Université de Montréal

Regulation of the inositol 1,4,5-trisphosphate receptor 1 (IP3R1) by microRNA-26a in atrial fibrillation

Par

Faezeh Vahdatihassani

Faculté de Médecine

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Ce mémoire intitulé

Regulation of the inositol 1,4,5-trisphosphate receptor 1 (IP3R1) by microRNA-26a in atrial fibrillation

Présenté par

Faezeh Vahdatihassani

A été évalué(e) par un jury composé des personnes suivantes

Dr. Yahye Merhi
Président-rapporteur

Dr. Stanley Nattel
Directeur de recherche

Dr. Alvin Shrier
Membre du jury

Résumé

Contexte: La physiopathologie de la fibrillation auriculaire (FA) a été caractérisée par des changements de concentration cellulaire de Ca^{2+} et des processus connexes menant à l'apparition et au maintien de la maladie. Les récepteurs de trisphosphate d'inositol (IP3R) sont des canaux calciques ligand-dépendants pour lesquels la surexpression dans la FA a été liée à un remodelage cardiaque. Les microARN (miR, miARN), petits ARN non codants, sont d'une longueur d'environ 22 nucléotides et régulent l'expression des gènes par déstabilisation de l'ARN ou inhibition de sa traduction. De plus en plus de preuves ont été apportées sur le rôle des miARN dans la physiopathologie des troubles cardiaques, y compris le remodelage défavorable induit par la FA.

Objectif: Notre laboratoire a montré que le niveau nucléaire IP3R1 est régulé à la hausse dans le modèle canin de FA, ce qui produit une augmentation de la charge nucléaire en calcium. Cette étude vise donc à étudier le rôle des miARN dans la régulation d'IP3R1 qui initie et/ou perpétue la FA dans les cardiomyocytes auriculaires du modèle de FA chez le chien.

Méthodes: Nous avons utilisé un modèle canin de AF établi par méta-cardiographie auriculaire pendant 600 bpm × une semaine; des cœurs perfusés par Langendorff pour isoler les cardiomyocytes auriculaires pour des expériences moléculaires; le criblage des miRs qui ciblent le gène *ITPR1*, codant IP3R1, en utilisant des bases de données en ligne; RT-qPCR pour mesurer l'expression de l'ARNm de *ITPR1* et confirmer le niveau d'expression des miARN criblés; l'analyse Western Blot pour évaluer le niveau de protéine d'IP3R1; le test de la double luciférase reporter, la surexpression et l'abattement des miARN en culture primaire de cardiomyocytes isolées ou de lignées cellulaires appropriées; et l'imagerie par fluorescence calcique Fluo-4 AM pour évaluer le rôle potentiel des miARN sur la manipulation du Ca^{2+}. Pour les expériences de manipulation des miARN, les cellules ont été transfectées avec 1) un miARN non codant (miR-NC, groupe témoin), 2) un miARN mimétique et 3) un inhibiteur du miARN (AMO). La signification statistique est calculée avec le test t de Student ou l'analyse unidirectionnelle de variance (ANOVA) suivie par le test de Tukey à comparaisons multiples en utilisant le logiciel GraphPad Prism version 6.00.

Résultats: Nos données indiquent une augmentation du niveau de la protéine IP3R1 sans changement apparent de l'expression du gène *ITPR1* dans les cardiomyocytes de l'oreillette gauche par rapport à notre modèle canin de FA. Sur la base de l'analyse informatique, il a été prédit que miR-26a ciblerait l'ARNm de l'*ITPR1*. La FA a considérablement réduit la régulation du miR-26a dans les cardiomyocytes de l'oreillette gauche. Le dosage de la double luciférase reporté dans les cellules H9C2 a montré que le miR-26a agissait directement sur la région non traduite 3' (3'UTR) de l'ARNm *ITPR1*. De plus, la surexpression de miR-26a a réduit le niveau de la protéine IP3R1 et a diminué le taux diastolique [Ca^{2+}] dans le noyau et le cytosol des cardiomyocytes de chien, des transistors de Ca^{2+} stimulés électriquement; tandis que le knockdown de miR-26a a inversé ces effets. L'expression de l'ARNm de l'*ITPR1* est restée inchangée dans les cardiomyocytes de chien isolées après la transfection avec l'imitateur et l'inhibiteur de l'ARNm.

Conclusion: La régulation à la hausse d'IP3R1 dans la FA est due à l'inhibition de la traduction par le miR-26a, qui est régulé à la baisse dans les cardiomyocytes auriculaires du modèle canin de FA. Ce changement est associé à une altération de la manipulation du Ca^{2+}, qui se traduit par une augmentation des taux de Ca^{2+} diastolique nucléaire. Nos résultats suggèrent que la régulation à la baisse de miR-26a augmente l'expression de l'IP3R1, contribuant au remodelage pro-arythmique dans la FA.

Mots-clés: microARN, homéostasie calcique, physiopathologie cardiaque, fibrillation auriculaire, remodelage cardiaque, IP3R1.

Abstract

Background: The pathophysiology of atrial fibrillation (AF) has been characterized by changes in the cellular concentration of Ca^{2+} and related processes leading to the initiation and maintenance of the condition. Inositol trisphosphate-receptors (IP3Rs) are ligand-gated calcium channels for which overexpression in AF has been linked to cardiac remodeling. microRNA (miR, miRNA)s, small non-coding RNAs, are around 22 nucleotides in length and regulate gene expression by mRNA destabilization or inhibition of its translation. A growing body of evidence has emerged about miRNA's role in the pathophysiology of cardiac disorders, including AF-induced adverse remodeling.

Objective: Our laboratory has shown that nuclear IP3R1 level is upregulated in the dog AF model, producing increased nuclear calcium loading. Hence, this study aims to investigate the role of miRNAs in the regulation of IP3R1 initiating and/or perpetuating AF in atrial cardiomyocytes of the dog AF model.

Methods: We used AF dog model established by atrial-tachypacing for 600 bpm × one week; Langendorff-perfused hearts to isolate atrial cardiomyocytes for molecular experiments; screening miRs that target *ITPR1* gene, encoding IP3R1, using online databases; RT-qPCR to measure *ITPR1* mRNA expression and confirm the expression level of the screened miRNAs; western blot analysis to evaluate the protein level of IP3R1; dual-luciferase reporter assay, overexpression and knockdown of miRNAs in primary culture of isolated cardiomyocytes or appropriate cell lines; and Fluo-4 AM calcium fluorescence imaging to assess the potential role of the miRNA on Ca^{2+} handling. For miRNA manipulation experiments, cells were transfected with 1) non-coding miRNA (miR-NC, control group), 2) miRNA mimic, and 3) inhibitor of the miRNA (AMO). Statistical significance is calculated with Student's t-test or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test using GraphPad Prism software version 6.00.

Results: Our data indicated a rise in IP3R1 protein level with no apparent change in *ITPR1* gene expression in left atrial cardiomyocytes from our dog AF model. Based on the computational

analysis, miR-26a was predicted to target the *ITPR1* mRNA. AF significantly downregulated miR-26a in left atrial cardiomyocytes. The dual-luciferase reporter assay in H9C2 cells showed that miR-26a directly acted on the 3' untranslated region (3'UTR) of *ITPR1* mRNA. In addition, miR-26a overexpression reduced the IP3R1 protein level and decreased the diastolic [Ca^{2+}] in both nucleus and cytosol of the electrically-stimulated Ca^{2+} -transients, dog cardiomyocytes, while miR-26a knockdown reversed these effects. *ITPR1* mRNA expression remained unaltered in isolated dog cardiomyocytes after transfection with the miRNA mimic and inhibitor.

Conclusion: IP3R1 upregulation in AF is due to translation inhibition by miR-26a, which is downregulated in the atrial cardiomyocytes of the dog AF model. This change is associated with altered Ca^{2+} handling, reflected as enhanced nuclear diastolic Ca^{2+} levels. Our results suggest that miR-26a downregulation enhances the IP3R1 expression, contributing to pro-arrhythmic remodeling in AF.

Keywords: microRNA, calcium homeostasis, cardiac pathophysiology, atrial fibrillation, cardiac remodeling, IP3R1.

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List of acronyms and abbreviations

2-APB: 2-Aminoethoxydiphenyl borate

3'UTR: 3' untranslated region

AF: atrial fibrillation

AGO: Argonaute

AMO: anti-miRNA oligonucleotide

AP: action potential

APD: action potential duration

AT-II: angiotensin II

BNP: brain natriuretic peptide

CABG: coronary artery bypass grafting

CACNA1C: voltage-gated channel subunit alpha1 C

CaMKII: Ca^{2+}/calmodulin-dependent protein kinase II

CaT: Ca^{2+} transient

CHD: congenital heart disease

CTL: control

Cx: connexin

DAD: delayed afterdepolarizations

DAG: diacylglycerol

DGCR8: DiGeorge syndrome critical region gene 8

DNA: deoxyribonucleic acid

ER: endoplasmic reticulum

ET-1: endothelin-1

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

HDAC: histone deacetylase

HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HF: heart failure

HPRT1: hypoxanthine-guanine phosphoribosyltransferase

I_{CaL}: L-type Ca^{2+} current

I_{K1}: inward rectifier background K^+ current

I_{KACh}: acetylcholine-regulated K^+ current

IP3: inositol 1,4,5-trisphosphate

IP3R: IP3 receptor

IV: intravenous

KCNJ2: potassium inwardly-rectifying channel subfamily J member 2

LA: left atrium

LTCCs: L-type Ca^{2+} channels

MEF2: myosin enhancer factor 2

MHC: myosin-heavy chain

miR, miRNA: microRNA

miR-NC: non-coding miRNA

mRNA: mature ribonucleic acid

NCX: sodium/calcium exchanger

NFAT: nuclear factor of activated T cells

PLC: phospholipase C

POAF: postoperative atrial fibrillation

RISC: RNA-induced silencing complex

RyRs: ryanodine receptors

SERCA2a: SR Ca^{2+}-ATPase 2a

SR: sarcoplasmic reticulum

TRPC3: transient receptor potential canonical-3

A ma famille, pour m'avoir toujours soutenue

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Chapter 1 –Introduction

Atrial Fibrillation: definition and potential consequences

Atrial fibrillation (AF) is the most common type of sustained arrhythmia, with an estimated lifetime risk of 22% to 26%. AF is associated with pronounced population morbidity and mortality and reduction in quality of life and functional status (1). According to the 2014 AHA/ACC/HRS Guideline, AF is defined as "a supraventricular tachyarrhythmia with uncoordinated atrial activation and consequently ineffective atrial contraction" (2). In the electrocardiogram, AF is recognized by the absence of repeating P waves replaced by irregular atrial activity and irregular R-R intervals unless the patient has atrioventricular (AV) block (2). Atrial fibrillation can result in hemodynamic changes that are often clinically significant. Potential consequences vary for individual patients and are fatigue, which is the most common symptom, palpitations, fall in blood pressure, syncope, and dyspnea (2). AF is associated with heart failure (HF), and individuals with either condition could develop the other (3). Patients with AF can develop either too rapid or too slow ventricular rate, uncoordinated atrial contraction, and beat-to-beat variability in the ventricular filling, leading to hemodynamic consequences (2).

AF risk factors

Numerous risk factors are associated with an increased risk of developing AF. Nevertheless, various studies have shown that many of these risk factors are reversible, making it possible to prevent and manage some cases of AF through risk modification. Non-cardiac and cardiothoracic surgery, obesity, pneumonia, hyperthyroidism, hypertension, diabetes, pericarditis, myocarditis, HF, electrocution, diabetes mellitus, binge drinking, tobacco use, obesity, and many more have been reported as reversible causes of AF (2, 4).

Age is regarded as the most critical risk factor for AF in the general population. Evidence suggests that over 30% of patients diagnosed with AF are 80 years of age and older, and up to 16% are 75 to 84 years old, while approximately 1% of affected individuals are aged <60 years (2).

The results of the ATRIA study in the US showed that the prevalence of AF in the study population (1.89 million) was 0.95%, of which 45% were 75 years of age. This study projects that more than 5.6 million people will have AF by the year 2050, and 50% will be ≥80 years old (5). There is also a gender-dependent difference in the incidence of AF and, accordingly, the Framingham Heart Study has reported that the lifetime risk for development of AF after 40 years of age is 26% for men and 23% for women (6). Similar findings were also reported by the ATRIA study (5).

AF and concomitant cardiac diseases

Although AF is known to be a final common endpoint of atrial remodeling resulting from heart diseases, it can also be, in turn, a cause of electrophysiological changes and cardiac remodeling that explain the progressive nature of arrhythmia (7-9).

AF often complicates or exacerbates the underlying heart diseases or non-cardiac illnesses (2, 10). It seems possible that other critical illnesses cause changes in the underlying rhythm, which complicate the patient's problem and triggers recurrent AF (Figure 1) (10, 11). In asymptomatic AF patients as initially, the ventricular rate is not adequately controlled; they may develop tachycardia-induced ventricular dysfunction and HF (12). Worsened HF increases atrial stretch and heightens sympathetic tone making the AF more resistant to rate-control or rhythm-control treatments (11).

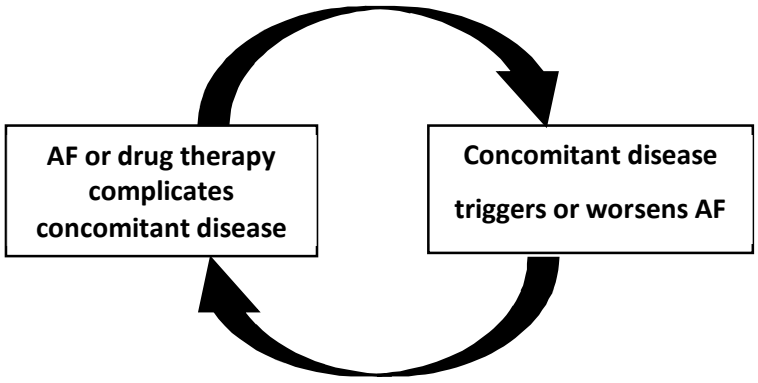

Figure 1: Interactions between atrial fibrillation (AF) and underlying illnesses (10, 11).

AF is a potent and independent risk factor for stroke, increasing its risk by approximately 5–fold throughout all ages. Detection of AF, even in its asymptomatic stage, is the basis of the decision

to implement anticoagulant therapy to prevent stroke (13). It has been shown that the left atrial appendage is the area where the thrombi are usually formed in AF (14). Impaired left ventricular (LV) ejection fraction (≤35%) and/or HF are important risk factors for stroke in AF patients as predicted by ACC/AHA/ESC guidelines (15).

AF can complicate valvular heart diseases, particularly left-side lesions. Aortic or mitral regurgitation/stenosis results in left atrial volume and pressure overload and, subsequently, progressive atrial dilation. The chronic structural changes in the left atrium promote AF by increasing atrial fibrosis and electrophysiological remodeling. Importantly AF emerges as a marker of adverse cardiovascular events, and AF patients with mitral valve disease face an elevated risk of mortality related to an increased risk of stroke (10).

Hypertrophic cardiomyopathy (HCM) is a genetic disorder that results from several mutations in genes involved in muscle contraction and characterized by unexplained LV hypertrophy (16). AF is also common in HCM patients and is related to left atrial dilatation and remodeling (17).

Patients with congenital heart disease (CHD), which affects 4-10 per 1000 live births (18), seem to be at higher risk of developing AF. Atrial arrhythmias occur in CHD patients due to comorbidities, persistent volume/pressure overload, and scars of surgical procedures and increase the risk of morbidity and mortality to great extent. Recent studies have reported a significantly higher risk of ischemic stroke, developing HF, and death in CHD patients who developed AF compared with patients who did not (19).

AF Pathophysiology

AF is maintained by multiple-circuit re-entry and/or repetitive and rapid firing of ectopic atrial foci, which mostly arise from delayed after-depolarizations (DADs) and, in some cases, early after-depolarizations (1). DADs are thought to be caused during diastole by the abnormal release of calcium from the sarcoplasmic reticulum (SR). Ryanodine receptors (RYRs) are the SR Ca^{2+} release channels that release Ca^{2+} during excitation-contraction coupling and are normally closed during diastole. Channel dysfunction and Ca^{2+} overload in SR contribute to the Ca^{2+} leakage from RyR2 (the predominant form of RyR in cardiac muscle) during diastole, which leads to the formation of

DAD. This diastolic calcium release by leaky RyR2 activates a net depolarizing inward positive-ion current and DADs through the sodium/calcium exchanger (NCX), which exchanges intracellular 1 Ca^{2+} ion for 3 extracellular Na^{+} ions (20).

Four major pathophysiological processes have been suggested to contribute to AF and account for the induction of focal ectopic activity and re-entrant circuit, and include electrical remodeling or ion channel dysfunction, structural remodeling, autonomic neural dysregulation, and Ca^{2+}-handling abnormalities (Figure 2) (1).

Findings of different studies imply that in addition to other cardiac diseases and conditions, AF itself can be the cause of these underlying contributors, which promotes the development of AF, and this vicious cycle is called “AF begets AF” (8). There has been a pile of evidence that confirms the central role of abnormal Ca^{2+}-handling in AF-pathophysiology. Regarding this, we review the studies in the area of AF-related Ca^{2+}-handling abnormalities, with particular focus on the contributions of IP3Rs. Also, the other contributing mechanisms are briefly summarized.

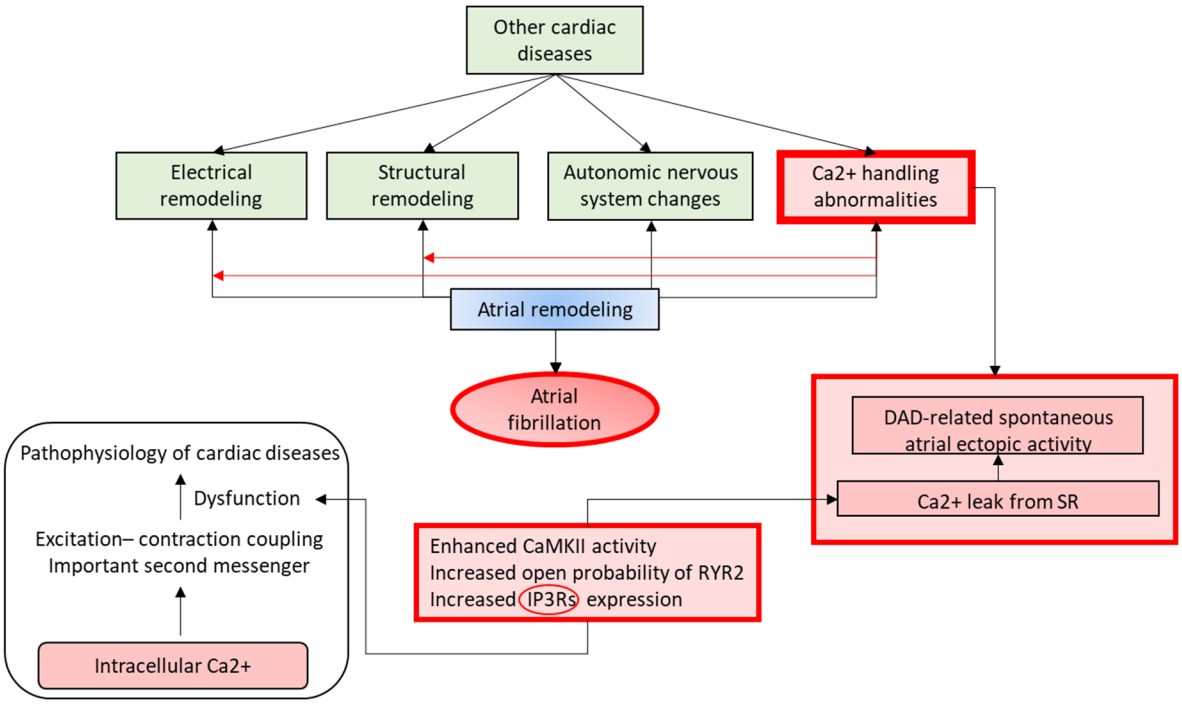

Figure 2: Major pathophysiological processes contributing to AF.
Ca^{2+} handling abnormalities have emerged as the central mechanism to the pathophysiology of AF, leading to delayed afterdepolarizations (DADs) that cause focal ectopic firing. Inositol trisphosphate-receptors (IP3Rs) are ligand-gated calcium channels overexpressed in AF,

contributing to cardiac remodeling. RyRs: ryanodine receptors; SR: sarcoplasmic reticulum; CaMKII: Ca^{2+}/calmodulin-dependent protein kinase II (8).

Autonomic neural dysregulation

The relationship between intrinsic cardiac nerve activity and spontaneous arrhythmias has attracted considerable attention. Some investigations have suggested that atrial arrhythmia can be generated due to the synergistic work and close anatomical association between muscle fibers and nerves. Accordingly, rapid atrial pacing in an animal model could produce autonomic remodeling (21). Shortening of the atrial effective refractory period by vagal and sympathetic activation can also be regarded as an important mechanism promoting AF induction and maintenance. Furthermore, left atrial hyperinnervation has been suggested to be important in AF induced by atrial tachycardia remodeling (22).

Structural remodeling

Extensive evidence indicates that AF-related structural remodeling is characterized by atrial enlargement and tissue fibrosis. Transforming growth factor (TGF)-β1, angiotensin II (AT-II), connective tissue growth factor, and platelet-derived growth factor are important profibrotic molecules involved in the atrial tissue fibrosis (23, 24). Although it has not been fully understood, several explanations have been put forward to justify the deleterious effects of atrial fibrosis in AF. Atrial fibrosis has been shown to cause local conduction disturbances and unidirectional block. It increases the number of fibroblasts, which produce large quantities of collagen, disturbing electrical continuity (25). Co-cultured preparations of fibroblasts and cardiomyocytes revealed that fibroblast coupling to cardiomyocytes could alter the electrical activity of cardiomyocytes leading to spontaneous impulse formation, abnormalities in cardiac conduction, and arrhythmias (25). Ca^{2+} signals are also essential for diverse functions in fibroblasts. For example, the receptor binding of AT-II activates the phospholipase C (PLC) pathway leading to diacylglycerol (DAG) and IP3 production. IP3 binds to the IP3R inducing Ca^{2+} release from the endoplasmic reticulum (ER is analogous to the SR in cardiomyocytes), and DAG can directly activate specific Ca^{2+}-permeable transient receptor potential (TRP) channels leading to Ca^{2+} entry into fibroblasts (26). Findings of a survey implied that increased Ca^{2+} entry via TRP canonical-3 (TRPC3) across the fibroblast cell

membrane or increased Ca^{2+} release from the ER cause enhancement of proliferation and fibroblast differentiation into myofibroblasts promoting fibrosis in AF (27).

Electrical remodeling or ion channel dysfunction

Pumps, ion channels, and exchangers are considered essential components of cardiac electrophysiology that can be altered by atrial remodeling.

Atrial tachycardia during AF has been shown to downregulate L-type Ca^{2+} current (I_{CaL}) carried by L-type Ca^{2+} channels (LTCCs). The Ca^{2+} overload, which is caused by a high atrial rate, activates the Ca^{2+}-dependent calmodulin–calcineurin–nuclear factor of activated T cells (NFAT) system and enhances NFAT translocation into the nucleus. NFAT is a transcription factor that decreases the transcription of the calcium voltage-gated channel subunit alpha1 C (CACNA1C) gene encoding *Cav1.2* LTCCs, thus reducing I_{CaL}. Downregulation of I_{CaL} mitigates inward Ca^{2+} current, which causes shortening of the action potential (AP) duration (APD) and thereby promotes re-entry (28). Another principal component of electrical remodeling is the upregulation of inward-rectifier K^+ current (I_{K1}) and acetylcholine-regulated K^+ current (I_{KACh}). I_{K1} is mainly composed of Kir2.1 subunits contributing to the resting potential and phase 3 repolarization. Evidence shows that I_{K1} is increased in chronic AF, which is an important determinant of AF-maintaining re-entry (29). As seen in AF, atrial tachycardia has been suggested to increase the I_{KACh} leading to reductions in APD (30). Alternation in expression and distribution of main atrial gap junction protein such as connexin (Cx) 40 and Cx43 connecting cardiomyocytes electrically can also occur in AF and contribute to electrical remodeling. Connexin gene transfer has been shown to improve atrial conduction and reduce AF (31).

Ca^{2+}-handling abnormalities

Despite the considerable physiologic potential, Ca^{2+} signaling has a central role in atrial remodeling and AF pathophysiology. During each AP in a healthy heart, Ca^{2+} enters cardiomyocytes through LTCCs and then triggers Ca^{2+} release from SR mainly through RYR2. During diastole NCX1 and SR Ca^{2+}-ATPase 2a (SERCA2a), remove Ca^{2+} from the cytosol (32).

Atrial tachycardia and increased reactive oxygen species in AF result in the persistent Ca^{2+} overload, which activates Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). This kinase regulates the activities of the proteins involved in Ca^{2+} handling LTCC, RyR2, and phospholamban (33, 34). CaMKII-dependent RyR2 hyperphosphorylation results in pronounced SR Ca^{2+}-leak, which, together with greater depolarizing inward NCX current and increased intracellular Ca^{2+} membrane voltage coupling gain, enhance the risk of potentially-arrhythmogenic DADs in AF (35).

Two forms of intracellular Ca^{2+}release channels regulate the intracellular Ca^{2+}concentration: RyR and IP3Rs. RYRs have been suggested to have a key role in Ca^{2+} release from SR during excitation-contraction coupling. IP3 induces intracellular Ca^{2+} release from IP3Rs that mediate the hormonal regulation of cardiac contraction. Cardiomyocytes express IP3Rs less abundantly compared to RYRs. However, the upregulation of IP3R expression in atrial tissue of patients with chronic AF is thought to contribute to intracellular Ca^{2+}abnormalities and Initiation and perpetuation of AF (36, 37).

As previously stated, the intracellular calcium level plays a central role in the activation of cell signaling, which mediates pro-fibrillatory atrial remodeling. In this regard, we aim to determine if a specific miRNA can contribute to the dysregulation of IP3Rs in AF. Below is a description that briefly summarizes the roles of IP3R in cardiac physiology and pathology with the emphasis on AF.

Inositol 1,4,5 trisphosphate receptor (IP3R)

Structure

The IP3R is a ligand-gated calcium channel activated by inositol trisphosphate (IP3). Molecular cloning studies have demonstrated that IP3R is comprised of three types that are encoded by distinct genes: *ITPR1*, *ITPR2*, and *ITPR3*. Each isoform is about 300 KDa and forms homo- and heterotetramers, divided into three functionally active domains: N-terminal ligand-binding domain, C-terminal helical domain, and modulatory domain. The N-terminus has a ligand-binding domain for IP3 and a suppressor domain. C-terminal domain has six transmembrane α-helices that are packed together to form the ion-conducting pore and C-terminal tail. The C-terminal tail

modulates gating, and It is also a site for post-translational modifications and binding of multiple ligands that modulate the channel function. The modulatory domain, located between N-terminus and the C-terminus channel domain, is a binding site for a variety of molecules regulating the channel function and for post-translational modifications. Three IP3R isoforms share similarities, and the channel domain is well conserved (65-70%) among them (38, 39).

Distribution

IP3Rs are ubiquitously expressed in all tissues and primarily localized to the ER/SR. It became apparent that most cell types express more than one type of IP3R, and the subcellular distribution of IP3Rs depends on the cell type. The Golgi apparatus, secretory vesicles, plasma membrane, or other specialized membranes such as perinuclear or nuclear membrane may contain IP3Rs (40).

Western blot, IP3-binding, and PCR analysis in Lipp et al. study indicated that the three IP3R isoforms are expressed in both atrial and ventricular myocytes, and types I and II IP3Rs are the most abundant species (41). It has been suggested that IP3R1 is dominant in human atrial myocytes and rat Purkinje myocytes, whereas IP3R2 is predominantly expressed in ventricular and atrial myocytes of the other animal species (42). Although all types of IP3Rs are expressed in the heart, only a few investigations assessed the role of type 1 and 3 (39).

Results of a co-immunostaining of atrial myocytes suggest that IP3R2 is mainly expressed in the subsarcolemmal region and is colocalized with the junctional RyR2. Also, in electrically paced atrial myocytes, the IP3 ester increased the action potential-evoked Ca^{2+} transient (CaT), indicating that IP3Rs could modulate excitation-contraction coupling (41). Electron microscopy results suggested that IP3R1 is highly found in the intercalated discs of the ventricular and atrial cardiomyocytes (43). Previous work has also shown that IP3Rs are highly concentrated in the nuclear envelope and perinuclear membranes. Local Ca^{2+} release from IP3Rs activates the gene expression or drives pathological cardiomyocyte growth (44, 45).

Results of a recent study suggest that IP3Rs are expressed in both nuclear and non-nuclear fractions of isolated canine atrial cardiomyocytes. Upon IP3 stimulation in this system, increased resting $[Ca^{2+}]_{nuc}$ and $[Ca^{2+}]_{Cyto}$ are observed (Figure 3).

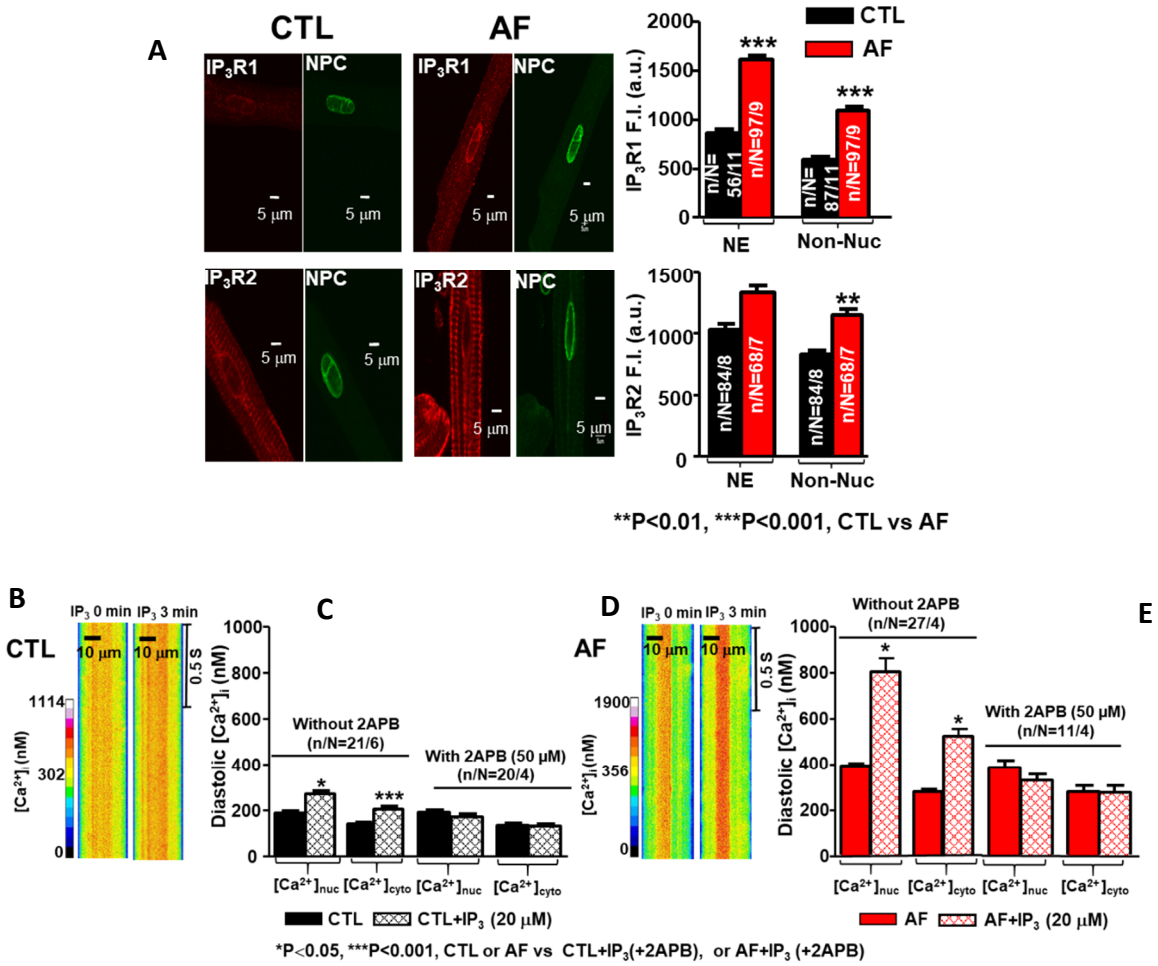

Figure 3: Upregulation of IP3R1/2 protein levels and alterations in resting $[Ca^{2+}]$ in atrial cardiomyocytes of the canine AF model.

(A): 2D images and fluorescence intensity from control (CTL) and atrial fibrillation (AF) atrial cardiomyocytes (CMs) after immunostaining for nuclear pore complex (NPC, green), IP3R1/2 (red). (B): Line-scan imaging of cytosolic and nucleoplasmic of resting Ca^{2+}in one permeabilized CTL atrial CM before and after 20 μM IP3. (C): Effect of IP3 and IP3+2-aminoethoxydiphenyl borate (2-APB) (50 μM) on CTL atrial CM resting $[Ca^{2+}]_{nuc}$ and $[Ca^{2+}]_{Cyto}$. (D): Line - scan imaging of cytosolic and nucleoplasmic CaTs in permeabilized AF atrial myocyte before and after IP_3. (F): Effect of IP_3 and IP_3 + 2APB (50 μM) on AF atrial CM resting $[Ca^{2+}]_{nuc}$ and $[Ca^{2+}]_{Cyto}$. Data are shown as mean±SEM. (Qi et al., Circ Res, in revision (46)).

IP3R role in cardiac function and pathological cardiac remodeling

IP3Rs are involved in the regulation of cardiomyocytes function and the pathogenesis of heart diseases. Although IP3Rs are the main channels regulating intracellular Ca^{2+} in many cells, RYRs are the dominant channels of calcium release in the heart (39). Therefore, many investigations

have focused on the RYRs signaling in cardiomyocytes and made the studies on IP3Rs roles challenging.

IP3 signaling cascade

In cardiomyocytes, hormonal factors such as endothelin-1 (ET-1), many transmitters, and stimuli such as stretch activate the IP3-induced Ca^{2+} release. IP3 is an intracellular messenger and is generated through the activation of plasma membrane receptors coupled to PLC. Receptors such as receptor tyrosine kinases and G protein-coupled receptors can activate PLCs and increase the Ca^{2+} release downstream of PLC activation. The PLC enzyme liberates the signaling molecules IP3 and DAG from phosphatidylinositol 4,5-bisphosphate (PIP2). The hydrophilic IP3 then translocates from the membrane to the cytoplasm and binds to its main target IP3R mostly on SR and causes a conformational change leading to Ca^{2+} release (42). The nuclear envelope is also able to store Ca^{2+} and has been shown to have the entire components of the IP3-mediated Ca^{2+} signaling network (47). Previous studies have revealed that G protein-coupled receptors are also found in the nuclear envelope and involved in Ca^{2+} release from the nuclear membrane into the nucleoplasm associated with IP3 signaling (48).

Role of IP3R signaling in the development of the heart

A plethora of investigations in embryonic stem cell or prenatal cardiac cells have suggested substantial roles for IP3Rs in cardiac development. IP3Rs have been demonstrated to be responsible for the early cycling of Ca^{2+} before the excitation-contraction coupling maturation in developing myocytes. There is evidence indicating that the periodic Ca^{2+} oscillation inducing spontaneous contractions in murine embryonic cardiomyocytes are dependent on the IP3 and sensitive to IP3R blocker 2-aminoethoxydiphenyl borate (2-APB) (49). In another survey on mice embryos using the IP3R1 probe, it was reported that IP3R mRNA is highly expressed in all tissues and the developing heart tube in the early stages of development (E8.5), while RYRs are not abundant. Data from the same study also suggests that the IP3-mediated Ca^{2+} signaling pathway during the early stage of embryogenesis may be involved in cellular differentiation and apoptosis as part of the developmental programs (50). In addition, IP3R-mediated Ca^{2+} release in embryonic stem cell-derived cardiomyocytes was shown to contribute to the spontaneous activity in

differentiating cardiomyocytes (51). The role of iP3Rs in nodal cell automaticity in the developing atria has also been observed in the isolated atrial cells from E14.5 mouse embryos (52). Also, IP3R1$^{-/-}$ and IP3R2$^{-/-}$ double-mutant mice could not survive due to heart defects showing that they play a crucial role in cardiogenesis (53).

IP3R function in cardiomyocytes

IP3Rs have been shown to modulate cytosolic calcium levels in the heart, despite the lower mRNA levels (~50-fold) than RYRs (54). Application of IP3 to permeabilized chick atrial muscle was shown to induce transient increase in tension, which is suggested to be involved in the positive inotropic effect and contraction in the atrium (55). Studies also have highlighted the role of IP3R-dependent Ca^{2+} release in positive inotropic effects in ventricles by enhancing the systolic CaTs. In permeabilized rabbit ventricular myocytes, IP3R activation by direct application of IP3 could increase the Ca^{2+} spark frequency (56). IP3Rs are located in the perinuclear and nuclear membranes of cardiomyocytes. This localization of IP3Rs restricts the CaT to the nuclear matrix and activates a variety of transcription factors, subsequently initiating cardiac remodeling in response to hormonal stimuli (e.g., ET-1) (44).

Role of IP3R signaling in cardiac diseases

The IP3Rs expression levels are altered during pathological remodeling. Owing to their functions in Ca^{2+} release from the SR and the nuclear resources, IP3Rs can trigger arrhythmias and hypertrophic gene transcription via several mechanisms.

Several lines of evidence have shown increased IP3R2 expression in hypertrophy observed in patients with ischemic dilated cardiomyopathy or animal models of hypertensive rats and aortically-banded mice. It is important to note that increased Ca^{2+} release through IP3Rs in hypertrophic cardiomyocytes sensitizes neighboring RyRs opening leading to enhancement of extrasystolic Ca^{2+} transients giving rise to cardiac arrhythmia (57). As previously discussed, IP3Rs are also responsible for the hypertrophic response of hormonal factors such as ET-1 and AT-II. The potent constrictor ET-1 activates the transcription of specific cardiac genes such as atrial natriuretic factor (ANF), a key marker of hypertrophy, and increases the assembly of myosin light chain-2 into sarcomeric units, which is a hallmark of the hypertrophic phenotype. Besides, these

effects of ET-1 are accompanied by the stimulation of inositol phospholipid hydrolysis, which results in the accumulation of inositol phosphates, including inositol monophosphate, inositol bisphosphate, and IP3 and DAG in cardiomyocytes (58). In a study by Higazi et al., induction of hypertrophic remodeling by ET-1 was shown to be dependent on IP3-induced Ca^{2+} release. They reported that ET-1 induced elevation of the nuclear Ca^{2+} content by stimulating the Ca^{2+} release from prenuclear IP3R2 and increased ANF expression by activating the calcineurin /NFAT pathway (59). In an investigation in IP3R2-deficient mice, the administration of ET-1 failed to increase diastolic Ca^{2+} concentration. It should be noted that no compensatory changes in protein expression of type 1 and 3 were also observed in the global knockout of IP3R-2. Therefore, lack of IP3R2 was protective against the positive inotropic and arrhythmogenic effects of ET-1 (60). However, IP3R-2 knockout in a mouse model of dilated cardiomyopathy could not alter the hypertrophic response in pressure overload (61). Cao et al. also indicated higher levels of IP3R1 mRNA in the atrial tissue of AF patients, which correlated with AF duration (62). Similar results were also reported by Yamada et al. on IP3R1 protein and mRNA levels in right atrial samples from AF patients with mitral valvular disease (37). 2-APB, an inhibitor of both IP3Rs and TRP channels, has been shown to suppress the incidence and probability of sustained cardiac arrhythmia in the rabbit AF model (63). In a recent investigation in our laboratory, assessment of IP3R expression in left atrial cardiomyocytes from AF dogs indicated that IP3R1/2 levels were higher in cytosolic and nucleoplasmic fractions compared with control samples. IP3R1 level was markedly increased in both nuclear and non-nuclear regions, while IP3R2 was significantly upregulated in the non-nuclear fraction. The physiological agonist IP3 increased the resting [Ca^{2+}] in cytoplasm and nucleus of control and AF saponin-permeabilized atrial cardiomyocytes. However, the effect was greater in AF cells, especially in the nuclear region. In the presence of 2-APB, IP3 failed to increase the resting [Ca^{2+}] in control and AF cardiomyocytes (Figure 3). These results suggest that atrial cardiomyocyte Ca^{2+} handling is deranged in AF and that the principle underlying mechanism is upregulation of IP3Rs.

miRNAs and post-translational regulation in cardiac disease

miRNAs are a class of genes that were discovered in *Caenorhabditis elegans* in 1993 by Lee et al. (64) and Wightman et al. (65). They suggested that lin-4 does not encode any protein and is

complementary to the LIN-14 3'UTR and negatively regulates its protein level and controls the postembryonic developmental events in *C. elegans* (64, 65).

Based on the latest data released by the miRBase database (v22), 38,589 hairpin precursor miRNAs have been found in 271 organisms. Precursor miRNAs (pre-miRNAs) are capable of producing 48,860 different mature miRNAs. The human genome is estimated to contain 1917 annotated pre-miRNAs and 2654 mature miRNAs sequences (66). miRNAs can be categorized based on their locations in the genome relative to intron and exon positions. Approximately half of the miRNAs are transcribed from polycistronic transcription units in which miRNAs loci are located in close proximity to each other. miRNAs loci are located in the intronic and exonic regions of protein-coding or non-protein-coding transcription units (67). The residing of the miRNAs within the introns of protein-coding genes depicts the dual performance of the genes and could explain the coordinated expression of mRNA and miRNAs modulating the same biological processes (68, 69).

miRNA nomenclature

According to a standard nomenclature system, the mature miRNAs are named with the prefix "miR-" followed by a number indicating the order of the miRNA discovery (e.g., miR-26). The first three letters, such as "hsa: human", before the prefix "miR", indicate the species (hsa-miR-26). A letter after the number reflects the identical sequences differing in only one or two nucleotides (e.g., miR-26a or miR-26b). An additional dash-number suffix shows different pre-miRNAs that are processed into an identical miRNA (e.g., miR-26-1). The 3p and 5p demonstrate the 3' and 5' miRNAs (e.g. miR-26a-5p). A uniform system to annotate miRNAs has been described in Ambros et al. short article (70).

Canonical biogenesis pathway and regulation of gene expression

miRNAs are a class of small (~22 nucleotides [nt] in length) non-coding RNAs that negatively regulate gene expression and play key roles in biological processes and pathological conditions in plants and metazoans. Most miRNAs have multiple isoforms or isomiRs that can be generated through different mechanisms modifying mature miRNAs and precursor miRNAs (pre-miRNAs) (67, 71). The first step in miRNAs biogenesis is the generation of a hairpin-shaped transcript called

primary miRNA (pri-miRNA, 100 to 1,000 base pairs). Transcription is mostly mediated by RNA polymerase II (Pol II) and for a minor group of miRNAs by Pol III. The hairpin structures are cleaved into a ∼65 nucleotide pre-miRNA by nuclear RNase III enzyme Drosha and with the help of the cofactor DiGeorge syndrome critical region gene 8 (DGCR8) and then exported from the nucleus into the cytoplasm by the exportin 5. The cytoplasmic RNase III Dicer catalyzes the pre-mRNA splicing producing a mature ∼22-nt miRNA duplex. The RNA duplex incorporates into the Argonaute (AGO) to generate RNA-induced silencing complex (RISC). The RNA duplex dissociates into a guide or seed strand, which remains in the AGO, and a passenger strand, which is degraded (8, 67). miRNA-induced silencing complex binds to 3' UTR of mRNA, leading to translational repression and/or RNA destabilization. When the base pairing between miRNA and mRNA is perfect, mRNA cleavage and degradation are induced. However, imperfect miRNA complementary with mRNA targets is the dominant case in metazoans and results in mRNA deadenylation, inhibition of translation initiation, elongation block, or proteolysis of the nascent polypeptides (72). miRNAs recognize the mRNA targets with the nucleotides 2–8 in their 5' region called "seed" region, which nucleates the miRNA–mRNA interaction. However, the seed region pairing is not always a necessity for miRNAs function (73).

Roles of miRNAs in cardiac development and function

One miRNA has numerous targets with different affinity, and each miRNA family that shares the same 7–8 nt in the seed region has 300 conserved targets (74). From the very beginning, miRNA roles in biological processes such as lin-4 and let-7 regulatory functions in *C. elegans* larval developmental timing have been investigated (75). Studies on cardiac-specific gene knockout and knockdown models have also suggested essential roles for miRNAs in modulating cardiac development and functions (69, 76). Results of a deep sequencing analysis in the murine adult heart revealed that a small number of miRNAs, including let-7 family members, miR-29a, miR-26a, and miR-133a were highly abundant, and miR-1 accounted for nearly 40% of all miRNAs in the heart (77).

Mice genetically deleted for DGCR8 displayed penetrant and severe phenotypes and developed ventricular dysfunction responsible for dilated cardiomyopathy and premature death. The

analysis of the gene expression profile of the hearts in DGCR8 mutant mice showed upregulation of 14% of the genes predicted to be targeted by 10 cardiomyocyte-enriched miRNAs. These observations emphasize the importance of miRNAs in maintaining cardiac function (77). Cardiac-specific deletion of the RNase enzyme Dicer gene essential for miRNA biosynthesis; was used to demonstrate the role of miRNAs in development. However, these results signify the overall functions of many miRNAs rather than a specific one in the cardiac system. In a survey by Zhao et al., the cardiac deletion of Dicer led to pericardial edema, deficiently developed ventricular myocardium, and death. Cardiac and muscle-specific miR-1-2 was shown to be affected abundantly in the Dicer mutant heart. Targeted deletion of miR-1-2 had profound consequences, including cardiac morphogenetic and electrophysiologic defects and cardiomyocyte hyperplasia induced by cell-cycle abnormality (78). Kwon et al. found that miR-1 was involved in the differentiation of cardiac progenitors and cardiac development in the *Drosophila* embryos. They reported that miR-1 overexpression in cardiac mesoderm reduced the number of cardiac cells. In addition, the deletion of miR-1 was lethal during embryogenesis and hatching (79). Chen et al. reported that lack of Dicer in the heart caused alterations in myocardial structure and cardiac conduction and reduced cardiac contractile proteins and heart rate. All these events led to dilated cardiomyopathy and death shortly after birth (80). miR-133 is another cardiac and muscle-specific miRNA. miR-1 and miR-133 are bicistronic miRNAs, and their expressions are controlled by the same regulators of muscle lineages such as myosin enhancer factor 2 (MEF2), serum response factor, and MyoD. Both miRNAs are present and enriched in early cardiac progenitors derived from embryonic stem cells. They have shown opposing effects during further differentiation of embryonic stem cells into cardiac muscle progenitors. miR-133 inhibited, whereas miR-1 promoted cardiomyocyte differentiation (81). Upon knockdown of miR-143 or disruption of the miR-143- adducin3 pathway, encoding an F-actin capping protein, dramatic changes in the cardiac chamber including atrial dilation and ventricular collapse were also displayed in zebrafish (82).

miRNAs in AF pathophysiology: electrical and structural remodeling

Several cardiovascular diseases are associated with differential gene expression driven by miRNAs function. Specific miRNAs are expressed and enriched in tissues and/or cells, reflecting their involvement in pathophysiologic mechanisms of diseases. In pathological conditions, up- or

downregulated miRNAs target genes of signaling pathways underlying arrhythmia, contractility defects, hypertrophy, cardiomyopathies, fibrosis, and other pathogenic phenotypes. Further analysis through molecular research, including miRNAs overexpression or inhibition of their function, has highlighted their essential roles as disease biomarkers and new therapeutic targets in cardiovascular disease treatments.

Several studies have reported regulatory roles for miRNAs in cardiac excitability, cardiac conduction, automaticity, and repolarization (83, 84). Deregulated miRNAs significantly altered the expression of cardiac ion channels such as calcium channels, sodium channels, potassium channels, and Cx43 in certain cardiac conditions associated with AF (83).

In the Luo et al. study, interesting results have been obtained on the potential roles of miR-26 in the pathophysiology of AF. miR-26 was shown to target potassium inwardly-rectifying channel subfamily J member 2 (KCNJ2) gene encoding Kir2.1 (IK1), which is upregulated in AF (85). They found that miR-26 expression was significantly decreased, and IK1 was upregulated in atrial samples of AF patients and the dog model of persistent AF. miR-26a overexpression and knockdown with miR-26 antisense were successful in suppressing and enhancing the KCNJ2/Kir2.1 expression. Adenovirus-mediated expression of miR-26 reduced AF vulnerability in mice, and the opposite effect was achieved upon knockdown of endogenous miR-26 by antagomiR. The transcription factor NFAT was found to be the repressor of the miR-26 gene. The results suggest that increased NFAT activity mitigates the transcription of miR-26, which results in the downregulation of KCNJ2/Kir2.1, creating a substrate for atrial remodeling and promoting AF (85). In another study, they showed that miR-26 could regulate TRPC3 in fibroblasts, contributing to atrial structural remodeling. TRPC3 protein expression was upregulated in freshly isolated left atrial fibroblasts from dogs and atria from AF patients, and miR-26 was decreased in canine AF atria. Experimental miR-26a overexpression in canine left atrial fibroblasts was shown to decrease TRPC3 protein expression and fibroblast-number (27).

The secretome of transfected primary murine cardiac fibroblast with pre-miR-29b was analyzed by proteomics. miR-29b was shown to target several proteins involved in fibrosis, such as collagen and matrix metalloproteinase-2 secretion, and blocked the response of fibroblasts to TGF-β as a

potent mediator in fibrosis (86). In another study, miR-29b expression was found to be decreased in the left atrial tissue and atrial fibroblasts of dogs with congestive heart failure (CHF) developing AF. Expression of the extracellular matrix genes, including collagen-3A1, collagen-1A1, and fibrillin, increased significantly in canine CHF fibroblasts and cultured left atrial fibroblasts subjected to miR29b knockdown (87). As mentioned in the text, I_{CaL} generated by LTCCs is reduced in AF, which is a hallmark of electrical remodeling. In a survey by Zhao et al., miR-29a was increased in atrial tissues of AF patients, whereas the expression of CACNA1C, a subunit of LTCC, was downregulated. miR-29a transfection into HL-1 cells negatively regulated CACNA1C expression and was successful in reducing I_{CaL} (88).

There is also evidence that miR-135a negatively regulates NCX1 expression and contributes to arrhythmogenic cardiac remodeling. As investigated in the Duong et al. study, the complete atrioventricular block was associated with miR-135 downregulation and increased NCX1 activity in left ventricular samples. miR-135a overexpression in neonatal rat ventricular myocytes reduced NCX1 expression and spontaneous beating frequency and altered caffeine-induced Ca^{2+} transients (89).

miR-1 is a muscle-specific miRNA and is expressed in high levels in cardiomyocytes. Girmatsion et al. reported that miR-1 could regulate Kir2 subunit expression, and I_{K1} density increased in the left atria of patients with persistent AF. Rapid pacing of the human atrial slices from SR patients led to increased Kir2.1 expression while decreased miR-1 expression (90). miR-1 was also increased in myocardial samples from patients with coronary artery disease and a rat model of myocardial infarction (MI). Transfection of miR-1 into healthy myocardium slowed cardiac conduction and resulted in potential abnormality leading to arrhythmias. Injection of the anti-miRNA oligonucleotide (AMO)-1 into infarcted hearts suppressed arrhythmias and alleviated the downregulated levels of Kir2.1 and Cx43 in MI rats (91).

miR-133 has been reported to play a crucial role in cardiac hypertrophy. Decreased expression of miR-133 was shown in samples from interventricular septum from patients with hypertrophic cardiomyopathy and different murine models of cardiac hypertrophy (92). In the study by Drawnel et al., IP3R2 expression was increased while the miR-133a level was diminished in

ventricular samples from rats with pressure overload-induced hypertrophy (93). In concordance with these results, miR-133a overexpression in neonatal rat ventricular myocytes downregulated IP3R2 expression. AMO-133a transfection prevented the repressive effect of miR-133a on IP3R2 expression and increased the cell surface area and expression of the hypertrophic marker ANF. The pro-hypertrophic effect of miR-133a was suggested to result from IP3-induced calcium release, which regulates the transcription of miR-133a. These results indicate that decreased miR-133a level in hypertrophic myocardium results in the upregulation of IP3R2, after which the excessive Ca^{2+} release from this receptor may promote arrhythmic events (93). Downregulation of miR-133a and miR-590 was observed in the canine model of nicotine-induced atrial fibrotic remodeling. Long term administration of nicotine to dogs increased AF vulnerability and duration. Nicotine also stimulated collagen content and fibrosis, upregulated TGF-β1 and TGF-β receptor type II, and decreased miR-133 and miR-590 expression. TGF-β1 and TGF-β receptor, which regulate collagen production and deposition, have been identified as the target genes of miR-133 and miR-590 (94).

miRNAs as biomarkers in AF

Very stable forms of miRNAs can be detected in plasma or serum in contrast to mRNAs (95). Therefore, this stability allows the retrospective analysis and detection of pathologic conditions using miRNAs as molecular biomarkers in conserved tissue and blood samples (96). It is not fully determined how miRNAs enter the circulation. Hypotheses have proposed that they are secreted in forms of membrane-bounded-vesicles such as exosomes, apoptotic bodies, or microvesicles. Another theory is that they are secreted as protected protein–miRNA complexes such as AGO or other RNA-binding proteins. miRNAs are also released as by-products of dead cells (97). A growing body of evidence indicates that circulating miRNAs might also be used as biomarkers in AF (95).

CHF-induced atrial fibrotic remodeling is a substrate for AF maintenance. An investigation by Dawson et al. has shown that miR-29b levels were decreased in atrial tissues of chronic AF patients and plasma of patients with CHF or AF and further reduced in plasma of patients with both conditions. The results suggested that miR-29b could serve as both biomarker and therapeutic target in AF and CHF (87).

In a pilot study by da Silva et al., expression levels of six miRNAs were measured in plasma from patients with acute new-onset AF and well-controlled AF. Results showed increased levels of miR-133b, miR-328, and miR-499 and decreased expression of miR-21 in patients with acute new-onset AF compared with well-controlled AF. Bioinformatics data demonstrated that these miRNAs target mRNA of genes such as Smad7 involved in fibrosis and directly related to AF (98).

Postoperative atrial fibrillation (POAF) is the most postoperative arrhythmia affecting approximately 1:3 patients undergoing coronary artery bypass grafting (CABG). Harling et al. indicated that among the 16 miRNAs differentially expressed in the atrial tissue of POAF patients, miR-483-5p and miR-208a were the most upregulated and downregulated miRNAs, respectively. miR-483-5p was also significantly overexpressed in the preoperative serum of POAF patients (99). In another study, serum levels of miR-23a and miR-26a were lower in POAF patients compared to non-POAF patients after CABG (100).

The expression levels of serum miRNAs in patients with hyperthyroidism (Graves' disease [GD]) with or without AF were also determined in Wang et al. study. Among the eight candidate miRNAs, miR-1a expression level was significantly decreased in GD + AF patients and negatively correlated with the increased left atrial diameter compared to the GD group (101). The association between plasma miRNAs levels and AF was also tested in the miRhythm study. Plasma and atrial tissue levels of miR-21 and miR-150 miRNAs, which have pro-fibrillatory potential and affect mRNAs of atrial fibrosis, were decreased in AF patients compared to those without AF. Plasma levels of both miRNAs increased after AF ablation. The Results also demonstrated a further decrease in plasma levels of miR-21 and miR-150 in persistent AF compared to paroxysmal AF, reflecting that miRNA levels were affected by the duration and intensity of AF (102). Similar findings on the effect of radiofrequency catheter ablation on miRNA expression profile have also been reported in AF patients. The authors in this study demonstrated that miR-409-3p and miR-432 levels were downregulated in plasma samples of AF patients and upregulated in the postoperative plasma samples. No significant difference was observed in miRNAs levels between healthy individuals and postoperative patients (103). In a recent investigation by Zhang et al., the plasma level of miR-155 was shown to be related to AF recurrence. miR-155 was remarkably

upregulated in the recurrent AF group compared to the non-recurrent group and positively correlated with the left atrial diameter (104).

Rationale, hypothesis, and objectives

AF is an important arrhythmia, and its mechanisms are still poorly understood. Levels of IP3Rs are significantly higher in the atrial tissue of patients with AF, as reviewed above (37, 62). In recent work from our laboratory in a canine AF model, IP3R1/2 was upregulated in nuclear and non-nuclear fractions prepared from cardiomyocytes isolated from left atrial tissue, producing increased nuclear calcium loading (Figure 3). Assessment of IP3R expression by immunostaining showed that in left atrial cardiomyocytes from AF dogs, IP3R1/2 expression is increased, particularly in nucleoplasmic fractions. IP3R1 was markedly upregulated in both nuclear and non-nuclear regions, while IP3R2 was significantly upregulated only in the non-nuclear fraction. Detailed analysis related increased nuclear Ca^{2+} loading to upregulated IP3R1. Furthermore, IP3R1 knockdown in atrial cardiomyocytes prevented the downregulation of L-type Ca^{2+} current, a central mechanism involved in AF-related remodeling. These findings lend direct support to the idea that IP3R upregulation plays a causal role in atrial arrhythmogenesis in AF.

Analysis of the changes of IP3R1 expression suggested greater increases in protein than in mRNA expression in the dog AF model. This observation suggests the possibility of underlying miRNA dysregulation because miRNA prominently interferes with mRNA translation. I designed this study to investigate the hypothesis that miRNAs play a role in the regulation of IP3R1 expression in atrial cardiomyocytes of dogs with atrial remodeling caused by AF.

The principal objectives were to:

- Confirm the protein expression of IP3R1 and expression level of the *ITPR1* gene, which encodes IP3R protein, in cardiomyocytes isolated from left atrial tissues of the AF dog model.
- Identify miRNA candidates that target the *ITPR1* gene through bioinformatic studies.
- Select a small number of candidate miRNAs for further study based on bioinformatics to test for biological effects.

- Assess the ability of selected candidate miRNAs to regulate ITPR1 expression and Ca^{2+} handling in cardiomyocytes isolated from left atrial tissues of control dogs.

Research Framework

Figure 4 is a schematic representation of the experimental procedures in the current study.

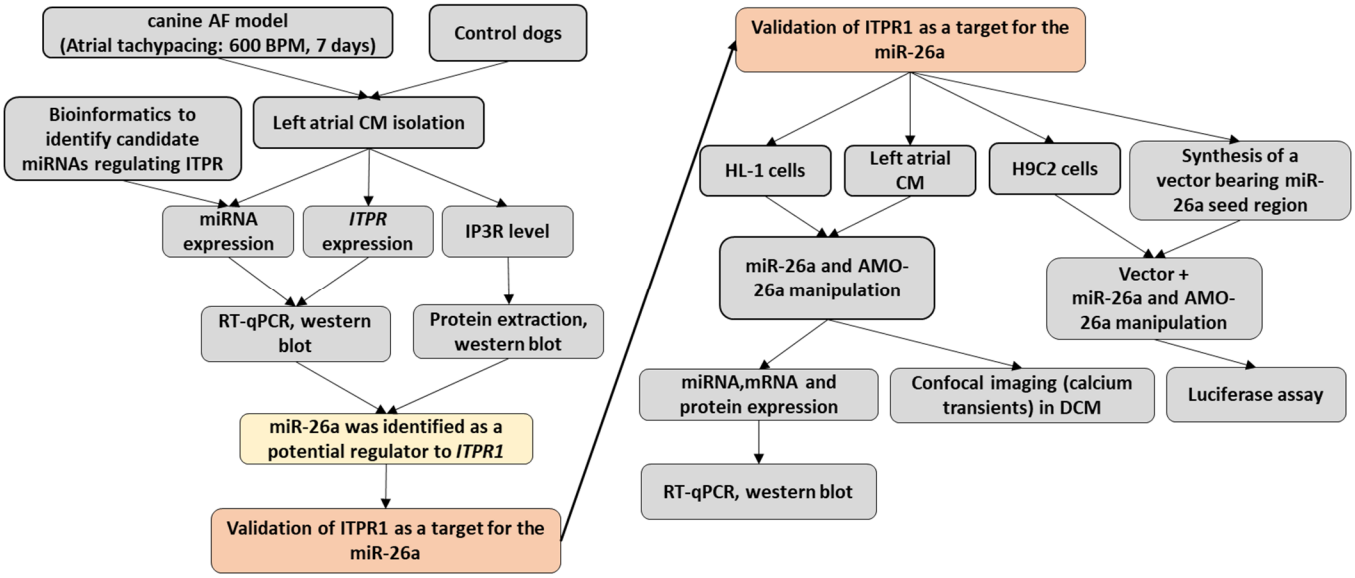

Figure 4: Schematic representation of the research framework.
AF: atrial fibrillation, DCM: dog cardiomyocytes.

Contribution of the Author

The AF-dog model for the present study is generated in the animal facility of the Montreal Heart Institute. Nathalie L'Heureux performed the surgeries on dogs, as described in Liu et al. publication (105). Xiaoyan Qi did all the work related to the left atrial cell isolation. I, together with Jiening Xiao, performed the construction of RNA/DNA sequences used in transfection studies. I determined the miRNAs targeting the *ITPR1* gene through the *in silico* studies. I cultured freshly isolated cardiomyocytes and H9C2 cells and performed the transfection and biochemistry and molecular biology experiments, including cloning, (micro)RNA and protein extraction, qRT-PCR, and western blot. I, together with Louis Villeneuve, performed the nucleoplasmic and cytoplasmic calcium transient experiment. I, together with Xiaoyan Qi and Feng Xiong, performed calcium transient analysis.

Chapter 2 – Methods

Canine atrial fibrillation model

Adult Mongrel dogs of either sex, weighing between 18 and 32 kg, were obtained from LAKA Inc (2016-47-01, 2019-47-03 for control dogs, 2015,47-01 for AF dogs) and assigned to atrial fibrillation (AF) or control (CTL) groups. Dogs used in this study were handled in accordance with the "Guide for the Care and Use of Laboratory Animals" established by the National Institutes of Health as approved by the Montreal Heart institute Ethics Committee. Animals were anesthetized with 0.07mg/kg acepromazine (IM), 5.3 mg/kg ketamine (IV), 0.25 mg/kg diazepam (IV), and then followed by 1.5% isoflurane and were ventilated. A bipolar pacing lead with fluoroscopic guidance was placed in the right atrial appendage via a jugular vein and connected to a subcutaneous pacemaker implanted in the neck (right side). Twenty-four to seventy-two hours after surgery, dogs in the AF group were subjected to atrial tachypacing at 600 bpm for seven days (Figure 5). In animals of the CTL group, no pacemaker was inserted (105).

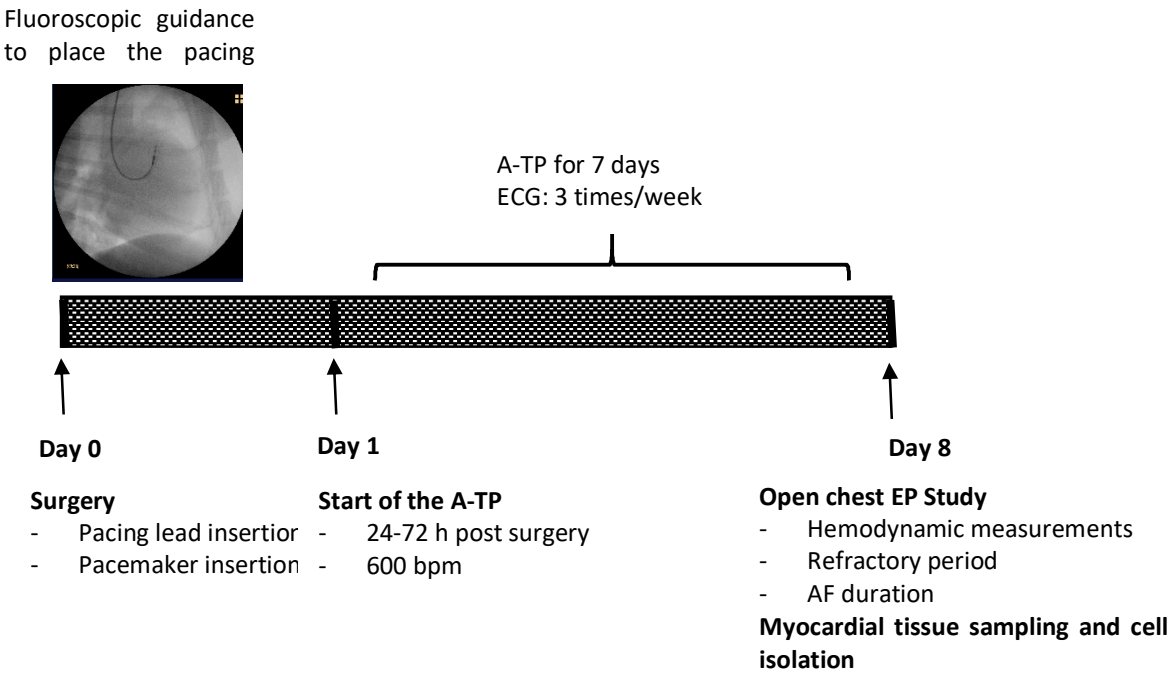

Figure 5: *In vivo* model of atrial fibrillation.
AF: atrial fibrillation, A-TP: atrial tachypacing; EP: electrophysiological.

Isolation of dog atrial cardiomyocytes

Cardiomyocytes were isolated from the left atrium (LA) with enzymatic digestion through the Langendorff system. Briefly, dogs were anesthetized with 2 mg/kg morphine (IV) and 120 mg/kg alpha-chloralose. Hearts were aseptically and quickly removed after intra-atrial injection of 10,000 U heparin and placed in Tyrode's solution containing 136 mM NaCl, 5.4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM dextrose, 5 mM HEPES, 0.33 NaH2PO4 (pH was adjusted to 7.3 with NaOH). The left coronary artery of the isolated heart was cannulated, and the LA was dissected free and perfused with 100% oxygenated Tyrode's solution (37°C, 1.8 mM Ca^{2+}). The arterial branches were ligated to have a leak-free system, and LA tissues were perfused with Ca^{2+}-free Tyrode's solution for 10 minutes, followed by 1-hour perfusion with 0.45 mg/mL collagenase (CLS II, Worthington) and 0.1% bovine serum albumin (MilliporeSigma) in Ca^{2+}-free Tyrode's solution for enzyme digestion. Digested tissue was removed from the cannula and cut into small pieces, and atrial cardiomyocytes were harvested (Figure 6) (105).

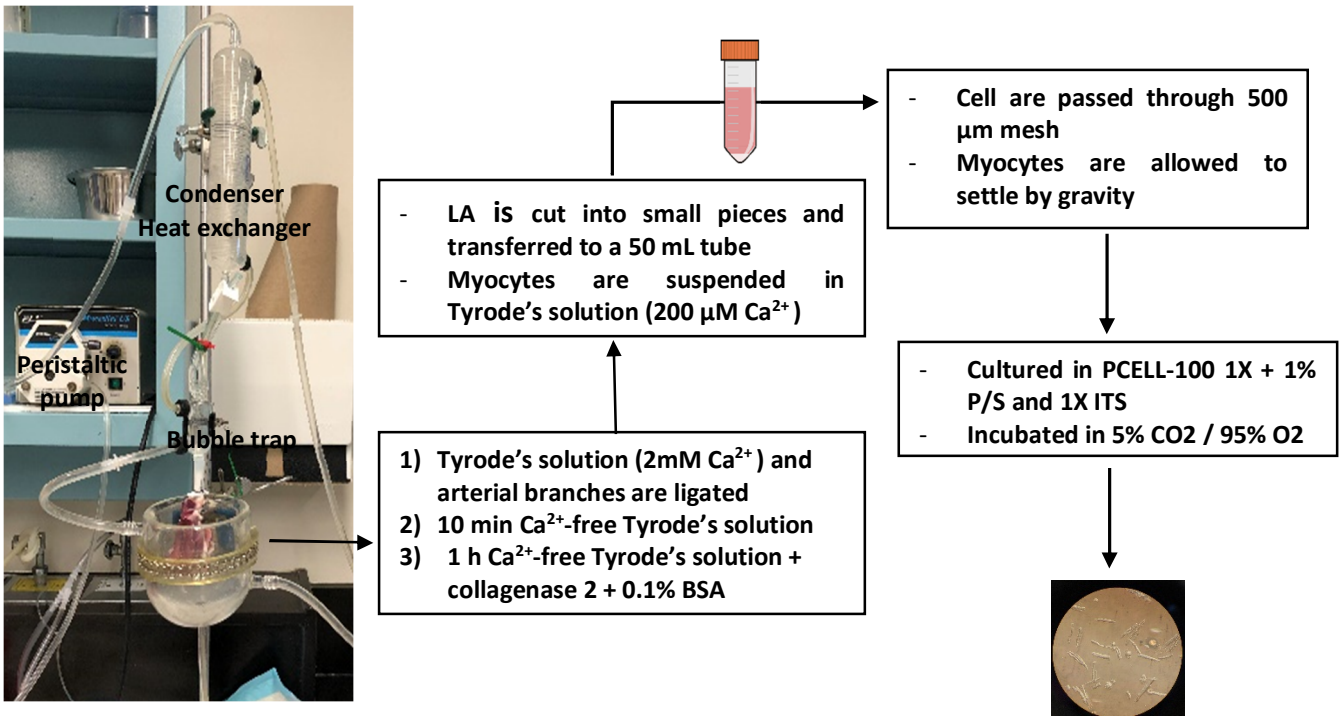

Figure 6: Left atrial cardiomyocyte isolation experimental setup and protocol
BSA: bovine serum albumin; ITS: insulin-transferrin-selenium-X.

Cell culture

The freshly isolated myocytes from the LA of control dogs were plated (~50% confluency) in 8-well plates in PCELL-100 1X (Multicell, USA) containing 1% penicillin/streptomycin (P/S) and 1X insulin-transferrin-selenium-X (Gibco, USA) and used for miRNA manipulation experiments. For calcium imaging, bovine serum albumin (Sigma-USA) was also added to the PCELL-100 1X medium. H9C2 cells were cultured in DMEM (Gibco, USA) that contained 1% P/S and 10% heat-inactivated fetal bovine serum (FBS) and seeded (3×10^4cells/well) in a 24-well plate for luciferase activity experiment. HL-1 cells were cultured in Claycomb media supplemented with 1% P/S, 10% FBS, 2-mM L-glutamine, and 100-µM norepinephrine and seeded (15-20 × 10^4cells/well) in a 6-well plate for miRNA manipulation experiments. Cells were seeded for all the cell lines when the cultures were 90% ± 5% confluent as assessed by microscopic observation. The H9C2 cell line was purchased from ATCC, USA, and the HL-1 cell line was obtained from Sigma-Aldrich, USA.

Synthesis and manipulation of miR-26a mimic and inhibitor

miR-26a was synthesized by Integrated DNA Technologies Inc., USA. Anti-miRNA oligonucleotide (AMO)-26a, which has 5 NT at both ends that are locked, was synthesized by Exiqon. The AMO-26a was a single-stranded DNA analog complementary to mature miR-26a. Sense and antisense strands of the miR-26 duplex and scrambled miRNA (miR-NC) duplex as negative control were diluted with RNase-free water to 200 µM and combined 1:1. The mixture was then heated for 5 minutes to 95°C and left overnight to cool slowly at room temperature. Freshly isolated myocytes cultured in the appropriate complete growth media were transfected with 100 nM miR-26a or miR-NC or 10 nM AMO-26a with Lipofectamine RNAiMAX (Invitrogen, USA) in reduced serum medium Opti-MEM (Gibco, USA). H9C2 cells were cultured in complete growth medium DMEM and transfected with 20 nM miR-26a or miR-NC, or AMO-26a (10 nM) + miR-26a (20 nM) along with 100 ng DNA vector using Lipofectamine 3000 (Invitrogen, USA). Samples were collected after 24 hours and kept at -80 °C for further studies.

Confirmation of miR-26a targeting site by luciferase assay

Based on the alignment of canine *ITPR1* 3'UTR and miR-26a with Clustal Omega, 100-bp DNA fragments (Table 1) containing miR-target site ("seed" region) were synthesized (Invitrogen). These inserts were amplified with forward and reverse primers containing an XhoI and XbaI restriction sites (Advantage-2 PCR Kit, Clontech) and then were digested with XhoI and XbaI (New England Biolabs) and then annealed and ligated into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega). The resulting DNA vector was transformed into the high-efficiency chemically competent DH5a (Thermo Fisher), diluted in SOC, and formed colonies were selected from ampicillin-containing plates, and the plasmids were purified with QIAprep Spin Miniprep Kit (Qiagen). The final product was sequenced at McGill University and Génome Québec Innovation Centre to confirm the generation of genomic sequences. Dual-Luciferase Reporter Assay System (Promega) was used to measure luciferase activity in H9C2 cultured in 24-well plates. Cells were lysed in 100 ul of 1× passive lysis buffer (Promega), and 10 µl of lysate was used to perform Luciferase reporter assays according to the manufacturer's protocol. Luciferase activity was normalized to Renilla luciferase internal control. Synergy 2 Multi-Mode Microplate Reader was used to measure the luminescence of a given sample (89).

Table 1 : List of RNA/DNA sequences (5'-sequence-3') used in luciferase activity assay and transfection studies.	
miR-NC_S	UCAUAAAGCUGAUAACCUCUAGAU
miR-NC_AS	CUAGAGGUUAUCAGCUUUAUGAAU
miR-26a_S	UUCAAGUAAUCCAGGAUAGGCU
miR-26a_AS	CCUAUUCUUGGUUACUUGCACG
miR-26b_S	UUCAAGUAAUUCAGGAUAGGU
miR-26b_AS	CCUGUUCUCCAUUACUUGGCUC
AMO-26a	AGCCTATCCTGGATTACTTGAA
*ITPR1*_T	CAAAAATATTAAAAATGCCTTTTTTGGAAGGACTAAAGAAAGCACCTGATTTGCACTT GAACCAGATTATAGATTTAAAAGTATATGACATGTATTTTGT
*ITPR1*_F	ACCGCTCGAGCGGCAAAAATATTAAAAATGC
*ITPR1*_R	GCTCTAGAGCACAAAATACATGTCATAT

Protein expression analysis

Protein-samples were extracted with a lysis buffer containing 0.15 M NaCl, 0.05 M Tris, 0.1% TritonX-100, 10% glycerol, 0.1% SDS, and protease inhibitor cocktails (Bimake, USA). Lysates were

centrifuged for 15 min at the highest speed (4 °C), and the supernatant was collected. Protein content was determined based on the Bradford dye-binding method using the Bio-Rad protein assay and bovine serum albumin as an internal standard. Minimum 25 µg of protein for each sample was combined with loading buffer and heated at 37 °C for 5 min. Protein samples were then separated at 120 V for ~60 min on 7.5% SDS-polyacrylamide gels (7.5% Mini-PROTEAN TGX Precast Protein Gels, Bio-Rad) and transferred electrophoretically to 0.45 µm pore size-Immobilon-P PVDF membranes 0.45-µm (Millipore) at 90 V, 4 °C for 70 min. Membranes were blocked with 5% w/v nonfat dry milk in Tris-buffered saline (TBS) containing 0.2% (v/v) Tween-20 (TBST) for 1 hour at room temperature and incubated with appropriate primary antibodies in TBST overnight at 4°C: 1:100 IP3R1 (73-035, NeuroMab), 1:1000 IP3R2 (ACC-116, Alomone), and 1:10000 GAPDH (10R-G109A, Fitzgerald). Membranes were washed with TBST and incubated with Donkey Anti-Mouse IgG, HRP-linked secondary antibody (715-035-151, Jackson ImmunoResearch Inc) for IP3R1 and GAPDH and Donkey Anti-Rabbit IgG, HRP-linked secondary antibody (715-035-152, Jackson ImmunoResearch Inc) for IP3R2 in TBST for 1 hour at room temperature. After washing with TBST, Protein bands were detected with enhanced chemiluminescence (PerkinElmer) or ultra-sensitive enhanced chemiluminescent (Thermo Fisher) and imaged with an X-ray Film Processor (model: 1186-3-4000, Ecomax), and quantified with Quantity One software (Bio-Rad). All Protein expression data were normalized to GAPDH.

Gene and miRNA expression analysis

Total RNA, including mRNA and miRNA, was purified with the miRNeasy Mini Kit (Qiagen), and RNA concentration was quantified using NanoDrop 2000 (Thermo Fisher). The primary cDNA was synthesized with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) using miRNA-specific TaqMan assay primers for miRNA, and High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using random primers for mRNA. Quantitative PCR was performed with TaqMan probes (Table 2) and TaqMan Universal PCR Master Mix (Applied Biosystems) on a StepOnePlus Real-Time PCR System for 40 cycles. RNA expression was measured in duplicate, and Fold change between RNA samples was calculated with the $2^{-\Delta Ct}$ method after normalizing to the internal control *HPRT1* for mRNA and U6 snRNA for miRNA.

Table 2 : List of TaqMan probes for RT-qPCR.			
Gene	Assay ID	Target species	Catalog number
HPRT1	cf02626258_m1	Dog	4331182
ITPR1	cf02675749_m1	Dog	4351372
ITPR2	cf02675776_m1	Dog	4351372
ITPR3	cf02630200_m1	Dog	4351372
ITPR1	Mm00439907_m1	Mouse	4331182
HPRT	Mm01545399_m1	Mouse	4331182
miR-26a	000405	Different	4427975
miR-26b	000406, 000407	Different	4427975
miR-92a	000431	Different	4427975
miR-130a	000454	Different	4427975
miR-130b	000456	Different	4427975
MiR-204	000508	Different	4427975
U6 snRNA	001973	Different	4427975

Confocal imaging of nucleoplasmic and cytoplasmic calcium transients

The freshly isolated myocytes cultured and transfected on laminin-coated 42-mm coverslips in Falcon® 60 mm TC-treated Cell Culture Dish. Twenty-four hours later, the medium was removed and coverslips mounted on a ZEISS LSM 5 LIVE confocal fluorescence microscope equipped with a 40x/1.3 N.A. Plan-Apochromat Oil Objective. Cells were incubated with 9 µM Fluo-4 AM (dissolved in Pluronic F-127, Invitrogen, USA) in Tyrode's solution containing 1.8 mM Ca^{2+} for 30 min at room temperature. After removing the Fluo-4 AM solution, cells were then perfused with Tyrode's solution (1.8 mM Ca^{2+}, 35 °C) for 10 minutes for de-esterification of the intracellular dye and paced at 1 Hz (sinus rate), 35 V, for an additional 5 min before imaging. Fluo-4 AM was excited by the 488-nm from an argon laser, and emissions were measured at >505 nm. Whole-cell imaging (512 x 256 pixels, pixel size = 0.31 um-X and 0.31 um-Y) of CaTs was performed every 10 ms during a 10-second window. Ca^{2+} transients are presented as the background-subtracted fluorescence intensity. The amplitude was calculated as $\Delta F = F - F0$, where F and F0 denote fluorescence intensity at peak and baseline CaTs, respectively (105).

Statistical analysis

Results are given as mean ± Standard error of the mean (SEM). Data analysis was performed in GraphPad Prism version 6.00. Multiple-group comparisons were carried out by one-way analysis

of variance (ANOVA) followed by Tukey's multiple comparisons test. Student's t-test was used to analyze two-group comparisons. P-value <0.05 was considered significant.

Chapter 3 – Results

ITPR1 shows higher expression in atrial cardiomyocytes

In this study, we first measured the expression levels of the three types of *ITPR* in cardiomyocytes isolated from the left atrium of control dogs. Results of the RT-qPCR demonstrated that *ITPR1* had the highest expression level, while type 3 was expressed to the least extent among the three isoforms (*ITPR1* > *ITPR2* > *ITPR3*, Figure 7). The expression levels of the ITPR2 and ITPR3 were 0.44 and 3 fold lower than that of *ITPR1*, respectively.

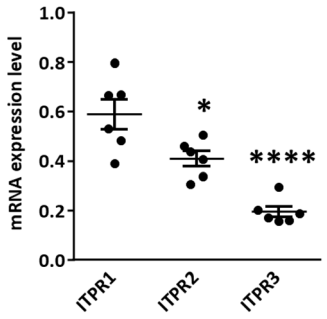

Figure 7: Differential expression of the 3 isoforms of *ITPR* in the left atrial cardiomyocytes of control dogs.

Data are given as mean ± SEM. Statistical analysis: ANOVA followed by Tukey's multiple comparisons test (*p<0.05, ****P<0.0001 vs. *ITPR1*). n=6 per group. *HPRT1* was used as the reference gene.

Upregulation of IP3R1 in atrial cardiomyocytes of the dog AF model

Adult cardiomyocytes were isolated from the left atrium of control and AF dogs, as described in detail in the methodology section and Figure 6. We investigated if IP3R protein expression levels could change in left atrial cardiomyocytes of our AF dog model. As shown in Figure 8, IP3R1 (2.47 fold change [FC] vs. control, P<0.05, Figure 8A) and IP3R2 (2.18 FC vs. control, P<0.05, Figure 8C) protein levels were significantly upregulated in AF cardiomyocytes. However, *ITPR1* (0.8 FC vs. control, P>0.05, Figure 8B) and ITPR2 (1.03 FC vs. control, P>0.05, Figure 8D) mRNA levels were unaltered in the AF group compared to the control group. Type 3 IP3R protein could not be detected in the cardiomyocyte samples, and this might be due to low expression or the probe

used to detect the respective antigen lacked specificity and/or affinity. The *ITPR3* expression level was similar in control and AF cardiomyocytes (1.09 FC vs. control, P>0.05, Figure 8E). Upregulation in IP3R protein levels within AF and control cardiomyocytes are shown in immunoblot images. These results point to the possibility of post-transcriptional regulation of IP3Rs by miRNAs.

Interesting results obtained in a recent study in our laboratory confirm that increased IP3R1-mediated Ca^{2+} release is involved in AF-induced atrial remodeling (Qi et al., Circ Res, in revision (46)). Nuclear IP3R1 level was upregulated in the left atrium of AF dogs. In isolated atrial cardiomyocytes, *ITPR1* knockdown decreased the nuclear diastolic [Ca^{2+}] and amplitude of electrically stimulated CaT. However, *ITPR2* knockdown did not significantly affect the CaT (Figure 16 in appendix). Therefore, in the current study, we investigated miRNA candidates contributing to the upregulation of IP3R1 during arrhythmogenic cardiomyocyte remodeling.

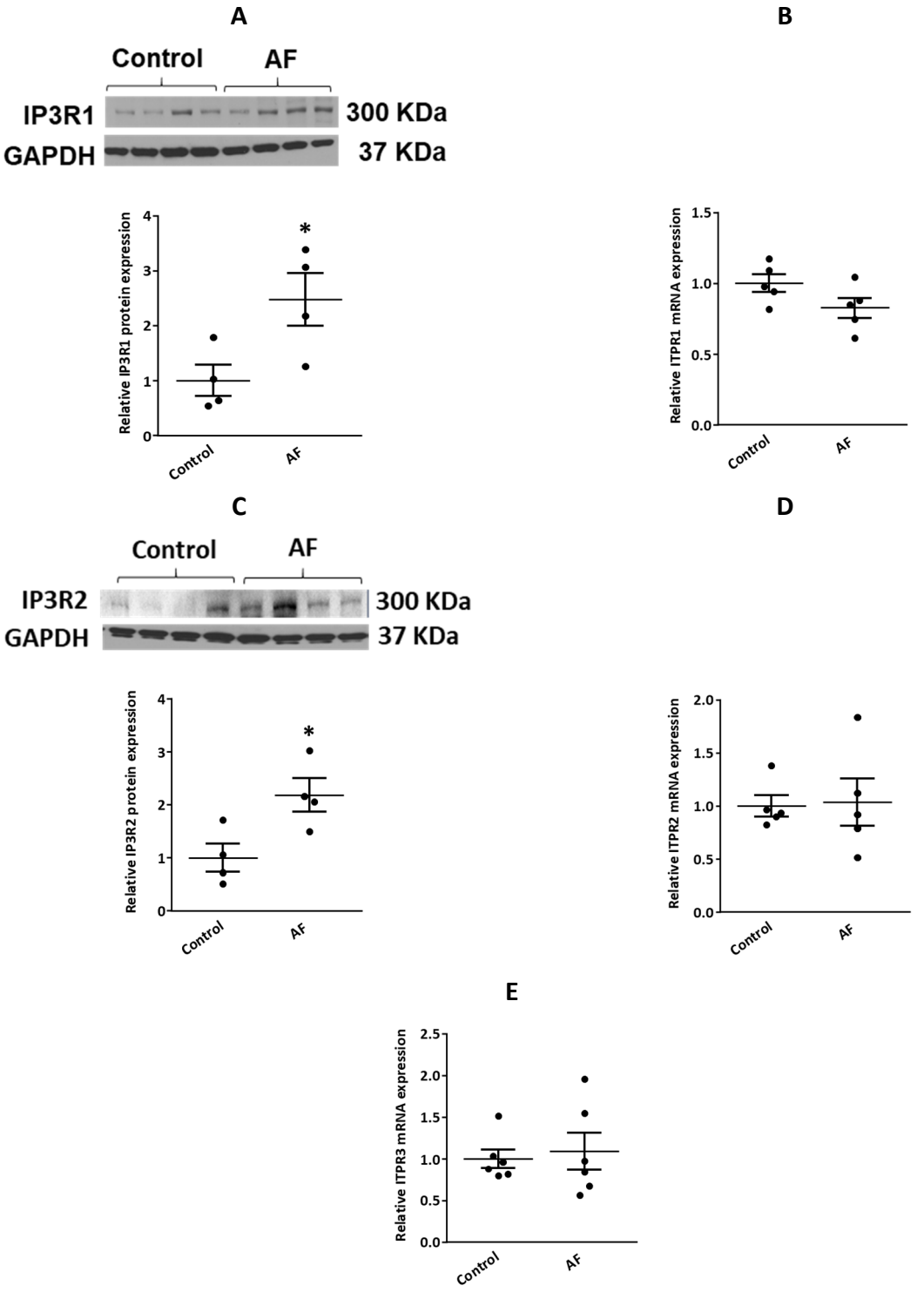

Figure 8: Upregulation of IP3R1/2 protein expression in the AF dog model.

(A) & (C): IP3R1/2 protein and (B) & (D) & (E): ITPR1/2/3 mRNA expression levels in freshly isolated cardiomyocytes from the left atrial tissue of the dog AF model. GAPDH was used as the loading control for western blot analysis, and HPRT1 was used as the reference gene for RT-qPCR analysis. Data are given as mean ± SEM relative to control. Statistical analysis: Unpaired

t-test (*p<0.05 vs. control group). n=4-6 per group. AF= atrial fibrillation; RT-qPCR= real-time-quantitative polymerase chain reaction.

Bioinformatic analysis of candidate miRNAs and selection of miR-26

We used three established miRNA target prediction algorithms MirTarget V3, PicTar, TargetScan7.2, and PubMed database for the bioinformatic analysis, and came up with many miRNAs predicted to target *ITPR1*. In order to narrow the list of the candidates, the following inclusion criteria were applied: target score>80, presence of conserved 8mer or 7mer site in 3'UTR of the *ITPR1* mRNA that matches the miRNA "seed" region, and miRNAs were predicted by 2 of the 3 algorithms. Out of the several candidates, few miRNAs were selected based on the strength of their expression in human LA. For this purpose, we used a database of miRNA expression in normal human tissues (106). Then we measured expression levels of the selected miRNAs in the left atrial cardiomyocytes of our control and AF dogs (TaqMan® probe was not available for cfa-miR-92b). Finally, we looked for the miRNAs that were downregulated in the AF dog cardiomyocytes, and its downregulation would cause upregulation in the IP3R1 protein level. Out of that analysis, we found only one miRNA, which was miR-26 (Figure 9).

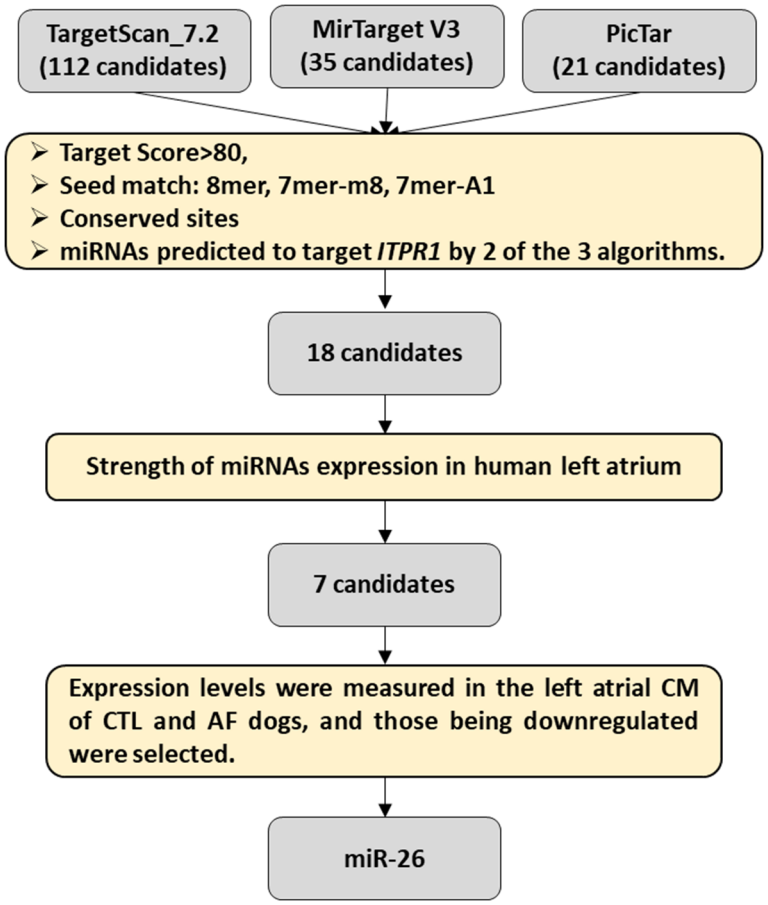

Figure 9: Schematic of selection criteria for candidate miRNAs predicted to target *ITPR1*.
AF: atrial fibrillation; CM: cardiomyocyte; CTL: control.

Figure 10 shows the expression level of the candidate miRNAs, including miR-26a/b, miR-92a, miR-130a/b, and miR-204, which were predicted to target ITPR1, in the left atrial myocytes of our control and atrial-tachypaced dog samples. Among these miRNAs, only miR-26a/b were significantly differentially expressed between control and AF samples (Figure 10). Therefore, we selected miR-26 as the primary candidate for *ITPR1* regulation. After this screening, we manipulated HL-1 cells with miR-26a/b and found similar results in western blot and RT-qPCR for both miRNAs (Figure 12). As miR-26a is significantly and highly expressed in the heart (~2 times higher than miR-26b) and shares the same seed sequence with miR-26b -suggesting the same target for these two miRNAs-, we continued our experiments in primary cell culture and luciferase activity with miR-26a.

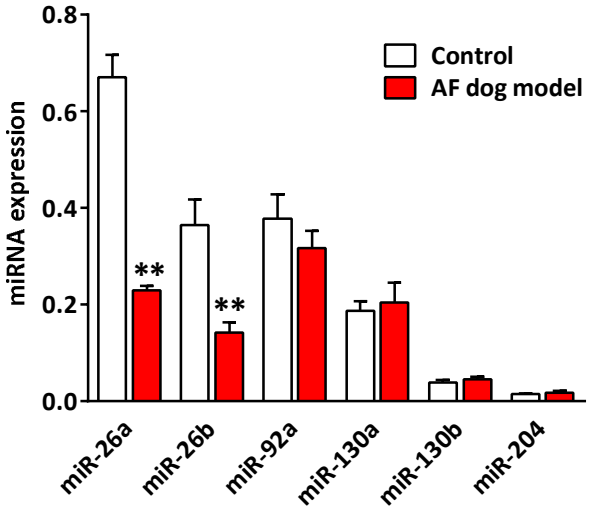

Figure 10: Expression of various miRNAs predicted to target ITPR1 in AF and control atrial cardiomyocytes.

miR-92b was also a candidate miRNA for *ITPR1*, but the TaqMan probe specific for the canine miR-92b sequence was not available to test its level. Data are given as mean ± SEM. U6 snRNA was used as the reference gene for RT-qPCR. n=5 per group. Statistical analysis: Student's t-test (**p<0.01 vs. control group). AF=atrial fibrillation; RT-qPCR=real-time- quantitative polymerase chain reaction.

Validation of miR-26a as a potential regulator of *ITPR1*

Based on in silico analysis of miRNA-binding sites to the 3' UTR of the mRNA, miR-26a was predicted to target *ITPR1*. TargetScan7 identified a 7mer-A1 site on the position 267-273 of *ITPR1* 3'-UTR in the dog that matches the seed region of miR-26a, and this binding site is conserved among different species (Figure 10B). Figure 11A shows the energetically best hit between the miR-26a and the 3'UTR of the *Canis lupus familiaris ITPR1*, transcript variant X10, predicted by RNA-hybrid. As presented in Figure 10, the miR-26a expression was significantly downregulated for approximately 66% in AF (0.34 FC vs. control, P<0.01). To better determine whether IP3R1 expression is post-translationally regulated with miR-26a, we performed a dual-luciferase reporter assay and RT-qPCR and western blot analysis.

A

cfa-miR-26a sequence
5’-UUCAAGUAAUCCAGGAUAGGCU-3’
3’UTR of *ITPR1*

Position 252: mfe: -20.2 kcal/mol

```
5' A    A              UGC         C 3'
    AGC     CCU  GAUU    ACUUGAA
    UCG     GGA  CUAA    UGAACUU
3'      GAUA    C                  5'
```

Cfa-miR-26a

B

```
          .250.......260.......270......
Dog       AGAAAGCACCUGAUUUGCACUUG-AACCAG
Human     AGAAAGCACCUGAUUUGCACUUG-AACCAG
Mouse     AGAAAGUACCUGAUUUGCACUUG-AACCAG
Rat       AGAAAGUACCUGAUUUGCACUUG-AACCAG
```

Figure 11: miR-26a is a potential candidate predicted to target *ITPR1.*
(A): Alignment of the sequences of miR-26a (lowe sequence) with its target site in the 3’-UTRs of dog *ITPR1* mRNA (upper sequence). RNAhybrid 2.2 was used to find the minimum free energy of hybridization of 3’UTR and miR-26a. (B): Conserved miRNA binding site within 3’-UTR of *ITPR1* mRNA among different species, predicted by TargetScan 7.2.

miRNA target identification is typically achieved by transfecting the cells using miRNA mimics to destabilize the mRNA or inhibit its translation or by anti-miRNA oligonucleotide to knockdown the endogenous miRNAs. The results of the miRNA manipulation experiments in the HL-1 cardiomyocyte cell line are shown in Figure 12. HL-1 cells are derived from the mouse atrial cardiomyocyte tumor lineage AT-1. This cardiac muscle cell can contract and maintain the characteristics of adult cardiac myocyte even after multiple passages (107). We transfected the cells with 100 nm miR-26a/b or miR-NC and confirmed the transfection efficacy by RT-qPCR measuring the miR-26a/b levels (Figure 12D&E). miR-26a/b–overexpression in HL-1 cells significantly decreased *ITPR1* mRNA expression (-27% for miR-26a and -22% for miR-26b vs. miR-NC, $p<0.05$, Figure 12A&B) and IP3R1 protein level (+48% for miR-26a and +61% for miR-26b vs. miR-NC, $P<0.01$, Figure 12C) when compared with control myocytes received miR-NC. These results showed an inverse relationship between the level of miR-26 expression and IP3R1 protein level in HL-1 cells.

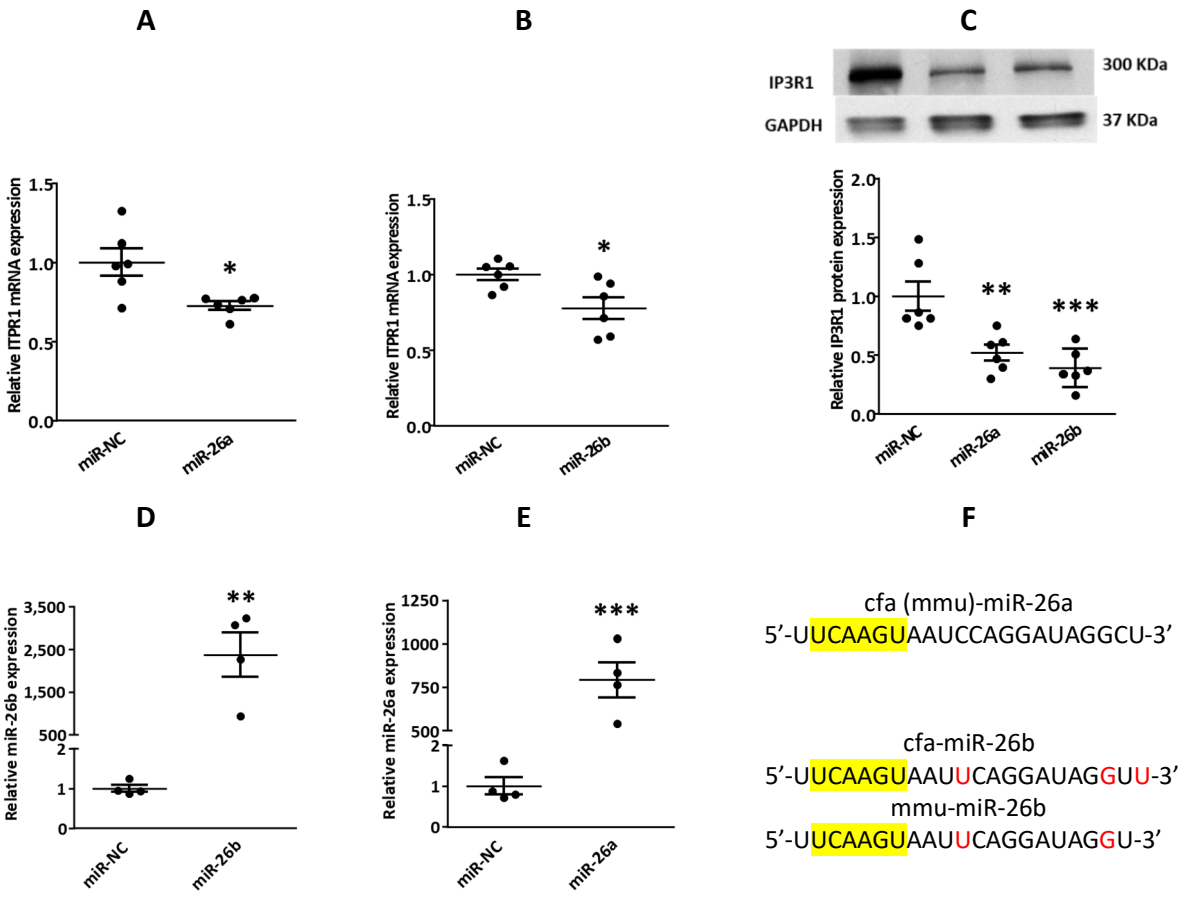

Figure 12: Confirmation of the regulation of IP3R1 expression by miR-26 in the HL-1 cell line. (A-B) *ITPR1* gene expression and (D-E): Changes in miR-26a/b expression was verified by RT-qPCR. (C): IP3R1 protein expression was verified by western blot. (F): miR-26a/b sequence (seed sequence is highlighted in yellow, and 3 (in mice 2) nucleotides difference between miR-26a and miR-26b in the dog are marked in red. GAPDH was used as the loading control for western blot, and *HPRT* (for mRNA) and U6 snRNA (for miRNA) were used as the reference genes for RT-qPCR. Data are given as mean ± SEM relative to miR-NC. Statistical analysis: Student's t-test for gene expression comparisons and ANOVA followed by Tukey's multiple comparisons test for protein expression comparisons (*p<0.05, **p<0.01, ***p<0.001 vs. miR-NC), n=4-6 per group. RT-qPCR= real-time- quantitative polymerase chain reaction; miR-NC=non-coding miRNA.

Primary cell cultures are preferable cellular models for *in vitro* studies and novel drug developments. Therefore, we assessed how miR-26a transfection affects the IP3R1 expression in primary adult canine atrial cardiomyocytes.

As shown in Figure 13A, transfection of miR-26a (100 nM) into the cardiomyocytes isolated from the left atrial tissue of the control dog significantly decreased IP3R1 protein expression (-40%, vs. miR-NC, P<0.01) compared to the miR-NC-treated cells. Knocking-down the endogenous miR-26a with antisense inhibitor AMO-26a (10 nM) to mimic the effect of AF on miR-26a level in cardiomyocytes resulted in the upregulation of IP3R1 protein (+35% vs. miR-NC, p<0.05, Figure 13A). We found no significant effects of miR-26a on the *ITPR1* mRNA expression (Figure 13B), reflecting the translational repression of the mRNA. This result is consistent with the observation that *ITPR1* expression is unaltered in cardiomyocytes of AF samples. The regulatory effect of miR-26a on *ITPR1* mRNA through binding to the 3'-UTR was confirmed by luciferase reporter assay (Figure 13D). The co-transfection of miR-26a or AMO-26a with the pmirGLO-*ITPR1* vector into H9C2 cells indicated that luciferase activity was significantly decreased by miR-26a (-22% vs. miR-NC, p<0.05) whereas increased by AMO-26a (+25% vs. miR-NC, p<0.01). The transfection efficacy of miR-26a and AMO-26a transfection in altering miR-26a expression was verified by RT-qPCR and presented in Figure 13C.

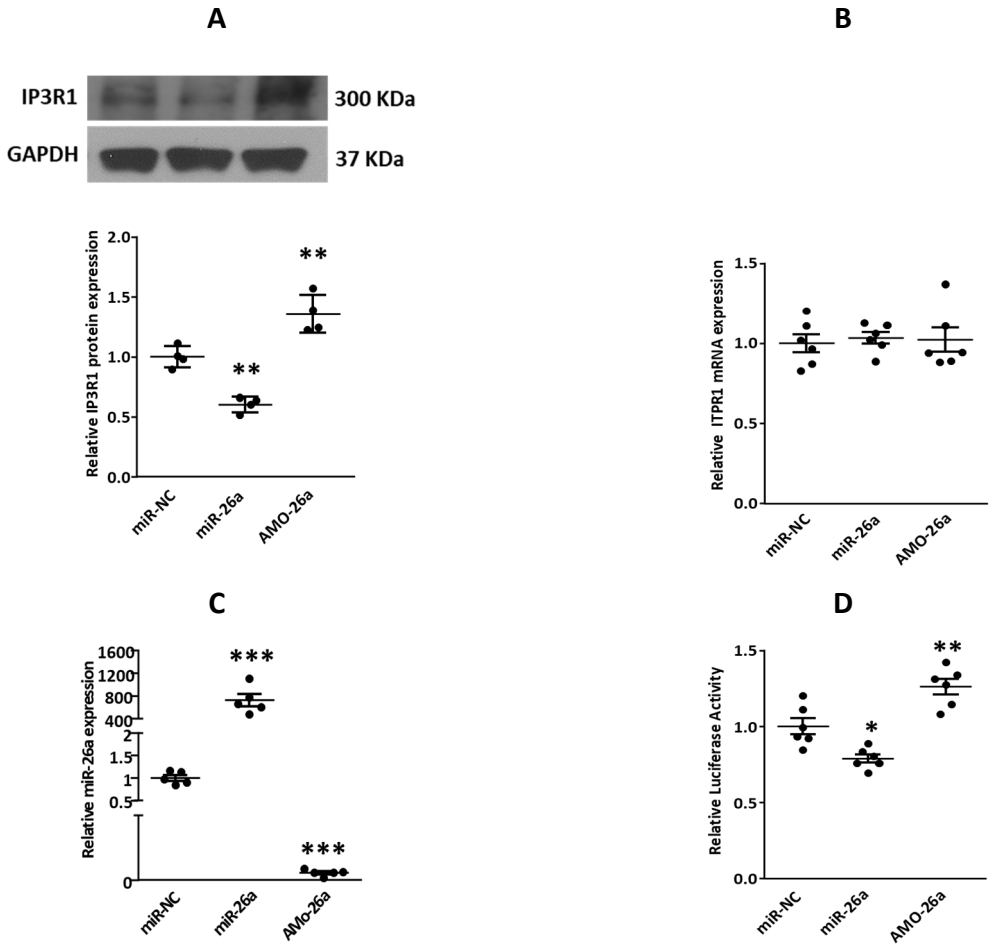

Figure 13: Confirmation of regulation of IP3R1 expression after transfection with miR-26a and AMO-26a in atrial cardiomyocytes.

(A): IP3R1 protein expression was verified by western blot. (B)-(C) *ITPR*1 gene expression and changes in miR-26a expression were verified by RT-qPCR. (D): Luciferase activity in H9C2 cells. GAPDH was used as the loading control for western blot, and HPRT1 (for mRNA) and U6 snRNA (for miRNA) were used as reference genes for RT-qPCR. Data are given as mean ± SEM relative to miR-NC. Statistical analysis: ANOVA followed by Tukey's multiple comparisons test (*p<0.05, **p<0.01, ***p<0.001 vs. miR-NC), n=4-6 per group. AF= atrial fibrillation, RT-qPCR= real-time-quantitative polymerase chain reaction; miR-NC=non-coding miRNA; AMO=anti-miRNA oligonucleotide.

miR-26a and Calcium transients

We investigated how miR-26a impacts CaTs in cardiomyocytes using cultured cardiomyocytes isolated from the left atrium of control dogs. For this purpose, we evaluated changes in CaTs in

cardiomyocytes transfected with miR-26a (100 nM) or with antisense inhibitor AMO-26a (10 nM). Figure 14A shows the representative recordings of electrically stimulated nucleoplasmic and cytoplasmic CaTs obtained with Fluo-4 AM imaging from each group, 24 h post-transfection. The results in Figure 14B show that nucleoplasmic diastolic Ca^{2+} level was substantially decreased in the cells treated with miR-26a ($p<0.01$, vs. miR-NC) as reflected by lower fluorescence intensity, while significantly enhanced with downregulation of the endogenous miR-26a with its inhibitor AMO-26a ($p<0.001$, vs. miR-NC). Diastolic Ca^{2+} level in the cytoplasmic compartment experienced similar changes after the application of miR-26a mimic or inhibitor ($p<0.05$, vs. miR-NC). Time to peak, amplitude, and time to 50% baseline were not altered by miR-26a overexpression or downregulation in both nucleus and cytosol (Figure 14C-E).

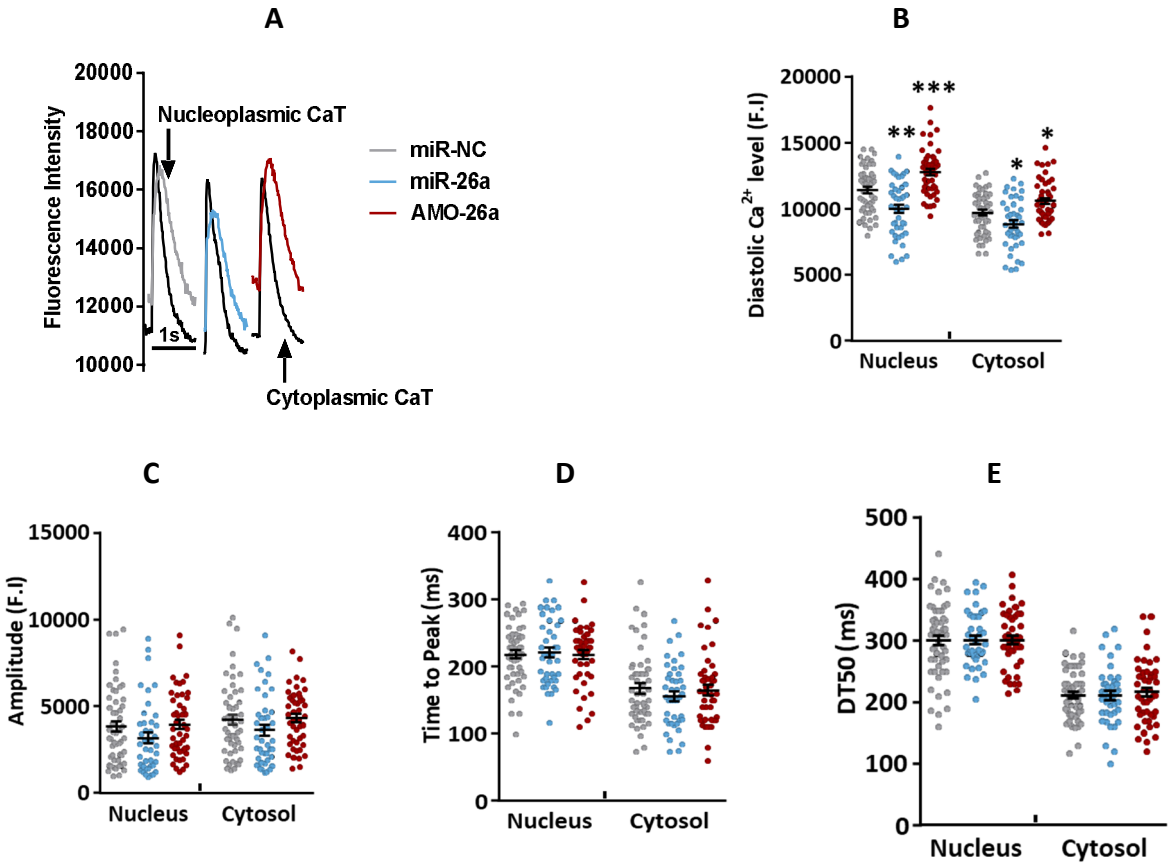

Figure 14: Effect of miR-26a overexpression and knockdown on Ca^{2+} transients (CaTs) in atrial cardiomyocytes.

(A): Representative Ca^{2+} transients, (B): Diastolic Ca^{2+} level, (C): Amplitude, (D): Time to peak, and (E): Time to 50% baseline (DT_{50}) of the nucleoplasmic and cytosolic CaTs in isolated cardiomyocytes from the left atrial tissue of the control dog subjected to miR-26a overexpression and knockdown (AMO-26a). Data are shown as mean ± SEM. Statistical analysis: ANOVA followed by Tukey's multiple comparisons test (*p<0.05, **p<0.01, ***p<0.001 vs. miR-NC). n/N=42-53/8. AF=atrial fibrillation, miR-NC=non-coding miRNA; AMO= anti-miRNA oligonucleotide.

Chapter 4 – Discussion

In this study, we have demonstrated that miR-26a is a potentially important regulator of IP3R1 upregulated in canine atrial cardiomyocytes after seven days of tachypacing. Among the miRNAs predicted to target IP3R1, the miR-26 family decreased significantly in the canine AF model. We showed that *in vitro* miR-26a knockdown reproduced AF-induced IP3R1 upregulation and increased diastolic Ca^{2+} levels while miR-26a overexpression reversed these effects. Therefore, miR-26a dysregulation of IP3R1 expression and Ca^{2+} handling is likely to be implicated in atrial arrhythmogenesis associated with AF.

Possible causative mechanism of IP3R upregulation in AF

In clinical practice, AF is the most common sustained arrhythmia associated with profound cardiovascular morbidity and mortality. AF-related remodeling has been implicated in the progressive nature of the arrhythmia and the complexity of treatment. Increased intracellular Ca^{2+} levels play a central role in the activation of cell signaling mediating profibrillatory atrial remodeling (8). Electrical stimulation, neurotransmitters, and hormones generate signals that trigger Ca^{2+} release from intracellular stores through intracellular Ca^{2+} release channels such as RyRs and IP3Rs (39, 54).

Our results indicated that three types of IP3R are expressed in canine atrial myocardial cells, and expression of the *ITPR1* gene encoding IP3R1 protein is more abundant than other isoforms (Figure 7). IP3R1 is also the dominant isoform in human atrial myocytes (41). However, in most animal species, IP3R2 is the predominant isoform in ventricular and atrial myocytes (42).

Additionally, our findings showed upregulation of type 1 and 2 IP3Rs in the left atrial cardiomyocyte of AF dogs. In the heart, IP3Rs expression is less abundant than RYRs; however, their expression levels increase in the diseased heart, such as in AF and cardiac hypertrophy (39). Increased expression of IP3R1 mRNA and protein is also observed in right atrial tissue samples of patients with chronic AF (37), and the IP3R1 mRNA levels correlate with the AF duration (62).

For the mechanism of IP3Rs dysregulation in arrhythmia, we searched the PubMed database with the following search terms ((ip3r"[All Fields]) AND (("arrhythmia"[All Fields]) OR ("atrial fibrillation"[All Fields])) AND ("dysregulation"[All Fields]) AND ("mechanism"[All Fields])) and Web of Science with the following search terms ALL=(IP3R AND dysregulation AND mechanism AND (atrial fibrillation OR arrhythmia)) and no publication was found regarding this subject. We also searched the same databases using the mentioned search terms without “atrial fibrillation” or “arrhythmia”. We found 9 articles in PubMed and 19 articles in Web of Science, but none of them discussed the modulation of IP3R1 at both transcriptional and post-transcriptional levels in other diseases or conditions.

Therefore, in the current study, we explored the mechanism of IP3R dysregulation in AF at the post-transcriptional level. For this purpose, we used an AF dog model and the isolated cardiomyocytes from healthy dogs. Despite the differences in physiology and complexity of human AF, the animal models enhance our understanding of this disease. Canine models have been widely used in the study of atrial electrophysiology and structural and electrical remodeling in AF. Spontaneous AF occurs naturally in dogs; however, when they are used in electrophysiology studies, AF is commonly established with atrial tachypacing (108).

Different AF models have been generated in dogs. AF-inducibility and duration, atrial fibrosis, and collagen-gene expression have been shown to increase in a model of rapid atrial pacing for three weeks (109). In another model, pacing right-atria at 600-bpm for one-week results in the upregulation of TRPC3 expression in fibroblasts and increases extracellular matrix gene expression (27). In a very recent study, our team has extensively investigated the role of IP3R1 in the electrical remodeling of the atrium in one-week atrial tachypacing model. In this model, IP3R1 expression is upregulated in cytosolic and nucleoplasmic fractions of atrial cardiomyocytes producing increased nuclear and cytoplasmic Ca^{2+} loading (Figure 3, Figure 16). These rapid pacing-related changes mirror the electrical remodeling observed in AF human atrial tissue.

miRNA dysregulation in AF and contribution to IP3R upregulation

miRNAs are a class of small genome-encoded RNAs regulating gene expression in eukaryotes referred to as RNA silencing. miRNAs recognize the mRNA targets with the “seed” region

(nucleotides 2–8 in the 5' region), which is not always a necessity for miRNAs function, and so post-transcriptionally regulates mRNA stability and translation. However, the precise mechanisms underlying the interaction between miRNA and their mRNA targets are complex and poorly understood. In the case of perfect base complementarity, mostly found in plants, miRNAs trigger mRNA cleavage and degradation. The imperfect complementary, which is dominant in vertebrates, results in repression of the translation (72, 73).

We performed a computational analysis of miRNAs suggesting that miR-26 has the potential to regulate *ITPR1* mRNA. miR-26 belongs to a cardiac-enriched miRNA family composed of 3 subtypes miR-26a-1, miR-26a-2, and miR-26b. The mature form of miR-26a-1 and miR-26a-2 has the same sequence. Both miR-26a and miR-26b have identical seed sequences and differ in two nucleotides suggesting that they target the same set of genes (85). The 3’ UTR of *ITPR1* mRNA contains a binding site for miR-26a, and consequently, we hypothesized that miR-26a contributes to AF pathophysiology by regulating *ITPR1* expression.

In our study, we considered post-transcriptional modification to explain the IP3R1 upregulation in AF cardiomyocytes. We identified downregulated miR-26 in AF atrial cardiomyocytes (Figure 10) as a potential regulator to IP3R1 upregulation. This regulatory effect was verified in the HL-1 cell line and primary culture of canine atrial cardiomyocytes. Luciferase analysis revealed that miR-26a binds to the 3’-UTR of ITPR1. miR-26a knockdown in isolated cardiomyocytes, to mimic the AF-induced miR-26a downregulation, increased the IP3R1 protein level, which was reversed by miR-26a overexpression (Figure 13A). Consistent with the observation in AF cardiomyocytes (Figure 8B), *ITPR1* mRNA expression was unaltered after miR-26a overexpression and knockdown (Figure 13B), reflecting miRNA-mediated translational repression. In the HL-1 cell line, miR-26a/b transfection decreased the expression of *ITPR1* and, to a greater extent, the IP3R1 protein level, possibly through mRNA degradation and translational repression.

The previous study of potential roles of miR-26 in AF suggests that miR-26 targets KCNJ2 gene encoding Kir2.1 (I_{K1}). The miR-26 expression is decreased in atrial samples of canine persistent AF model and AF patients, and I_{K1} is upregulated. Adenovirus-mediated expression of miR-26a reduces AF vulnerability in mice, and the opposite effect is achieved upon knockdown of

endogenous miR-26a. Increased NFAT activity in AF suppresses the inhibitory effect of miR-26a on KCNJ2/Kir2.1, creating a substrate for atrial remodeling and promoting AF (85). miR-26a is also downregulated in left atrial fibroblasts of CHF dogs and contributes to fibroblast I_{K1} enhancement. Larger I_{K1} is observed after knockdown of miR-26a in cultured atrial fibroblasts (110).

miR-26 also plays a substantial role in atrial structural remodeling by targeting the gene encoding TRPC3 upregulated in atrial fibroblasts of AF dogs and atrium of AF patients. Experimental miR-26a knockdown in cultured canine left atrial fibroblasts increases TRPC3 expression and fibroblast-proliferation indexes. NFAT translocation into the nucleus is also increased in left atrial fibroblasts of AF dogs, suppressing miR-26 transcription (27).

miRNAs post-transcriptionally regulate gene expression in almost all physiological processes to control development and homeostasis. Under disease conditions, altered miRNA expression has been investigated as biomarkers and novel therapeutic targets (69, 111). Many prior studies suggested that miRNA dysregulation plays a pathophysiological role in AF (Table 3 summarizes these miRNAs and their regulatory effects). None of these mechanisms works through the dysregulation of nuclear $Ca^{2+,}$ as was observed in the current study.

The following search terms were used in PubMed database (76 results) to find the miRNA presented in Table 3 : ((("miRNA"[Title/Abstract] OR "microRNA"[Title/Abstract] OR "miR"[Title/Abstract]) AND ("arrhythm*"[Title/Abstract] OR "atrial fibrillation"[Title/Abstract]) AND ("target*"[Title/Abstract]) AND ("atrial"[Title/Abstract] OR "atrium"[Title/Abstract])) NOT ("biomarker*"[Title/Abstract])) NOT ("review"[Title/Abstract]). Studies with poor quality, those published in languages other than English or in which the effect of a miRNA on its target was not directly evaluated were excluded. I could not discuss all of the miRNAs here in detail so I only discussed the ones that I think were the most important or relevant.

Table 3 : List of miRNAs that their roles have been described in AF					
miRNA	**Changes in AF**	**AF model**	**Main target gene**	**Function This miRNA**	**Reference**
miR-210	Up	Right atrial tissue and serum from AF patients	Foxp3	suppresses regulatory T cell function and promotes atrial fibrosis	(112)
miR-96	Up	Atrial tissues from AF patients	KLF13	promotes Ang-II-induced proliferation, migration, and collagen production	(113)
miR-320d	Down	Cardiomyocytes from a C57BL/6 mice AF model	STAT3	promotes apoptosis in cardiomyocytes	(114)
miR-221/222	UP	Cardiomyocytes from EGFR KO mice* with extensive hypertrophy	CACNA1C and KCNJ5	reduces the I_{CaL} current reduces ion flux through GIRK1/4	(115)
miR-27b-3p	Down	Atrium from $CaCl_2$-induced AF Sprague-Dawley rat	Wnt3a	Promotes atrial fibrosis and occurrence of AF	(116)
miR-23	Up	Right atrial appendage tissues from AF patients	TGF-β1	Is positively correlated with TGF-β1 expression and promotes the proliferation of cardiomyocytes	(117)
miR-133a/b	Down	ZFHX3-KD HL-1 mouse atrial myocytes	Different targets such as DVL2, CaMKII, JNK, CX43, and FGFR1	plays an essential role in controlling cardiac calcium homeostasis and cardiac remodeling	(118)
miR-23b-3p and miR-27b-3p	Up	Right atrial appendages from AF patients	TGF-βR3	promotes atrial fibrosis	(119)
miR-206	Up	Hearts from a transgenic CD-1 mice model with inducible miR-206 overexpression	Cx43	induces abnormal heart-rate and PR interval	(120)
miR-155-5p and miR-24-3p	Up	Serum from AF patients and myocardial tissue from a swine AF model	eNOS	is involved in the pathogenesis of AF by regulating the expression of eNOS and the production of NO	(121)
miR-125a	UP	Samples from patients with late recurrence of AF after catheter ablation**	IL-6R	promotes the recurrence of AF	(122)
miR-138-5p	Down	Right atrial appendage tissue from AF patients	CYP11B2	inhibits cell proliferation	(123)
miR-27b	Down	Left atrium from a C57BL/6 mice AF model	ALK5	decreases AngII-induced atrial fibrosis and increases AF incidence, AF duration, and interatrial conduction time	(124)
miR-34a	Up	Right atrial tissues from AF patients	Ank-B	enhances Ca^{2+} signaling	(125)

miR-30c	Down	Atrium from a Sprague-Dawley rat model of atrial fibrosis induced by pressure overload via AAC	TGF-βRII	reduces atrial fibrosis	(126)
miR-208a-3p	Up	Right atrial appendage tissues from AF patients	Cx40	involved in Cx40 remolding in AF.	(127)
miR-132	Down	Right atrial tissue from AF patients and left atrium from a dog AF model	CTGF	modulates fibrosis	(128)
miR-328	Up	Right atrium from a New Zealand rabbit AF model	CACNA1C	modulates electrical remodeling	(129)
miR-21	Up	Atrial tissue and fibroblasts from AF patients	WWP-1	increases fibroblast proliferation	(130)
miR-21	Up	Left atrial appendages from AF patients and heart tissue specimens from a Sprague-Dawley rat AF model	CADM1	Increases fibroblast proliferation	(131)
miR-499	Up	Right atrial appendages from AF patients	CACNB2	contributes to the electrical remodeling	(132)
miR-29a-3p	Up	Tip of the right atrial appendages from AF patients	CACNA1C	reduces I_{CaL}	(88)
miR-31	Up	Right atrial appendages from AF patients and left atrial tissues of a goat AF model	Dystrophin and nNOS	Shortens APD and abolishes rate-dependent adaptation of the APD	(133)
miR-30d	Up	Right atrial appendages from AF patients	KCNJ3	reduces I_{KACh}	(134)
miR-21	Up	Left atrium from a New Zealand rabbit AF model	Smad7	increases fibrosis	(135)
miR-30a	Down	Left atrium from a New Zealand rabbit AF model	snail 1	modulates fibrosis	(136)
miR-192-5p	Up	Left atrial appendage tissues from AF patients	SCN5A	reduces I_{Na}	(137)
miR-206	Up	Left superior fat pad from a dog AF model	SOD1	shortens the atrial effective refractory period increases reactive oxygen species	(138)
miR-146b-5p	Up	Left atrial appendages from patients with nonvalvular paroxysmal AF	TIMP-4	modulates fibrosis Increases collagen content	(139)
miR-106b-25 Cluster***	Down	Right atrial appendages of AF patients	RyR2	increases total SR Ca^{2+}-leak, Ca^{2+}-spark frequency, atrial ectopy, and AF susceptibility	(140)
miR-21	Up	Atrial myocytes isolated from right atrial appendages of AF patients	CACNA1C, CACNB2	reduces I_{CaL}	(141)

miR-17-92 and miR-106b-25 clusters	Down	Hearts from a Pitx2$^{null/null}$ mutant CD-1 mice model	Shox2, Tbx3	regulates sinoatrial node development	(142)
miR-130a	Up	Hearts from a miR-130a overexpression CD-1 mice model	Cx43	contributes to gap junction remodeling	(143)
miR-1	Up	Right atrial cardiomyocytes from a New Zealand rabbit AF model	KCNE1, KCNB2	reduces atrial effective refractory period, prolongs the duration of AF, enhances I_{Ks}	(144)
miR-499	Up	Right atrial appendage tissues from AF patients	KCNN3	contributes to the electrical remodeling	(145)
miR-21	Up	Left atrial tissue from a Wistar rat MI model	Sprouty-1****	promotes atrial fibrosis	(146)
MiR-328	Up	Atrial samples from AF patients and a dog AF model	CACNA1C, CACNB1	enhances AF vulnerability, decreases I_{CaL}, shortens atrial APD.	(147)
miR-133, miR-590	Down	Right atrial appendage tissues from AF patients	TGF-β1, TGF-βRII	modulates collagen content and fibrosis	(94)

AAC: abdominal aortic constriction; AF: atrial fibrillation; ALK5: type I receptor/activin like kinase 5; AngII: angiotensin II; Ank-B: Ankyrin-B; APD: action potential duration; SCN5A: sodium voltage-gated channel alpha subunit 5; CACNA1C: calcium voltage-gated channel subunit alpha1 C; CACNB2: calcium voltage-gated channel auxiliary subunit beta 2; CADM1: cell adhesion molecule 1; CTGF: connective tissue growth factor; Cx: connexin; eNOS: endothelial nitric oxide synthase; GIRK1/4: G-protein-activated inwardly-rectifying potassium channel; KCNJ3: potassium inwardly-rectifying channel subfamily J member 3: I_{CaL}: L-type Ca^{2+} current; I_{KACh}: acetylcholine-regulated K^+ current; I_{Na}: sodium current; IL: interleukin; IL-6R: IL-6 receptor; Iks: slowly activating delayed rectifier potassium current; KCNB2: potassium voltage-gated channel subfamily B member 2; KCNE1: potassium voltage-gated channel subfamily E member 1; KCNN3: potassium calcium-activated channel subfamily N member 3; MI: myocardial infarction; nNOS: neuronal nitric oxide synthase; NO: nitric oxide; Pitx2: paired-like homeodomain transcription factor 2; RyR2 : ryanodine receptor type-2; SERCA2: sarcoplasmic reticulum-Ca2 + pump, Shox2: short-stature homeobox 2; SOD1: superoxide dismutase 1; TBX3: T-Box transcription factor 3; TGF-β1: transforming growth factor-b1; TGF-βR3: TGF-β1 receptor 3; TIMP-4: tissue inhibitor of metalloproteinase 4 WWP-1: WW domain-containing protein 1; ZFHX3: zinc finger homeobox 3.

* The EGFR KO mice had altered electrocardiography parameters.

**Authors in the article stated that "atrial, auricle, and peripheral blood samples were obtained from each AF patient."

*** miR-106b, miR-93 and miR-25

**** Adam et al. suggested similar results (148)

miRNA dysregulation of calcium in cardiomyocytes: possible roles in AF pathophysiology

Available evidence suggests that most cell types express more than one type of IP3R localized in cytoplasmic compartments, mainly at the ER/SR membranes. In cardiomyocytes, IP3Rs are highly concentrated in the nuclear envelope and perinuclear compartments (39, 40). IP3Rs are also colocalized with the junctional RyR2, where they participate in the action potential-evoked CaT and modulate excitation-contraction coupling (41). In cardiomyocytes, Ca^{2+} release from IP3Rs

within or around the nucleus activates CaMKII and transcription factors leading to cardiac remodeling (39, 40).

Our results indicated that the electrically stimulated dog cardiomyocytes overexpressing miR-26a had lower nuclear diastolic [Ca^{2+}], while miRNA knockdown produced the opposite result (Figure 14B). This effect was consistent with the observed reduction of IP3R1 protein expression in AF (Figure 8A). A similar but less profound effect was observed on diastolic [Ca^{2+}] in the cytoplasmic compartment after miRNA transfection (Figure 14B).

During cardiac hypertrophy, Ca^{2+} signaling remodeling is associated with increased IP3R expression and function (57). Increased cardiac IP3R2 expression in HF patients underlies the Ca^{2+} signaling remodeling leading to ventricular arrhythmia (149). 2-APB, a membrane-permeable inhibitor of IP3Rs and TRP channels, has been shown to suppress the incidence and probability of sustained cardiac arrhythmia in the animal model of AF (63). A recent study in our laboratory by Xiaoyan Qi suggests that IP3Rs are expressed in both nuclear and non-nuclear fractions of isolated canine atrial cardiomyocytes. Upon IP3 stimulation in this system, increased resting $[Ca^{2+}]_{nuc}$ and $[Ca^{2+}]_{Cyto}$ are observed (Figure 3). She has also found that atrial cardiomyocyte nuclear Ca^{2+} handling is dysregulated, with enhanced nuclear diastolic [Ca^{2+}] and slowed decay in AF, causing selective nuclear CaMKII hyperphosphorylation and histone deacetylase-4 (HDAC4) export. The underlying mechanism of nuclear Ca^{2+} dysregulation is suggested to be increased the IP3R1 expression (Qi et al., Circ Res, in revision (46)).

Few studies in AF or other cardiac diseases pointed to the involvement of dysregulated miRNA in the molecular changes of Ca^{2+}-related signaling. miR-133 downregulation has been shown in cardiac samples of patients with hypertrophic cardiomyopathy (92) and ventricular samples of a rat model of hypertrophy and is inversely correlated with IP3R2 expression and pro-hypertrophic calcium release (93). miR-135a is downregulated in the mice model of complete atrioventricular block and alters Ca^{2+} handling and spontaneous automaticity consistent with regulation of NCX1 (89). miR-208b upregulation is observed in human AF atrial tissue and negatively regulates the expression and function of LTCC (CACNA1C and CACNB2 subunits) and SERCA2a (150).

Novel elements and significance

This is the first study to indicate the mechanism of IP3R1 upregulation in AF and the first to look at the miRNA regulation of nuclear Ca^{2+} in atrial cardiomyocytes. We were able to show that downregulated miR-26a in cardiomyocytes negatively controlled the IP3R1 translation. Furthermore, we found that knockdown of endogenous miR-26 by antagomiR mimics the reduction of miR-26a in AF and enhances the nuclear diastolic Ca^{2+} level. The currently available treatment options for AF are far from ideal, highlighting the need for new therapeutic strategies focused on specific targets to control the complex remodeling process. This study was directed to evaluate the mechanism of IP3R dysregulation and Ca^{2+}-handling abnormalities in AF. The present findings suggest a potential new clinical therapeutic strategy to prevent or treat AF via miR-26 controlling *ITPR1* expression. The role of downregulated miR-26 in AF has been recognized in atrial electrical and structural remodeling of the heart in different canine AF models (27, 85). Our results indicated the involvement of miR-26 in abnormal Ca^{2+}-handling, which has emerged as the central mechanism in atrial remodeling. It is known that miRNA-mRNA interaction results in translational repression and/or mRNA degradation. In our study, we observed that miR-26 showed different behavior in regulating the expression of *ITPR1* in two specious; in dog cardiomyocytes by inhibition of translation and in HL-1 mice cardiomyocytes by affecting the mRNA level in addition to translational repression.

Our study, along with previous investigations (27, 85) on miR-26, reveals novel molecular mechanisms contributing to the pathophysiology of AF, as illustrated schematically in Figure 15.

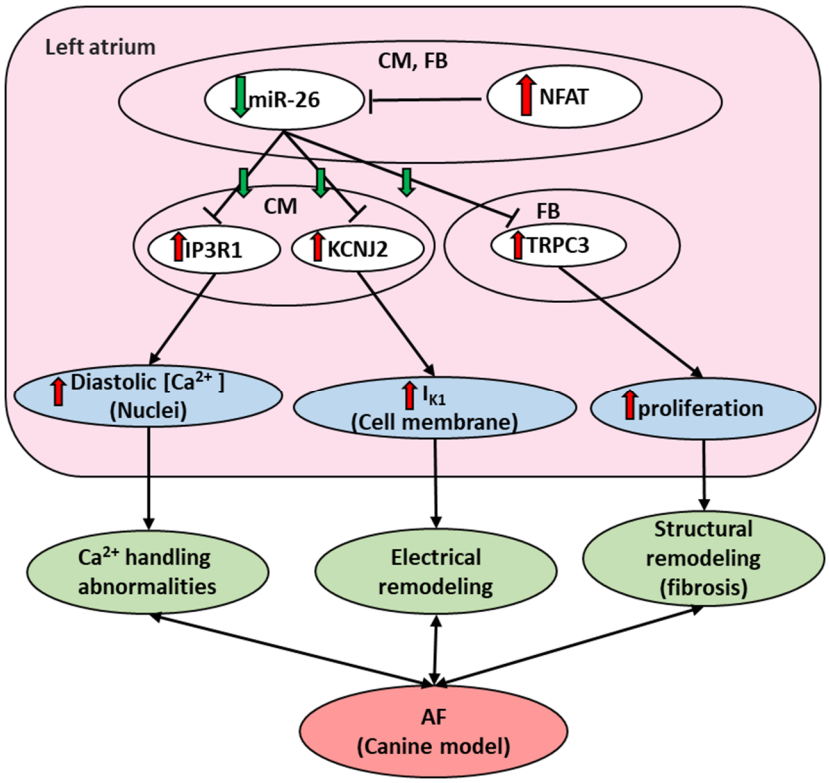

Figure 15: Molecular consequences of downregulated miR-26a in AF.
Increased activity and translocation of NFAT into the nucleus decrease the expression of miR-26. Downregulated miR-26 potentially derepresses the target genes KCNJ2 and IP3R1 in cardiomyocytes and TRPC3 in fibroblasts at the protein and/or mRNA levels. Consequently, the upregulation of each gene results in particular changes (components illustrated in green) contributing to pathophysiological mechanisms (components illustrated in blue) underlying AF. AF= atrial fibrillation; CM: cardiomyocyte; Fib: fibroblast; KCNJ2: potassium inwardly-rectifying channel subfamily J member 2; TRPC3: Ca^{2+}-permeable transient receptor potential canonical-3; IP3R1: inositol 1,4,5-trisphosphate receptor 1; I_{K1}: Inward-rectifier K^+ current.

Limitations

The current study was subject to several potential limitations. The damages to the cells during the isolation process and culture could not be avoided. Isolated cardiomyocytes could be maintained *in vitro* only for a limited time and were more difficult to culture and more sensitive than cell lines. Although primary cells are closer to *in vivo* settings, they do not entirely mimic the tissue of origin. The cells were taken from mongrel dogs. We cannot exclude the possibility that cells could behave differently in culture. After isolation, some cardiomyocytes in suspension could

attach and form clumps, so we could not estimate the cell number and cell viability using Trypan Blue. Therefore, to ensure the study of viable cells, cardiomyocytes were cultured with confluency of ~50%, meaning approximately half of the culture plate's surface was covered.

Chapter 5 – Future research and Conclusions

Future research

In vivo consequences of miR-26a dysregulation and role in AF:

Different studies have suggested critical roles for the miR-26 family in cardiovascular disease (CVD) (151). Increased miR-26a expression has been shown in a mice model of acute MI, which inhibits angiogenesis by targeting bone morphogenic protein/Smad1 in endothelial cells (152). miR-26a/b level is reduced in rat cardiac hypertrophy and regulates structural changes associated with suppressing the glycogen synthase kinase-3β (GSK3β) signaling pathway in cardiomyocytes (153). miR-26b down-regulated in mouse hearts after transverse aortic constriction (TAC) is required for the GATA4 upregulation and cardiac hypertrophy induced by pressure overload (154). miR-26a is downregulated in fibroblasts of a TAC mouse model and targets collagen type I and CTGF, which are critical in cardiac fibrosis (155). miR-26a/b is downregulated in fibroblasts and left atrial tissue from a canine AF model causing upregulation of Kir2.1 in cardiomyocytes and TRPC3 in fibroblasts (27, 85). Among the mentioned studies, Icli et al. (152) and Luo et al. (85) used global overexpression and silencing *in vivo* to demonstrate miR-26a regulatory roles in MI and AF. In both miR-26 manipulation studies, mice were tail-vein injected with miR-26a mimics or inhibitors (85, 152). In addition, as reviewed elsewhere, a single miRNA can affect complex genetic networks may be an advantage over conventional drug intervention therapies. The present study was conducted *in vitro*. Targeted *in vivo* miRNA delivery into the myocardium in preclinical studies is a major challenge. Systemic administration of miRNA mimics and inhibitors face a number of barriers, including low efficacy, toxicity problems, activating the innate immune system leading to side effects, and affecting different organs (156, 157). To overcome the challenges in miRNA manipulation, different strategies such as local administration of the miRNA (e.g., intracardiac injection (158) or viral vectors (159)) and targeted nanoparticle-based delivery systems (e.g., PLGA-based dual-targeted nanoparticles in hepatic carcinoma (160)), might be used in future studies investigating the role of miR-26a in AF.

In addition, tissue-specific promoter/locus through a Cre/loxP approach can be used to generate *in vivo* model of tissue-specific miRNAs knockout and overexpression (161). Evidence indicates that structural remodeling in AF is characterized by an increased number of fibroblasts, which produce large quantities of collagen, leading to tissue fibrosis (25). Cardiac fibroblasts have complicated roles in AF, and manipulation of their activities is favorable to limit their deleterious effects. With the development of Cre/loxP-mediated cardiac fibroblast lineage-tracing mouse lines, it is possible to track the expression level of the reporter in fibroblast, which reflects the expression of the specific gene and also understand the origin of cardiac and cell fate changes of fibroblasts and ultimately treat heart diseases through manipulating fibroblast activities (162). In this regard, Nishiga et al. generated a cardiac fibroblast (CF)-specific knockout (KO) mice (Pn-Cre+miR-33flox/flox) using the promoter of mouse Periostin (Pn-Cre) induced in CFs during pressure overload. They found that Pn-Cre-induced deletion of miR-33 in cardiac fibroblasts in mice reduced fibroblast proliferation and cardiac fibrosis after TAC (163). Furthermore, Ca^{2+} signaling abnormalities in cardiomyocytes also have a central role in AF pathophysiology (32). The Cre/loxP system offers the possibility to generate cardiomyocyte-specific KO or overexpression mice using α-myosin-heavy chain (α-MHC) (164). Huang et al. generated a mouse model of miR-128 overexpression in cardiomyocyte using α-MHC promoter under temporal regulation by doxycycline (α-MHC-tTA; miR-128^{TetRE}). They found that overexpression of this miRNA in cardiomyocyte impairs cardiac homeostasis (164). To date, no *in vivo* model of fibroblast and cardiomyocyte-specific -miR-26a knockout and overexpression has been generated to study the role of miR-26a in AF, so this might be interesting in future studies.

Probing the consequences of nuclear Ca^{2+} loading and HDAC export for the molecular control of AF

IP3Rs are highly concentrated in the nuclear envelope and perinuclear membranes of cardiomyocytes. This localization of IP3Rs restricts the Ca^{2+} release to the nuclear matrix, which in response to stress activates transcription factors such as NFAT or alters pro-hypertrophic gene expression mediated by HDACs (44, 45). Xiaoyan Qi from our laboratory has found that IP3R1 upregulation enhanced nuclear diastolic Ca^{2+} causing CAMKII activation leading to HDAC4 nuclear export in atrial cardiomyocytes in a canine model of atrial tachypacing (Qi et al., Circ Res, in

revision (46)). Arantes et al. used an adenovirus construct (Ad-IP3-buffer-NLS) to import the IP3 into the nuclear compartment of neonatal cardiomyocytes in order to investigate the role of nuclear IP3 and IP3R signaling in hypertrophy (45). This plasmid contains the ligand-binding domain of the IP3R1 and a nuclear localization signal (NLS) for nuclear import tagged with a fluorescent protein. They showed that the buffering of IP3 in the nucleus inhibited nuclear HDAC5 export and CaMKIIδ phosphorylation upon ET-1 treatment (45). In future studies, to see whether the increased nuclear Ca^{2+} release induced by nuclear IP3R1 is directly involved in HDAC4 export observed in AF cardiomyocytes, we can use the Ad-IP3-buffer-NLS in isolated canine atrial cardiomyocytes subjected to tachypacing.

In a recent study, Zhang et al. found class I (HDAC1, HDAC2, HDAC3, and HDAC8) and class IIa (HDAC4, HDAC5, HDAC7, and HDAC9) HDACs have opposite roles in the progression of AF (165). Overexpression of HDAC3 in HL-1 cells significantly reduces CaT amplitude in normal-paced (1 Hz) and tachypaced (5 Hz) cells, and its knockdown protects against contractile dysfunction in tachypaced HL-1 cells. In contrast to HDAC3, HDAC5 and HDAC7 overexpression is protective against tachypacing-induced CaT loss in HL-1 myocytes. Furthermore, tachypacing also increases the phosphorylation and nuclear export of HDAC5, resulting in the derepression of MEF2 and upregulation of its related fetal gene (β-MHC, brain natriuretic peptide [BNP]). Overexpression of HDAC4 is not protective against reduced CaT amplitudes induced by tachypacing (165). Although Zhang et al. study evaluated the role of HDACs in an *in vitro* model of tachypacing-induced CaT loss (165)., the upstream pathways of the HDAC activation need further study. In the canine AF model, which was also used in the current study, IP3R upregulation is accompanied by increased HDAC4 export; it might be interesting to see the expression profile of different classes of HDAC in IP3R1 overexpression and knockdown cardiomyocytes under sinus rhythm and tachypacing conditions. We also showed in the current study that miR-26a is a regulator of IP3R1 expression in cardiomyocytes. In addition, in a previous study on miR-26, knockdown of endogenous miR-26 in a mouse model could enhance AF vulnerability and suggested to be due to the upregulation of its target I_{K1}/Kir2.1 (85). Therefore, investigating the regulatory effects of miR-26a gene transfer in preventing AF in a broader sense, for instance, on increased HDAC export or expression of the transcription factor MEF2, are also of potential interest to future studies.

Conclusions

Our study demonstrates for the first time that downregulated miR-26a in AF regulates IP3R1 expression in cardiomyocytes, indicating that it may play a critical role under pathological conditions. The knockdown of miR-26a reproduces AF-induced IP3R1 upregulation and enhances diastolic [Ca^{2+}] in cytoplasmic and nuclear compartments, which is reversed by miR-26a overexpression. These findings reveal a regulatory pathway leading to IP3R1 upregulation in AF and provide potential new strategies to prevent or treat this prevalent and challenging clinical arrhythmia.

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Appendix

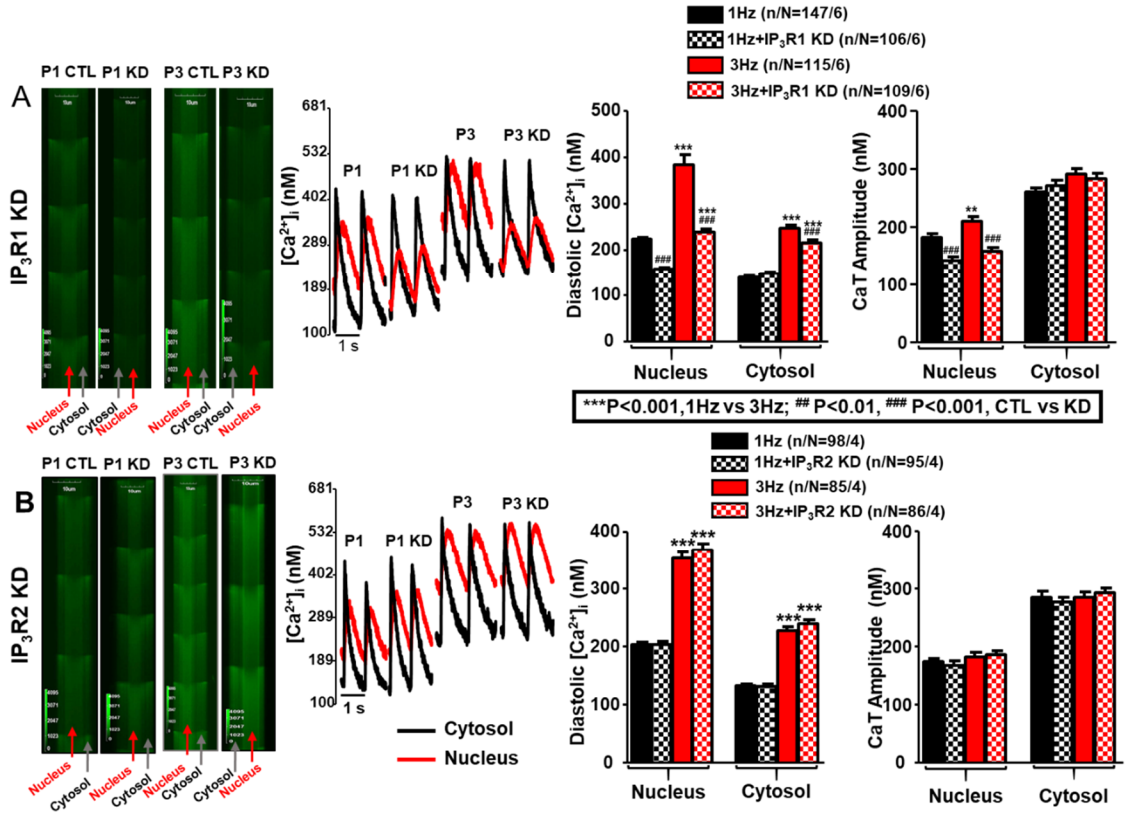

Figure 16: Effect of IP3R1/2 knockdown on Ca^{2+} transients (CaTs).

(A)& (B): Line-scan imaging, representative CaTs, diastolic Ca^{2+} level, and amplitude of the nucleoplasmic and cytosolic CaTs in control and IP3R1/2 knockdown atrial cardiomyocytes. Data are shown as mean ± SEM. KD: knockdown (Qi et al., Circ Res, in revision (46)).