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The Role of MicroRNA Regulation of Cardiac Ion Channel in Arrhythmia

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RÉSUMÉ

La fibrillation auriculaire (FA) est le trouble du rythme le plus fréquemment observé en pratique clinique. Elle constitue un risque important de morbi-mortalité. Le traitement de la FA reste un défi majeur en lien avec les nombreux effets secondaires associés aux approches thérapeutiques actuelles. Dans ce contexte, une meilleure compréhension des mécanismes sous-jacents à la FA est essentielle pour le développement de nouvelles thérapies offrant un meilleur rapport bénéfice/risque pour les patients. La FA est caractérisée par *i)* un remodelage électrique délétère associé le plus souvent *ii)* à un remodelage structurel du myocarde favorisant la récurrence et le maintien de l'arythmie. La diminution de la période réfractaire effective au sein du tissu auriculaire est un élément clef du remodelage électrique. Le remodelage structurel, quant à lui, se manifeste principalement par une fibrose tissulaire qui altère la propagation de l'influx électrique dans les oreillettes. Les mécanismes moléculaires impliqués dans la mise en place de ces deux substrats restent mal connus. Récemment, le rôle des microARNs (miARNs) a été pointé du doigt dans de nombreuses pathologies notamment cardiaques. Dans ce contexte les objectifs principaux de ce travail ont été i) d'acquérir une compréhension approfondie du rôle des miARNs dans la régulation de l'expression des canaux ioniques et ii) de mieux comprendre le rôle de ces molécules dans l'installation d'un substrat favorable a la FA.

Nous avons, dans un premier temps, effectué une analyse bio-informatique combinée à des approches expérimentales spécifiques afin d'identifier clairement les miARNs démontrant un fort potentiel de régulation des gènes codant pour l'expression des canaux ioniques cardiaques humains. Nous avons identifié un nombre limité de miARNs cardiaques qui possédaient ces propriétés. Sur la base de ces résultats, nous avons démontré que l'altération de l'expression des canaux ioniques, observée dans diverse maladies cardiaques (par exemple, les cardiomyopathies, l'ischémie myocardique, et la fibrillation auriculaire), peut être soumise à ces miARNs suggérant leur implication dans l'arythmogénèse.

La régulation du courant potassique I_{K1} est un facteur déterminant du remodelage électrique auriculaire associée à la FA. Les mécanismes moléculaires sous-jacents sont peu connus. Nous avons émis l'hypothèse que l'altération de l'expression des miARNs soit

corrélée à l'augmentation de l'expression d'I_{K1} dans la FA. Nous avons constaté que l'expression de miR-26 est réduite dans la FA et qu'elle régule I_{K1} en modulant l'expression de sa sous-unité Kir2.1. Nous avons démontré que miR-26 est sous la répression transcriptionnelle du facteur nucléaire des lymphocytes T activés (NFAT) et que l'activité accrue de NFATc3/c4, aboutit à une expression réduite de miR-26. En conséquence I_{K1} augmente lors de la FA. Nous avons enfin démontré que l'interférence *in vivo* de miR-26 influence la susceptibilité à la FA en régulant I_{K1}, confirmant le rôle prépondérant de miR-26 dans le remodelage auriculaire électrique.

La fibrose auriculaire est un constituant majeur du remodelage structurel associé à la FA, impliquant l'activation des fibroblastes et l'influx cellulaire du Ca^{2+}. Nous avons cherché à déterminer *i)* si le canal perméable au Ca^{2+}, TRPC3, jouait un rôle dans la fibrose auriculaire en favorisant l'activation des fibroblastes et *ii)* étudié le rôle potentiel des miARNs dans ce contexte. Nous avons démontré que les canaux TRPC3 favorisent l'influx du Ca^{2+}, activant la signalisation Ca^{2+}-dépendante ERK et en conséquence activent la prolifération des fibroblastes. Nous avons également démontré que l'expression du TRPC3 est augmentée dans la FA et que le blocage *in vivo* de TRPC3 empêche le développement de substrats reliés à la FA. Nous avons par ailleurs validé que miR-26 régule les canaux TRPC3 en diminuant leur expression dans les fibroblastes. Enfin, nous avons montré que l'expression réduite du miR-26 est également due à l'activité augmentée de NFATc3/c4 dans les fibroblastes, expliquant ainsi l'augmentation de TRPC3 lors de la FA, confirmant la contribution de miR-26 dans le processus de remodelage structurel lié à la FA.

En conclusion, nos résultats mettent en évidence l'importance des miARNs dans la régulation des canaux ioniques cardiaques. Notamment, miR-26 joue un rôle important dans le remodelage électrique et structurel associé à la FA et ce, en régulant I_{K1} et l'expression du canal TRPC3. Notre étude démasque ainsi un mécanisme moléculaire de contrôle de la FA innovateur associant des miARNs. miR-26 en particulier représente apres ces travaux une nouvelle cible thérapeutique prometteuse pour traiter la FA.

Mots clés : Arythmie, fibrillation auriculaire, microARN, miR-26, I_{K1} et TRPC3

ABSTRACT

Atrial fibrillation (AF) is the most frequently-encountered arrhythmia in clinical practice and constitutes a major cause of cardiac morbidity and mortality. The management of AF remains a major challenge as current therapeutic approaches are limited by potential adverse effects and high rate of AF recurrence/persistence. A better understanding of the mechanisms underlying AF is of great importance to improve AF therapy. AF is characterized by impaired electrical and structural remodeling, both of which favors the recurrence and maintenance of the arrhythmia. A key feature in electrical remodeling is the reduced atrial effective refractory period, due to ion channel alteration. Structural remodeling, on the other hand, mainly results from atrial fibrosis. However, the precise molecular mechanisms underlying these remodeling processes are still incompletely understood. The importance of microRNAs (miRNAs) in various pathophysiological conditions of the heart has been well established, but little is known with regard to cardiac arrhythmias. Emerging evidence suggests that dysregulation of miRNAs may underlie heart rhythm disturbances. The aim of the present work was to acquire a comprehensive understanding of miRNA-mediated regulation of ion channels in cardiac arrhythmias. Notably, we will focus on the mechanistic insights of miRNAs related to the control of AF.

Currently available experimental approaches do not permit thorough characterization of miRNA targeting. For this purpose, we performed bioinformatic analyses in conjunction with experimental approaches to identify miRNAs from the database that potentially regulate human cardiac ion channel genes. We found that only a subset of miRNAs target cardiac ion channel genes. Based on these results, we further demonstrated that the dysregulation of ion channel gene expression observed in various cardiac disorders (e.g. cardiomyopathy, myocardial ischemia, and atrial fibrillation) can be explained by the dysregulation of miRNAs. These findings further support the potential implication of miRNAs in arrhythmogenesis under these cardiac conditions.

The upregulation of the cardiac inward rectifying potassium current, I_{K1}, is a key determinant of adverse atrial electrical remodeling associated with AF. The molecular mechanisms underlying this ionic remodeling are poorly understood. We hypothesized that altered miRNA expression is responsible for I_{K1} upregulation in AF. We found that miR-26

is significantly downregulated in AF and regulates I_{K1} by controlling the expression of its underlying subunit Kir2.1. Moreover, we demonstrated that miR-26 is under the transcriptional repression of the nuclear factor of activated T cells (NFAT) and enhanced activities of members of the NFAT family, NFATc3/c4, results in miR-26 downregulation, which accounts for I_{K1} enhancement in AF. Furthermore, we observed that *in vivo* interference of miR-26 affects AF susceptibility via the regulation of I_{K1}, suggesting an important role of miR-26 in atrial electrical remodeling.

Atrial fibrosis is a major constituent in AF-associated adverse atrial structural remodeling, involving the activation of fibroblasts and cellular Ca^{2+} entry. Here, we sought to determine whether the Ca^{2+} permeable channel, TRPC3, plays a role in AF-induced fibrosis by promoting fibroblast activation. Furthermore, we investigated the potential role of miRNAs in this context. We found that TRPC3 channels promote Ca^{2+}-entry, which results in activation of Ca^{2+}-dependent ERK-signaling and consequently fibroblast activation. We also demonstrated that TRPC3 is upregulated in AF and in vivo TRPC3 blockade suppresses the development of AF-promoting substrate. Furthermore, we observed that miR-26 regulates TRPC3 channels via controlling the expression of the underlying channel subunit and is downregulated in AF-fibroblasts. Finally, we showed that the reduced expression of miR-26 is also due to the enhanced NFATc3/c4 activities in AF-fibroblasts and accounts for AF-induced upregulation of TRPC3, suggesting the potential contribution of miR-26 in AF-related adverse structural remodeling process.

In conclusion, our findings emphasize the importance of miRNAs in the regulation of cardiac ion channels. Notably, miR-26 plays a crucial role in AF-associated electrical and structural remodeling via the regulation of I_{K1} and TRPC3 channel genes. Thus, our study unravels a novel molecular control mechanism of AF at the miRNA level, suggesting miR-26 as a new and promising therapeutic target for AF.

Keywords : Arrhythmia, atrial fibrillation, microRNA, miR-26, I_{K1} and TRPC3

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LIST OF ABBREVIATIONS

2’-OMe	2’-O-methyl
3’-UTR	3’-untranslated region
AAVs	Adeno-associated viruses
AAV9	Adeno-associated viruses serotype 9
ACC	American College of Cardiology
ACE	Angiotensin converting enzyme
AF	Atrial fibrillation
AFFIRM	Atrial Fibrillation Follow-up Investigation of Rhythm Management
AHA	American Heart Association
AngII	Angiotensin II
AP	Action potential
APD	Action potential duration
Arl2	ADP-ribosylation factor-like 2
ATP	Atrial tachypacing
ATR	Atrial tachycardia remodeling
AT1R	Angiotensin II type 1 receptor
Ba^{2+}	Barium ion
Bcl2	B-cell lymphoma 2
Bim	BH3-only protein
Ca^{2+}	Calcium ion
$[Ca^{2+}]_i$	Intracellular calcium concentration
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CAMKIIδ	Ca^{2+}/calmodulin-dependent protein kinase II delta
C elegans	*Caenorhabditis elegans*
CH	Cardiac hypertrophy

CHF	Congestive heart failure
CMs	Cardiomyocytes
Col1A1	Collagen 1A1
Cs^+	Cesium ion
CTGF	Connective tissue growth factor
CV	Conduction velocity
Cx40	Connexin 40
Cx43	Connexin 43
DAD	Delayed afterdepolarization
DAG	Diacylglycerol
Dyrk1a	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1a
EAD	Early-afterdepolarization
EADs	Early-afterdepolarizations
ECM	Extracellular matrix
ECs	Endothelial cells
ESC	European Society of Cardiology
ERK1/2	Extracellular signal-regulated kinase 1/2
ERP	Effective refractory period
FasL	Fas ligand
Gd^{3+}	Gadolinium ion
GPCR	G protein-coupled receptor
HEK293	Human embryonic kidney cell line
HF	Heart failure
Hif-1α	Hypoxia-inducible factor 1 alpha
I_{CaL}	Inward L-type calcium current

IGF-1	Insulin-like growth factor-1
I_{K1}	Inward rectifier potassium current
I_{KACh}	Acetylcholine-activated inward potassium current
I_{Kr}	Rapidly activated delayed rectifier potassium current
I_{Ks}	Slowly activated delayed rectifier potassium current
I_{Kur}	Ultra-rapidly activating delayed-rectifier K^+-current
I_{Na}	Inward sodium current
IP_3	Inositol 1,4,5-triphosphate
IR	Ischemia reperfusion
ITGA5	alpha-5 integrin
I_{to}	Transient outward potassium current
La^{3+}	Lanthanum ion
LNA	Locked nucleic acid
Mg^{2+}	Magnesium
MEF2A	Myocytes enhancer factor 2A
MFs	Myofibroblasts
miRNAs	microRNAs
MMP-2	Metalloproteinase-2
MuRF1	Muscle specific ring finger protein 1
Na^+	Sodium ion
NCX	Na^+/ Ca^{2+} exchanger
NFAT	Nuclear factor of activated T cells
PAK4	p21-activated kinase-4
PDCD4	Programmed cell death 4
PDGF	Platelet-derived growth factor

PIP2	Phosphatidylinosital-4, 5-bisphosphate
PKA	Protein kinase A
PKC	protein kinase C
PLC	Phospholipase C
Ppp2r5a	Protein phosphatase 2A
PTEN	Phosphatase and tensin homolog
Pyr3	Pyrazole
RISC	RNA-induced silencing complex
ROCE	Receptor-operated Ca^{2+} entry
ROCK1	Rho-associated coiled-coil containing protein kinase-1
RP	Refractory period
RT	Reverse transcription
RyR2	Ryanodine receptor 2
SA	Sinoatrial
SERCA	SR Ca^{2+}-ATPase
siRNAs	Short interfering RNAs
SMCs	Smooth muscle cells
SOCE	Store-operated Ca^{2+} entry
Spred-1	Sprouty-related EVH1 domain-containing protein 1
Spry1	Sprouty homolog 1
SR	Sarcoplasmic reticulum
SRF	Serum response factor
TAC	Transverse aortic constriction
TFIIB	Transcription factor II B
TFs	Transcription factors

TGF-β_1	Transforming growth factor β_1
TGF-βRII	TGF-β receptor type II
THRAP1	Thyroid hormone receptor associated protein 1
TM	Transmembrane domains
TRP	Transient receptor potential
TRPA	Transient receptor potential ankyrin
TRPC	Transient receptor potential canonical
TRPM	Transient receptor potential melastatin
TRPML	Transient receptor potential mucolipin
TRPP	Transient receptor potential polycystin
TRPV	Transient receptor potential vanilloid
US	United States
VSMCs	Vascular smooth muscle cells
WL	Wavelength

DEDICATION

This thesis is dedicated to:

My parents, for their unconditional love and support, endless patience and understanding. Were it not for their sacrifice, this thesis may have never been completed.

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First of all, I would like to express my deepest appreciation to my supervisor, Dr. Stanley Nattel, for providing me the opportunity to be part of the lab. Were it not for his invaluable support, persistent encouragement, and pertinent guidance, my graduate studies and this thesis would have never been completed. I also want to extend my sincere gratitude to him for inspiring me not only with his rich scientific thoughts but with his philosophy of life.

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Xiaobin Luo, August 2012

STATEMENT OF AUTHORSHIP

Here is a statement regarding the contribution of coauthors and myself to the three papers included in this thesis.

Chapter 2:

Luo X, Zhang H, Xiao j, Wang Z. Regulation of Human Cardiac Ion Channel Genes by MicroRNAs: Theoretical Perspective and Pathophysiological Implications. ***Cell. Physiol. Biochem.*** 2010;25(6):571-86.

I designed the experiments, performed the miRNA microarray and real-time RT-PCR experiments, partially contributed to bioinformatic works, and analyzed the data. Haijun Zhang took part into the analysis of the bioinformatic results and helped in generating the figures. Dr. Jiening Xiao participated in the miRNA microarray and real-time RT-PCR experiments. Dr. Zhiguo Wang generated the original idea, supervised the work, wrote the manuscript, and finalized the manuscript for publication. More specifically, I generated the Fig. 1A, which is based on the re-analysis of the experimental results of a previous paper (Liang et al, BMC Genomics, 2007), showing the relative expression of all miRNAs in different organs in humans by Realtime RT-PCR. In addition, in collaboration with Dr. Jiening Xiao, we performed Realtime RT-PCR to verify the relative abundance of the cardiac-enriched miRNA reported in Liang's paper in the healthy human ventricular sample. These data are presented as supplementary figure 1. I also prepared Fig. 1B, which summarizes the total number of ion-channel target genes each cardiac-enriched miRNAs shown in Fig. 1A. Moreover, together with Haijun Zhang, we prepared Fig. 2, which is the cartoon illustration of the cardiac-enriched miRNAs and their corresponding predicted ion-channel target genes. For Fig. 3, I was responsible for the summary and the comparison of all the previously-reported deregulated miRNAs under cardiac hypertrophy and congestive heart failure. For Fig. 4, I compared and summarized the miRNAs changes in ischemic hearts which were reported by three previous publications as well as the miRNA microarray analysis we performed in the hearts of MI rat (the cardiac tissuses of the rat MI model were kindly provided by Dr. Baofeng Yang) and these data are presented in supplementary figure 2. Furthermore, together with Dr. Jiening Xiao, we conducted the

microarray analysis of miRNAs expression profile in the left atrium of a canine AF model (The AF dog atrial samples were also kindly provided by Dr. Baofeng Yang) and I performed Realtime RT-PCR to verify the deregulated miRNAs and prepared Fig. 5. Finally, I summarized the list of ion channel genes selected in the study with their detailed descriptions and prepared Table 1.

Chapter 3:

Luo X*, Pan Z*, Shan H*, Xiao J, Sun X, Wang N, Lin H, Xiao L, Maguy A, Qi X-Y, Li Y, Gao X, Dong D, Zhang Y, Bai Y, Ai J, Sun L, Lu H, Luo X, Wang Z, Lu Y, Yang B, Nattel S. MicroRNA-26 governs profibrillatory inward-rectifier potassium current changes in atrial fibrillation. ***J. Clin. Invest.*** (accepted for publication, in press).

Along with Dr. Zhenwei Pan, I designed all the experiments, conducted majority of the luciferase, western blot, real-time RT-PCR, EMSA, and a portion of in vivo experiments, and analyzed the data. During the subsequent revision work of this paper Dr. Hongli Shan has made significant contribution to the in vivo NFAT-overexpression study as well as AF time-course study. Therefore, Dr. Zhenwei Pan, Dr. Hongli Shan and I were considered to have an equal contribution to the present study. Xiao-Yan Luo and Drs. Xuelin Sun, Jiening Xiao, Huixian Lin, Ling Xiao, Deli Dong, Jing Ai, Ange Maguy, Xiaoyan Qi, and Ning Wang performed parts of the luciferase, real-time RT-PCR, CHIP and Western blot analyses. Drs. Hongli Shan, Xuelin Sun, Lihua Sun, and Yunlong Bai conducted patch-clamp recordings. Dr Hang Lu provided the human samples. Drs. Yanjie Lu, Xu Gao and Y.Z. conducted parts of the animal studies. Dr. Zhiguo Wang helped in the conceptualization and design of the studies. Dr. Stanley Nattel and Dr. Baofeng Yang supervised the project and wrote the manuscript. More specifically, I proposed and designed the experiments presented in Fig.1A-F. I designed and performed the Western blot and Realtime RT-PCR experiments as shown in Fig.2A, 2B, 2C and 2F. Together with Dr. Jiening Xiao, we designed and generated the wild-type and mutant luciferase report constructs containing the sequences correspond to the miR-26 binding sites in 3'UTR of KCNJ2 mRNA (Fig. 2E). In addition, I conceived and designed the experiments related to the in vivo assessment of miR-26 overexpression or knockdown on AF vulnerability and worked collaboratively with Dr. Zhenwei Pan on the AF induction experiments in mice

subjected to in-vivo miRNA-26 interference as shown in Fig. 3A. Moreover, I designed the miR-mimics and miR-masks constructs and worked collaboratively with Dr. Pan for the measurement of AF induction rate as well as duration in mice received these constructs (Fig 4A and 4C). Furthermore, I proposed, designed and performed the majority of the experiments related to molecular mechanism underlying the transcriptional control of miR-26 in AF (Fig. 6A, 6C and 6D and Fig. 7A and 7C). I also partially contributed to the works in Fig. 6B and Fig. 7B together with Drs. Jiening Xiao and Huixian Lin. Finally, I carried out bioinformatic analysis or western blot experiments and prepared Suppl. Fig. 1, 2, 3, 4, 9, 11, 12, and 13.

Chapter 4:

Harada M, **Luo X**, Qi X, Tadevosyan A, Maguy A, Ordog B, Ledoux J, Kato T, Naud P, Voigt N, Shi Y, Kamiya K, Murohara T, Kodama I, Tardif J, Schotten U, Van Wagoner D, Dobrev D, Nattel S. TRPC3-dependent Fibroblast Regulation in Atrial Fibrillation. ***Circulation.*** 2012 Oct 23; 126(17):2051-64.

In this work, I proposed, designed, performed, and analyzed all the experiments related to miRNAs (Figure 6 and Suppl. Figure 12). Dr. Masahide Harada was the primary investigator of this study, performed most of the in vivo and in vitro experiments, and wrote the manuscript. Dr. Xiao-Yan Qi performed the patch clamp experiments. Artavazd Tadevosyan conducted the NFAT immuno-staining study. Dr. Ange Maguy was involved in experiments of western blotting. Dr. Balazs Ordog helped in producing TRPC3 knockdown viruses. Dr. Jonathan Ledoux participated in recording of Ca^{2+} transient. Dr. Takeshi Kato helped in electrophysiological study of the animal model. Dr. Patrice Naud participated in real-time RT-PCR experiments. Drs. Niels Voigt, Ulrich Schotten, David R. Van Wagoner, and Dobromir Dobrev provided atrial samples from human, goats, and CHF dogs. Drs. Yanfen Shi and Jean-Claude Tardif were involved in echocardiographic measurements. Drs. Kaichiro Kamiya, Toyoaki Murohara, and Itsuo Kodama consulted on the manuscript. Dr. Stanley Nattel generated the original idea, supervised all the aspect of the work, and finalized the manuscript for publication. As the second author of this paper, my major contribution to this paper is to unveil the role of miR-26 in regulation of TRPC3 in AF, for which I proposed the original idea. My findings are important for the understanding of how

TRPC3 is dysregulated in AF and help importantly to the final acceptance of the paper in Circulation. More specifically, I proposed, designed, and performed the experiments (Real-time RT-PCR and bioinformatic analysis) related to the identification of miR-26 as the candidate to regulate TRPC3 in AF, as shown in Figure 6A; I designed and helped Dr. Harada with the cloning of the luciferase constructs of 3'UTR of TRPC3 mRNA bearing the wild-type or mutated miR-26 binding sites. In addition, I designed the miR-26 mimic duplex and miR-26 knockdown oligos and helped in the luciferase experiments, together with Dr. Harada, we measured the luciferase activities of the wild-type and mutated constructs in response to miR-26 overexpression and knockdown in HEK293 cells, the results of which generate Figure 6B. Moreover, I designed, proposed, and, partially contributed to the cell proliferation as well as the Western blot experiments in freshly-isolated fibroblasts subjected to miR-26 over-expression or knockdown and these results are shown in Fig. 6C, 6D and 6E. Furthermore, I proposed and helped in the design of study related to the NFAT-regulation of miR-26 in AF, for which, I partially performed the Realtime RT-PCR and Western blot experiments as shown in Fig 7D and 7E, respectively. Finally, I performed experiments looking at the relative expression of miR-26 in both cardiomyocytes and freshly-isolated fibroblasts and generated the Suppl. Fig. 12.

CHAPTER 1. INTRODUCTION

1.1 Overview of Atrial Fibrillation

Atrial fibrillation (AF) represents the most clinically-encountered sustained cardiac arrhythmia and contributes significantly to cardiac morbidity and mortality. AF increases the risk of developing congestive heart failure (CHF) and stroke, leading to an increased demand on healthcare, and thereby posing a significant socioeconomic burden [1, 2].

1.1.1 Epidemiology of AF

Approximately 0.5% to 1% of the general population is affected by AF [3, 4]. According to a retrospective study in the United States (US), it is estimated that 3.03 million Americans were suffering from AF in 2005 and this number will reach 7.56 million by 2050 [5]. Several epidemiological studies have suggested that the prevalence and incidence of AF increases dramatically in aging population [6-9]. The prevalence of AF increases from 1% in people under 60 to 8% in the population aged 80 or older [4]. It is predicted that the number of AF patients will likely increase by two or three fold within the next two or three decades [10]. The incidence of AF also displays a gender-specific disparity. Based on the Framingham Heart Study, the likelihood of developing AF in men is 1.5 fold greater than that in women with the corrections for age and other predisposing conditions [6]. However, the total number of female AF patients is actually equal to or greater than that of male AF patients because women have longer lifespan [11]. The racial discrepancy of AF has also been reported in several studies, showing that AF is more prevalent in whites than in blacks among the populations either with or without cardiac complications [12-14]. However, the reason for this discrepancy is complex and remains unclear [12, 13, 15].

1.1.2 Classification of AF

The classification of AF was well defined in the 2006 guideline laid out by the joint effort of ACC (American College of Cardiology), AHA (America Heart Association) and ESC (European Society of Cardiology) [16-18]. According to this guideline, AF is classified based on the duration and responses to treatments, which generally falls into three main

categories: paroxysmal, persistent and permanent [16-18]. Paroxysmal AF occurs periodically and is able to self-terminate within a short period (as short as a few seconds or as long as a few hours or days). Persistent AF does not terminate spontaneously but it can be ceased upon proper treatments (either pharmacologic or direct-current electrical cardioversion). Permanent AF, which is irreversible, shows no response to either medication or electrical cardioversion, and hence lasts indefinitely. Permanent AF can arise from paroxysmal and persistent AF [19]. Paroxysmal AF is believed to represent the natural origin of AF. It can gradually develop into persistent or permanent forms through a process termed "atrial remodeling", in which, the changes in the electrical and structural properties of the atria is caused by AF itself and/or the underlying cardiac conditions [19, 20]. In addition to the above classification, AF can also be classified by the features of the patients: lone AF (AF patients aged under 60 in the absence of underlying cardio-pulmonary disease), valvular AF (AF in these patients is caused by structural changes in the mitral valve or congenital heart diseases), non-valvular AF (AF patients with no sign of rheumatic mitral valve disease, prosthetic heart valve, or mitral valve repair), and secondary AF (AF occurs as a secondary event under pre-existing cardiac disorders) [16-18].

1.1.3 Signs, Symptoms and Predisposing Factors of AF

1.1.3.1 Signs and Symptoms of AF

AF patients can be symptomatic or asymptomatic depending on their awareness of the rapid and irregular heart rate. Clinical presentations of symptomatic AF patients include palpitation (the most common symptom in AF), dizziness, fainting, weakness, fatigue, breath shortness, or angina (chest pain caused by lack of blood supply) [16-18].

1.1.3.2 Predisposing Factors for AF

Substantial evidence has indicated that age is one of the most important risk factors for AF [4-11, 21-23]. Meanwhile, at any given age, gender is also considered as a predisposing factor for AF [8, 9, 21-25]. Other important risk factors predisposing to AF include hypertension [8, 9, 26-29], coronary artery disease [8, 30-32], congestive heart failure [8, 9,

33, 34], valvular heart disease [8, 9, 35, 36], cardiomyopathies [37, 38], myocardial infarction [9, 39-41], diabetes mellitus [8, 9, 42, 43], pulmonary disease [8, 44], postoperative state [45, 46] and hyperthyroidism [47, 48]. AF-promoting phenomenon as seen under the above cardiac conditions is likely due to the accompanied augmentation of atrial pressure and/or atrial dilation [49]. Nonetheless, detailed mechanisms remain largely unknown and merit further investigations.

1.1.4 Current Management and Challenges in Treatment of AF

The goals of AF treatment and management are to correct chaotic and irregular atrial contraction and to prevent thromboembolism and stroke [16-18]. In clinical practice, two main strategies (rhythm control and rate control) are commonly applied to restore normal atrial activities [16-18, 50]. Rhythm control aims to restore and maintain sinus rhythm, whereas rate control focuses on maintenance of normal ventricular response (or the effective heart rate). Furthermore, anticoagulation therapy serves as an important strategy to prevent thromboembolism and stroke during AF treatment and is recommended as a mandatory procedure for both rhythm and rate control treatments [16-18, 50]. Approaches adopted in AF treatment generally fall into two main categories: pharmacological and non-pharmacological. Pharmacological approaches primarily involve using anti-arrhythmic drugs that preferentially control rhythm (Class I and III) or ventricular rate (Class II and IV) or, in some cases, control both rhythm and rate depending on the principle actions of the drug. Non-pharmacological approaches, on the other hand, refer to interventions with attempts to terminate arrhythmia without using pharmacological agents; these include direct current cardioversion (electrical cardioversion), catheter ablation, and other surgical therapies (maze procedure and left atrial appendage obliteration). In general, pharmacological approaches (except amiodarone) are considered as the first-line treatments of AF because of their non-invasive properties, whereas most of the non-pharmacological approaches involve invasive intervention, and thus are considered as the second-line treatment for AF.

In spite of the availability of various approaches for rhythm and rate control strategies, there has been a long debate for clinicians in the choice of intervention. From the

theoretical point of view, rhythm control seems to be a better option. This is based on a notion that reduced ventricle function manifested during AF is able to be reversed once the regular cardiac rhythm is restored, and consequently, the normal heart rate would be retained and thromboembolism would be prevented [51]. However, data from several comparative studies [52-56] and meta-analysis [57] have suggested that rhythm control is suboptimal in terms of mortality due to adverse cardiovascular events [52-56]. In fact, none of these studies showed the expected outcomes from rhythm control [52-56]. Instead, in Atrial Fibrillation Follow-Up Investigation of Rhythm Management (AFFIRM) study, lack of anticoagulants appeared to be the strongest risk factor for stroke, with increased incident of stroke in patients for whom the oral anticoagulation treatment was discontinued while their sinus rhythm was restored [56]. The suboptimal outcome of rhythm control in AF treatment is, probably, in part due to the poor tolerability, limited efficacy, and the potential pro-arrhythmic effect of presently available anti-arrhythmic drugs. Moreover, re-analysis of the cause-specific mortality in AFFIRM study further revealed that several of the anti-arrhythmic drugs used for rhythm control were also correlated with significant increase in mortality due to non-cardiovascular causes [58]. Collectively, it seems that rate control is more preferable than rhythm control in AF treatment. Nevertheless, this conclusion might be oversimplified, given the fact that the currently available population study is limited and that none of these studies shows clear advantages between the two strategies [59]. Taken together, it appears that neither of rate-control, rhythm-control, or anticoagulation strategies is mutually exclusive of each other.

1.2 Mechanisms of AF

1.2.1 Physiological Basis of Cardiac Action Potential

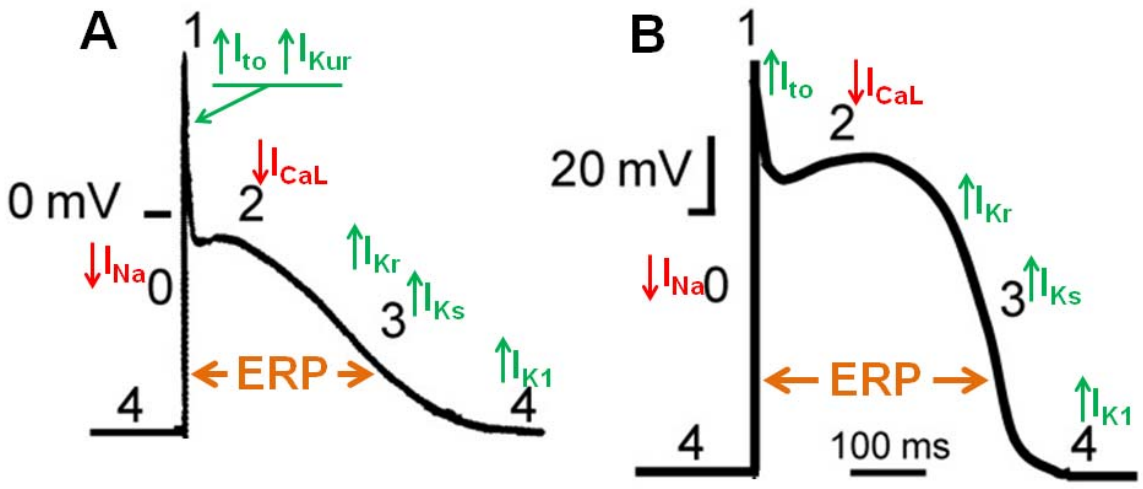

Figure 1. Schematic representation of atrial (A) and ventricular (B) action potentials with underlying principle ionic currents. Inward currents (with downward arrows): I_{Na}-sodium current; I_{CaL}-L-type calcium current; Outward currents (with upward arrows): I_{to}-transient outward current; I_{Kur}-ultra rapidly activating delayed rectifier current; I_{Kr} and I_{Ks}-rapidly and slowly activating delayed rectifier current; I_{K1}-inward rectifier current; Note that I_{Kur} is presented only in the atria. Numbers in black indicate the different phases of action potential. ERP: effective refractory period. (Adapted from Ravens U. et al. [60] with modifications).

The normal electrophysiological behavior of the heart is determined by the orderly propagation of electrical impulses resulting in rapid depolarization and slow repolarization, thereby generating action potentials in individual cardiac cells. In order to better understand AF mechanisms, it is essential to review the basic physiology of the cardiac action potential (AP). As the key determinant of the rhythmical contraction of cardiac cells, cardiac AP reflects the alterations of transmembrane potentials which are determined by inward (depolarizing) and outward (repolarizing) currents (Figure 1). Both atrial (Figure 1A) and ventricular (Figure 1B) APs last several hundred milliseconds and consist of five phases: Phase 0, Phase 1, Phase 2, Phase 3, and Phase 4. A rapid depolarization occurs during the phase 0 of AP, which is the result of a large inward current carried by voltage-gated sodium channels (I_{Na}) (Figure 1). This rapid depolarization is followed by a phase 1 early repolarization, which is primarily due to the inactivation of I_{Na} as well as the activation of the transient outward potassium current (I_{to}) and the ultra-rapidly activating delayed

rectifier potassium current (I_{Kur}) (in atria) (Figure 1A). Repolarization continues in phase 2 of AP, where inward L-type calcium current (I_{CaL}) counteracts with a gradually-increasing outward repolarizing potassium currents that are mainly composed of the rapid delayed rectifier potassium current (I_{Kr}) (Figure 1A&B). This results in a long lasting repolarization, constituting a morphological "plateau" shape in cardiac AP (Figure 1A&B). It is noteworthy that the shape of atrial AP (Figure 1A) is normally triangular because of the relatively short plateau phase compared with ventricular action potential (Figure 1B). Phase 3 is the final repolarization step in AP. Both I_{Kr} and slow delayed rectifier potassium current (I_{Ks}) contribute to terminate the repolarization during this phase. Once repolarization is complete, cardiac cells return to resting membrane potential during phase 4 of AP. The maintenance of the resting membrane potential in phase 4 is determined by the inward rectifier potassium current (I_{K1}). Collectively, increase in inward currents will tend to prolong the action potential duration (APD), whereas increased outward currents abbreviate it. It is important to note that cardiac cells are refractory to the initiation of new APs during phases 0, 1, 2, and part of phase 3 (Figure 1A&B). This is termed the effective refractory period (ERP). During this period, no stimulus regardless of its strength is able to initiate a propagated AP until cells return to phase 4. The ERP acts as a protective mechanism to keep the heart rate in check and coordinate the cardiac muscle contraction, thereby preventing arrhythmia. It is directly correlated with APD. For example, the shorter ERP is often associated with shorter APD.

1.2.2 Overview of AF Pathophysiology

AF is defined as a supraventricular tachyarrhythmia with characteristic chaotic and uncoordinated contraction of atrium, which results in a mechanical dysfunction of the atria. The first modern notions about the mechanism of AF was introduced in the early twentieth century [49]. Over the past century, researches have greatly increased our knowledge in understanding of AF mechanisms; it is now believed that focal ectopic activity (or triggered activity) and reentry (single-circuit reentry or multiple-circuit reentry) are the underlying pathophysiological mechanisms for AF initiation and maintenance [49, 61, 62], as shown in Figure 2.

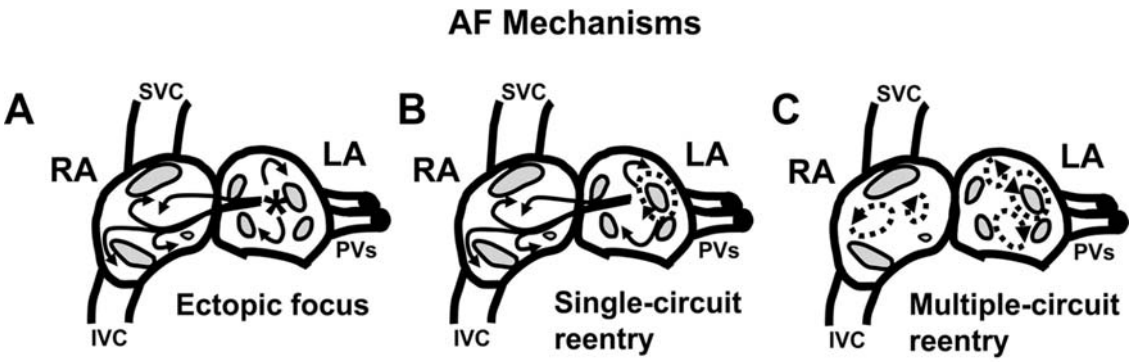

Figure 2. Arrhythmogenic mechanisms underlying AF. A. Ectopic focus. B. Single-circuit reentry. C. Multiple-circuit reentry. (Adapted from Iwasaki Y. et al. [61]).

1.2.2.1 Ectopic Mechanism

Up until Haissaguerre et al. [63] discovered that ectopic beats originating in the pulmonary veins played a pivotal role in AF initiation, the multiple-circuit reentry remained the sole dominant theory for AF mechanism. Abnormal automaticity, delayed afterdepolarization (DAD), and early afterdepolarization (EAD) constitute the principle mechanisms underlying focal ectopic activity as demonstrated in Figure 3 [62].

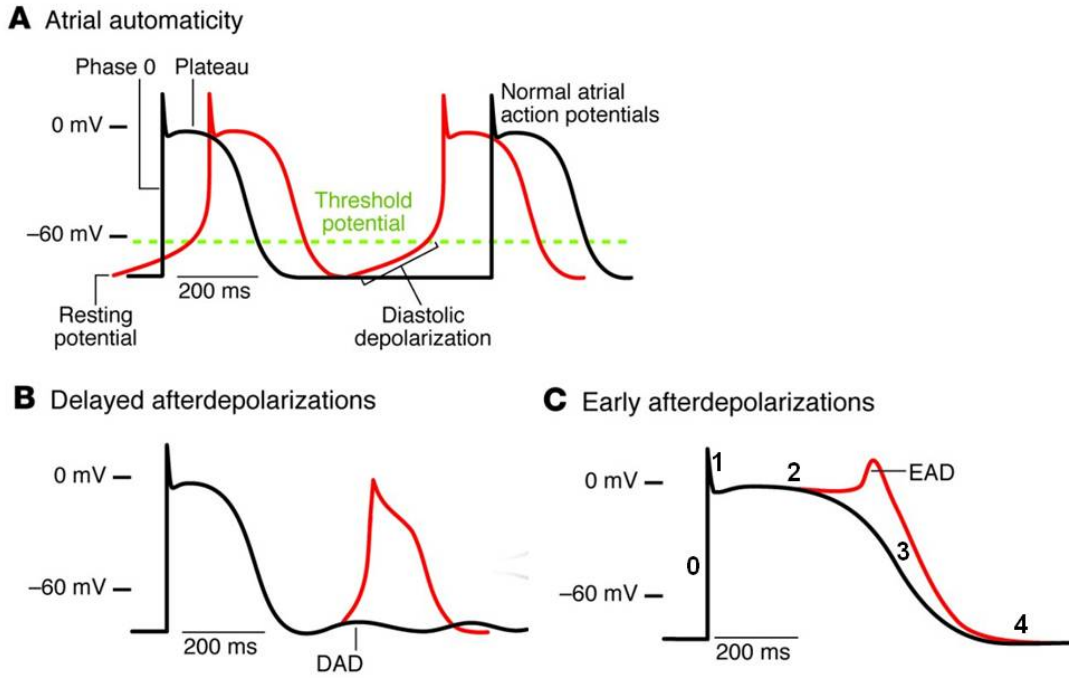

Figure 3. Cellular mechanisms underlying focal ectopic activity. A. Abnormal automaticity. B. Delayed afterdepolarization. C. Earlier afterdepolarization. Numbers in panel C indicate

the different phases of action potential. (Adapted from Wakili R. et al. [62] with modifications).

1.2.2.1.1 Abnormal Automaticity

Normal atrial cells are fired through the sinoatrial (SA) node pacemaking system, and then return to negative potential (resting potential, ~ -80mV) until the next firing from SA node (Figure 3A, black line). However, when the cell membrane reaches critical potential (or threshold potential, ~ -60mV) before the next normal impulse from SA node, an abnormal spontaneous depolarization may occur, resulting in abnormal automaticity (Figure 3A, red line). It is worth noting that the role of abnormal automaticity as a proarrhythmic mechanism during AF remains unclear [62].

1.2.2.1.2 Delayed Afterdepolarization

Delayed afterdepolarizations (DADs) occur after the complete repolarization of the triggering action potential and account for the most important mechanism underlying focal ectopic activity in AF (Figure 3B) [62]. DADs are caused by excessive diastolic Ca^{2+} released from sarcoplasmic reticulum (SR, the main cardiac Ca^{2+} storage organelle). The excessive diastolic Ca^{2+} are handled by transmembrane Na^{+}/ Ca^{2+} exchanger (NCX) in an electrogenic manner, extruding one Ca^{2+} ion while pumping 3 Na^{+} into the cell. This creates a net inward current, which can depolarize the cell, resulting in an afterdepolarization. Therefore, both increased diastolic Ca^{2+} and enhanced NCX activity may contribute to DADs. The DAD-related AF is associated with congestive heart failure [64] and genetic defect [65].

1.2.2.1.3 Early Afterdepolarization

Early afterdepolarizations (EADs) occur during phase 2 or 3 of the action potential (Figure 3C). This type of afterdepolarization is primarily caused by recovering of L-type Ca^{2+}

channels from inactivation during the plateau phase due to the excessively-prolonged APD. EAD-related AF has been seen in patients with congenital long-QT syndrome [66].

1.2.2.2 Re-entrant Mechanisms

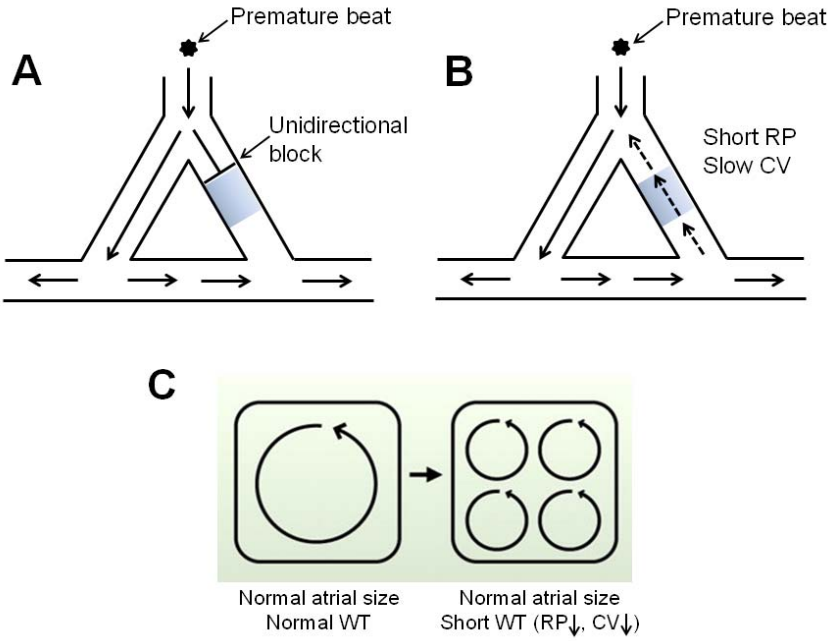

Figure 4. Mechanisms for reentry. A. An unidirectional block occurs when an ectopic impulse dies out in a still-refractory region. B. Conditions for maintenance of reentry. C. Conditions favoring multiple-circuit reentry. RP: refractory period; CV: conduction velocity. Blue-shadow area represents still-refractory region. (Panel C adapted from Nattel S et al. [67] with modifications).

Reentry is conventionally initiated by a premature ectopic beat (Figure 4A). This ectopic beat fails to conduct through a still-refractory region in one direction ("unidirectional block") (Figure 4A), while conducting in the other direction through a region that is no longer refractory (Figure 4A). However, if enough time has elapsed for the recovery of excitability in the refractory region, the impulse can re-enter this region, resulting in reentry (Figure 4B). Accordingly, shorter refractory period (allowing faster recovery of excitability) and/or slower conduction velocity (gaining more time for the non-refractory region to regain excitability) may favor the maintenance of reentry (Figure 4B). Reentry can sustain either as a single circuit form (Figure 1B) or a multiple-circuit form (Figure 1C). For single circuit reentry, the irregular activity is maintained by rapid regular firing

(Figure 1B), whereas for multiple-circuit reentry, the irregularity is determined by the co-existence of multiple dyssynchronous reentry circuits (Figure 1C). There are two leading theoretical models that conceptualize the mechanism of reentry: leading circuit [68] and spiral wave [69]. While both models predict the presence of vulnerable substrates as the key determinant in reentry sustainability, the leading circle theory fails to explain the clinical observation that blocking of Na^+ channel effectively terminates AF [70]. Nevertheless, leading circle theory still remains the widely-accepted notion to explain reentry mechanism. An important concept of leading circle theory is the "wavelength of reentry" [49, 68]. The wavelength (WL) of a reentry circuit refers to the shortest pathlength by which a reentry can be established. It is given as the distance that an electrical impulse travels in one refractory period and is critically determined by refractory period (RP) and conduction velocity (CV), as shown in the equation: WL=RP × CV [70, 71]. Accordingly, a shorter wavelength (as the result of abbreviated RP and/or reduced CV) may allow more available reentry circuits to be accommodated in a given atria, favoring multiple-circuit reentry that sustains AF (Figure 4C).

1.2.2.3 Relation of basic AF mechanisms to different forms of clinically-encountered AFs

The natural course of AF is believed to begin as the paroxysmal form, which is triggered by the ectopic foci originated from pulmonary vein sleeves [63].With time, AF tends to become persistent if ectopic firing is sustained or reversible reentry substrates are developed [62]. If the reentry substrates further become irreversible or fixed, AF becomes permanent [62].

1.3 Atrial Remodeling in AF

The introduction of the concept "atrial remodeling" over the past two decades has greatly advanced our understanding of AF mechanisms. Atrial remodeling refers to the process by which AF, once initiated, alters atrial electrophysiological (in a short term) and/or structural (over a long term) properties in ways that sustain itself [72].

1.3.1 Electrical Remodeling in AF

AF-induced electrical remodeling, which results in trigger activity and functional reentry substrates by altering ion channel/transporter expression and/or function, occurs normally within a short time after AF initiation [73]. Both focal ectopic activity and functional reentry are primarily caused by increased Ca^{2+} loading due to the very rapid atrial rate (tachycardia) during AF [73]. Therefore, more recently, AF-induced electrical remodeling has been termed as atrial tachycardia remodeling (ATR) [67]. Evidence from animal models and clinical studies has highlighted the importance of ATR in pathogenesis of AF and in the transition from paroxysmal AF to persistent AF [72, 74-81]. A prominent finding in ATR is the abbreviation of refractoriness as the result of APD shortening. Consistently, a shorter atrial APD is found in AF patients compared to patients with sinus rhythm [78-81]. Similar observations have also been seen in animal models of AF [72, 74-76].

The ionic mechanisms underlying APD shortening primarily involve downregulation and/or inactivation of L-type calcium current (I_{CaL}) and enhancement of inward rectifier potassium currents (I_{K1} and/or I_{KACh}). The functional implication of the deregulation of other ion channels in APD shortening remains obscure. Details regarding the pathophysiological significance of these changes will be described below.

1.3.1.1 Upregulation of I_{K1}

One of the most important electrophysiological changes in AF-related ATR is the upregulation of inward-rectifier potassium channel, particularly, I_{K1}. Increased I_{K1} has been consistently observed in isolated atrial cardiomyocytes from AF patients [82-89] and experimental AF animal models [90-92]. Interestingly, the functional change of I_{K1} in AF is in parallel with increased expression of its underlying subunits Kir2.1 at both mRNA and protein levels [83, 85]. The pathophysiological significance of I_{K1} upregulation in AF is believed to enhance AF sustainability by substantiating reentrant substrates [20, 73]. This is likely attributable to the crucial roles of I_{K1} in maintenance of resting potential and termination of action potential [67, 73]. Increased I_{K1} hyperpolarizes the atrial cells, which, on one hand, inhibits the abnormal automaticity, whereas on the other hand, shortens APD and ERP, thereby favoring the reentrant substrates for AF [67, 73, 93, 94]. Recently,

genetic evidence from familial AF as the result of a Kir2.1 gain-of-function mutation further indicates the pivotal role of I_{K1} upregulation in AF pathogenesis [95]. Nevertheless, the underlying molecular mechanism responsible for enhanced I_{K1} in AF is largely unknown. Interestingly, a new class of recently-discovered small non-coding RNA, namely microRNAs (miRNAs), has been suggested to be responsible for the upregulation of I_{K1} in AF [96].

1.3.1.2 Downregulation of I_{CaL}

Another key finding in AF-related ATR is the downregulation of I_{CaL}. I_{CaL} has been consistently found to be downregulated in both clinical and experimental AF paradigms [76, 82, 87, 97-100], accompanied by corresponding decreases of its α-subunit Cav1.2 at mRNA level [101-107]. However, discrepant changes for Cav1.2 protein have been reported [99, 101, 103, 104, 107, 108], implying the complicated underlying mechanisms as well as uncontrollable variables in clinical studies [67]. The observed I_{CaL} reduction in AF is believed to be an adaptive response of atrial cardiomyocytes to calcium overload as a consequence of rapid atrial rate during AF [49]. This adaptive response, in a short term, tends to reduce channels activity, but over a longer term, tends to decrease the expression of the channels at the gene level [49]. As a result, reduced I_{CaL} decreases APD and wavelength, creating reentry substrate for AF perpetuation [49]. Although the molecular mechanism underlying ATR-induced I_{CaL} downregulation has been extensively investigated by many research groups, it is still not completely understood. While some studies indicated that the transcriptional downregulation of Cav1.2 α-subunit is likely due to the activation of Ca^{2+}/calmodulin/calcineurin/NFAT signaling pathway caused by Ca^{2+} overload [107, 109], others suggested the possible involvements of the downregulation of accessory β_1-, β_{2a}-, β_{2b}-, β_3- and $\alpha_2\delta_2$-subunits, impairment of Cav1.2 protein trafficking, and dephosphorylation of the channels due to enhanced PP1 and PP2A activities [85, 99, 102, 110-112]. Furthermore, a recent study demonstrated that miRNAs may potentially contribute to the downregulation of I_{CaL} in AF through a post-transcriptional regulatory mechanism [113]. Taken together, the underlying molecular mechanism for AF-induced I_{CaL} downregulation appears to be a very complex process, involving both transcriptional and post-transcriptional regulations.

1.3.1.3 Abnormal Ca^{2+} Handling

In addition to the reduction of I_{CaL}, AF-induced electrical remodeling also causes abnormal intracellular Ca^{2+} handling, which primarily promotes spontaneous diastolic SR Ca^{2+} release that enhances NCX-mediated Ca^{2+} extrusion. The NCX-mediated Ca^{2+} extrusion is an electrogenic process, which produces a net inward (depolarizing) current, causing DADs [62, 73]. Several studies have suggested that the spontaneous diastolic SR Ca^{2+} release is attributed to the dysfunction of ryanodine receptor 2 (RyR2, cardiac Ca^{2+} release channel on SR) [114-117], which is likely due to hyperphosphorylation caused either by increased activities of protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent kinase II (CaMKII) [115-118] or by decreased activities of phosphatases [119]. Additionally, abnormal RyR2 release can also be a result of excess SR Ca^{2+} loading due to abnormal enhancement of SR Ca^{2+} uptake mediated through SR Ca^{2+} ATPase (SERCA, Ca^{2+} uptake pump on SR), which is activated by hyperphophorylated phospholamban [119].

1.3.1.4 Alterations of other K^+ currents

I_{KACh}, another important inward rectifier potassium current, is activated upon the stimulation of acetylcholine released from vagal nerve and is responsible for cardiac vagal effects. Activation of the channels hyperpolarizes the cell membrane and shortens APD, contributing to AF pathogenesis. Several studies have shown that the agonist-dependent I_{KACh} is reduced, whereas constitutively-active I_{KACh} (agonist-independent) is significantly enhanced during AF-induced ATR [83, 91, 120, 121]. However, mRNA and protein levels of its underlying subunit Kir3.1 and Kir3.4 are found unaltered in experimental AF animal models [120, 121] but decreased in AF patients [122]. The enhanced constitutively-active I_{KACh} is attributable to increased single channel open probability, which is controlled by the balance between different protein kinase C (PKC) isoforms [123, 124].

Consistent reduction of I_{to} along with the mRNA and protein of its underlying α-subunit Kv4.3 is found in both AF patients and experimental AF animal models [76, 86, 101, 104]. However, the functional implication of I_{to} reduction in AF remains to be unclear [67, 73].

Inconsistent changes of I_{Kur} have been reported in both experimental AF animal models and AF patients [67], indicating the lack of pathological relevance of this channel in AF. However, given the atrial specific property of I_{Kur}, it still remains as a very attractive therapeutic target for the treatment of AF [49].

No changes of delayed rectifier currents I_{Ks} and I_{Kr} were observed in a canine model of AF [76] and there is lack of information regarding the changes of these currents in humans due to the technical difficulty in acquiring human samples and cell isolation [125].

1.3.2 Structural Remodeling in AF

Atrial structural remodeling (ASR) is another major aspect of atrial remodeling in AF. It is a relatively slower remodeling process compared to electrical remodeling, primarily comprising morphological changes to atrial myocardial structure and atrial architecture [20, 126]. ASR has been observed in AF from both clinical settings and experimental models, and significantly contributes to form the reentry substrates for AF. Consequences of ASR include increased atrial fibrosis, altered connexin expression, myocyte hypertrophy, myocyte apoptosis, and atrial dilation, among which, the first three aspects have been extensively studied.

1.3.2.1 Atrial Fibrosis

Atrial fibrosis, although not exclusively related to AF but appears as a common feature of clinical AF. It is one of the most important arrhythmogenic contributors to AF [73]. Atrial fibrosis has been commonly observed in lone AF [127, 128] and AF-associated pathophysiological conditions, including congestive heart failure (CHF) [129, 130], valvular diseases [131], rheumatic heart disease [132, 133], dilated and hypertrophic cardiomyopathy [134, 135], and senescence [136, 137]. Moreover, a reduced AF stability and delayed atrial structural remodeling process have been observed in some experimental and clinical studies using compounds with known anti-fibrotic effect, such as statins (HMG-CoA reductase inhibitors) [138, 139], angiotensin II type 1 receptor (AT1R) blocker

[140], and fish oil [141]. However, the observed beneficial outcome of these compounds may be a result of the improvements in hemodynamic [73]. Together, these findings suggest an important association between atrial fibrosis and AF but fail to establish a causal significance of atrial fibrosis in AF occurrence and perpetuation. It is also important to note that AF may itself promote atrial fibrosis, which in turn sustains AF [142, 143]. Evidence supporting this notion came from the observations that the quantity of fibrosis positively correlates with the persistence of AF in patients [144], and that atrial tachypacing alone causes atrial fibrosis in a canine ATP model with well-controlled ventricular rate [145].

Cardiac fibrosis is either a reparative or reactive process, which primarily involves excessive accumulation of extracellular matrix (ECM) proteins secreted by myofibroblasts (cells derived from fibroblasts in the presence of various stimuli) [142, 143]. Reparative fibrosis maintains tissue structural integrity by replacing dead cardiomyocytes, whereas reactive fibrosis occurs in response to various cardiac insults and causes interstitial expansion, separating muscle bundles [142, 143]. The resultant fibrotic tissue creates an obstacle to interfere with normal impulse propagation and thus causes conduction abnormalities [129]. In addition, recent studies have suggested that cardiac fibroblasts can electrically influence adjacent cardiomyocytes and thus alter cardiac electrical function [143]. Taken together, the fibrotic remodeling manifested during AF may cause conduction abnormalities and increase fibroblast-cardiomyocyte electrical interaction, favoring AF occurrence and maintenance.

1.3.2.1.1 Profibrotic Factors and Atrial Fibrosis

The precise molecular mechanisms underlying ECM formation during atrial fibrosis remain incompletely understood. Emerging evidence has suggested that several cardiomyocyte and/or fibroblast secreted factors with known profibrotic effects are critically involved [142]. Among these factors, angiotensin II (AngII) and its downstream mediator transforming growth factor β_1 (TGF-$\beta_{1)}$ have been well characterized to contribute to fibroblast differentiation and proliferation, whereas platelet-derived growth factor (PDGF) and connective tissue growth factor (CTGF) have just emerged as potential fibrosis mediators [20, 142].

It has been shown that increased production of AngII as a consequence of cardiac-specific overexpression of angiotensin converting enzyme (ACE) may cause severe atrial dilation accompanied by atrial fibrosis and AF in mice [146]. AngII promotes cardiac fibrosis via signaling cascades coupled to AT1R and AT2R, which have distinct and sometimes opposite effects (eg. AT1R facilitates fibrosis while AT2R counteracts the effect of AT1R); the net outcome of these opposite effects is an activation of mitogen-activated protein kinase (MAPK) that is considered as an important mediator for fibrotic remodeling [142, 147]. In addition, stimulation of AT1R can also activate protein kinase C (PKC) and increase intracellular Ca^{2+} release, together contributing to AngII-mediated fibrotic remodeling [147].

TGF-β_1 has been suggested to play a central role in signaling cascades involved in cardiac fibrosis [142, 148]. TGF-β_1 is a primary downstream mediator upon AngII stimulation and is secreted by both fibroblasts and cardiomyocytes [149]. Cardiac-specific overexpression of constitutively-active TGF-β_1 in mice causes atrial fibrosis and atrial conduction heterogeneity and increases AF vulnerability [150]. TGF-β_1 primarily acts through the SMAD signaling pathway, stimulating fibroblast activation and collagen production [151].

More recently, a study showing the presence of cardiac fibrosis in mice with cardiac-specific PDGF overexpression has indicated the potentially-important role of PDGF in the genesis of cardiac fibrosis [152, 153]; however, the link between PDGF and fibrosis-related AF susceptibility was missing, given that AF vulnerability was not assessed in these animals [142, 152]. CTGF is also found to be an important mediator in cardiac fibroblast activation upon AngII stimulation [154]. Moreover, pathway analysis in a genomic study of CHF-related AF substrate has suggested a possible involvement of CTGF in this process [155].

1.3.2.1.2 Potential role of TRP Channels in Atrial Fibrosis

There is a growing body of evidence suggesting that fibrotic susceptibility is more profound in atria than in ventricle [129, 146, 150, 156, 157]. Atrial fibroblasts show greater proliferative response and faster myofibroblast differentiation upon growth stimuli, as

compared to ventricular fibroblasts [149]. Fibroblast proliferation and differentiation into myofibroblasts are the characteristics of fibrogenesis and numerous studies have suggested that Ca^{2+} entry is essential for fibroblast proliferation and fibroblast function [158-162]. Interestingly, however, the absence of voltage-gated Ca^{2+} channels in cardiac fibroblasts suggests the existence of other Ca^{2+}-permeable ion channels that are responsible for Ca^{2+} entry in these cells [163]. The presence of transient receptor potential (TRP) channels in cardiac fibroblasts has shed light on this matter. TRP channels are well-known to be responsible for Ca^{2+} entry in a variety of cell types and are activated upon mechanical stretch, oxidative stress, and metabolic disturbance, conditions commonly observed in AF [164-166]. Hence, it is very likely that TRP channels may participate in atrial fibrogenesis in AF, particularly via stimulation of fibroblast proliferation and differentiation. To date, 28 mammalian TRP channel genes have been identified [164]. Based on sequence homology, they are categorized into six subfamilies: TRPC subfamily (canonical, TRPC1-7), TRPM subfamily (melastatin, TRPM1-8), TRPV subfamily (vanilloid, TRPV1-6), TRPA subfamily (ankyrin, TRPA1), TRPP subfamily (polycystin, TRPP1-3), and TRPML subfamily (mucolipin, TRPML1-3). However, only subsets of these families are detectable in cardiac fibroblasts. For instance, TRPC1-3, TRPC5-7, TRPM7 and TRPV4 are expressed in rat and human cardiac fibroblast as measured by RT-PCR [167, 168]. Of note, a recent study has highlighted the potentially important role of TRP channels in AF fibrogenesis [168]. This study showed that atrial fibroblasts from AF patients are more prone to myofibroblast differentiation with concomitant increases of TRPM7 expression and Ca^{2+} influx. Moreover, in vitro knockdown of TRPM7 largely attenuates AF fibroblast differentiation, suggesting an essential role of TRPM7 in AF fibrogenesis [168].

1.3.2.2 Dysregulation of Gap junction protein

The conduction abnormality in AF can also result from altered and/or heterogeneous expression of the gap junction proteins, namely connexins, which are responsible for cell-to-cell conduction [67, 73]. Connexin40 (Cx40) and connexin43 (Cx43) are two main subunits in atria, whereas, in ventricular tissue, Cx43 appears to be the sole underlying subunit [169, 170]. Several studies have reported inconsistent changes of Cx40 and Cx43 in

AF, as some found increased while others found unaltered or decreased expression [67, 73]. Nevertheless, heterogeneous expression of Cx40 and Cx43 has been consistently documented in both experimental and clinical AF [171-174]. The increased connexin heterogeneity may contribute to abnormal conduction, thereby favoring reentry substrates for AF.

1.4 Inward Rectifier Potassium Channel (I_{K1})

1.4.1 Biophysical Properties and Cellular Functions of I_{K1}

I_{K1} is the main component of inward rectifier basal currents in human atrial myocytes [175, 176]. It plays two key roles in cardiac electrophysiology: 1) to maintain the cellular resting membrane potential; and 2) to terminate repolarization of cardiac AP [93, 177]. These are largely due to its "inwardly rectifying" property, which allows the channels selectively support the flow of positively-charged K^+ into the cells (inward current), when membrane potential is negative to potassium equilibrium potential (or reversal potential) [93, 177]. A simpler way to understand this is that the channels pass inward current more easily than outward current. Because the inward rectification of I_{K1} is strong, this allows the channels to carry substantial inward current at negative potentials (even more negative than the K^+ reversal potential), thereby stabilizing the membrane resting potential [177, 178]. On the other hand, when cell membrane depolarizes or is positive to K^+ reversal potential, the large conductance for inward current is progressively shut down as a result of "rectification", eliciting a relatively small but significant outward current, which helps to terminate the cardiac AP [177, 178]. The phenomenon of inward rectification of I_{K1} is the result of the block of the channel pore by intracellular Mg^{2+} and polyamines at positive potentials, resulting in a decrease of outward current [179, 180]. I_{K1} is constitutively active and extracellular Ba^{2+} and Cs^+ selectively block the channels in a voltage-dependent manner. This selective blockade by Ba^{2+} and Cs^+ is often used to distinguish I_{K1} from other currents in voltage clamp experiments [181].

1.4.2 Molecular Basis of I_{K1}

Substantial evidence has indicated that native cardiac I_{K1} is carried by channels that appear as heterotetramers consisting of Kir2 subunits (Kir2.1, Kir2.2, and Kir2.3) [182, 183]. Each of these subunits consists of two transmembrane domains (TM1 and TM2) [184]. As defined by human genome organization, genes encoding Kir2 subunits are given name *KCNJ*, i.e., *KCNJ2* (encoding Kir2.1), *KCNJ12* (encoding Kir2.2), and *KCNJ4* (encoding Kir2.3) [185]. In human, Kir2.1 is the most abundantly-expressed subunit underlying I_{K1} [186, 187]. The predominant role of Kir2.1 as the main molecular identity underlying I_{K1} was elucidated by using Kir2.1 and Kir2.2 knockout mice [188, 189]. No detectable I_{K1} was recorded in the isolated cardiomyocytes from Kir2.1 knockout mice, whereas, ~ 50% reduction of I_{K1} was observed in the myocytes from Kir2.2 knockout littermates, suggesting a crucial role of Kir2.1 in genesis of I_{K1} and functional I_{K1} channels are likely heteromeric [188, 189]. Indeed, this is supported by subsequent biochemical studies, confirming the co-assembly of different Kir2 subunits in cardiomyocytes [190, 191].

1.4.3 Regulation of I_{K1}

In addition to the direct regulation of the channel pore by intracellular Mg^{2+} and polyamines, native I_{K1} can also be regulated by many other factors including phosphatidylinositol-4, 5-bisphosphate (PIP2), adrenergic stimulation, protein kinases (PKA and PKC), and pH [192]. While circumstantial evidence has suggested that I_{K1} can be activated by PIP2 through its direct interaction with a group of positively charged residues located in the transmembrane domain 2 (TM2) region of Kir2.1 [193-195], other studies have suggested that residues harbored in cytoplasmic NH_2 and COOH termini also contribute to PIP2 activation of Kir2.1 [196]. Adrenergic stimulation is also reported to regulate the native I_{K1} [197, 198]. In isolated human cardiomyocytes, stimulations of both α1- and β-adrenergic receptors strongly inhibite native I_{K1} [197, 198]. This inhibitory effect upon stimulation of α1- adrenergic receptors is attributable to PKC activation [197], whereas β-adrenergic inhibition of the channel primarily is mediated by a PKA-dependent pathway [198]. Furthermore, modulation of I_{K1} can be achieved via phosphorylation of the channel subunits by protein kinases such as PKA and PKC [192]. PKC activators were

shown to repress I_{K1} associated with Kir2.2 and Kir2.3 but not that with Kir2.1 [123, 199]. Activation of PKA, however, increases currents carried by Kir2.1 or Kir2.2 [200, 201]. The native I_{K1} is also affected by intracellular pH. Decreased intracellular pH was shown to cause significant reduction of I_{K1} [202]; this is likely due to the abolishment of channel-PIP2 interaction [203]. Interestingly, a recent study has clearly demonstrated an involvement of microRNA in the regulation of I_{K1}. In this study, the authors showed that microRNA-1 (a type of cardiac-enriched muscle-specific non-coding small RNA) is able to repress native I_{K1} current through a post-transcriptional repression of the channel subunit Kir2.1, suggesting a potentially-important novel regulatory mechanism for I_{K1} [204].

1.4.4 Dysregulation of I_{K1} and its pathophysiological implication in AF

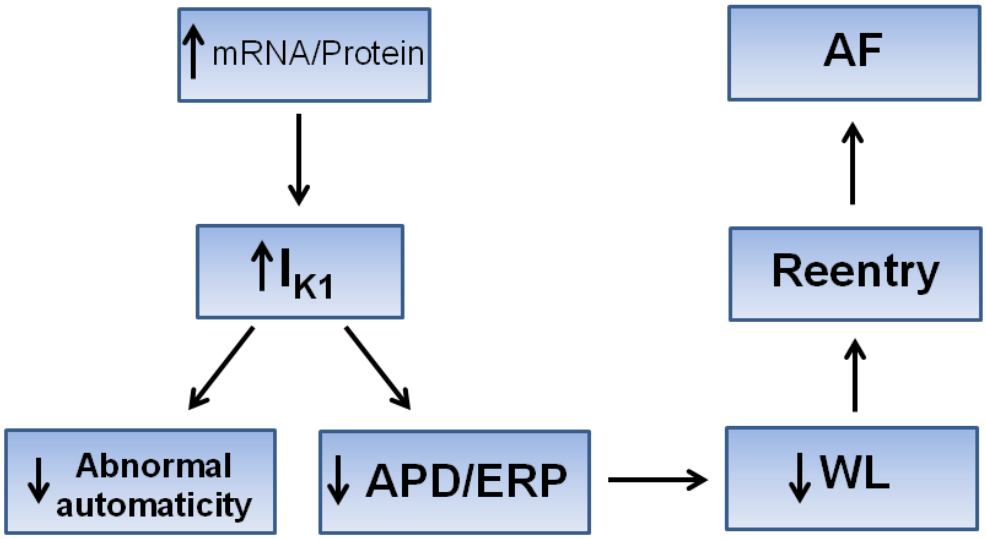

Figure 5. Schematic representation of pathophysiological significance of I_{K1} upregulation in AF. APD: action potential duration; ERP: effective refractory period; WL: wavelength; AF: atrial fibrillation.

A schematic illustration of the pathophysiological significance of I_{K1} upregulation in AF is shown in Figure 5. As discussed in Section 3.1.1, upregulated I_{K1} has been consistently observed during AF [82-89], and this upregulation was accompanied with the increased expression of its underlying subunit Kir2.1 at both mRNA and protein levels [83, 85]. Given the crucial role of I_{K1} in setting the cellular membrane resting potential and terminating cardiac AP, the upregulation of I_{K1} in AF, may have two pathophysiological

consequences: 1) the increased I_{K1} may favor the maintenance of cellular membrane resting potential, thereby preventing the abnormal automaticity (Figure 5); 2) the enhanced I_{K1} may contribute to the shortening of APD and ERP, favoring the maintenance of reentrant substrates for AF (Figure 5). However, little is known about the molecular mechanism underlying AF-induced upregulation of I_{K1}. Recently, a study provided a novel insight into this matter. In this study, the authors evaluated the correlation between the changes of miR-1 and the alterations of I_{K1} as well as Kir2.1 expression in the left atrium of the valvular heart disease patients with persistent AF [96]. I_{K1} was found to be significantly upregulated with a corresponding increase in Kir2.1 protein expression in the atrial samples from these AF patients [96]. Conversely, miR-1 levels were found to be significantly reduced [96]. The authors proposed that AF-induced upregulation of I_{K1} and its underlying subunit (Kir2.1) expression is likely attributable to the reduction of miR-1 [96], because miR-1 was previously reported to post-transcriptionally repress *KCNJ2* (gene encoding Kir2.1) [204]. The findings of this study are valuable in the sense that they suggest a potentially-important novel regulatory mechanism for AF-induced I_{K1} upregulation. However, the evidence provided in this study to support the role of miR-1 downregulation in AF-induced I_{K1} upregulation is only suggestive. Further studies are required to establish a direct role of miR-1 in AF. Moreover, it is important to determine whether there are other miRNAs involved in this AF-induced ionic remodeling process.

1.5 Transient Receptor Potential Canonical Channel Type 3 (TRPC3)

TRPC3 belongs to a group of TRPC channels, a classical or canonical subclass in TRP channels superfamily. The human TRPC family consists of six isoforms (TRPC1, 3-7), which can be grouped into two subfamilies: TRPC1/4/5 and TRPC3/6/7, based on sequence homology and functional similarities [205-207]. For example, TRPC3 shares ~75% sequence identity with TRPC6 and TRPC7 [208].

1.5.1 Biophysical Properties and Cellular Functions of TRPC3

Owing to the high degree of similarities among all TRPC isoforms and the lack of specific pharmacological blockers, it is technically difficult to record the currents carried by TRPC3 channels in a native environment; thus, the biophysical properties of TRPC3 channels are mainly characterized in either homologous or heterologous expression systems [205, 209, 210]. TRPC3 as well as its homologues TRPC6 and TRPC7 typically assemble as homo- or heterotetramer to form functional channels permeable to cations (Ca^{2+} and Na^{+}) [205, 209, 210]. The resultant currents show voltage independence and dual rectifications in both inward and outward directions [208, 211]. Moreover, TRPC3 channels are constitutively active and are effectively blocked by La^{3+} and Gd^{3+} ions, which also block the other TRPC channels. Interestingly, a recent study found a pyrazole compound (Pyr3) to be a selective blocker for TRPC3 channels [212]. This compound may serve as a powerful tool for the in vivo functional study of TRPC3 channels [212].

TRPC3 channels broadly exist in a variety of cell types including excitable and non-excitable cells and are predominantly located on the plasma membrane [205, 206, 209]. Their primary cellular function is thought to be non-voltage-gated Ca^{2+} entry [205-207, 213]. There are two types of Ca^{2+} entry mechanisms: store-operated Ca^{2+} entry (SOCE) and receptor-operated Ca^{2+} entry (ROCE). However, the participation of TRPC channels in these mechanisms is controversial [205-207, 213].

1.5.2 Molecular Basis of TRPC3

Functional TRPC3 channels are comprised of four TRPC subunits [209]; however, it is unclear whether the native TRPC3 channels exist as homotetramers or heterotetramers [209]. Typical structure of TRPC3 subunit includes six transmembrane domains (TM1-TM6), a putative pore region constituted by TM5 and TM6 together with their connecting loop, and intracellular N and C termini that are functionally important for channel trafficking, anchoring, localization, and gating [214, 215]. Of note, the lack of voltage sensing in TM4 makes the channels non voltage-dependent [206, 209, 215]. The *TRPC3* gene was originally cloned from human embryonic kidney cell line (HEK293) and consists of 11 exons that locate on chromosome 4 in the human genome [215]. The regional

expression profile of *TRPC3* in the heart is incompletely known, although evidence has indicated its broad expression in a variety of cardiac cells such as cardiac endothelial cells, cardiac smooth muscle cells, and cardiomyocytes [206, 216].

1.5.3 Regulation of TRPC3

The activity of TRPC3 channels can be regulated by mechanical stretch and various ligands and plasma membrane receptors [206, 209]. Substantial evidence suggests that mechanical stress may enhance TRPC3 channel activation and/or expression [217, 218]. It has been shown that TRPC3 channels are constitutively active and this basal activity of TRPC3 channel is attributable to the glycosylation status, which is increased upon stimulation of G protein-coupled receptor (GPCR) or receptor tyrosine kinase [211, 219-221]. Consequently, ligands such as AngII and ET-1, which can stimulate GPCR, were found to increase TRPC3 channel activity [222, 223]. Increased phospholipase C (PLC) activity as a result of activated GPCR-dependent signaling also enhances TRPC3 channel activity [224, 225]. This is primarily due to a direct activation of TRPC3 channels by diacylglycerol (DAG), a lipid mediator that is generated by the GPCR-PLC signaling pathway [224-226]. In addition to GPCR, several other studies have suggested that activation of inositol 1,4,5-trisphosphate (IP_3) receptor may indirectly activate TRPC3 channels through a displacement of inhibitory calmodulin from a common binding domain on the channel [227, 228]. Despite the regulation by plasma membrane receptors, TRPC3 channel activity as well as its expression can also be regulated by a redox-dependent alteration in membrane lipids, more specially, cholesterol [229-232]. Mechanistically, the agonist effect of cholesterol on TRPC3 channels is attributable to the association between TRPC3 and scaffolding protein, caveolin-1 [233]. Additionally, an interaction between immunophilin and TRPC3 protein has been suggested to be essential for channel activation, as there is a direct binding domain for immunophilin in the C-terminal proline-rich region of TRPC3 protein [234]. Finally, protein kinases have also been suggested to regulate TRPC3 channel activity [235, 236]. Indeed, PKC was found to negatively regulate TRPC3 through direct phosphorylation of serine at position 712 [236], whereas enhanced PKA activity was able to increase TRPC3 expression [235].

1.5.4 Dysregulation of TRPC3 and its potential pathophysiological implication in AF

As discussed in Section 3.2.1.2, TRP channels may likely contribute to Ca^{2+}-mediated atrial fibrogenesis in AF. Indeed, a recent study has highlighted an important role of TRPM7 channels in AF-associated fibrotic remodeling [168]. However, it is important to determine whether other TRP channels also participate in this remodeling process, given that several TRP channels including TRPM7 are abundantly expressed in cardiac fibroblasts and many of them are functionally and structurally similar [164, 168]. Interestingly, it has been shown in a recent study that, unlike other TRP members, TRPC3 is mainly enriched in freshly-isolated atrial fibroblasts and this enrichment diminishes upon fibroblast differentiation to myofibroblasts [237]. These findings suggest a potentially-important role of TRPC3 in mediating fibroblast function, which contributes importantly to fibrogenesis. In this regard, any deregulation of TRPC3 occurring during AF may potentially contribute to the AF-associated atrial fibrosis. Further studies are required to determine the changes of TRPC3 in AF as well as the underlying mechanisms responsible for this deregulation.

1.6 MicroRNA Biology

1.6.1 Historical View of MicroRNAs

MicroRNAs (miRNAs) belong to a new class of non-coding RNAs. The discovery of the first microRNA (miRNA), lin-4 occurred almost twenty years ago [238, 239]. However, this discovery was initially considered an anomaly. These small non-coding RNAs were not brought into attention until the identification of a second miRNA, let-7, which was found to be crucially involved in the development of *Caenorhabditis elegans* (*C elegans*) [240]. Subsequent studies soon revealed that let-7, as well as many other newly-identified miRNAs, broadly exist in various vertebrate species including humans, suggesting that these miRNAs are not only evolutionarily conserved but also ubiquitously expressed across different species [241-244]. Since then, miRNAs have increasingly attracted interest, leading to the discovery of many more mammalian miRNAs. A pioneer study showing the correlation between miRNAs and human disease appeared in 2002 [245]. 3 years later, in

2005, the first cardiac miRNA study was conducted, revealing that miRNA is essential during cardiogenesis [246]. During the past 6 years, the roles of miRNAs have been increasingly appreciated in the cardiovascular system by researchers, as these tiny molecules appear to be essential in controlling a wide range of biological processes. Recent studies in the field have clearly demonstrated that aberrant expression of miRNA is tightly correlated with the onset and progression of cardiovascular diseases [247, 248]; and in some cases, correcting an aberrantly-expressed miRNA is able to prevent or attenuate the progression of certain cardiac diseases [247, 248]. Together, these findings have greatly advanced our current understanding of the potential pathophysiological implications of miRNAs in various cardiac conditions [247, 248]. Hence, miRNAs may potentially serve as new diagnostic tools and therapeutic targets for cardiovascular diseases.

1.6.2 MiRNA Biogenesis

MiRNA biogenesis is a multi-step process that requires synergistic participation of a variety of enzymes and regulatory proteins [249, 250]. Similar to protein-coding genes, the primary transcripts of miRNAs, namely pri-miRNAs, are initially transcribed from genomic sequences by RNA polymerase II in the nucleus [251, 252], as shown in Figure 6. Of note, depending on the locations of original miRNA-coding genes, miRNAs can be classified into three different categories: intergenic miRNAs (miRNA coding genes not belonging to any annotated transcripts), intronic miRNAs (miRNA coding genes falling within an intron of a host protein coding gene), and exonic miRNAs (miRNA coding genes overlapping with an exon of a known protein coding gene) [247, 253] (Figure 6). For intronic and exonic miRNAs, an extra step of trimming by spliceosome is required to release the pri-miRNAs from their host gene transcripts [253] (Figure 6). In addition, pri-miRNAs can give rise to either a single or multiple miRNAs, so-called “polycistronic miRNAs”, a cluster of miRNAs with similar sequences [247, 253]. Unlike protein-coding genes, the primary transcripts of miRNAs (or pri-miRNAs), with length ranging from hundreds to thousands of nucleotides (nt), subsequently fold into imperfectly base-paired stem-loop structures that are further excised by RNase III endonuclease Drosha and the double-stranded RNA binding protein DGCR8, resulting in ~70- to 100-nt hairpin-shaped

precursor miRNA (pre-miRNA) [254-256] (Figure 6). It is noteworthy that the processing of intronic miRNAs from their pri-miRNAs to pre-miRNAs escapes the Drosha pathway, but uses an alternative splicing-mediated mechanism and the resultant hairpin pre-miRNAs are named "mirtron" [257, 258] (Figure 6). Through incorporating with the nuclear export factor exportin 5 [259-261], the pre-miRNAs are then translocated from the nucleus to the cytoplasm, where they are further cleaved by another RNase III endonuclease Dicer to generate ~19- to 25-nt mature miRNA duplex [252, 262] (Figure 6).

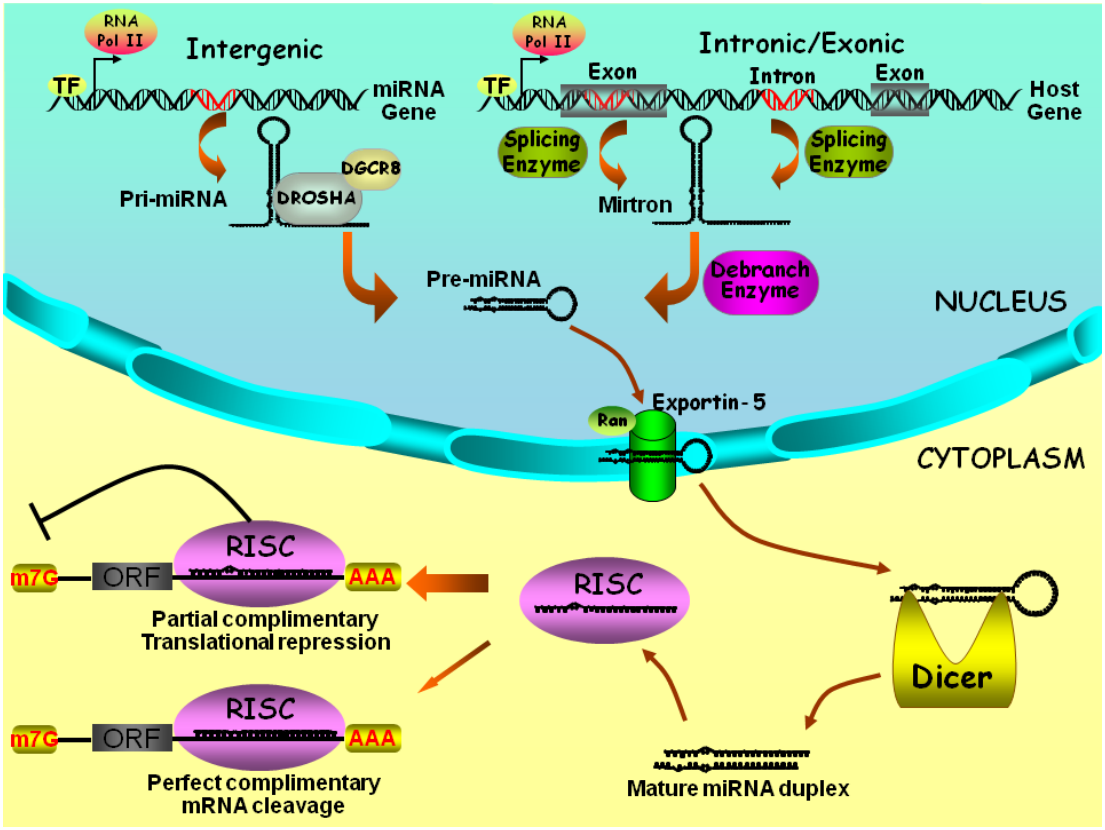

Figure 6. Biogenesis and actions of miRNA. TF: transcription factor; Pri-miRNA: primary transcript of miRNA; Pre-miRNA: precursor miRNA; RISC: RNA-induced silencing complex; ORF: open reading frame. Adapted from Wang Z et al. [263] with modification.

1.6.3 Action and Biological Functions of MiRNAs

1.6.3.1 Action of MiRNAs

The primary action of miRNAs is to repress the translation output from the target mRNAs [252]. As the first step leading to this action, the mature miRNA duplex is incorporated into

the protein complex known as RNA-induced silencing complex (RISC) [264, 265] (In Figure 6). Only one strand of the mature miRNA (guide strand) remains in RISC, whereas the other strand (passenger strand) is often degraded [264, 265] (Figure 6). This single-stranded miRNAs-RISC primarily binds to the 3'-untranslated region (3'UTR) of their target mRNAs according to Watson and Crick base-paring rule [266, 267]. Unlike short interfering RNAs (siRNAs), the majority of miRNAs **:** mRNAs bindings are partially complementary [266-269]. However, a perfect complementary between the first 2- to 8- nt 5' sequence of a miRNA (also known as the "seed site" region of a miRNA) and the target mRNA is essential for the miRNA action [266-269]. Finally, depending on the degree of complementarity between miRNAs and their target mRNAs, miRNAs either inhibit the translation of the encoded proteins (by partial complementarity, Figure 6) or lead to mRNA degradation (by perfect complementarity, Figure 6) [269, 270].

1.6.3.2 Biological Functions of MiRNAs

Since the discovery of the first functional miRNA, research over the past twenty years has generated substantial evidence showing the essential roles of miRNAs in various fundamental biological processes such as development, apoptosis, cell differentiation and proliferation, cell growth and cell death, and aging [249, 250]. Another major advancement in our understanding of the biological functions of miRNAs is that miRNAs have emerged not only as indicative but also causative factors in numerous diseases, including cancer, heart diseases, diabetes, and neurological diseases [248-250, 271-273].

1.6.4 MiRNA Nomenclature

Table 1. MiRNA nomenclature.

miRNA	Nomenclature	Example
Mature form of miRNA	**The prefix "miR" is followed by a dash and a number, which is designated sequentially**	**miR-1 was likely discovered before miR-26**
miRNAs with nearly identical sequences	**Denoted with an additional lowercase letter**	**miR-26a/miR-26b; miR-29a/miR-29b**
miRNA originated from different species	**The three-letter prefix representing the species of origin is followed by "miR"**	**has-miR-26 (*Homo sapiens*)** **mmu-miR-26 (*Mus musculus*)**

According to the guideline of miRNA registry [274], the designation of mature miRNAs is given as number that follows a chronological order based on the time of their discoveries (Table 1). Exceptions are seen for some of the earliest discovered miRNAs, such as lin-4 and let-7, whose name are defined according to their target genes. Similar miRNAs that differ only in one or two oligonucleotides in 3' region of their sequences are classified as a family and are denoted with an extra letter for their designations (Table 1). Since the majority of miRNAs are highly conserved across different species, a three-letter prefix representing their species of origin is adopted in miRNA nomenclature (Table 1).

1.6.5 Determination of MiRNA Targets

To date, over 900 human miRNAs have been identified [248]; it has been predicted that the human genome may encode more than 1000 miRNAs, which may regulate two thirds of all human protein coding genes [250, 253, 275, 276]. Given that a single miRNA could theoretically target hundreds or thousands of potential targets, and conversely, one mRNA could be simultaneously targeted by multiple miRNAs, identification and validation of authentic miRNAs targets inevitably represents the major challenges in miRNA research

[250, 275]. Current practice to overcome these problems involves a two-step procedure, including an initial computational target prediction followed by an experimental validation.

1.6.5.1 Computational Prediction of MiRNA Targets

There is an array of tools that are currently available for miRNA target prediction, which are presented as website-based interfaces for an easy-access purpose. Of note, most of these tools are developed by using seemingly distinct but actually similar algorithms that follow a series of important standards in regards to the identification and ranking of potential targets [247, 250]. For example, most of the programs primarily rely on sequence complementarity between the 5' seed region (2-8 nt of the 5' sequence) of a miRNA and the 3'UTRs of target mRNAs to determine the likelihood of miRNA targets [247, 250, 275]. The ranking of these predicted targets is based on the degree of overall complementarity as well as the conservation of both miRNA and 3'UTR target sequences across species [247, 250, 275]. Currently, three most commonly and widely used programs are TargetScan [267], PicTar [277], and miRanda [278], which differ in prediction sensitivity and specificity [250]. A practice to combine the results generated from different programs could theoretically enhance the sensitivity and/or specificity and, hence, becomes increasingly favorable for miRNA researchers [247]. There are two advantages of using in silico approach for miRNA target prediction prior to the experimental identification. First, the inherent high throughput feature allows researchers to easily portray a more thorough picture of the miRNA candidates for a given gene or the genes targeted by a given miRNA. Second, it serves as a powerful mean to predict a potential cellular function of a miRNA or a cluster of miRNAs.

1.6.5.2 Experimental Validation of MiRNA Targets

A common scheme to experimentally validate miRNA targets includes two parts [247, 279]: 1) to verify the binding ability of a miRNA to its predicted 3'UTR binding site by using luciferase reporter assay; i.e., insert the partial or full length of 3'UTR of a target gene containing the predicted binding site for a given miRNA into the luciferase reporter vector; by this way, any physical interaction between the miRNA and the 3'UTR can affect

the production of the luciferase protein; therefore, the effectiveness of the binding can be determined by measuring of the fluorescent signal generated by the luciferase [247, 279]. 2) to observe whether an endogenous protein encoded by a given miRNA target gene, is indeed affected by the miRNA in a native cellular environment [247, 279].

Two strategies are commonly used in the luciferase study to validate the miRNA targets [247, 279]. The first strategy involves the construction of a luciferase report vector that contains either the wild-type 3'UTR (bearing the intact putative binding for a specific miRNA) or the mutant 3'UTR (bearing the mutated binding site for the same miRNA); these constructs are then separately transfected into a cell line that abundantly expresses the miRNA of interest; if the luciferase activity from cells transfected with wild-type 3'UTR is reduced and indeed this reduction is absent in cells transfected with mutant construct, the binding site is likely to be authentic for the miRNA [247, 279]. Of note, an additional experiment to inhibit the endogenously expressed miRNA by anti-miRNA oligos (miRNA inhibitor) in the same settings can further prove the specificity [247]. The second strategy relies on a cell line, in which the miRNA of interest is modestly or poorly expressed [247, 279]. Instead of knocking down the endogenous miRNA, a specific miRNA is overexpressed in the cells by using the synthetic "miRNA mimic" or the miRNA overexpression vector [279]. Consequently, if cells transfected with wild-type 3'UTR show a dose-dependent reduction of the luciferase activity in response to the miRNA overexpression while this effect is absent in cells transfected with the mutant 3'UTR, the tested binding site is considered to be responsive to the given miRNA [247, 279].

Unlike luciferase reporter assay, western blotting allows a direct measurement of the effect of a miRNA on the level of its target protein. Similar to luciferase study, transfection of the synthetic miRNA mimic or the miRNA overexpression vector can be used as an overexpression approach, whereas inhibition can be achieved by using anti-miRNA oligos [279, 280]. It should be noted that choosing a proper cell type with relatively-high expression levels of the target protein and the corresponding miRNA is important for a successful western blotting verification [279, 280]. Ideally, the selected cell type should modestly express the miRNA of interest because the changes of the protein expression in response to either miRNA knockdown or overexpression can be more accurately reflected when the background level of the studied miRNA in the cells is modest.

1.6.6 MiRNA Expression in the Heart

1.6.6.1 Cardiac Selectivity of MiRNA Expression

Under the normal conditions, expression of miRNAs in heart displays a distinct pattern comparing to the other organs [281]. Of ~ 900 miRNAs identified in human, only a subset of these miRNAs is abundantly expressed in heart. Based on a recent study, the top 20 most abundant miRNAs in human heart are miR-1, miR-133a/b, miR-26a/b, miR-125a/b, let-7a/b/c/f/g, miR-16, miR-100, miR-126, miR-145, miR-195, miR-199, miR-20, miR-21, miR-23, miR-24, miR-29a/b, miR-27a/b, miR-30a/b/c, miR-92a/b, and miR-99 [281]. Interestingly, the majority of these cardiac-enriched miRNAs are ubiquitously expressed in various organs except for miR-1 and miR-133a/b [281], both of which are preferentially expressed in cardiac and skeletal muscle [282]. In addition to miR-1 and miR-133a, some less abundantly-expressed miRNAs, miR-208a/b and miR-499, are also found to be cardiac-specific [283], as they are co-transcribed with the cardiac-specific genes, MYH6 (encoding the α-myosin heavy chain, miR-208a), MYH7 (encoding the β-myosin heavy chain, miR-208b), and MYH7b (encoding isoforms b of the β-myosin heavy chain, miR-499), respectively [283].

1.6.6.2 Cell Type Specification of MiRNA Expression in the Heart

Although the majority of cardiac-enriched miRNAs are ubiquitously expressed in various organs, many of them exhibit cell-type preferential expression. For example, miR-1 and miR-133a/b are muscle-specific miRNAs that are preferentially expressed in cardiac and skeletal muscle cells [282]; miR-21 and miR-29a/b are preferentially expressed in fibroblasts [284, 285]; miR-126, miR-24, and miR-92a are largely enriched in endothelial cells [286]; miR-145 is detectable mainly in smooth muscle cells [287].

1.6.6.3 MiRNA Detection and Quantification

Current methods for miRNA detection primarily focus on the detection of mature miRNAs. This is due to the fact that the expression level of pri-miRNAs is generally not in linear

relation with the expression level of their corresponding mature miRNAs [247]. To date, a variety of techniques have been employed to determine the abundance and/or presence of miRNAs. Microarray and deep sequencing serve as profiling approaches to determine the global expression of miRNAs, whereas the expression of individual miRNA can be more precisely quantified by real-time RT-PCR and Northern blotting.

1.6.6.3.1 Detection of MiRNAs by Microarray

One of the most significant advantages of using microarray for miRNA detection is that it allows a large number of miRNAs in a given tissue or cell sample to be simultaneously analyzed [288, 289]. Owing to this high through-put feature, microarray analysis is generally considered as the most practical approach for miRNA screening. However, given that the principle for microarray is based on the hybridization between capture probes (synthetic oligonucleotides) and targeted miRNAs, and that the binding affinities towards the capture probes differ among miRNAs, microarray should not be considered as a quantitative method to determine the expression level of miRNAs [290-292]. Instead, it can serve as a comparative approach to show the relative changes of miRNAs expression between 2 states or the presence of miRNAs between different tissues or cell-types [290-292]. For example, it will be ideal to use microarray analysis to compare the miRNA expression profiles between diseased and non-diseased hearts [247, 289]. Currently, there are several commercially available miRNA microarray platforms, which differ in sensitivity and specificity due to the different designs of capture probes [290]. Although microarray represents a practical and easy approach for miRNA screening, there are several inherent limitations: 1) relatively low sensitivity of the capture probes may fail to detect the low abundant miRNAs; 2) owing to the low specificity of the hybridization, a false positive result is unavoidable; and 3) microarray results represent the relative changes between samples and are unable to conduct quantitative analysis. Therefore, the microarray results should be confirmed by other detection methods [288, 289, 293].

1.6.6.3.2 Detection and Quantification of MiRNAs by Deep Sequencing

Similar to microarray analysis, deep sequencing is also a high through-put approach for profiling a large number of miRNAs [292]; however, unlike microarray that is solely based on hybridization, deep sequencing relies on the PCR-based parallel sequencing, which can capture miRNAs with low copy numbers (few molecules per cell) that are normally undetectable by microarray [292, 294]. In addition to the superior sensitivity, deep sequencing can measure the absolute abundance of the miRNAs, and thus the result is quantitative [294]. Despite the advantages over microarray as a miRNA profiling method, deep sequencing can also serve as a powerful tool for new miRNA discovery [294, 295]. This is due to the fact that this sequencing based-technique can theoretically capture any known and unknown miRNAs or even structurally-similar RNA fragments during the robust PCR reactions [294, 296]. However, a proper interpretation of the massive information generated by deep sequencing poses a great challenge for subsequent bioinformatic analysis and experimental verification, which are normally costly and time-consuming [292, 294]. Therefore, deep sequencing still remains as a second option for miRNA profiling in comparison to microarray.

1.6.6.3.3 Quantification of MiRNAs by Real-time RT-PCR

Although the overview of global miRNA expression provided by microarray or deep sequencing is useful and informative, the accuracy of the results needs to be further validated by more specific approaches that allow individual quantification of the miRNA expression [297, 298]. To date, real-time RT-PCR is the most commonly used quantitative method for the assessment of individual miRNA expression [299-301]. Unlike detection of protein-coding RNA, detection of miRNA by real-time RT-PCR is bound with difficulties, particularly for the synthesis of first strand cDNA (reverse transcription). These are largely due to the unique features of miRNAs, i.e., the lack of poly (A) tail, short length (~22nt), and the presence of mature miRNA sequence in its precursor and primary transcript [300-302]. Therefore, over the past several years, researchers in the field have developed two different methods for reverse transcription (RT) of miRNAs, namely miRNA-specific RT or universal RT [300]. During miRNA-specific reverse transcription, miRNAs are reversely

transcribed individually by using miRNA-specific primers, which are designed to form a "stem loop" structure, as shown in Figure 7A [300, 302]. Each stem loop primer is composed of three parts: 1) a short single-stranded part of the stem containing sequence that is complementary to the 3' end of a target miRNA; 2) double-stranded part of the stem; and 3) a loop region that contains the sequence for binding of the universal primer during real-time PCR [300, 302]. The purpose of this stem-loop design is to reduce the annealing of the primers to pre- or pri-miRNAs, thereby enhancing the specificity of the primers [300, 302]. Of note, as each stem-loop RT primer is specifically designed for its targeted miRNA, the RT reactions should be performed individually for the corresponding miRNAs [300, 302]. For universal reverse transcription, all miRNAs are first tailed with a common sequence (normally a ploy (A) tail) and then are reversely transcribed with a universal primer (Figure 7B) [298, 300]. Based on this principle, one reaction by universal methods could theoretically transcribe all the miRNAs in a given sample [298, 300].

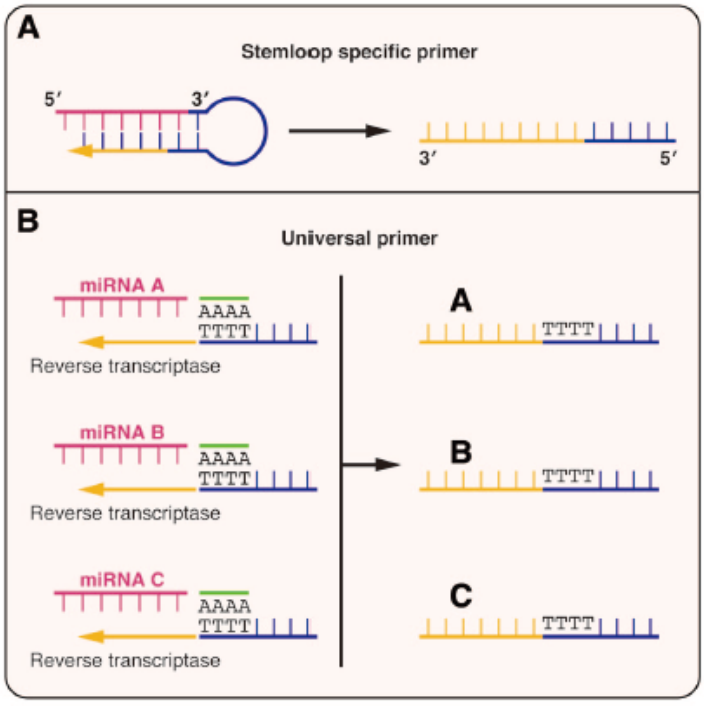

Figure 7. Different approaches for miRNA quantification by real-time RT-PCR. A. Reverse transcription of miRNAs by "stem loop" RT primers. B. Reverse transcription of miRNAs by universal RT primers; note that miRNAs are first tailed with poly "A" sequence prior to reverse transcription. Adapted from van Rooij E et al. [247].

1.6.6.3.4 Quantification of MiRNAs by Northern blot

Northern blotting is another technique that allows individual quantification of specific miRNAs [303-305]. One obvious advantage of using northern blotting to detect a given miRNA is that it allows the visualization of both mature miRNA and precursor miRNA [304, 305]. However, this technique is generally time-consuming and requires large amounts of RNA sample (8ug or more), which poses a challenge for experiments with limited amount of samples [303]. Another potential drawback of the technique is the cross reaction of the detection probe with miRNAs that share high sequence homology [303]. Consequently, results obtained from northern blotting should ideally be verified by other detection methods, for example, real-time RT-PCR.

1.6.7 Regulation of MiRNA Expression and MiRNA Interference

1.6.7.1 Transcriptional Regulation of MiRNA Expression

Similar to protein-coding genes, the transcription of miRNA genes is also tightly controlled by their 5' proximal regulatory sequences (promoters) in coordination with a variety of DNA-binding factors (transcription factors, TFs) [252, 306]. However, depending on the type of miRNAs, miRNA genes can be transcribed by using either their own promoters (for intergenic miRNAs) or their host gene promoters (for exonic and intronic miRNAs) [307, 308]. Interestingly, it appears that the promoters of miRNA genes and protein coding genes share many similarities, such as initiator elements, the presence of TATA box, transcription factor II B (TFIIB) recognition, the occurrence of CpG islands and histone modifications [252, 306, 308]; and more importantly, the TFs that regulate the transcription of miRNA genes or protein coding genes can largely overlap [252, 253, 306]. For example, the proto-oncogene c-myc is found to positively regulate transcription of miR-17, while it can also promote the transcription of E2F1 [309]. Another example is p53, a well defined pro-apoptotic factor, which has been found to control both the transcription of miR-34 genes and various tumor genes [310].

In cardiomyocytes, several cardiac-specific TFs have been implicated in the control of miRNA transcription. For example, serum response factor (SRF) and myocytes enhancer

factor 2 (MEF2) have been well characterized to transcriptionally activate the expression of two muscle-specific miRNAs, miR-1 and miR-133, during cardiac development [246, 282, 311]. GATA-4, another important TF in the heart, directly binds to the promoters of miR-144 and miR-451 and promotes the transcription of these two miRNAs [312]. Nkx2.5 regulates the transcription of miR-143 and miR-145, which are important for the differentiation of cardiac progenitor cells [287].

In addition to the cardiac-specific TFs, many of other non cardiac-specific TFs have also been found to regulate the transcription of miRNAs in cardiomyocytes. A typical example is nuclear factor of activated T cells (NFAT). A recent study has shown that NFAT transcriptionally activates miR-23a expression [313], and similarly, in another recent study, NFAT was found to be a direct transcriptional activator for miR-199b [314].

1.6.7.2 MiRNA Interference

1.6.7.2.1 In Vitro MiRNA Interference

Transient overexpression or inhibition of miRNA in a cell-based system is the initial step towards functional studies of miRNAs. Several transfection-based approaches are currently used to overexpress miRNA in vitro: synthesized double-stranded miRNA mimics [279, 280, 282, 315], miRNA precursors [316, 317] or vectors that overexpress the miRNA of interest [247, 315]. For cells with low transfection efficiency, such as adult cardiomyocytes, a virus-based infection approach can be used [248, 279, 315]. For in vitro miRNA inhibition, the most commonly used strategy is to transfect cells with the miRNA antisense, namely antimiRs, which are the modified DNA oligos with the perfect complementarity to the guide-strand of a given miRNA that can antagonize the endogenous mature miRNAs and prevent them from binding to their targets [279, 282, 315, 317]. Alternatively, knockdown can be achieved by using either a regular vector or a viral-vector that expresses transcripts containing multiple miRNA target sites, by which the miRNA of interest can be functionally blocked or decoyed thereby being prevented from affecting its endogenous targets [318-321].

1.6.7.2.2 In Vivo MiRNA Interference

The in vivo function of a specific miRNA can be elucidated by gain- and/or loss-of-function studies, which generally rely on three different approaches: 1) generation of transgenic animals with genetic modifications (either overexpression or knockout/down) of the targeted miRNA; 2) application of virus-based overexpression or knockdown techniques; and 3) the oligos-based antimiRs knockdown technique [247, 248, 321, 322]. Although many studies have revealed that genetic manipulation is a powerful approach to define the function of miRNA in vivo, some potential drawbacks with this approach should be taken into account. In some cases, forced overexpression of a miRNA could result in off-target effects due to the supraphysiological level of the miRNA expression [247, 248, 315, 317]. In other cases, genetic deletion of a miRNA could cause fetal lethality because the deletion might also disrupt a protein-coding gene, given that ~40% of miRNA genes are located within the introns of the protein coding genes that are potentially important for development [247, 248, 315, 317]. To overcome these problems, one alternative strategy is to overexpress or knockdown a miRNA by systemic delivery of the adenoviral vector expressing transcripts that contain either the miRNA precursor or multiple miRNA binding sites in the adult animals [247, 248, 315, 317]. However, due to the lack of tissue-specific expression inherent to the regular adenoviral approach, there is a growing interest of using different serotypes of adeno-associated viruses (AAVs) to achieve the tissue-specific expression, among which, AAV serotype 9 (AAV9) has been found to preferentially target cardiac tissues [248, 321, 322]. In addition to the genetic manipulation and viral expression approaches, a series of recently-developed miRNA knockdown oligos (antimiRs) have emerged as potent in vivo miRNA inhibitors with great therapeutic potential [248, 322]. Generally, these synthetic antimiRs are chemically-modified antisense oligos bearing the full or partial reverse complementary sequence of a mature miRNA that can either degrade or functionally block the endogenous miRNA upon binding [321, 323, 324]. The most commonly-used chemical modifications include 2'-O-methyl (2'-OMe)-modified oligonucleotides (Figure 8B) and locked nucleic acid (LNA)-modified oligonucleotides (Figure 8C), in which the 2'-O-oxygen is connected to the 4' position by a methylene linker to form a tight bicycle and is locked into the C3'-endo (RNA) sugar conformation, favoring the formation of a thermodynamically strong duplex with the complementary RNA [323,

324]. Both 2'-OMe and LNA modifications aim to increase the stability of oligonucleotides, improve the nuclease resistance, and enhance binding affinity to the target RNA [323, 324]. They can also be combined with some other chemical modifications to further improve cellular stability or uptake [247, 321]. For example, addition of phosphosrothioate backbone (Figure 8D) between nucleotides makes the oligonucleotides more resistant to nuclease [325]; the use of cholesterol conjugated oligonucleotides is proven to facilitate cellular uptake [326].

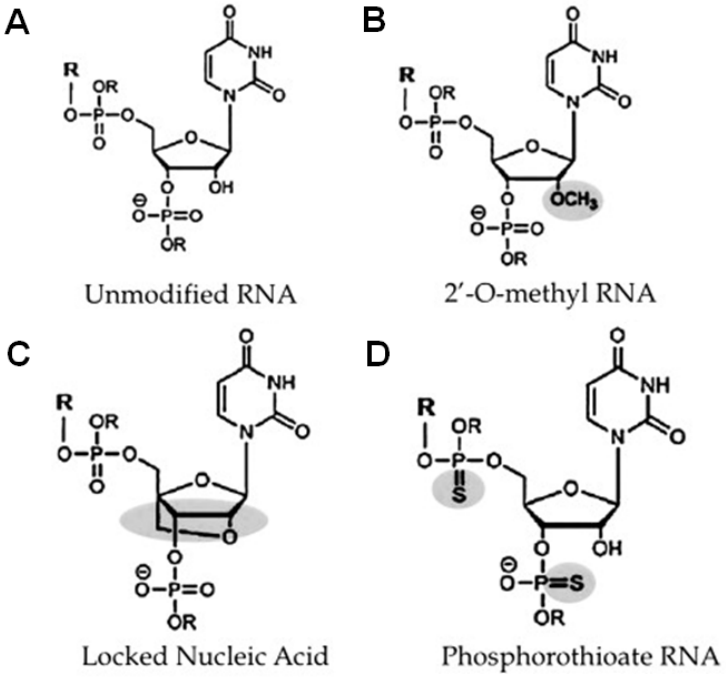

Figure 8. Different chemical modifications of in vivo miRNA knockdown oligos. A. Conformation of unmodified RNA. B. Conformation of 2'-O-methyl RNA (a methyl group bound to the 2′ oxygen of the ribose, highlighted in grey). C. Conformation of LNA monomer (methylene bridge between 2'-O and 4' in the ribose, highlighted in grey). D. Conformation of phosphorothioate RNA (S indicates sulfur substitution of a non-bridging oxygen to make a phosphorothioate linkage between nucleotides). (Adapted from Ruberti F et al. [327] with modifications).

1.6.8 Role of MiRNAs in the Heart

The complexity of the cardiac system is manifested by the fact that the normal function and formation of the heart are finely controlled by networks of transcriptional factors and signaling systems that govern the expression of cardiac genes that are essential for cardiogenesis, morphogenesis, and contractility [328]. The discovery of miRNAs as novel gene regulators during various biological processes has added a new layer of complexity to the cardiac system [322, 329]. It has now become clear that miRNAs tightly interact with a variety of cardiac signaling and transcriptional pathways to regulate cardiac development, function, and disease [275, 322, 329].

1.6.8.1 Implications of MiRNAs in Cardiac Development

Table 2. Summary of miRNAs related to cardiac development.

miRNAs and cardiac development			
miRNA	Cell Type	Functions	Refs
miR-1	Cardiomyocytes and skeletal muscle cells	Promotion of myocardial differentiation and negative regulation of cardiomyocyte proliferation	333; 334
miR-133	Cardiomyocytes and skeletal muscle cells	Repression of myocardial differentiation and cardiomyocyte proliferation	334; 335
miR-17~92	Cardiomyocytes	Promotion of myogenic differentiation	336; 337
miR-126	Endothelial cells	Promotion of vasculogenesis	286; 338; 339
miR-143	Smooth muscle cells	Promotion of smooth muscle cell differentiation and proliferation; vessel formation	287; 340; 341
miR-145	Smooth muscle cells	Promotion of smooth muscle cell differentiation and proliferation; vessel formation	287; 340; 341
miR-138	Cardiomyocytes	Cardiac morphogenesis	342

The first direct evidence supporting the requirement of miRNAs for cardiac development and function came from studies using mice with cardiac-specific deletion of *Dicer* genes, which encode a protein that is essential for miRNAs processing [330-332]. The germline deletion of *Dicer* in myocardium or cardiovascular smooth muscle resulted in an aberrant expression of cardiac contractile proteins and embryonic lethality, indicating the crucial roles of miRNAs in earlier cardiac development [330, 331]. Interestingly, a subsequent study, which aimed to circumvent the embryonic lethality with germline deletion of *Dicer* by using mice with conditional *Dicer* knockout in postnatal myocardium, also observed the premature death of the animals shortly after inducing the deletion [332]. The lethal consequence of *Dicer* knockout is likely due to a joint-function of multiple miRNAs rather than a single miRNA, because, thus far, no embryonic lethality was observed when a specific miRNA was deleted [322].

To date, many of the cardiac-enriched miRNAs have been individually investigated for their potential roles in various aspects of cardiac development (e.g. myocardial differentiation and vasculogenesis), as summarized in Table 2. Muscle-specific miR-1, the most abundantly-expressed miRNA in cardiac tissue, was found to promote the formation of cardiac or skeletal muscle cells, whereas another muscle-specific miRNA, miR-133, was reported to negatively regulates myocardial differentiation [333-335]. Interestingly, both miR-1 and miR-133 were found to negatively regulate cardiomyocyte proliferation [334, 335]. Several miRNAs that belong to the miR-17-92 cluster, have also been reported to play an important role in mediating the myocardial differentiation of cardiac progenitor cells [336, 337]. In addition to myocardial differentiation, the involvement of miRNAs has also been seen in vasculogenesis during cardiac development. MiR-126, a cardiac-enriched miRNA that is selectively expressed in endothelial cells (ECs), was found to be crucially involved in angiogenic signaling and contribute importantly to vascular formation [286, 338, 339]. Interestingly, evidence supporting the potential contribution of miRNAs to vascular development has been found not only in ECs but also in smooth muscle cells (SMCs). SMC-specific miRNAs, miR-143 and miR-145, were reported to cooperatively regulate differentiation and proliferation of vascular smooth muscle cells (VSMCs) and determine VSMC cell fate, thereby contributing to the blood vessel formation [287, 340, 341]. Apart from the above various aspects of cardiac development, miRNAs are also

involved in the regulation of cardiac patterning. Evidence from a recent study using a genetic approach to delete miRNAs in zebrafish heart suggested that miR-138, a broadly conserved miRNA in mammals, is required for the proper formation of heart chambers through controlling chamber-specific gene expression patterns during cardiac morphogenesis [342].

1.6.8.2 Implications of MiRNAs in Cardiac Pathologies

Although tremendous efforts have been devoted to deciphering the underlying mechanisms responsible for a variety of cardiovascular diseases (e.g. cardiac hypertrophy, heart failure, ischemia, and arrhythmia), miRNAs have only recently been shown to play important roles during these pathological conditions [322, 329, 343]. It has become clear that there is a distinct expression pattern of miRNAs associated with different cardiac diseases and in some cases, the deregulation of a single miRNA is sufficient to result in a cardiac pathological condition [275, 322, 329, 343-346].

1.6.8.2.1 Roles of MiRNAs in Cardiac Hypertrophy and Heart Failure

Table 3. Summary of miRNAs related to cardiac hypertrophy and heart failure.

miRNAs in cardiac hypertrophy and failure				
miRNA	Disease/Expression	Targets	Function	Refs
miR-195	CH and HF / Upregulated	Unknown	Induction of cardiac hypertrophy and failure	347
miR-208a	CH / Unaltered	Myostatin; THRAP1	Essential for hypertrophic response	283; 348
miR-1	CH / Downregulated	IGF1; Calmodulin; Mef2a	Inhibition of cellular hypertrophy	320; 349-352
miR-133	CH and HF / Downregulated	RhoA; CTGF; Col1A1	Anti-hypertrophic (? Inconsistent *); anti-fibrotic	320; 334; 347; 353; 355-357

miR-23a	CH and HF / Upregulated	MuRF1	Promotion of cellular hypertrophy	313
miR-199b	CH and HF / Upregulated	Dyrk1a	Promotion of cellular hypertrophy	314
miR-21	CH / Upregulated	Spry1	Profibrotic	284; 354
miR-29b	CH and HF / Downregulated	Col1A1; Col1A2; Col3A1; FBN1; ELN	anti-fibrotic	285

CH: Cardiac hypertrophy; HF: Heart Failure.
* Note that inconsistent results were reported for the anti-hypertrophic role of miR-133.

To date, the contribution of miRNAs to pathogenesis of cardiac hypertrophy and heart failure has been highlighted in numerous studies, as summarized in Table 3. The first study was done in mice subjected to transverse aortic constriction (TAC) or cardiac overexpression of activated calcineurin [347]. A series of deregulated miRNAs exhibiting a distinct expression pattern was observed in the cardiac tissues from these mice [347]. Interestingly, among these deregulated miRNAs, the cardiac-specific overexpression of miR-195 recapitulated the phenotypic changes associated with cardiac hypertrophy and heart failure, suggesting the pathogenetic role of miRNAs in these disease conditions [347]. Subsequently, a cardiac-specific miRNA, miR-208a, was found to play an essential role in cardiac hypertrophy [283, 348]. Mice with genetic deletion of miR-208a failed to demonstrate hypertrophic response to stress and hypothyroidism [283], whereas cardiac-specific overexpression of miR-208a induced cardiac hypertrophy [348]. Subsequent experiments demonstrated that the pro-hypertrophic effect of miR-208a is attributable to the repression of its targets, thyroid hormone receptor associated protein 1 (THRAP1) and myostatin, both of which function as negative regulators of muscle growth and hypertrophy [283, 348]. In addition to miR-208a, other cardiac-specific miRNAs, miR-1 and miR-133, have been reported to be involved in cardiac hypertrophy and failure [320, 349, 350]. The expression of miR-1 and miR-133 has been consistently found to be downregulated during cardiac hypertrophy in animal models and humans [320, 349, 350]. In vivo and in vitro inhibition of miR-1 in cardiomyocytes resulted in the increases in cell size and cell mass, suggesting an anti-hypertrophic role of miR-1 [320, 350]. The anti-hypertrophic effect of miR-1 may be attributed to its targeting of several signaling factors that are known to be

important to the development of cardiac hypertrophy, including insulin-like growth factor-1 (IGF-1) [351, 352], calmodulin [349], and myocytes enhancer factor 2A (MEF2A) [349]. As for miR-133, several studies have consistently documented a reduced expression of this miRNA in hypertrophied heart [320, 347, 353]. In vivo knockdown of miR-133 by systemic administration of its antagomir in the normal mouse hearts, was found to induce cardiac hypertrophy, presumably through the release of inhibition on its target, RhoA, a guanosine diphosphate-guanosine triphosphate exchange protein that can induce cardiac hypertrophy when its expression is elevated [320]. However, the exact role of miR-133 in cardiac hypertrophy remains controversial, as a subsequent study reported that mice with cardiac-specific knockout of miR-133 fail to develop cardiac hypertrophy [334]. The causes for this obvious discrepancy remain yet to be determined. More recently, two separate studies have suggested that miR-23a and miR-199b might also play important roles in cardiac hypertrophy and failure [313, 314]. The expression of both miRNAs was found to be profoundly increased in hypertrophied and failing hearts [313, 314]. In vivo inhibition of miR-23a completely abrogated the hypertrophic response upon isoproterenol stimulation [313]. The pro-hypertrophic effect of miR-23a is thought to be mediated by an anti-hypertrophic protein, muscle specific ring finger protein 1 (MuRF1), which is experimentally established as a target for miR-23a [313]. Similarly, miR-199b was also reported to positively regulate cardiac hypertrophy and failure, presumably through a relief of its repression on nuclear NFAT kinase dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1a (Dyrk1a), which functions as a negative regulator for cardiac hypertrophy [314]. Indeed, transgenic mice with overexpression of miR-199b demonstrated an enhanced hypertrophic response to pressure overload, whereas in vivo inhibition of miR-199b was able to reverse the phenotypic changes (cellular hypertrophy and fibrosis) observed in the mouse models of heart failure [314].

In addition to the direct pathogenetic roles in cardiac hypertrophy and failure, miRNAs have also been found to participate in fibrogenesis during cardiac hypertrophy and failure. The first documented fibrosis-related miRNA during cardiac hypertrophy is miR-21 [284]. A significant increase of miR-21 expression was observed during cardiac hypertrophy and this increase was found more profound in myofibroblasts than in myocytes [284]. In vivo inhibition of miR-21 with its antagomir in mice successfully prevented the fibrosis and

cardiac hypertrophy in response to pressure overload, suggesting a profibrotic role of miR-21 during cardiac hypertrophy [284]. The profibrotic effect of miR-21 is likely attributable to its targeting of sprouty homolog 1 (Spry1), which functions as a negative regulator for extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation and myofibroblast survival [284]. However, a recent study questioned the necessity of miR-21 in hypertrophy-induced fibrosis. In this study, in vivo interference of miR-21 in the hearts was found to have no impact on fibrosis in three different mouse models of cardiac hypertrophy [354]. Thus, further studies are needed to resolve these obvious contradictions. Expression levels of miR-133 and miR-29b have also been repeatedly reported to be downregulated in hypertrophied and failing hearts [285, 355, 356]. Restoration of miR-133 to normal levels in mouse models of cardiac hypertrophy was able to prevent the fibrosis [356]. The anti-fibrotic effect of miR-133 is likely due to its repression on collagen 1A1 (Col1A1) and connective tissue growth factor (CTGF), a well-known profibrotic factor [355, 357]. Similarly, miR-29b has also been found to play an anti-fibrotic role during cardiac hypertrophy through direct targeting of multiple collagen isoforms, fibrillin, and elastin [285]. Accordingly, in vivo inhibition of miR-29b in mice was able to promote fibrogenesis in the heart [285].

1.6.8.2.2 Roles of MiRNAs in Myocardial Ischemia

Table 4. Summary of miRNAs related to myocardial ischemia.

miRNAs in cardiac ischemia				
miRNA	Expression	Targets	Function	Refs
miR-199a	Downregulated	Hif-1α	Anti-apoptotic	358
miR-320	Downregulated	HSP-20	Anti-apoptotic	359
miR-21	Downregulated (in CMs)	PDCD4; PTEN; FasL	Anti-apoptotic	360; 361
	Upregulated (in MFs)	PTEN	Profibrotic	370
miR-494	Downregulated	PTEN; ROCK1; CAMKIIδ	Anti-apoptotic	362

miR-24	Downregulated (in CMs)	Bim	Anti-apoptotic	364
	Upregulated (in ECs)	GATA2; PAK4	Proapoptotic and anti-angiogenic	363
miR-15	Upregulated	Bcl2; Arl2	Proapoptotic	365-367
miR-29	Downregulated	Col1A1; Col1A2; Col3A1; FBN1; ELN	Anti-fibrotic	285
miR-92a	Downregulated (in ECs)	ITGA5	Anti-angiogenic	369
miR-126	Upregulated (in ECs)	Spred1	Proangiogenic	286; 368

CMs: cardiomyocytes; MFs: myofibroblasts; ECs: endothelial cells

Aberrant miRNA expression has been repeatedly observed in cardiac ischemia. Cardiac ischemia is a result of insufficient supply of blood flow to the heart, which may cause many abnormalities, affecting apoptosis, angiogenesis, fibrosis and contractile dysfunction in the myocardium. Several miRNAs that affect cell viability were reported to be perturbed during cardiac ischemia, including miR-199a [358], miR-320 [359], miR-21 [360, 361], miR-494 [362], miR-24 [363, 364], and miR-15 [365], as summarized in Table 4. Among these deregulated miRNAs, reduced expression of miR-199a, miR-21, miR-494, and miR-24 was found within the infarct zone [358, 360-362, 364]. Interestingly, when restoring the expression of these miRNAs in the ischemic heart, a reduced infarct size and improved cell viability were observed [358, 360-362, 364]. At the cellular level, overexpression of miR-199a, miR-21, miR-494, or miR-24 alone was able to inhibit hypoxia-induced apoptosis in cardiomyocytes [358, 361, 362, 364]. The underlying mechanisms responsible for their anti-apoptotic effects are likely attributable to the inhibition of various proapoptotic factors, such as hypoxia-inducible factor 1 alpha (Hif-1α, target of miR-199a) [358], phosphatase and tensin homolog (PTEN, target of miR-21 and miR-494) [361, 362], Fas ligand (FasL, target of miR-21) [361], programmed cell death 4 (PDCD4, target of miR-21) [361], Rho-associated coiled-coil containing protein kinase-1 (ROCK1, target of miR-494) [362], calcium/calmodulin-dependent protein kinase II delta (CAMKIIδ, target of miR-494) [362], and BH3-only protein (Bim, target of miR-24) [364]. Unlike the decrease of miR-199a, miR-21, miR-494, or miR-24, which deteriorates cell viability during ischemia, the observed downregulation of miR-320 during myocardial ischemia appears to be

cardioprotective, as in vivo suppression of this miRNA was found to reduce infarct area after ischemia reperfusion (IR) [359]. The cardioprotective effect of miR-320 downregulation during IR is likely due to an increased expression of heat shock protein 20 (HSP20), a well known cardioprotective protein that is targeted by miR-320 [359]. Upregulated miRNAs are also seen within the infarct zone, one example is miR-15b. In vivo inhibition of miR-15b during ischemia resulted in reduced infarct size and improved cardiac function [365]. The mechanism underlying the beneficial role of miR-15b knockdown is likely related to the increased levels of cell death suppressor proteins, ADP-ribosylation factor-like 2 (Arl2) and B-cell lymphoma 2 (Bcl2), both of which are validated targets of miR-15b [366, 367].

Deregulated miRNAs seen in myocardial ischemia are implicated not only in cell viability but also in angiogenesis. A typical example is miR-126, which was found to be highly enriched and upregulated in endothelial cells during cardiac ischemia [368]. The enhanced expression of miR-126 was proven to be required for reparative angiogenesis in response to ischemic insult through repression of an anti-angiogenic protein, sprouty-related EVH1 domain-containing protein 1 (Spred-1) [286, 368]. Interestingly, for miR-24, in contrast to its downregulation in cardiomyocytes during myocardial ischemia, a significant increase in its expression was observed in endothelial cells, where it induced apoptosis of these cells [363]. Accordingly, in vivo inhibition of miR-24 during myocardial ischemia was found to prevent endothelial cells from undergoing apoptosis and increases angiogenesis, suggesting an anti-angiogenic role of miR-24 [363]. The observed anti-angiogenic effect of miR-24 is likely caused by an increased expression of miR-24 conserved targets, GATA2 and p21-activated kinase-4 (PAK4), both of which are known to positively regulate angiogenesis [363]. MiR-92a was also found to be downregulated during myocardial ischemia [369]. This downregulation was shown to promote reparative angiogenesis, which is likely due to an increased level of alpha-5 integrin (ITGA5), a proangiogenic protein that is targeted by miR-92a [369].

Similar to cardiac hypertrophy, miRNAs are also involved in fibrogenesis during myocardial ischemia. Two miRNAs have so far been implicated in control of fibrosis during cardiac ischemia: miR-21 and miR-29. Both of these miRNAs are highly enriched in fibroblasts [285, 370]. While miR-21 was reported to be dramatically increased in the

infarcted region during cardiac ischemia [370], downregulation of miR-29 was consistently observed in the same context [285]. Increased level of miR-21 in cardiac fibroblasts during cardiac ischemia was found to promote fibrosis by repressing the expression of PTEN (a validated target of miR-21), which in turn stimulates the synthesis of metalloproteinase-2 (MMP-2), a well-known profibrotic factor [370]. Likewise, decreased expression of miR-29 in the ischemic heart is also thought to promote fibrosis [285]. This is likely attributable to an increased expression of various ECM proteins that are validated as targets of miR-29, including collagens, fibrillins, and elastin [285].

1.6.8.2.3 Roles of MiRNAs in Arrhythmia

Table 5. Summary of miRNAs related to cardiac arrhythmia.

Roles of miRNAs in cardiac arrhythmia				
miRNA	Disease/Expression	Targets	Function	Refs
miR-1	Ischemia / Upregulated	Cx43; KCNJ2	Proarrhythmic	204
	AF / Downregulated	KCNJ2	To be determined	96
	HF / Upregulated	Ppp2r5a;	Abnormal Ca^{2+} handling	373; 374
miR-133	CH / Downregulated	KChIP2	Prolongation of QT interval	356
	Nicotin-induced AF / Downregulated	TGF-β1; TGF-βRII	Atrial fibrosis	375
miR-590	Nicotin-induced AF / Downregulated	TGF-β1; TGF-βRII	Atrial fibrosis	375
miR-328	AF / Upregulated	CACNA1C; CACNB1	Overexpression promotes AF	113

CH: Cardiac hypertrophy; HF: Heart Failure; AF: Atrial Fibrillation.

Accumulating evidence has suggested that miRNAs are implicated in arrhythmogenesis under different disease paradigms such as cardiac hypertrophy, heart failure, myocardial ischemia, and atrial fibrillation, as summarized in the Table 5. The first arrhythmia-related miRNA was discovered in a study aimed at elucidating the molecular mechanism responsible for ischemia-related arrhythmia [204]. MiR-1 was found to be dramatically

upregulated during coronary artery disease in humans and ischemic rat hearts, which coincided with an increased incidence of arrhythmia [204]. Inhibition of miR-1 in the ischemic rat heart resulted in a reduced occurrence of arrhythmia, whereas overexpressing miR-1 in the healthy rat heart induced arrhythmia [204]. This proarrhythmic effect of miR-1 in ischemic conditions is attributed to its targeting and suppression of two proteins, Connexin 43 (encoded by *GJA1*, the main form of cardiac gap junction proteins) and Kir2.1 (encoded by *KCNJ2*, subunit of K^+ channels carrying I_{K1}) [204], as the deregulation of these proteins was reported to promote arrhythmia [371, 372]. Likewise, a significant increase of miR-1 was also observed in cardiomyocytes isolated from both rat and dog failing hearts [373, 374]. Subsequent in vitro studies demonstrated that this increased level of miR-1 could result in abnormal Ca^{2+} handling, which may contribute to arrhythmogenesis in failing hearts [373, 374]. In this case, miR-1 exerts its effect via suppressing the regulatory subunits of protein phosphatase 2A (Ppp2r5a), which is important for Ca^{2+}/calmodulin-dependent protein kinase (CaMKII)-mediated phosphorylation of ryanodine receptors (RyR2) [374].

In cardiac hypertrophy, one consistent finding is the downregulation of miR-133. In fact, a recent study suggested that this downregulation is responsible for the reduced expression of I_{to} channel accessory subunit, KChIP2, which may indirectly contribute to prolonged QT interval, thereby promoting arrhythmogenesis [356].

The first evidence indicating a direct role of miRNAs in AF was obtained from a study using nicotine-induced AF dogs [375]. Severe fibrosis was observed in the atrial tissue from these dogs, accompanying with significant increase of transforming growth factor-β1 (TGF-β1) and TGF-β receptor type II (TGF-βRII), and reduced expression of miR-133 and miR-590 [375]. Further in vivo and in vitro experiments confirmed that the reduction of miR-133 and miR-590 is responsible for the enhanced expression of TGF- β1 and TGF- β RII, as these proteins are validated as targets of miR-133 and miR-590 [375]. While the above study highlights the potential role of miRNAs in atrial structural remodeling under AF [375], evidence from two other studies suggests miRNAs may also contribute to atrial electrical remodeling associated with AF [96, 113]. Upregulation of I_{K1} and downregulation of I_{CaL} represent the two most prominent findings during ionic remodeling associated with AF. Interestingly, a recent study indicated that miR-1 levels are significantly reduced in

persistent AF patients and this reduction may contribute to upregulation of Kir2.1 subunits (validated target of miR-1), thereby leading to increased I_{K1} [96]. Nonetheless, further in vivo experiments are needed to establish a potential mechanistic link between miR-1 and AF. In another study, miR-328 was found to be upregulated in human AF patients with rheumatic disease, as well as in a canine model of AF [113]. Subsequent in vivo experiments in dogs demonstrated that overexpression of miR-328 by intracardial injection of miR-328-overexpressing adenovirus increases AF susceptibility [113]. Likewise, transgenic mice with miR-328 overexpression were more susceptible to AF induction, whereas inhibiting the overexpression of miR-328 in these mice reduced AF inducibility [113]. Mechanistically, the increased incidence of AF is attributable to the reduction in I_{CaL}, as both the α1c and β1 subunits of the L-type calcium channel were experimentally established as the targets of miR-328 [113].

1.7 Rationale for Present Studies

Atrial fibrillation is the most clinically-encountered arrhythmia, representing a major cause of morbidity and mortality. Over the past two decades, a major advancement in our understanding of the pathogenesis of AF is the recognition that AF induces both electrical and structural remodeling in the atria, which in turn promotes the recurrence and maintenance of AF. A key feature in electrical remodeling is the abbreviation of atrial effective refractory period, which is primarily due to shortening of action potential as the result of ion channel alterations. Structural remodeling, on the other hand, is mainly manifested by atrial fibrosis which may also result from the deregulation of ion channels. Interventions that prevent deregulation of ion channels and/or atrial fibrosis may adequately serve as an effective approach to prevent AF occurrence. However, the precise molecular mechanisms underlying these remodeling processes are still poorly understood. Of note, the recent discovery of miRNAs as an important regulator in various cardiac pathologies has shed light on this matter. Emerging evidence has suggested that miRNAs can directly regulate the expression of ion channel genes. More importantly, deregulation of ion channels as a result of the aberrant miRNA expression has been implicated in arrhythmogenesis under several cardiac pathological conditions. Therefore, the goals of the

studies presented herein were to gain an appreciation for the importance of miRNA in regulation of cardiac ion channel and its pathophysiological implication in AF, to improve our understanding of the basic mechanisms for electrical and structural remodeling pertaining to AF, and to ultimately identify a potential therapeutic target for the treatment of AF. To this end, three separate studies with specific focus were carried out as below:

1. As discussed in Section 1.6.5 of the introduction, currently-available experimental approaches do not permit thorough characterization of miRNA targeting. In order to acquire a comprehensive view of the miRNA regulation of cardiac ion channel in arrhythmia, a rationally-designed bioinformatics analysis was performed in conjunction with experimental approaches to identify the miRNA from the currently available miRNA databases which have the potential to regulate human cardiac ion channel genes and to validate the analysis with several pathological settings associated with the deregulated miRNAs and ion channel genes in the heart.
2. As discussed in Section 1.3.1 and 1.4.4 of the introduction, upregulation of I_{K1} is a key component of AF-related electrical remodeling. In order to study the role of miRNAs in this AF-related ionic remodeling process. Both in vitro and in vivo approaches were used to identify a deregulated miRNA in experimental and clinical AF, to test whether the deregulated miRNA can target *KCNJ2* (gene encoding Kir2.1 subunit for I_{K1}), to investigate the arrhythmogenic potential of the deregulated miRNA, and to explore the molecular mechanisms underlying the deregulation of the selected miRNA.
3. As discussed in Section 1.3.2 and 1.5.4 of the introduction, TRP channels may contribute to fibroblast proliferation and differentiation, which are central to AF-induce fibrotic remodeling. In order to test whether TRPC3 channels play a role in AF-induce fibrosis and whether miRNAs are involved in this process. Both in vitro and in vivo approaches were used to investigate whether TRPC3 channels are deregulated during AF and whether this deregulation play a role in AF-induced fibrotic remodeling, to identify a miRNA candidate responsible for the deregulation of TRPC3, and to study the molecular mechanisms underlying the change of the selected miRNA.