waveform. From Reuter H et al., Circ Res, 2002.}
\end{figure}

\subsection*{1.4 Atrioventricular Block}

Atrioventricular block (AVB) involves impaired impulse conduction from the atria to the ventricles, which can lead to both ventricular bradyarrhythmia and tachyarrhythmia.\textsuperscript{6} Third-degree AVB, or complete AVB (CAVB), occurs when atrial electrical activity is completely dissociated from the ventricles, due to conduction block through the AVN or the His-Purkinje system.\textsuperscript{1} An idioventricular escape rhythm is generated by a subsidiary pacemaker that is fully isolated from the SAN.

\subsubsection*{1.4.1 Clinical Perspective}

CAVB exists in 0.04\% of the population world wide, but incidence becomes relatively higher in infancy and with advancing age.\textsuperscript{54} Congenital CAVB occurs in one of 20-20000 births, resulting
from maternal antibody mediated damage, usually in association with autoimmune diseases like systemic lupus erythematosus or rheumatoid arthritis.\textsuperscript{55} Acquired CAVB in young and elderly adults is often caused by anatomical lesions. Common sources are coronary artery disease and the associated myocardial infarction (MI), degenerative diseases, dilated cardiomyopathies, infections, autoimmune disorders and infiltrative processes.\textsuperscript{1} Familial lamin A/C and Na\textsuperscript{+} channel mutations, as well as excessive vagal tone, can also contribute to CAVB.\textsuperscript{1} CAVB can also be induced by individual or combinations of medications, such as class 1-4 antiarrhythmics and cardiac glycosides.\textsuperscript{56} CAVB therapy typically involves pacemaker implantation, since the underlying lesions are often irreversible; for example, revascularization of the myocardium following an infarct does not improve CAVB.\textsuperscript{57} If caused by excessive vagal tone or an adverse drug event, CAVB can be reversed by addressing the causal factor(s), as indicated.

Consequences of CAVB without ventricular pacing depend on the rate of the idioventricular escape rhythm, since functional impairment is limited in congenital cases where the rate is nearly normal.\textsuperscript{6} In other cases, the rate can be severely slow and may even cease, reducing CO and contributing to syncopal attacks that make up Stokes-Adams syndrome.\textsuperscript{6} The idioventricular rhythm that exists is a bradyarrhythmia, to which the myocardium adapts with a time course of electrical and structural remodeling that promotes decompensated HF and a polymorphic ventricular tachyarrhythmia termed torsades de pointes (TdP). This pattern of remodeling has been extensively studied in animal models with induced CAVB by radiofrequency or chemical ablation of the His-bundle, primarily in dog but also rabbit and mouse.\textsuperscript{2, 58, 59}
1.4.2 Electrical Remodeling

Electrical remodeling refers to alterations in the electrophysiological properties of the myocardium, which can be visualized at the tissue level with electrocardiogram recordings or in isolated cells with AP and current recordings (Figure 7). Remodeling is typically the result of changes in transcriptional expression or post-translational modification of ion channels or Ca\(^{2+}\) handling proteins, though the underlying causes may be tied to other regulatory factors.

It is well established that electrical remodeling proceeds independently of structural remodeling following CAVB.\(^2,3,60\) Electrical remodeling may cause an acquired long QT syndrome, indicating a slower repolarization rate across the myocardium. This is caused by a rapid reduction in expression of the ion channel subunits responsible for the predominant repolarizing current in cardiomyocytes. In dogs and rabbits, \(I_{Kr}\) (Kv11.1/\(KCNH2\)) and \(I_{Ks}\) (Kv7.1/\(KCNQ1\)) are diminished,\(^{61,62}\) while in mice, \(I_{to,fast}\) (Kv4.2/\(KCND2\) and accessory protein KCHIP2/\(KCNIP2\)) is decreased.\(^2,63\) The ventricular rate following CAVB determines the extent of electrical remodeling, such that maintenance of a physiological rate with a pacemaker prevents any changes in the major repolarizing currents.\(^64\) A reduction in repolarization that prolongs the APD allows for more Ca\(^{2+}\) influx from LTCCs, leading to greater CICR and a more substantial contraction.\(^{65,66}\) In conjunction, an increase in NCX activity may be another contractile adaptation facilitating CICR.\(^67\)
Acquired QT prolongation and torsades de pointes in rabbits with complete atrioventricular block (CAVB). A) Electrocardiogram recording indicate an idioventricular rhythm that emerges following AVB, with time-dependent increases in RR (top), QT (bottom) and B) QRS intervals. C) Spontaneous torsades de pointes (TdP)-like ventricular tachyarrhythmias are evident. Prolonged QT and QRS intervals are explained by increases in D) action potential duration (APD) caused by reductions in E) total repolarizing $K^+$ current. Adapted from Tsuji Y et al., Circulation, 2002.

Acquired long QT syndrome is a clinical feature that predisposes patients to TdP arrhythmias and sudden cardiac death. TdP ventricular tachyarrhythmias result from remodeling that promotes triggered activity caused by afterdepolarizations (Figure 8) that occur either in early (EAD) or late (DAD) repolarization. EADs occur with reduced repolarization reserve, a phenomenon caused by diminished repolarizing currents and/or enhanced depolarizing currents. $I_{Ca,L}$ has an activation-inactivation window between -30mV to -10mV; if repolarization
within this range is sufficiently delayed, $I_{Ca,L}$ can reactivate and depolarize the cell in phase 2 or 3 of the AP.\textsuperscript{69} Thus, EADs are more common at slow rates. NCX can facilitate EADs, since local Ca\textsuperscript{2+} influx via $I_{Ca,L}$ will activate forward mode NCX and thereby induce an inward current during Ca\textsuperscript{2+} efflux.\textsuperscript{70} NCX can also initiate an EAD by generating inward current during efflux of SR Ca\textsuperscript{2+} following CICR or spontaneous release, delaying repolarization and minimizing Ca\textsuperscript{2+}-dependent inactivation so that $I_{Ca,L}$ can reactivate.\textsuperscript{70} While $I_{Ca,L}$ current density is unchanged, inactivation has been found to be shifted to more positive potentials several weeks post-CAVB, increasing the activation-inactivation window that can contribute to EADs.\textsuperscript{66,67} These afterdepolarizations are most likely to occur in Purkinje and midmyocardial cells, the ventricular cells that have the longest APDs.\textsuperscript{6}

On the other hand, DADs are initiated by spontaneous SR Ca\textsuperscript{2+} releases that are products of Ca\textsuperscript{2+} overload, thus they are more common at more rapid rates.\textsuperscript{69} In response to high intracellular [Ca\textsuperscript{2+}], NCX forward mode generates a depolarizing inward current as it extrudes Ca\textsuperscript{2+}, promoting depolarization following full repolarization.\textsuperscript{70}
1.4.3 Structural and Mechanical Remodeling

The structural and mechanical remodeling following CAVB is geared towards maintaining CO and emerges at a time point later than electrical remodeling. Despite stroke volume increasing, there is a reduction in CO immediately following CAVB onset.\textsuperscript{63, 71} The myocardium soon adapts, such that LV and RV systolic function increase to an extent that CO returns to baseline.\textsuperscript{72} The improved contractile performance has been attributed to enhanced NCX activity that leads to pronounced SR Ca\textsuperscript{2+} loading and AP prolongation.\textsuperscript{67} Biventricular eccentric hypertrophy is also evident at this time point; LV and RV mass are greater\textsuperscript{72} and individual myocytes are increased in length.\textsuperscript{67} This state of compensated hypertrophy is associated with increased levels of phosphorylated Akt, which is typically linked to physiological hypertrophy.\textsuperscript{73} While CaMKII activity is also heightened following CAVB, it is uncoupled from the histone
deacetylase/myocyte enhancer factor 2 pathway that promotes deterioration into heart failure; however, CaMKII activity still exerts proarrhythmic effects post-CAVB.\textsuperscript{73}

Long term CAVB is eventually associated with decompensated hypertrophy, which refers to a failure to maintain appropriate CO, though there is a species and rate dependent sensitivity to this outcome.\textsuperscript{74} This is linked to a decreased SERCA to NCX expression ratio that affects contractility and relaxation.\textsuperscript{75, 76} Necrotic and apoptotic cell loss and tissue fibrosis are also common in this late stage remodeling. Overall, it is more so the electrical remodeling, rather than structural, that predisposes to arrhythmic risk following CAVB; in fact, structural alterations have been shown to be reversible while the electrical remodeling and arrhythmias continue to persist.\textsuperscript{77}

1.5 MicroRNA
MicroRNAs (miRNAs) are small non-coding nucleotides involved with epigenetic regulation of gene expression. They are part of a family of non-coding RNAs that are linked to the control of many cell functions and have emerged as important players in cardiac physiology and pathology.\textsuperscript{78} Around 2000 human miRNAs have been catalogued to date, though they display tissue- and developmental stage-specific patterns of expression.\textsuperscript{79, 80}

1.5.1 Mechanisms of Biogenesis and Regulation
Biogenesis\textsuperscript{81} involves DNA transcription of the miRNA encoding sequence by the typical machinery (RNA polymerase II and transcription factors) to produce a primary miRNA (pri-miRNA). Based on their location, miRNAs can be defined as intergenic, which are under the control of their own promoter, or intronic/exonic, which are controlled by their host gene
promoters. Pri-miRNAs are hundreds or thousands of nucleotides long; within them, ~100 nucleotide sequences containing the mature miRNA fold into stem loop structures via imperfect base pairing. These stem loops are excised by the Microprocessor complex containing RNase III endonuclease Drosha and double stranded RNA binding protein DiGeorge syndrome critical region 8, forming the ~60 nucleotide pre-miRNA intermediate. They are transported out of the nucleus by exportin 5 and once in the cytosol, the stem loops are processed by another RNase III endonuclease Dicer, resulting in mature miRNA duplexes.

Argonaut proteins bind a single miRNA duplex to form the miRNA induced silencing complex (miRISC). One strand dissociates and is typically degraded, while the remaining ~18-24 nucleotide seed strand in the miRISC binds to the 3’ untranslated regions (3’UTRs) of mRNA via complementary Watson-Crick base pairing. Canonical binding interactions involve near perfect complementarity of the miRNA seed region (5’ nucleotides 2 through 8) to the miRNA response element (MRE) of the targeted mRNA. This induces translation repression and/or mRNA degradation, depending on overall miRNA-mRNA complementarity, number of MREs in the 3’UTR of the target mRNA and 3’UTR secondary structures surrounding MREs. Given this flexible mechanism (Figure 9), it is possible for multiple miRNAs to target the same mRNA and for one miRNA to target multiple different mRNAs. This enables a highly complex and redundant network of gene regulation that plays crucial roles in development and disease. The relevance of a single miRNA, however, relies on its expression as well as its (in)direct targets that can be predicted by computational methods.
1.5.2 miRNAs in Cardiac Development and Disease

Expression profiling indicates that the 18 most abundant miRNAs in the heart account for 90% of all cardiac miRNAs.\textsuperscript{86} miR-1 and miR-133 are among the most highly expressed; their expression is mediated by cardiac specific transcription networks and thus they play a role in cardiomyocyte differentiation, size and function.\textsuperscript{87} Several miRNAs have stress dependent activation and are linked to structural and electrical remodeling in cardiac disease. miR-208a, miR-208b and miR-499 are encoded within the contractile proteins \textit{MYH6}, \textit{MYH7} and \textit{MYH7B}, respectively, and regulate transcriptional repressors such that they exacerbate pathological remodeling in response to hemodynamic stress.\textsuperscript{88} Conversely, miR-21 and miR-29 are downregulated by cytokines secreted in atrial fibrillation or MI, thereby promoting cardiac fibrosis by reduced translational regulation of ECM proteins including \textit{COL1A1} and \textit{COL3A1}.\textsuperscript{89-91} Nuclear factor of activated T cells (NFAT) signaling is commonly activated in response to chronic elevated intracellular [Ca\textsuperscript{2+}] and is associated with significant remodeling processes.\textsuperscript{81} It was demonstrated that NFAT directly represses miR-26, which is a regulator of I\textsubscript{K1} and promoter of atrial fibrillation.\textsuperscript{92} miR-1\textsuperscript{93} and miR-328\textsuperscript{94} target \textit{CACNA1C} that forms I\textsubscript{Ca,L}, while miR-1, miR-133, miR-212 affect AP repolarization by targeting \textit{KCND2} (I\textsubscript{to}) and \textit{KCNJ2} (I\textsubscript{K1}).\textsuperscript{95} Furthermore, miR-1 and miR-133 are both altered in ischemic heart disease and HF; they have been demonstrated to target protein kinases (\textit{CAMK2D}) and phosphatases (\textit{PP2A}) which regulate LTCC and RYR phosphorylation, Ca\textsuperscript{2+} handling and thus, arrhythmogenesis.\textsuperscript{81} Overall, miRNA control of protein expression exerts a strong influence on structural and electrical remodeling in various cardiac pathologies, the scope of which has yet to be fully determined.
1.5.3 Biomarkers and Therapeutics

miRNAs in the miRISC can be bound by endolysosomes, which serve as vehicles for degradation or secretion.\(^9_6\) As a result, miRNAs can enter the circulation and remain stable and protected from enzymatic degradation. This allows for long distance cell-cell communication when the miRNAs are internalized. Circulating miRNAs also make for good biomarkers by possessing high sensitivity, high specificity and long half lives; many have already been studied for the diagnoses of HF,\(^9_7\) MI\(^9_8\) and numerous pathological states not necessarily related to cardiovascular function.

Potential therapeutic use of miRNAs has seen extensive exploration in recent years. Specific overexpression can be achieved with oligonucleotide mimics or viral vectors to enhance beneficial miRNAs, while inhibition can be attained with anti-sense oligonucleotides to sequester and degrade disease promoting miRNAs.\(^9_9\) Anti-miRNA technologies continue to improve; they have been effective in animal models of HF and atherosclerosis and have shown promise in early clinical phases of research as well.\(^1_0_0\)
Figure 9. Concepts of microRNA function. A) miRNA biogenesis involves transcription and several splicing steps before the mature form is able to affect protein expression. Key characteristics of miRNAs are the B) multiplicity of targets and the C) cooperativity and redundancy, which allow for a robust and diverse regulatory network. D) These characteristics also allow miRNAs to act as buffers against perturbations to biological processes; loss or gain of miRNA function can then have pathological consequences. From Small EM and Olson EN, Nature, 2011.
1.6 Thesis Rationale

It has become increasingly clear that specific cardiac pathologies are accompanied with characteristic changes in the cardiac miRNA expression profile. As negative regulators of gene expression, miRNAs have significant roles in such remodeling processes. Time course studies of miRNA expression in animal models of cardiac disease can provide important and novel mechanistic insights that can contribute to their treatment. While miRNA contributions have been studied previously in cardiac hypertrophy, HF, ischemic heart disease, atrial fibrillation and many other disease states, it has yet to be explored in the context of bradyarrhythmia associated electrical and structural remodeling. The compensatory changes in cardiomyocyte repolarization and mechanics are unique, promoting polymorphic TdP ventricular tachyarrhythmias and an eccentric hypertrophy where cardiac function is largely maintained. Thus, the main goal of this thesis was to investigate the miRNA expression profile in the LV following CAVB in mice and validate relevant targets for any altered miRNAs. miRNA expression was measured at the 24 hour and 4-week time points to coincide with early- and late-phase remodeling following CAVB. miR-135a, previously unexplored in the cardiovascular field, was the only miRNA downregulated within 24 hours, and with a multitude of in vitro assays, several of its predicted, cardiac relevant targets were validated, including NCX1.
CHAPTER 2 – SCIENTIFIC ARTICLE
MicroRNA-135a Regulates Sodium-Calcium Exchanger Gene Expression
and Cardiac Electrical Activity

Eric Duong, BSc,1,2 Jiening Xiao, PhD,2 Xiao Yan Qi, PhD,2 Stanley Nattel, MD1,2,3

From 1Department of Pharmacology and Therapeutics, McGill University, Montreal, Canada,
2Department of Medicine, Montreal Heart Institute and Université de Montréal, and 3Institute of
Pharmacology, West German Heart and Vascular Center, Faculty of Medicine, University
Duisburg-Essen, Essen, Germany

Short title: Duong—MicroRNA-135a, NCX and cardiac electrophysiology

Word count: 4752

Correspondence to: Stanley Nattel, Montreal Heart Institute Research Centre, 5000 Belanger
St. E., Montreal H1T 1C8, Quebec, Canada. Fax: 514-376-1355; Tel: 514-376-3330; e-mail:
stanley.nattel@icm-mhi.org.

A revised manuscript was accepted for publication by Heart Rhythm on January 24, 2017.
Abstract

**Background:** Complete atrioventricular block (CAVB) causes arrhythmogenic remodeling and increases the risk of Torsades de Pointes (TdP) arrhythmias. MicroRNAs (miRNAs) are key regulators of gene-expression that contribute to cardiac remodeling.

**Objective:** To assess miRNA changes after complete atrioventricular block (CAVB) and identify novel candidates potentially involved in arrhythmogenic cardiac remodeling.

**Methods:** CAVB was induced in mice via His bundle ablation. Expression of microRNAs was evaluated by pan-miRNA microarray with qPCR confirmation, on samples obtained 24 hours and 4 weeks post-CAVB. MiRNA target-prediction algorithms were used to identify potential target genes. Targets confirmed by luciferase assays in HEK293 cells were followed up with overexpression studies in neonatal rat ventricular myocytes (NRVMs) to evaluate regulation using RT-qPCR, Western blots, cell shortening measurements and fura-2 Ca$^{2+}$ fluorescence imaging.

**Results:** Of >400 miRNAs assayed, only miRNA-135a was altered at 24 hours, downregulated 78% ($p<0.001$). Algorithms predicted miRNA-135a regulation of the sodium-calcium exchanger type-1 (NCX1). miRNA-135a transfection suppressed NCX1 3’UTR reporter activity by 42% ($p<0.001$), mRNA expression by 34% ($p<0.001$) and protein levels by 45% ($p<0.001$), versus noncoding miRNA control. miR-135a overexpression reduced spontaneous beating frequency of NRVMs by 63% ($p<0.001$), while slowing decay (by 56%, $p<0.05$) of caffeine-induced Ca$^{2+}$ transients. miR-135a also suppressed the Ca$^{2+}$-loading effects of ouabain and ouabain-induced spontaneous Ca$^{2+}$-release events (SCREs).
Conclusions: NCX1 is negatively regulated by miR-135a, a microRNA that is downregulated in the heart following CAVB in mice. By controlling NCX1 expression, miR-135a modulates cardiomyocyte automaticity, Ca^{2+}-extrusion and arrhythmogenic Ca^{2+}-loading/SCREs. miR-135a may therefore contribute to pro-arrhythmic remodeling following CAVB.

Keywords: microRNAs, miR-135a, Na^{+}/Ca^{2+} exchanger, post-transcriptional regulation, atrioventricular block
**Introduction**

MicroRNAs (miRNAs) are small non-coding nucleotide sequences that act as negative regulators of gene expression.¹ Substantial evidence suggests that miRNAs are stress responsive and regulate electrical, hypertrophic, apoptotic and fibrotic alterations in pathological cardiac remodeling.²⁻⁴

Complete atrioventricular block (CAVB) results in a slow ventricular escape rhythm, which is followed by electrical and structural remodeling.⁵ Electrical remodeling reduces repolarization reserve and promotes Torsades de Pointes (TdP) ventricular tachyarrhythmias. Dilation and hypertrophy allow for maintenance of cardiac output in the face of severe bradycardia. The cellular basis for these electrophysiological and structural changes have been studied extensively in several animal models,⁶⁻⁸ but miRNA involvement has yet to be investigated.

Here, we performed left-ventricular miRNA expression-profiling in the hearts of mice with CAVB for 24 hours and 4 weeks. We identified miR-135a as a miRNA of interest, and explored the predicted targets and functional consequences with *in vitro* models, identifying a role in the regulation of the Na⁺-Ca²⁺ exchanger (NCX) and associated electrical activity.

**Methods**

*Mouse Model of Complete Atrioventricular Block*

The atrioventricular node mouse model has been described previously.⁸ Briefly, under general anesthesia a radiofrequency ablation catheter inserted fluoroscopically via a jugular vein was used to produce CAVB. All animal experimentation protocols followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (2011) and were approved by the Animal Research Ethics Committee of the Montreal Heart Institute.
**miRNA Microarray**

Left ventricular free wall samples were collected at baseline, 24 hours and 4 weeks after CAVB, and flash-frozen in liquid-N2. RNA was extracted with miRNeasy MiniKit (Qiagen, Germany) and concentrations measured with Nanodrop-2000 (ThermoFisher, USA). RNA quality was verified with an Agilent-2100 Bioanalyzer (Agilent Technologies, USA). 750-ng total RNA-samples were labeled with Hy3TM and Hy5TM (miRCURY LNA™ miRNA Hi-Power Labeling Kit, Exiqon, Denmark). Hy3TM- and Hy5TM-labeled RNA were mixed pair-wise and hybridized to the miRCURY LNA™ miRNA Array 7th Gen (Exiqon, Denmark, containing capture-probes targeting all miRNAs for human, mouse or rat in miRBASE 18.0) with a Tecan HS4800™ hybridization station (Tecan, Austria). Microarray slides were scanned with the Agilent G2565BA Microarray Scanner System (Agilent Technologies, USA) and image-analysis obtained with ImaGene® 9 (Exiqon, Denmark). Signals were background-corrected and normalized via global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm.

**Cell Culture**

H9c2 cells (ATCC, USA) were cultured in DMEM containing 10% heat-inactivated fetal bovine serum (FBS) and 1%-penicillin/streptomycin. HL-1 cells (Sigma-Aldrich, USA) were cultured in Claycomb media supplemented with 10% FBS, 1% P/S, 100-µM norepinephrine and 2-mM L-glutamine. Neonatal rat ventricular myocytes (NRVMs) were isolated from 2-3 day old Wistar rats (Charles River, USA). Following decapitation, their hearts were extracted and washed with Ca2+- and Mg2+-free Hank’s Balanced Salt Solution. The atria were removed and ventricles were minced. The tissue was digested with 50-µg/mL trypsin overnight at 4°C. The next day, 2000-µg soybean trypsin inhibitor was added, followed by further digestion with 1500 U purified collagenase reconstituted in 5 mL Leibovitz L-15 medium and constant agitation for 20 mins at
37°C. Digested tissue was tritured and centrifuged at 60×g for 5 mins. Cells were re-suspended in complete growth medium (Medium 199 with 10% FBS, 1% P/S, 0.2% ITS) and plated on Cell+ culture dishes (Sarstedt, Germany) for 3 hours to limit fibroblast content. NRVMs were seeded at a density of 5.5X10^4 cells/cm^2, media were changed after 24 hours and cells were transfected at 48 hours. Enzymes and reagents used for isolation/culture procedure were obtained from Worthington Biochemical (USA).

**Manipulation of miR-135a Expression**

Sense and anti-sense strands of the miR-135a duplex were synthesized (Invitrogen, USA), diluted to 200-µM with RNase-free water, combined 1:1, heated to 95°C for 5 mins and left to cool at room temperature. Scrambled negative control noncoding (NC) miRNA was prepared in an identical manner. H9c2 cells, HL-1 cells and NRVMs were transfected with 30-nM miRNA with Lipofectamine RNAiMAX (Thermo Fisher, USA) in a 1:1 ratio with the appropriate complete growth media and Opti-MEM (Thermo Fisher, USA). The control treatment consisted of no Lipofectamine or miRNA with the new media. The appropriate complete growth medium was re-introduced after 24 hours and cells were studied after 72 hours.

HEK-293 cells (ATCC, USA) were cultured in DMEM containing 10% FBS and 1% P/S, and transfected with 100-ng vector DNA using Lipofectamine 3000 (Thermo Fisher, USA), along with the appropriate miRNA (10-nM). Samples were collected after 24 hours.

**Confirmation of miR-135a Targets**

Prediction algorithms (TargetScan, microT-CDS and TarBase) and PubMed database searches were used to identify potential cardiac-relevant targets based on 8-mer or 7-mer miR-135a specific miRNA recognition elements (MREs). Based on miRNA and target gene 3’UTR alignment with Clustal Omega, ~100bp DNA-sequences with the target gene MREs were
synthesized (Invitrogen, USA). Sequences were amplified with forward primers containing an XhoI restriction-site and reverse primers containing an XbaI restriction-site (Advantage-2 PCR Kit, Clontech, USA). These sequences, as well as pmirGLO Dual Luciferase miRNA Target Expression Vectors (Promega, USA), were digested with XhoI and XbaI (New England Biolabs, USA), then annealed and ligated into the vector. Ligated vectors were transformed using high efficiency chemically competent DH5α (Thermo Fisher, USA), selected from ampicillin-containing plates and purified with QIAprep Spin Miniprep Kit (Qiagen, Germany). DNA concentration was determined with Nanodrop 2000. 24 hours after HEK-293 transfection, the Dual-Luciferase Reporter Assay System (Promega, USA) was used to measure luciferase activity (normalized to control renilla luciferase activity) with a Synergy 2 Multi-Mode Reader (Biotek, USA). All sequences are listed in Supplemental Table S1.

**Determination of miRNA, mRNA and Protein Expression**

mRNA was extracted with NucleoSpin RNA (Machery-Nagel, Germany) and miRNA with the miRNeasy Mini Kit (Qiagen, Germany). First-strand cDNA was synthesized with the High Capacity cDNA Reverse Transcription Kit or TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, USA), using random primers and 100-ng RNA for mRNA, or miRNA-specific TaqMan assay primers and 10-ng RNA for miRNA, followed by thermal cycling (2720 Thermal Cycler, Applied Biosystems, USA). Expression was measured in duplicate with the Applied Biosystems StepOne Real-Time PCR System and 6-carboxy-fluorescein labeled TaqMan primers (Applied Biosystems), TaqMan Fast Advanced Master Mix and 5-ng mRNA or 0.5-ng miRNA. Thermal cycling began at 95°C×20 seconds, followed by 40 cycles of 95°C×1 second and 60°C×20 seconds. Relative expression was calculated with the ΔC_T and ΔΔC_T.
method, with the geometric mean of \textit{GAPDH, HPRT1} and \textit{B2M} as reference for mRNA and \textit{U6} for miRNA.

Protein was collected with RIPA-buffer containing (mM) 150 NaCl, 50 Tris-HCl (pH 7.4, NaOH), 10 NaF, 5 EDTA, 1 EGTA, 0.5% Na deoxycholate, 1% Triton X-100, 0.1% SDS and a cocktail of protease and phosphatase inhibitors. Protein concentrations were determined via Bradford assay (Bio-Rad, USA) on an Ultrospec 2100 spectrophotometer (GE Healthcare, USA). Minimum 10-µg total protein in Laemmli buffer, preheated at 95°C for 5 mins, was separated via SDS-PAGE on 4-20% Mini-PROTEAN TGX Precast Gels (Bio-Rad, USA; 120 V, 90 mins), followed by wet transfer to Immobilon-P 0.45-µm polyvinylidene difluoride membranes (Millipore, USA) at 90 V for 90 mins. Membranes were blocked with 5% non-fat milk in TBST for 1 hour and incubated with primary antibodies in TBST overnight at 4°C: 1:500 HCN2 (S71-37, Abcam, UK), 1:500 HIF-1A (EP1215Y, Abcam, UK), 1:500 mineralocorticoid-receptor (H-300, Santa Cruz Biotechnology, USA), 1:500 MEF2C (6H2G2, Thermo Fisher, USA), 1:1000 NCX1 (6H2, Abcam, UK), 1:10000 GAPDH (6C5, Fitzgerald, USA). Membranes were then washed and incubated with secondary antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch Inc, USA) at 1:1000 in 1% non-fat milk in TBST for 120 mins at room temperature. Signals were detected by chemiluminescence (PerkinElmer, USA), and quantified with Quantity-One 1-D (Bio-Rad, USA). Protein expression was normalized to GAPDH.

\textit{Measuring \textit{Ca}^{2+} and Cell Contraction}

NRVMs cultured on borosilicate glass coverslips were incubated with M199 medium containing 2.5-µM fura-2 AM in Pluronic F-127 (ThermoFisher, USA), for 30 mins, followed by 30-min de-esterification at 37°C. NRVMs mounted on a TH4-100 microscope (Olympus, Japan) were perfused with normal Tyrode’s solution (NT) containing (mM) 136 NaCl, 5.4 KCl, 1 MgCl$_2$, 5
HEPES, 0.3 NaH$_2$PO$_4$ (pH 7.38, NaOH), heated to 33°C and field-stimulated (25 V, 2 Hz). Fura-2 was excited by the μStep Light Source and emission measured at 510-nm; the 340/380-nm excitation ratio was calculated following background subtraction. Cell-shortening was recorded with a video camera at 240 Hz. Spontaneous rate was calculated based on spontaneous contractions during a 15-second window. Regularity of beating was defined as the coefficient of variation of beating-interval (variance/mean). Ca$^{2+}$-transient signals (CaTs) were filtered with a low-pass 2$^{nd}$-order Butterworth filter (10-Hz); at least 5 CaTs were averaged for each recording. Caffeine (20-mM) in NT was rapidly applied to cells with a pre-heated superfuser (Cell MicroControls, USA). NRVMs were treated with vehicle (H$_2$O) or 100-µM ouabain (Tocris, UK) during the de-esterification step (30 mins), then perfused with vehicle or ouabain in NT while recording.

**Statistical Analysis**

GraphPad Prism 7.0 was used for data analysis. Data are presented as mean±standard error unless otherwise indicated. One-way and two-way ANOVA were used for multiple-group and multiple-category comparisons, respectively, followed by specified post-hoc multiple-comparisons tests. Fisher exact tests were used to compare observed and expected event-frequencies. Two-tailed $p<0.05$ indicated statistical significance.

**Results**

**miRNA Expression Profile Post-CAVB**

Figure 1 shows expression changes in 433 miRNAs on the microarray, represented as a volcano plot of fold-change (relative to baseline) versus P-value for the change. At 24 hours (Fig. 1A), only miR-135a showed a statistically-significant change compared to baseline (decreased 4.6-
fold, \( p<10^{-6} \) after adjustment for multiple comparisons). At 4 weeks post-CAVB (Fig. 1B), 4 miRNAs were significantly downregulated at least 1.25-fold and 10 miRNAs were upregulated at least 1.25-fold. Microarray results were confirmed by RT-qPCR for miRNAs potentially involved in cardiac function and/or pathology: miR-135a, miR-499, miR-150, miR-24-2, miR-486 and miR-31 (Fig. 1C).

**miR-135a and Cardiac-Relevant Targets**

Because miR-135a was the only miRNA dysregulated at 24 hours (Fig. 1A, C) and continued to show highly-significant changes by qPCR at 4 weeks (Fig. 1C), we selected miR-135a for further investigation. Based on target prediction, we identified 7 potential cardiomyocyte-relevant targets: Ca\(^{2+}\)-calmodulin dependent kinase-2 (*CAMK2D*), the pacemaker-current subunit gene *HCN2*, the transcription-factor genes *HIF-1A* and *MEF2C*, the signaling-molecule encoding gene *JAK2*, the mineralocorticoid-receptor gene *NR3C2* and the Na\(^+\)-Ca\(^{2+}\)-exchanger type-1 gene *SLC8A1*. We then measured the relative activity of luciferase reporters linked to predicted miR-135a 3’UTR MREs expressed in HEK-293. The results (Fig. 2) indicated that *JAK2* (-64% vs miR-NC, \( p<0.01 \)), *MEF2C* (-38%, \( p<0.01 \)), *NR3C2* (-69%, \( p<0.001 \)) and *SLC8A1* (-42%, \( p<0.001 \)) luciferase activities were significantly reduced by miR-135a, while *CAMK2D*, *HCN2* and *HIF-1A* were unaffected. miR-135a targeting was then further confirmed by mRNA and protein quantification in H9c2 or HL-1 cells. *MEF2C* mRNA and protein levels were not affected (Figure 3), while *NR3C2* protein expression was significantly decreased (-28% vs miR-NC, \( p<0.01 \)), as were *SLC8A1* mRNA (-47%, \( p<0.05 \)) and protein (-35%, \( p<0.05 \)). As a final control, a mutation was performed in the miR-135a binding site on *SLC8A1*, and eliminated the miR-135a effect in the luciferase assay (Figure 2).
Effects of miR-135a Overexpression In Vitro

Because of the stronger regulation of NCX1 than NR3C2 and its importance in cardiac electrophysiology, we selected NCX1 for further detailed study as a potential target of miR-135a in native cardiomyocytes, choosing NRVMs for their ease of transfection and spontaneous pacemaker activity. RT-qPCR and Western blot confirmed NCX1 mRNA (reduced by about 34%) and protein (reduced by about 45%) regulation by miR-135a in NRVMs (Fig. 4). NCX1 plays a significant role in cardiac pacemaking: treatment of NRVMs with 10-µM SN-6, a selective blocker of NCX1 function, abolished their spontaneous beating (Supplemental Fig. S1). NRVMs had a basal spontaneous beating frequency of 150±8 bpm (Figure 5). Compared to the negative-control miRNA (147±8 bpm), miR-135a overexpression decreased the rate by 63% (to 55±6 bpm, p<0.001, Fig. 5A, B). Significant beat-to-beat interval irregularities occurred following miR-135a overexpression, with bursts of contractions followed by periods of quiescence (Fig. 5A, C). Thus, miR-135a overexpression significantly alters NRVM spontaneous beating, as expected from NCX1-downregulation.

We then evaluated changes in Ca\(^{2+}\)-handling in miR-135a overexpressing NRVMs. Representative recordings obtained with fura-2 imaging are illustrated in Figure 6A. Action potential-triggered Ca\(^{2+}\) transients (2 Hz) were not altered by miR-135a overexpression, with no changes seen in the decay rate, amplitude, diastolic baseline or time to peak (Fig. 6B-E).

With regular action potential-evoked Ca\(^{2+}\)-transients, beat-to-beat removal of Ca\(^{2+}\) from the cytosol occurs primarily via uptake into the sarcoplasmic reticulum (SR) through the SR Ca\(^{2+}\)-ATPase (SERCA). Caffeine causes SR Ca\(^{2+}\)-release and a transient that is not followed by effective SR Ca\(^{2+}\)-uptake, so that the Ca\(^{2+}\)-transient decay corresponding to removal of Ca\(^{2+}\) from the cytosol depends principally on NCX1 function. Under these conditions (Figure 7A), miR-
Overexpression of miR-135a resulted in a significant increase in the decay time-constant (Fig. 7B), time to return to 50% baseline (Fig. 7C) and Ca\(^{2+}\)-transient amplitude (Fig. 7D), changes consistent with an impairment of NCX1-mediated removal of Ca\(^{2+}\) from the cytosol.

The positive inotropic and SR Ca\(^{2+}\)-loading effects of cardiac glycosides like ouabain have been attributed to NCX1 action secondary to Na\(^+\)/K\(^+\)-ATPase inhibition and Na\(^+\)-loading.\(^{12}\)

Furthermore, NCX1-current is a major contributor to delayed afterdepolarization (DAD) generation and associated arrhythmogenesis,\(^{13}\) as produced by digitalis glycoside toxicity. Thus, given that miR-135a downregulates NCX1, we tested whether miR-135a overexpression might limit the toxic effects of ouabain. Ouabain infusion enhanced Ca\(^{2+}\)-transient amplitude and caused post-pacing spontaneous Ca\(^{2+}\)-release events (SCREs, Figure 8). Compared to results in the presence of the control NC miRNA, miR-135a suppressed the Ca\(^{2+}\)-loading (Fig. 8B) and proarrhythmic (Fig. 8C) effects of ouabain. Cessation of pacing in the presence of ouabain and miR-NC miRNA produced SCREs in 11/15 cells, versus 1/13 cells with miR-NC and ouabain-free vehicle (\(p<0.001\)). Ouabain-induced SCREs were abolished by miR-135a (0/11 cells with miR-135a/ouabain; \(p<0.001\) versus ouabain with NC-miRNA).

**Discussion**

In this study, a rapid reduction was observed in miR-135a expression in the left ventricles of mice with CAVB; miR-135a was the only microRNA downregulated within 24 hours of CAVB onset. We therefore evaluated potential biologically-significant targets and identified NCX1 (encoded by *SLC8A1*) as a prime candidate. An in-depth exploration of the biological implications revealed alterations in spontaneous automaticity, Ca\(^{2+}\)-handling and DAD-related arrhythmogenic risk consistent with significant regulation of NCX1 by miR-135a.
NCX1 is a bidirectional exchanger that exchanges 3 Na\(^+\) for 1 Ca\(^{2+}\). NRVMs overexpressing miR-135a had slowed Ca\(^{2+}\)-decay in the presence of caffeine (Fig. 7), indicating a reduction in NCX activity consistent with the observed suppression of mRNA and protein expression (Fig. 4). NCX is also a key component of the mechanisms of automaticity in embryonic cardiomyocytes and adult pacemaking cells, in which rhythmic release of Ca\(^{2+}\) induces depolarizing currents,\(^{14,15}\) consistent with observed effects of miR-135a overexpression on spontaneous beating (Fig. 5), Following ouabain, SR Ca\(^{2+}\) load is enhanced, leading to larger Ca\(^{2+}\)-transient amplitudes and SCREs; these effects were suppressed by miR-135a overexpression. The only previous study of miR-135a in cardiac-related systems suggested that miR-135a protects against ischemia-reperfusion injury in HL-1 cells and regulates thioredoxin-interacting protein TXNIP.\(^{16}\) Protection was interpreted as being due to changes in TXNIP. However, miR-135a mediated reduction of NCX1 may also have contributed to the attenuation of ischemia-reperfusion injury, as previously shown with antisense inhibition\(^{17}\) and pharmacological blockade\(^{18}\) of NCX1 function.

Increased NCX-expression is an adaptation in cardiac hypertrophy to maintain contractile function, but this adaptive alteration can also cause proarrhythmia, since NCX1 contributes to both early and delayed afterdepolarization generation.\(^{13}\) Downregulation of miR-135a in CAVB, decreasing the inhibitory regulation of SLC8A1 by miR-135a, may contribute to the increase in NCX1 activity that participates in arrhythmogenesis following CAVB.\(^{19,20}\) NCX expression and function changes follow pressure overload by transverse aortic constriction\(^{21}\) and myocardial infarction,\(^{22}\) two other models in which miR-135a regulation might be altered. Of note, miR-135a is most highly expressed in the brain,\(^{23}\) where NCX1 plays a particularly important role in Ca\(^{2+}\) homeostasis under both physiological and pathological conditions such as ischemia-
reperfusion during stroke.\textsuperscript{24} In this context, manipulations that upregulate miR-135a may have therapeutic potential for conditions with pathological expression of enhanced NCX1-function.\textsuperscript{20} The results of this study clearly point to NCX1 as a functionally-relevant target of miR-135a. We also validated \textit{NR3C2} regulation by miR-135a with luciferase assay, RT-qPCR and Western blot; these results are in agreement with a recent study of miR-135a in the amygdala.\textsuperscript{25} \textit{NR3C2} encodes the mineralocorticoid receptor, previously implicated in proarrhythmia\textsuperscript{26} and sudden cardiac death.\textsuperscript{27} Further studies of the significance of this regulation are warranted, along with investigations of other potential miR-135a targets like \textit{NKX2-5,\textsuperscript{28} HAND2\textsuperscript{29} and ATP2B2\textsuperscript{4,30}}. We noted that a number of additional miRNAs are changed after 4 weeks of CAVB. Thus, a variety of miRNAs may be involved in the progressive cardiac remodeling response to CAVB. miR-499 is highly abundant in the myocardium and negatively regulates the cardiac stress response,\textsuperscript{31} while miR-150 expression is inversely related to hypertrophy\textsuperscript{32} and miR-486 regulates PI3K/AKT hypertrophic signaling.\textsuperscript{33} Additionally, miR-31 is thought to enhance arrhythmias,\textsuperscript{34} while miR-24 is endothelium enriched and regulates angiogenesis.\textsuperscript{35} Further studies of the role of these other miRNAs would be of potential interest.

\textbf{Conclusions}

The present study establishes miR-135a as a miRNA that is rapidly downregulated in CAVB, and identifies NCX1 as a potentially important functionally-relevant target. miR-135a altered spontaneous automaticity, Ca\textsuperscript{2+}-handling and DAD-related arrhythmic risk in a fashion consistent with significant regulation of NCX1. In addition, this work points to other potential targets and later-responding miRNAs that may be of interest in future research on the mechanisms underlying cardiac remodeling caused by CAVB and other cardiac pathologies.
Acknowledgements

We thank Chantal St-Cyr, Nathalie L’Heureux and Audrey Bernard for their technical assistance, and Jennifer Bacchi for secretarial help with the manuscript.

Sources of Funding

This work was supported by the Canadian Institutes of Health Research and the Quebec Heart and Stroke Foundation.

Disclosures

None
References


8. Le Quang K, Benito B, Naud P, Qi XY, Shi YF, Tardif JC, Gillis MA, Dobrev D, Charpentier F, Nattel S. T-type calcium current contributes to escape automaticity and


Figure 1

Left-ventricular miRNA expression after CAVB. A, B. Volcano plots of microarray results for miRNA fold-change vs statistical significance at 24 hours and 4 weeks post-CAVB. Only one miRNA is significantly altered at 24 hours, miR-135a. C. RT-qPCR confirmation for selected miRNAs. Data are presented relative to baseline, as mean±SD. n=4/group. Statistical comparison for microarray results was determined with multiple-testing correction following moderated t-statistics, and with Dunnett’s post-hoc comparisons for RT-qPCR. *p<0.05, ***p<0.01 for 24h vs corresponding baseline; #p<0.05, ##p<0.01, ###p<0.001 for 4w vs corresponding baseline.
Figure 2

miR-135a targeting assessed by luciferase assays in HEK-293 cells. Data are mean±SEM relative to miR-NC. n=4-8/group. **p<0.01, ***p<0.001, Tukey’s post-hoc comparisons. Mut = mutation in miR-135a binding site.
Figure 3

Potential miR-135a targets assessed at the levels of mRNA (top) and protein (bottom) expression. miR-135a was overexpressed in HL-1 (MEF2C, HIF-1A) or H9C2 (HCN2, NR3C2, SLC8A1) cells. n=3-6/group. Data are mean±SEM relative to miR-NC. *p<0.05, **p<0.01, Dunnett’s post-hoc comparisons.
Confirmation of miR-135a regulation of *SLC8A1* (NCX1 gene) in neonatal rat ventricular cardiomyocytes. RT-qPCR results are shown above, while representative Western blots and mean data are shown below, with GAPDH being used as the loading control. Data are presented relative to miR-NC, as mean±SEM. n=5-10/group. ***p<0.001, Tukey’s post-hoc comparisons.
miR-135a overexpression in neonatal rat ventricular cardiomyocytes alters their beating frequency and interbeat interval variability. **A.** Cell shortening recordings. **B.** Beating frequency. **C.** Interbeat interval variability. Data are mean±SEM. n=10-20/group. ***p<0.001, Tukey’s post-hoc comparisons.
Figure 6

Effects of miR-135a overexpression on Ca\(^{2+}\)-transients. **A.** Recordings of Ca\(^{2+}\)-transients in field-stimulated neonatal rat ventricular cardiomyocytes. **B.** Decay time-constant. **C.** Time to 50\%-baseline. **D.** Ca\(^{2+}\)-transient amplitude. **E.** Diastolic Ca\(^{2+}\)-level. **F.** Time to Ca\(^{2+}\)-transient peak. Data are mean±SEM. n=25-50/group.
miR-135a overexpression in NRVMs alters caffeine-induced Ca\(^{2+}\)-transients. A. Representative recordings. B-E. Average data for properties of caffeine-induced Ca\(^{2+}\)-transient recordings: (B) decay time-constant; (C) time to 50%-baseline; (D) amplitude; (E) and time to peak. Data are mean±SEM. \(n=8-16/\text{group}\). **\(p<0.01\), ***\(p<0.001\), Tukey’s post-hoc comparisons.
Figure 8

Consequences of miR-135a overexpression in neonatal rat ventricular cardiomyocytes for effects of a toxic concentration of ouabain. 
A. Original recordings of Ca\(^{2+}\)-transients. \(↓\) indicate Ca\(^{2+}\) transients generated following electrical field stimulation. B. Amplitude of Ca\(^{2+}\)-transients. C. Spontaneous Ca\(^{2+}\)-release events (SCREs). Data are mean±SEM. n=8-12/group. ***p<0.001 vs vehicle, #p<0.05, ###p<0.001 vs miR-NC, Tukey’s post-hoc comparisons.
Supplementary Data

**Table S1**: List of RNA/DNA sequences used in luciferase and transfection studies.

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56
Figure S1

Spontaneous beating is abolished within 2.5 min of infusion of 10µm SN-6, a selective blocker of NCX1. 1 min washout restores the spontaneous beating activity. Representative cell shortening recording from 1 NRVM is shown.
CHAPTER 3 – GENERAL DISCUSSION
3.1 Summary of Novel Findings

The investigations undertaken in this study were the first to explore miRNA expression following CAVB. A significant observation was a rapid downregulation of miR-135a, a miRNA that has received little consideration in the context of cardiovascular disease. The major finding of this thesis is that miR-135a regulates expression of NCX1 encoded by \( SLC8A1 \), such that miR-135a overexpression in NRVMs reduces spontaneous activity, \( Ca^{2+} \) extrusion and the potential for \( Ca^{2+} \) overload and arrhythmic risk.

3.2 Potential Significance and Limitations

3.2.1 NCX1 Regulation by miR-135a

Following a series of validation methods, we confirmed that miR-135a regulates the expression of NCX1 via mRNA degradation and/or translational repression. Overexpression of miR-135a led to a marked reduction in NCX1 protein expression in NRVMs and the functional effects were similar to the effects of gene knockout in many ways. The reduction and irregularity of spontaneous beating frequency is comparable to observations made in NCX1 knockout mice. While homozygous, global knockout is embryonically lethal due to lack of a heartbeat and cardiomyocyte apoptosis,\(^{102}\) cardiac-specific knockout mice survive until maturity but exhibit slower and irregular beating rates.\(^{52, 53, 103}\) These studies demonstrated that NCX forward mode drives membrane depolarization following SR \( Ca^{2+} \) release and confirmed NCX1 involvement in cardiac automaticity. Similarly, adenoviral mediated NCX1 knockdown also reduced spontaneous beating in NRVMs,\(^{104}\) further supporting the observed functional effect of miR-135a mediated NCX1 knockdown.
Despite the downregulation of NCX1 by miR-135a overexpression, we observed no significant alterations in the diastolic baseline, amplitude, time to peak nor decay rate of electrically evoked Ca\(^{2+}\) transients in NRVMs. This observation is consistent with data from Henderson et al.\(^{50}\) and Pott et al.,\(^{51}\) who demonstrated that compensation for NCX1 suppression occurs because NCX1 knockout forces an adaptive decrease in Ca\(^{2+}\) influx and increase in Ca\(^{2+}\) reuptake by SERCA.\(^{51}\) Our data on caffeine evoked Ca\(^{2+}\) transients revealed that miR-135a overexpression reduces the decay rate and enhances SR Ca\(^{2+}\) content, which is indicative of a compensatory shift towards SERCA-mediated Ca\(^{2+}\) removal.\(^{51}\) A study by Hurtado et al. using adenoviral mediated knockdown of NCX1 in NRVMs also showed no change in the Ca\(^{2+}\) transient shape with a compensatory increase in PMCA expression, which may be expected with miR-135a overexpression.\(^{104}\)

Furthermore, we observed that the inotropic effect of ouabain was eliminated by miR-135a overexpression. As Na\(^{+}/K^{+}\)-ATPase inhibitors, ouabain and other cardiac glycosides cause an increase in intracellular [Na\(^{+}\)]. NCX typically handles this disturbance with reverse mode activity, leading to greater intracellular [Ca\(^{2+}\)], larger Ca\(^{2+}\) transient amplitudes and stronger contractions. Similar to our results, Reuter et al. used embryonic heart tubes of global NCX1 knockout mice to illustrate that the effectiveness of ouabain is dependent on NCX1 expression, even at toxic concentrations.\(^{48}\) Thus, miR-135a limits the potential for Ca\(^{2+}\) overload, which is a relevant pro-arrhythmic and deleterious phenomena associated with faster heart rates, ischemia/reperfusion (I/R) injury and HF.\(^{105}\) Aside from MI, I/R injury is a significant complication after stroke. NCX1 is highly expressed in the brain,\(^{106}\) as is miR-135a.\(^{107}\) In concordance with our results in NRVMs, miR-135a may be a key player in mediating Ca\(^{2+}\) overload sensitivity in neurons as well. Ultimately, the investigations of miR-135a
overexpression in this thesis yielded changes to NRVM function that are all consistent with reduced NCX1 expression.

Given its regulation of NCX1, miR-135a may be an interesting subject in a cardiovascular context. Previous reports have indicated that miR-135a expression decreases in the mouse heart in response to experimental stressors. Zhu et al. demonstrated a reduction in miR-135a following ischemia/reperfusion (I/R) injury,\textsuperscript{108} and Kalsotra et al. pointed to a decline following traverse aortic constriction (TAC).\textsuperscript{109} Additionally, miR-135a was found to be downregulated in rats exposed to psychological and physical stress.\textsuperscript{110} Thus, in conjunction with the findings of this thesis following CAVB, miR-135a may, in fact, be a stress responsive cardiac miRNA; however, this is only currently attributable to mice and rats, since we have observed no reduction post-CAVB in rabbits (data not shown) and no reports have been made in other species. As a negative regulator of NCX1, a decrease in miR-135a may facilitate upregulation of NCX1 expression via stress related increases in neurohumoral signaling, including α- and β-adrenergic signaling.\textsuperscript{111} Enhanced NCX1 expression is a typical adaptation during cardiac hypertrophic remodeling to maintain or improve contractility,\textsuperscript{112} and is observed following CAVB,\textsuperscript{67} I/R\textsuperscript{113} and TAC.\textsuperscript{114} Whether miR-135a knockdown enhances pro-hypertrophic signaling and NCX expression is a hypothesis worth exploring in the future. However, despite the benefits towards contractility, increased NCX activity is a double edged sword because it enhances the risk of both EADs and DADs, and is ultimately pro-arrhythmic.\textsuperscript{70, 115}

3.2.2 miRNAs in CAVB

While many reports of the molecular and cellular changes have been published thus far,\textsuperscript{2-4, 116} the specific alterations in miRNA expression following CAVB have yet to be characterized. Cardiac
adaptation to the bradycardic escape rhythm involves mechanistically and temporally distinct processes: 1) early electrical remodeling, and 2) delayed structural remodeling. The miRNA signature at the 24-hour time point post-CAVB was largely unchanged compared to baseline, with only miR-135a expression being drastically reduced. The electrical remodeling in mice involves immediate reductions in Kv4.2/KCND2, Kv4.3/KCND3 and KCHIP2/KCNIP2 (protein/mRNA). These ion channel subunits assemble to produce the transient outward K⁺ current (Ito,fast), such that the reduction prolongs the action potential and allows for enhanced Ca²⁺ influx and contractility, which are useful adaptations to CAVB as well as in human HF. Reduction in transcript expression is thought to be mediated by stretch induced signaling, neurohormonal signaling via PKC pathways and/or members of the transcription factor families calcineurin/NFAT, IRX, GATA, FOG and MEF2 that are activated by elevated intracellular [Ca²⁺] or autocrine/paracrine signaling. miR-135a is not predicted to directly target KCND2, KCND3, KCNIP2 or most of the implicated transcription factors, making it an unlikely mediator of the early electrical remodeling.

Significant structural remodeling becomes evident 2 days post-CAVB, and at 4 weeks, increases in LV end diastolic diameter, LV mass and fractional shortening are all evident, along with alterations in contractile apparatus components (proteins α-MHC, β-MHC, MLC2V, cTnI). These compensatory changes maintain CO, while a fibrotic response is typically absent. The significantly altered miRNAs at 4 weeks may contribute to the ventricular phenotype. miR-499 is highly abundant in the myocardium, is located in the intron of MYH7B, a ventricular myosin heavy chain, and has been previously implicated in the early gene response to cardiac stress. miR-150 expression is inversely related to hypertrophy and miR-486 regulates PI3K/AKT hypertrophic signaling. Additionally, miR-31 is thought to enhance the
arrhythmogenic substrate\textsuperscript{130} and I/R,\textsuperscript{131} while miR-24 is endothelium enriched and regulates angiogenesis.\textsuperscript{132} The altered miRNAs likely play individually intricate roles post-CAVB, but a limitation in interpreting these data is that cell type or transmural cardiomyocyte differences were not explored.

In contrast to the mouse, CAVB in rabbits and dogs leads to reductions in $I_{Kr}$ and $I_{Ks}$ in the initial electrical remodeling process, while $I_{io}$ is left unchanged.\textsuperscript{3, 61} RT-qPCR analysis of the LV miRNA profile in a rabbit CAVB model indicated no change in miR-135a at the 2 week time point, which may suggest that miR-135a downregulation is a product of a species-specific remodeling process tied to $I_{io}$.

Of the seven target genes predicted by our initial search, only $NR3C2$ and $SLC8A1$ were successfully validated as miR-135a regulatory targets by luciferase assays, RT-qPCR and Western blot, and both have been linked to arrhythmogenic cardiac remodeling. $NR3C2$ encodes the mineralocorticoid receptor, the molecular mediator for aldosterone signaling that is involved with proarrhythmia\textsuperscript{133} and sudden cardiac death.\textsuperscript{134} $SLC8A1$ encodes NCX1, an electrogenic Ca\textsuperscript{2+} exchanger that modulates cardiac electrical activity, Ca\textsuperscript{2+} flux, contractility and afterdepolarization generation post-CAVB.\textsuperscript{115} Numerous other predicted targets that are linked to cardiac function and have yet to be studied include the transcriptional factors $NKX2-5$\textsuperscript{135} and $HAND2$,\textsuperscript{136} Ca\textsuperscript{2+} transporters $ATP2B2-4$,\textsuperscript{137} ion channels $TRPM4$\textsuperscript{138} and $TRPM7$,\textsuperscript{139} and neurohormone receptor $ADRA2A$.\textsuperscript{140} Several more miR-135a regulatory targets have been previously validated, albeit in other cellular contexts where miR-135a has largely been studied as an oncogene or tumor suppressor, including $ROCK1/2$,\textsuperscript{141} $KLF4$,\textsuperscript{142} $TRPC1$\textsuperscript{143} and $TXNIP$.\textsuperscript{108} Given the potentially extensive network of miR-135a regulation in the heart, the significant and
immediate downregulation of miR-135a post-CAVB may play a broader role in remodeling, beyond the changes in NCX1 explored here.

3.3 Future Directions

To the best of our knowledge, miR-135a has received limited attention in the cardiovascular field. The brunt of research on miR-135a overexpression has been conducted in cancer contexts, where it has been shown to enhance metastatic potential in hepatocellular carcinoma, colorectal and breast cancers, as well as inhibit proliferation of prostate and gastric cancers. In the heart, Uppugunduri discussed the potential involvement of miR-135a in HF that is associated with a base-pair deletion in MYBPC3, a gene encoding cardiac myosin binding protein C. Zhu et al. conducted the most relevant study, where miR-135a mimics delivered to diabetic db/db mice limited infarct size and apoptosis. The protective effects seen were attributed to TXNIP targeting, but as discussed above, regulation of NCX1 could play a significant role.

A model to overexpress miR-135a in vivo would be an elegant tool to confirm the results of this thesis and further define the role of miR-135a in cardiac physiology and disease. Such an approach would offer flexibility for both in vivo and in vitro experimentation, as described below.

1) Cardiac specific miR-135a overexpression in mice. Overexpression of miR-135a can be achieved via transgenic, viral or mimicry methods. Transgenic mice could be generated by flanking pre-miR-135a-1 (chromosome 9, 90 bp) or pre-miR-135a-2 (chromosome 10, 100 bp) with a cardiomyocyte specific promoter, such as MYH6 and MYL2. Adeno-associated viral (AAV) vectors have been used extensively for gene therapy purposes in
AAV9 carrying pre-miR-135a-1 or -2 expressed under a cardiomyocyte specific promoter could allow for overexpression through intravenous injection or direct injection to the heart. Similar to those used in our transfection studies in vitro, miRNA mimics can also be used in vivo following delivery by injection. miR-135a mimics can be developed by and purchased from various biotechnology companies. For the purposes of the studies proposed below, targeted overexpression of miR-135a with an AAV vector may be the optimal method, because overexpression will be cardiomyocyte targeted, long term and cost effective.

2) **Confirmation of NCX1 downregulation in miR-135a overexpressing mice.** Once targeted overexpression is successfully validated, NCX1 expression in various chambers of the heart can be measured via RT-qPCR and Western blot. Ventricular cardiomyocytes can be isolated via collagenase digestion, and NCX1 activity can be measured in individual cells using fluorescence microscopy, as described in the manuscript, or via the patch clamp technique. Applying caffeine to cardiomyocytes induces NCX1 forward mode and a detectable inward current. We would expect that cardiomyocytes overexpressing miR-135a will have a reduced NCX1 current that is sensitive to block by SN-6 or Ni²⁺.

3) **Exploring the role of miR-135a in afterdepolarizations and triggered activity.** Membrane voltage of isolated cardiomyocytes can be measured using the perforated patch clamp method, such that both the amplitude and occurrence of DADs and EADs can be quantified following proarrhythmic stimulation. DADs, EADs and the resulting spontaneous APs can be elicited by rapid, irregular pacing in the presence of isoproterenol. We would expect reduced occurrence of DADs, EADs and/or
spontaneous APs in cardiomyocytes overexpressing miR-135a; this may be tied to a reduction in LTCC current, which can be measured in the voltage clamp configuration.

4) Exploring the role of miR-135a in CAVB. Given its abrupt reduction following CAVB induction, exploring how maintained/elevated miR-135a levels affects the CAVB pathology may give significant insight into its role. Implanted telemetry devices offer 24 hour electrocardiogram recordings and can be used to determine the effect of miR-135a on sinus rhythm following infection and on idioventricular escape rhythm following CAVB. The effect on the incidence and severity of TdP-like ventricular arrhythmias post-CAVB can also be monitored in this manner. Combined pharmacological NCX and LTCC inhibition has been shown to reduce TdP occurrence in CAVB dogs\textsuperscript{153} and rabbits;\textsuperscript{154} while no such studies have been conducted in mice, we might expect a similar decrease after miR-135a overexpression. Furthermore, echocardiographic studies at baseline, following miR-135a overexpression and after CAVB induction can illustrate changes to cardiac function through measures of LV end diastolic diameter, LV ejection fraction, CO and fractional shortening. Histology and ratio of LV mass to body weight can be used to investigate myocardial injury and hypertrophy, respectively, post-CAVB.

5) Exploring the role of miR-135a in MI. miR-135a overexpressing mice can be subjected to left coronary artery or left anterior descending artery ligation to induce MI or I/R.\textsuperscript{108} Determination of MI region size in the LV through histological methods can be made at acute (within hours) or chronic (within weeks) time points following artery occlusion. Such an approach would allow us to test the hypothesis that miR-135a overexpression and its related NCX1 downregulation is protective against MI and I/R, as well as confirm the results of the Zhu et al. study.
The mechanism of miR-135a regulation in the CAVB mouse heart is also a question left unanswered. Several factors have been implicated in miR-135a transcription, including forkhead box M1, androgen receptor, phosphoinositide-3-kinase (PI3K)/Akt signaling, transforming growth factor beta 1 and signal transducer and activator of transcription 5a. Of these, PI3K/Akt signaling was shown to reduce miR-135a expression in PC-3 cells. PI3K/Akt is well known for its regulation of cardiomyocyte size, survival, angiogenesis and inflammation in physiological and pathological hypertrophy and is activated via growth factor and G-protein coupled receptors present during cardiac stress. Whether PI3K/Akt regulation of miR-135a occurs in cardiomyocytes remains to be seen.

Moreover, the collection of knowledge surrounding miR-135a in human cardiac tissue is severely limited. To the best of our knowledge, only one report has been made to date, where an increase in circulating miR-135a was reported in coronary artery disease patients. Evaluation of the clinical relevance of miR-135a depends on subsequent studies of its expression in larger animal models of cardiac disease, as well as in human samples; however, given its regulation of NCX1, an important modulator of cardiac physiology and pathology, miR-135a may be an interesting target moving forward. This would also require a more detailed analysis of the range of miR-135a regulatory targets.

3.4 Conclusions

The findings of this thesis are some of the earliest investigations of miR-135a function in the mammalian heart. Here, we identified that miR-135a is immediately downregulated following CAVB in mice, and established that NCX1 is a functionally relevant regulatory target. Subsequent overexpression in NRVMs revealed that miR-135a altered spontaneous activity, Ca^{2+}
handling and DAD-related arrhythmic risk in a fashion consistent with downregulated NCX1 expression. Further investigation of \textit{in vivo} miR-135a overexpression would be necessary to gain a better appreciation of its role in CAVB and other cardiac pathologies including MI. Additionally, our study implicated several other miRNAs in the CAVB remodeling process. These miRNAs, as well as the other potential miR-135a targets discussed here, may help elucidate the mechanisms of remodeling following CAVB or other cardiac pathologies.
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