Analysis of miRNAs in cardiovascular diseases and cardiac aging

Análisis de los miRNAs en enfermedades cardiovasculares y envejecimiento cardíaco

Trabajo de Fin de Master en Biología Molecular y Celular

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Abbreviations

β1AR	β-adrenergic receptor
3´UTR	3´ Untranslated region
5´UTR	5´ Untranslated region
Acta1	Actin Alpha 1
AF	Atrial fibrillation
AGO	Agonaute
AGTR1	Angiotensin II receptor type 1
Akt	Protein kinase B
AMI	Acute myocardial infarction
AngII	Angiotensin II
ANP	Atrial Natriuretic Peptide
Azin1	Antyzyme Inhibitor 1
Bcl2	B-cell lymphoma 2
BNP	B-type natriuretic peptide
Ca2+	calcium ion
CACNA1C	Calcium Voltage-Gated Channel Subunit Alpha1 C
CACNB1	Calcium Voltage-Gated Channel Auxiliary Subunit Beta 1
Cdc42	Cell division control protein 42
CaM	Ca^{2+}/calmodulin-dependent calcineurin
CAMK2D	Ca2 + / CaM- dependent protein kinase II δ
CaN	Calcineurin
Casp9	Caspase 9
Ccnd1	Cyclin D1
CH	Cardiac hypertrophy
Check1	Check-point kinase 1
Cited2	Cbp/P300 Interacting Transactivator With Glu/Asp Rich Carboxy-Terminal Domain 2
Clcn3	Chloride voltage-gated Channel 3
Clic5	Chloride Intracellular Channel 5
COL1α1	Collagen type I alpha 1
COL1α2	Collagen type I alpha 2
COL3α1	Collagen type III alpha 1
cTNI	Cardiac troponins
CTGF	Connective Tissue Growth Factor
CX43	Connexin 43
DCM	Dilated cardiomyopathy
DGCR8	DiGeorge Syndrome Critical Region 8
Drp1	Dynamin-related protein 1
Dyrk1a	Dual- specificity tyrosin- (Y) -phosphorylation regulated kinase 1a
ECM	Extracellular Matrix
eIF4E	Eukaryotic Translation Initiation Factor 4E

Eln1	Elastin 1
Eng	Endoglin
Erbb4	Erb-B2 Receptor Tyrosine Kinase 4
ERK/MAPK	extracellular signal-regulated kinase /mitogen-activated protein kinase
FoxP1	Forkhead Box P1
FoxO3	Forkhead box protein O3
Gata4	GATA binding protein 4
GDP-GTP	Guanosine triphosphate-Guanosine diphosphate
Gsk3β	Factor glycogen synthase kinase 3b
HCM	Hypertrophic cardiomyopathy
HDL	high-density lipoprotein
hERG	Human éter-a-go-go related gene
HF	Heart Failure
Hopx	Homeodomain-only protein/Homeobox only protein
IGF-1	Insulin-like growth factor 1
IGF1R	Insulin-like growth factor receptor 1
I_{KR}	Cardiac Rapidly Activating Delayed Rectifier Potassium Channels
I/R	Ischemia/reperfusion
IRX5	Iroquois Homeobox 5
Itga5	Integrin Subunit Alpha 5
Jnk1	c-Jun N-terminal kinases 1
JPH2	Junctophilin 2
KCND2	Potassium Voltage-Gated Channel Subfamily D Member 2
KCNH2	Potassium Voltage-Gated Channel Subfamily H Member 2
KCNH4	Potassium Voltage-Gated Channel Subfamily H Member 4
KCNJ2	Potassium inwardly rectifying channel subfamily J member 2
KO	Knock-out
LQTS	Long QT syndrome
MAPK	Mitogen-activated protein kinase
Mfn2	Mitofusin 2
MHC	Myosin heavy chain
MI	myocardial infarction
mTOR	Mammalian target of rapamycin
miRISC	miRNA induced silencing complex
Murf1	Muscle ring-finger protein 1
MYBPC3	myosin binding protein C gene
Myh7b	Myosin 7b
ncDNA	non-coding DNA
Ncx1	Sodium / calcium exchanger 1
NFAT	Nuclear Factor Of Activated T Cells
Npm1	Nucleophosmin 1
NRAS	Neuroblastoma RAS viral oncogene homolog
nt	nucleotide

Oga	O-GlcNAcase
P-bodies	Apoptotic bodies
Pcg-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3k	Phosphatidylinositol-3-kinase
Pnuts	Phosphatase 1 Nuclear Targeting Subunit
Pp-1	Protein phosphatase 1
Pp2a	Protein phosphatase 2A
Pparα	Peroxisome proliferator-activated receptors
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA transcript
Pten	Phosphatase and tension homolog
RAAS	Renine-angiotensin-aldosterone system
RanGTP	Ran-guanosine triphosphate
RhoA	Ras Homolog Family Member A
RNApol II	RNA polimerase II
RNApol III	RNA polimerase III
ROS	Reactive oxygen species
RP	Ribosomal proteins
rRNA	Ribosomic RNA
RYR2	Ryanodine receptor 2.
SERCA2A	Sarco/endoplasmic reticulum Ca2+-ATPase
Sirt1	Sirtuin 1
Smad3	Mothers Against Decantaplegic homolog 3
Smad7	Mothers Against Decantaplegic homolog 7
Sox6	Sex-determining region Y-box 6
SPRED-1	sprouty related EVH1 domain containing 1
Spry 2	Sprouty 2
Spry1	Sprouty 1
ssRNA	single strand RNA
Stat3	Signal transducer and activator of transcription
SUMO1	Small ubiquitin-related modifier 1
TGF-β	Transforming growth factor beta
TGF-βR1	Transforming growth factor beta receptor 1
Thrap1	Thyroid-hormone-receptor-associated protein 1
TRBP	Transactivation-responsive RNA-binding protein
TRPC3	Ca2 + -permeable transient receptor potential canonical-3
TRPM3	Transient receptor potential cation channel subfamily M member 3
VEGF	Vascular endothelial growth factor
Wee1	WEE1 G2 Checkpoint Kinase
WHO	World Human Organization
Xpo5	Exportin 5
YAP	Yes-associated protein 1

1. Abstract

Cardiovascular diseases (CVD) are one of the main causes of death that affect worldwide. There are several risk factors for the development of CVD, among them, **aging** is important, which implies the anatomical and functional deterioration of the heart. CVD constitute a great burden in advanced societies. There is a need for the development of therapies and non-invasive prognostic and diagnostic biomarkers to effectively tackle them. **microRNAs (miRNAs)** are small RNAs known to regulate main biological process. For this reason, miRNAs have been proposed as a powerful therapeutic targets or biomarkers.

Here the role of miRNAs in the development of CVD and cardiac aging has been reviewed. In addition, the translation level of results in animal models to human has been investigated. As result, a complete compilation of miRNAs that contribute to CVD and cardiac aging is offered. This literature compilation points out that the great majority of the described miRNAs remain unstudied in human. Based in the high degree of miRNA sequence conservation between species and their target sequences, similar functions could be expected in human. Yet, other physiological differences in comparison with animal models could suggest the contrary. To assess the contribution of miRNA in cardiac related pathophysiology, cardiomyocytes derived from human induced pluripotent stem cells (iPSC-CMs) emerge as a model for their study avoiding this translation difficulties.

In addition, the BSICoS group has built a bioinformatic regulatory network controlled by **miRNAs associated with biological age (BIO-AGEmiRNAs)** of the human myocardium. Experimental validation of the interactions within this network is required. A new cost-effective validation tool has been built using fluorescence. Last, luciferase reporter assays (DualGlo assay) are used to study interaction of cardiac-related genes with miR-3916. As a result, positive interaction trends have been observed for *CASQ2*, *ACTN2* and *DSP* with miR-3916. This fact highlights the potential of miRNAs in the regulation of genes associated with human cardiac aging and CVD and their powerful value as therapeutic targets.

2. Introduction

Currently, **cardiovascular diseases (CVD)** are the leading cause of death around the world, especially in developed countries. According to the World Health Organization (WHO), 17,9 million people died because of CVD (31% of all deaths worldwide) and it is the cause of 37% of deaths in people under the age of 70.

The main risk factors for this type of diseases are high blood pressure (hypertension), hyperlipidemia, diabetes, obesity and overweight, smoking, physical inactivity, sex, inheritance and **age**, but from all of them, aging becomes one of the most important (Quiat and Olson 2013, WHO 2017). For people with CVD or with a high cardiovascular risk, early detection and early treatment are essential (WHO 2017), so it is necessary to investigate about therapies or non-invasive biomarkers to be able to predict, diagnose and reduce this type of disease in the world population.

microRNAs (miRNAs) are small RNA molecules that regulate gene expression at the post-transcriptional level in many tissues and physiological conditions including the correct function of the cardiovascular system and even development of CVD [2], [3]. So far, many miRNAs have been proposed as biomarkers or as targets to develop new therapeutic strategies [4]–[6].

The group BSICoS studies the role that miRNAs exert on the control of biological age-related transcriptional alterations in the human heart since aging is a main risk factor of the CVD. For this, a transcriptomic analysis of the human left ventricle across aging was performed and a bioinformatics network of gene regulation controlled by **miRNAs associated with biological aging (BIO-AGEmiRNAs)** was created [7].

3. Hypotheses and goals

The working hypothesis of this Master's Thesis is that miRNAs, as important post-transcriptional regulators, can alter expression of genes involved in CVDs and cardiac aging. Therefore, they postulate as putative therapeutic targets. Also as biomarkers, since they can be secreted to biological fluids. To investigate the role of miRNA in CVD development and aging, a deep bibliographical revision has been conducted. The focus has been set in the translation of results in animal models to humans, in order to gain understanding of therapeutic potential of the current investigations. Two specific aims have been pursued:

1 To identify the principal miRNAs that have been described in the main CVDs and aging.
2 Set the focus on the translation of such investigations into humans, an essential step to postulate miRNA as candidates for the development of novel therapies or as biomarkers.

A second hypothesis, in continuation with previous work of the group BSICoS, is that BIO-AGEmiRNA can regulate genes involved in cardiac functions and, therefore, contribute to age-related cardiac dysfunction in humans. The experimental goal is to extend the BIO-AGEmiRNA-target genes interaction analysis initiated already in the group. Also, to create a new tool for interaction analysis for reproducible and cost effective testing. Specific aims are:

3 Creation of a reporter assay to study miRNA-mRNA interactions based in fluorescence.
4 Cloning of new putative targets of miR-24-2, miR-4435 and miR-3916 BIO-AGEmiRNAs for interaction analysis.

5 Analysis of the interaction of genes of interest (GOI) previously cloned with miR-3916.

4. Content

4.1. microRNA

4.1.1. miRNA biology

miRNAs are non-coding single chain nucleic acids, with approximately 21-25 nt in length expressed in multicellular organisms [8] and many of them are evolutionarily conserved [9].

Their principal function is to regulate genes at post-transcriptional or co-transcriptional level by binding to their complementary sites within the coding sequences, 3´UTRs and 5´UTRs of mRNA target resulting in translational repression or transcript degradation. In addition, at a lower extent, miRNAs have been also related to translation activation [10].

miRNAs can modulate hundreds of mRNA targets and a mRNA can be regulated by several miRNAs [11]. Approximately, in human genome, 2500 miRNAs have been identified [3] and the 60% of protein-encoding genes are regulated by miRNAs at post-transcriptional level [12]. Due to this regulation ability, these small RNAs are involved in a wide variety of physiological and biological processes in living organisms like cell proliferation, differentiation, survival, metabolism, genome stability, inflammation, invasion, angiogenesis…, meaning that a failure in their expression or in its processing can be associated with many diseases including CVD [13], [14]. For that reason, miRNAs are proposed as therapeutic targets and are investigated as such (reviewed in the Master Thesis of Blanca Pina, 2020).

Moreover, miRNAs can be secreted into extracellular fluids. They are delivered to target cells and may operate in an autocrine, paracrine, and/or endocrine way. Their location in extracellular fluids, make of miRNAs putative biomarkers. Indeed, different studies have been demonstrated that these molecules are biomarkers for a variety of diseases [13].

4.1.2. miRNA biogenesis

miRNAs are predominantly expressed from intronic regions of protein coding genes, but they can also be expressed from intergenic regions or from specific miRNA gene loci [11], [14]. Half of the known microRNAs have genomic locations very close to other microRNAs. Then, sets of miRNAs can be expressed as polycistronic primary transcripts in addition to as monocistronic (Figure 1) [14], [15].

The main miRNA processing pathway is the canonical pathway [13].

First, pri-miRNAs, which contain at least a hairpin-like structure and extends 1kb or much longer, are transcribed by RNApol-II, or less frequently by RNApol-III [8], [9]. After transcription, they are processed into 60 to 70 nt pre-miRNAs in the nucleus by the microprocessor complex [16]. Microprocessor is composed of Drosha, a RNA III ribonuclease and DGCR8, and a RNA binding protein that acts as an essential cofactor. DGCR8 interacts with the ssRNA leading Drosha to process the pri-miRNA duplex into the pre-miRNA cleaving at the base of the hairpin structure.

Consecutively, pre-miRNAs are exported into the cytoplasm in order to be processed by the RNase III endonuclease Dicer in complex with TRBP. This process removes the terminal hairpin, resulting in a 18- to 25-nt mature miRNA duplex (miRNA:miRNA*) (Figure 1) [13], [14].

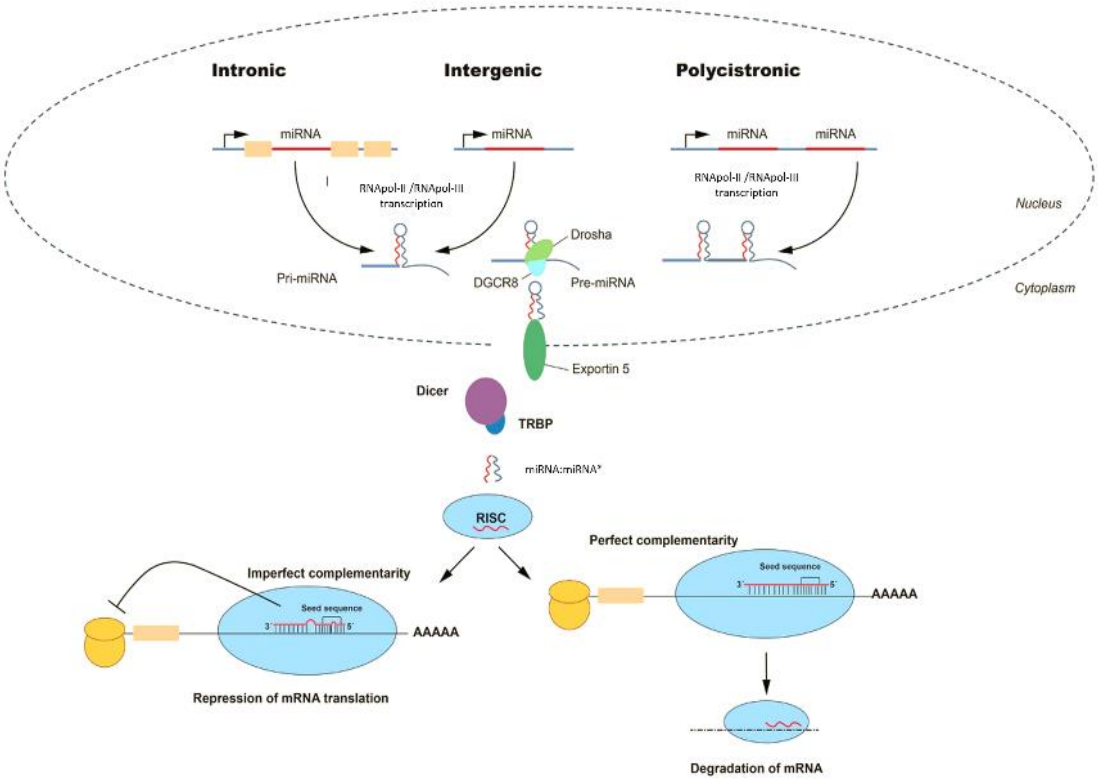

Figure 1. *miRNA biogenesis. Image modified from* [11]

After the formation of the mature miRNA:miRNA*, one of the strands derived from the duplex is loaded into AGO proteins to form the miRISC complex. The selection of one strand depends on the thermodynamic stability of the two ends of the duplex. This process suggests that a helicase-like enzyme (not yet identified) promotes relaxation and exposes both the ends of the miRNA duplex [8], [15]. Generally, the strand with lower end stability is preferentially loaded into AGO. The strand loaded (guide strand, miRNA) lead miRISC to the mRNA target and the unloaded strand (passenger strand, miRNA*) will be unwound from the guide strand and degraded by cellular machinery [13].

4.1.3. Mechanisms of miRNA-mediated gene regulation

The association between miRNAs and their targets creates a three-dimensional miRNA-mRNA interactome. miRNA can bind in different mRNA regions: in the 3'UTR region, where involves degradation of the target transcript or inhibition of translation, in the 5´UTR and in coding regions, which leads to the repression of gene expression, and finally, in promoter regions which results in the induction of transcription, although more studies are necessary for understand this last action [13], [17].

The strand loaded into AGO guides miRISC to miRNA response elements (MRE) on target mRNA. The degree of complementarity determines the future of the transcript. Perfect match between MREs:miRNA results on its degradation. [8], [15]. Imperfect MREs:miRNA complementarity because of central mismatches leads to mRNA silencing by distinct mechanisms of translation inhibition [13], [15].

Some studies have also demonstrated that certain miRNAs can act without miRISC complex although the mode of action of these miRISC-free miRNAs is not completely known [12], [18].

4.2. Association of microRNA with main cardiac diseases and aging

The spatial-temporal expression and performance of different miRNAs determines the correct anatomical development and physiology of different organs and tissues. In other words, their expression levels must be accurately regulated. In fact, the loss or gain of expression of some of key miRNAs could lead to a pathological and be even lethal [19].

Regarding CVDs, many miRNAs have been identified as upregulated or downregulated in different cardiac pathogenic conditions.

Moreover, miRNA expression patterns in each cell type present in the heart will be specific and probably different in cardiomyocytes, endothelial cells, smooth muscle cells, fibroblasts or immune cells [20]. Next, a detailed revision of the contribution of miRNA in CVD is presented (Figure 2).

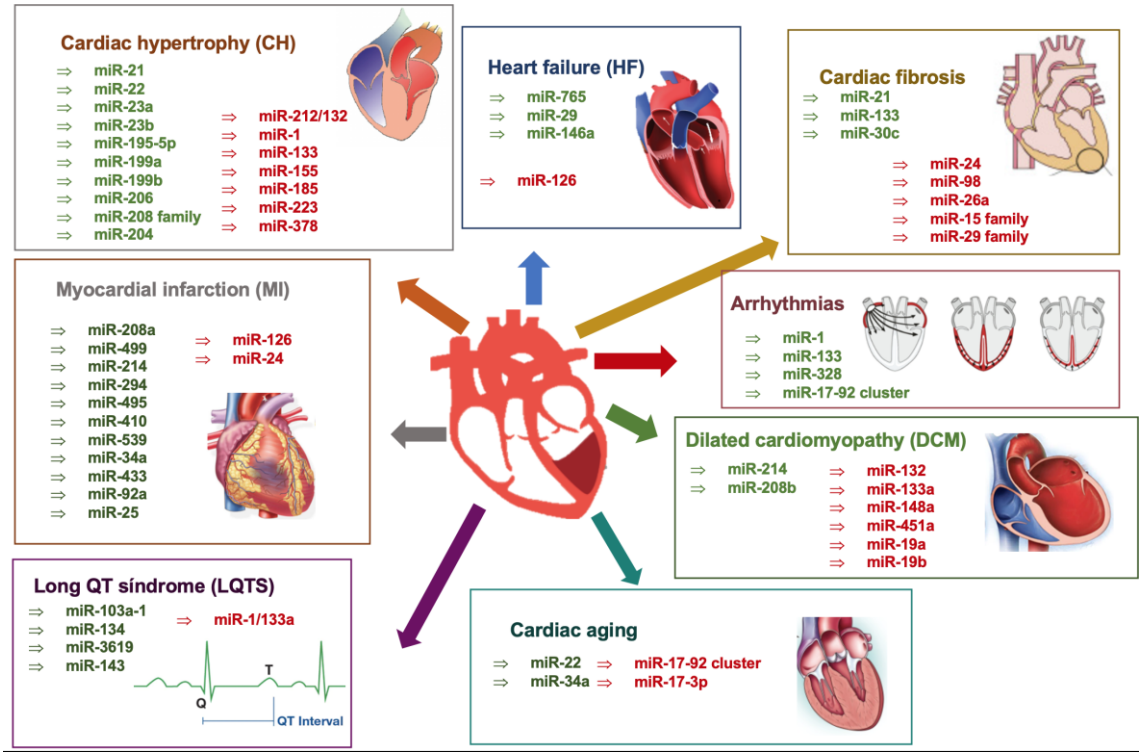

Figure 2. *miRNAs in CVD. miRNA coloured in green are up-regulated miRNAs, miRNA coloured in red are down-regulated miRNAs.*

4.2.1. Cardiac hypertrophy

Maladaptive **cardiac hypertrophy (CH)** is a heart pathology observed in many CVD. At the cellular level, it involves the increase of cardiomyocytes size, protein synthesis and organization of the sarcomere [21]. Gene expression dysregulation, unbalance of the excitation-contraction system and dysfunction of the energy and metabolic balance lead to the growth of cardiomyocytes. In initial stages, hypertrophic process is an adaptive response, but prolonged hypertrophy can lead to cardiomyocyte degeneration, which could imply heart failure (HF) and death [3].

CH is one of the most characteristic events in hypertrophic cardiomyopathy (HCM). This disease can be caused by genetic mutations in protein-encoding genes relevant in contractile function, sarcomeric or Z disk proteins. Moreover, the development of CH is a common feature in different diseases such as MI and HF [22].

The two most important pathways leading to CH are (1) calcium-dependent pathway, which is regulated by CaM and in turn, activates the kinase CaMKII and CaN/NFAT and (2) PI3K/AKT pathway. Other pathways like ERK/MAPK have also been related to hypertrophy (Figure 3) [21], [23]. Some miRNAs have been reported to target hypertrophic-related molecular mechanisms to promote or inhibit hypertrophic growth (Figure 2, Table 1).

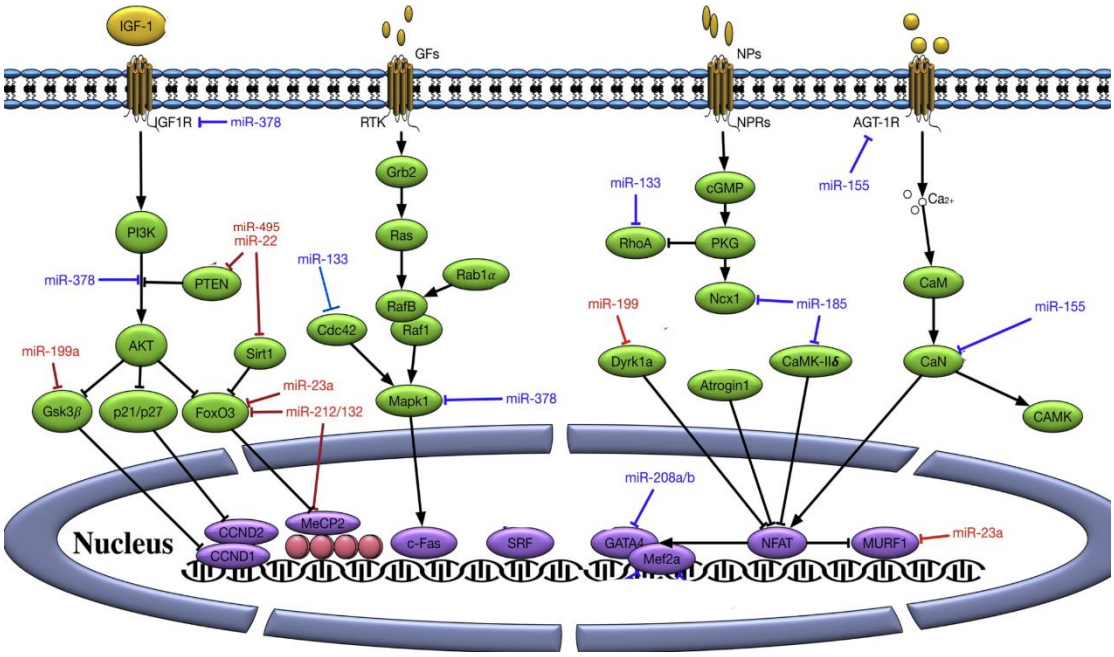

Figure 3. *miRNAs in cardiac hypertrophy. miRNAs coloured in blue have an anti-hypertrophic function, miRNAs coloured in red have a pro-hypertrophic function.*
Image adapted from [23]

miR-21, miR-21, up-regulated in hypertrophy, in pressure-overloaded hearts, promotes these processes in cardiomyocytes. This miRNA can enhance cardiomyocyte growth inhibiting Spry2, which controls ERK/MAPK signalling pathway (da Silva et al., 2018a; Kura et al., 2020; Wojciechowska et al., 2017).

Pten, which inhibits PI3K/AKT pathway, is negatively regulated by **miR-22**, up-regulated in CH. Furthermore, miR-22 exerts a positive regulation on the production of hypertrophic markers for ANP, BNP and β-MHC [3], [23]. *Pten* is also targeted by miR-495 [3].

The high expression levels of **miR-23a** and **miR-23b** during CH indicate their pro-hypertrophic capacity under stress conditions. NFAT regulates miR-23a translation levels and at the same time, this miRNA suppresses the translation of Murf1, a protein with anti-hypertrophic properties [26]. It has also been proposed that it may have a role in the down regulation of FoxO3, which is a transcription factor with anti-hypertrophic and pro-autophagic properties because of its influence on PI3K/AKT signalling pathway [23]. FoxO3 is also downregulated in

cardiomyocytes because of its down-regulation by miR-212/132, pro-hypertrophic miRNAs [3], [23].

Table 1. *miRNAs, their targets, pathways or processes affected and final effects involved in cardiac hypertrophy. Targets coloured in red are down-regulated targets by miRNAs.*

miRNA	*Targets*	*Affected pathway/process*	*miRNA final effect*	*References*
miR-21	*Spry2*	ERK/MAPK	Pro-hypertrophy	[2], [19], [25]
miR-22 ***miR-495***	*Pten*	PI3K/AKT	Pro-hypertrophy	[3], [23]
miR-23a	*Murf1* *FoxO3*	PI3K/AKT	Pro-hypertrophy	[23], [26]
miR-195-5p	*Mfn2* *Fbxw7*	PI3K/AKT	Pro-hypertrophy	[23]
miR-199a	*Gsk3β*	PI3K/AKT	Pro-hypertrophy	[3], [20]
miR-199	*Dyrk1a*	CaN/NFAT	Pro-hypertrophy	[3], [23]
miR-206	*FoxP1*	CaN/NFAT	Pro-hypertrophy	[3]
miR-208a	*Hopx* *Cx40*	Cardiac conductivity deficiences	Pro-hypertrophy	[2], [23], [27]
miR-208a ***miR-208b***	*Thrap1* *myostatin* *Gata4* *Sox6*	Cell growth and contractility	Pro-hypertrophy	[2], [23], [27]
miR-212/132	*FoxO3*	PI3K/AKT	Pro-hypertrophy	[3], [23]
miR-499	*Myh7b* *Acta1*	Reduction in contrability function	Pro-hypertrophy	[2], [28]
miR-17-5p	*Stat3*		Anti-apoptosis in HCM	[3]
miR-1	*Clcn3*		AntI-hypertrophy Cardiac and vascular remodelling	[29]
miR-133	*RhoA,* *Cdc42*		Anti-hypertrophy	[28], [30]
miR-155	*AGTR1*	AGTR CaN	AntI-hypertrophic	[30]
miR-185	*CAMK-2D,* *Ncx1 NFAT*	NFAT	AntI-hypertrophic	[30]
miR-378	Several effectors	MAPK PI3K/AKT	Anty-hypertrophic	[30]

MiR-195-5p is up-regulated in AngII-induced cardiac hypertrophy [23], [25]. This miRNA down-regulates their targets Mfn2 and Fbxw7. Mfn2, a GTP-ase located in the mitochondrial membrane and Fbxw7, that promotes indirectly the inactivation of Akt [23].

MiR-199a negatively regulates the expression of *Gsk3β*, which in turn, inhibits mTOR, a negative regulator of autophagy which is part of PI3K/AKT pathway [3], [20]. The target of miR-199 is *Dyrk1a*. This miRNA activates this kinase which is involved in the phosphorylation of NFAT factors, and therefore, is responsible for the activation of the CaN/NFAT signalling pathway [3], [23].

MiR-206 is regulated by YAP, an important apoptosis-inducing and pro-hypertrophic molecule in the Hippo pathway. Its target is *FoxP1*, which negatively regulates cardiac hypertrophy through inhibition of NFAT [3].

The **miR-208** family, composed of miR-208a, miR-208b and miR-499, is encoded in intronic regions of the *α-MHC* and *β-MHC* genes. These miRNAs are capable of inducing hypertrophy. miR-208a and miR-208b induce hypertrophic processes in response to stress and hypothyroidism through the negative regulation of their targets: Thrap1, myostatin and Gata4, related to cell growth and contractility. Tharp1 negatively regulates the expression of *β-MHC*. This implies that miR-208 positively regulates *β-MHC* indirectly. Besides, up-regulation of miR-208 is also related to hypertrophy trough negative regulation of *Sox6* [2], [23], [27].

High levels of **miR-499** expression in mice heart lead to a hypertrophic phenotype and a reduction in contractibility function, since it acts on two important proteins for this function: Myh7b and Acta1 [2].

As previously mentioned, HCM can have a genetic origin in some patients. For instance, patients with mutations in the *MYBPC3* presented upregulation of **miR-204** and *TRPM3* gene which encodes for channel involved in calcium entry and embeds miR-204. So, miR-204 and *TRPM3* could participate in this disease derived from *MYBPC3* mutations [3].

miR-17-5p, up-regulated also in HCM murine model, regulates *Stat3* which is a cellular survival factor since it protects the cell from apoptosis during oxidative stress [3].

During cardiac hypertrophy, low expression levels of **miR-1** and **miR-133** have been demonstrated in mouse and human. Therefore, both have an important role in the negative modulation of hypertrophic growth [6], [27]. Other studies have pointed out that the expression of miR-1-3p is inversely correlated to left ventricle (LV) end diastolic diameter and directly correlated to LV ejection fraction in human left ventricular heart samples. One of its targets, *Clcn3*, has been shown to have a role in cardiac and vascular remodelling during hypertrophy [29].

On the other hand, **miR-133** is the responsible of the negatively regulation of anti-hypertrophic genes in mice and humans with CH such as RhoA and Cdc42, both are GTP-binding proteins involved in this disease. What is more, this miRNA is negatively regulated by CaN in *in vivo* and *in vitro* CH models [28], [30] .

miR-155, an anti-hypertrophic miRNA, has also been found down-regulating *AGTR1* and suppressing CaN pathway [23].

miR-185 have also demonstrated anti-hypertrophic properties in a TAC-induced hypertrophy mouse model. This miRNA regulates various genes involved in calcium signalling pathways such as *CAMK-2D, Ncx1* and *NFAT* [23].

MiR-378, an anti-hypertrophic miRNA, is capable of repressing MAPK signalling targeting different effectors of this pathway, and suppressing PI3K/AKT signalling pathway [23].

4.2.2. Cardiac fibrosis

Cardiac fibrosis is the result of abnormal accumulation of **ECM proteins** secreted by fibroblasts that causes cardiac dysfunction. Cardiac fibroblasts are the main effectors in a fibrotic heart, but there are other cells such as cardiomyocytes, vascular cells or immune cells that can contribute through the secretion of pro-fibrotic factors like TGF-β, cytokines and chemokines and/or proteases [32]. The development of HCM, DCM, arrhythmias and MI may be associated with fibrotic processes in the heart [3]. miRNAs are capable of modulate these fibrotic processes trough target gene modulation (Figure 2, Figure 4 and Table 2).

The **miR-15** family is important in fibrotic processes since it suppresses ECM remodelling and inhibits the TGF-β pathway through regulation of genes involved in it [19], [23], [25].

miR-21, expressed in fibroblasts, is one of the most important miRNAs in fibrotic processes. This miRNA is able to induce the pro-fibrotic ERK/MAPK signalling pathway (downstream of TGF-β receptor) in cardiac fibroblasts through the inhibition of Spry1. Its association with TGF-β and TGF-βR1 trough the inhibition of Smad7 leads to fibrosis due to TGF-β-mediated endothelial to mesenchymal transition. Other negatively regulated target is Pten which negatively regulates the PI3K/AKT pathway in fibroblasts [19], [27], [33].

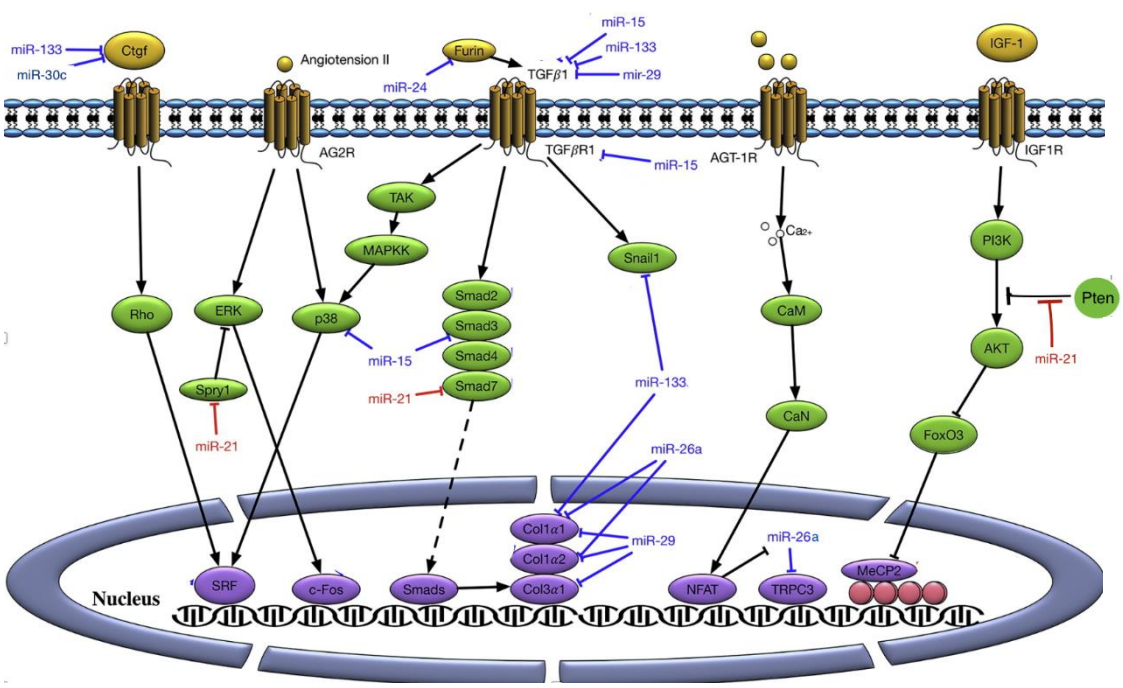

Figure 4. *miRNAs in cardiac fibrosis. miRNAs coloured in blue have an anti-fibrotic function, miRNAs coloured in red have a pro-fibrotic function. Image adapted from [23].*

Reduced expression levels of the **miR-29** family, composed of miR-29a, miR-29b and miR-29c, has been associated with fibrosis after MI since their targets correspond to pro-fibrotic genes encoding collagen and extracellular matrix proteins and *TGF-β,* also related to matrix proteins [2], [23], [27], [34]

The expression levels of **miR-24** in cardiac fibrosis are also reduced. It has been described that could inhibit the pro-protein convertase Furin, necessary for the secretion of *TGF-β,* down-regulating its levels in cardiac fibroblasts in a post-MI model [3], [23].

Another downregulated miRNA in cardiac fibrosis is **miR-26a**. This miRNA has several targets: *COL1α1* and *COL1α2*, thereby increasing collagen deposition; *KCNJ2*, affecting the remodelling process in fibroblasts and *TRPC3* channels, affecting the proliferation and differentiation of fibroblasts [23], [25].

The increased expression of **miR-133** and **miR-30c** fibroblasts and cardiomyocytes leads to a down-regulation of *CTGF* in rodent models and in left ventricular biopsies of patients. *CTGF* promotes the ECM and collagen deposition [2]. On the other hand, miR-133 directly inhibits *TGF-β* in a canine model of atrial fibrosis and *COL1α1* in rats with cardiac fibrosis induced by angiotensin II [35]–[37].

Table 2. *miRNAs, their targets, pathways or processes affected and final effects involved in cardiac fibrosis Targets coloured in red are down-regulated targets by miRNAs.*

miRNA	*Targets*	*Affected pathway/process*	*miRNA final effect*	*References*
miR-15 family	*TGF-βR1* *P38* *Smad3* *Smad7* *Eng*	ECM remodelling TGF-β	Anti-fibrotic	[19], [23], [25]
miR-21	*Smad7*	TGF-β	Pro-fibrotic	[19], [27], [33]
	Spry1	ERK/MAPK		
	Pten	PI3K/AKT		
miR-29 family	*COL1α1* *COL1α2* *COL3α1* *FBN1* *ELN1* *TGF-β*	Collagen deposition ECM remodelling TGF-β	Anti-fibrotic	[2], [23], [27], [34]
miR-24	*Furin*	TGF-β	Anti-fibrotic	[3], [23].
miR-26a	*COL1α1* *COL1α2* *KCNJ2* *TRPC3*	Collagen deposition Fibroblasts remodelling Fibroblasts proliferation and diferenciation	Anti-fibrotic	[23], [25]
miR-133, miR-30c	*CTGF*	ECM synthesis Collagen deposition	Anti-fibrotic	[2]
miR-133	*TGF-β* *COL1α1*	Collagen deposition TGF-β	Anti-fibrotic	[35]–[37]

4.2.2.1. Heart failure

The development of heart failure (HF) is due to the inability of the heart to provide enough force to pump blood. It is caused by different physiology alterations in circulatory system including failures in calcium handling [19], activation of neurohormonal mechanisms or cardiac remodelling. Different pathologies are observed in HF hearts: hypertrophy, apoptosis and excessive myocardial fibrosis. Furthermore, the incidence and prevalence of heart failure are known to increase with aging [38]. There are miRNAs involved in these processes, and they can promote or suppress the development of HF (Figure 2, Table 3).

miR-765 is increased in human failing hearts. In HEK293 cells and in mouse ventricular myocytes it has been seen that the increase in miR-765 leads to a suppression of its target, inhibitor-1 levels and promotion of PP-1 activity, involved in sarcomeric function. For this reason this miRNA is involved in cardiomyocyte contractility [39]

In patients with HF, **miR-29** could participate in ECM remodelling and in apoptotic processes in the advanced stage of the disease. This miRNA acts by repressing the expression of genes that encode ECM proteins, so it is crucial for cardiac function [2].

The expression induction of **miR-146a** in endothelial cells showed its role in the reduction of proliferation and viability of endothelial cells trough targeting *NRAS*. Consequently this miRNA promote the decrease in microvasculature density in the heart [19]. Another of its target is *SUMO1,* which is a regulator of the sumoylation process in the heart. Overexpression of miR-146a leads to a reduction in *SUMO1* levels, a reduction of *SERCA2A* sumoylation and a decrease in contractile function in human and animal models with failing hearts [40].

Another miRNA related to promote the breakdown of microvasculature is **miR-126** which targets *SPRED-1.* This miRNA, expressed in endothelial cells, is down-regulated during right ventricle (RV) failure in patients with pulmonary arterial hypertension [19].

Table 3. *miRNAs, their targets, pathways or processes affected and final effects involved in heart failure. Targets coloured in red are down-regulated targets by miRNAs.*

miRNA	***Targets***	***miRNA final effect***	***References***
miR-765	*inhibitor-1* *PP-1*	Dysregulated cardiomyocyte contractility	[39]
miR-29	ECM proteins	ECM remodelling Apoptotic processes	[2]
miR-146a	*NRAS* *SUMO-1*	Reduce microvasculature density Sumoylation reduction	[19] [40]
miR-126	*SPRED-1*	Reduce microvasculature density	[19]

4.2.2.2. Arrhythmias

Arrhythmias are deviations from the normal heart rate and / or rhythm pattern. They are mostly due to electrophysiological failures in the heart caused by abnormal conduction, action potential, or by a combination of both. The correct **ionic balance** is essential for a correct action potential and **cell-cell communication** is required for a correct signal conduction [3]. Certain

miRNAs have been shown to regulate these processes by modulating the expression of channels and receptors (Figure 2, Table 4).

miR-1 is up-regulated in ischemic myocardium, AF and arrhythmias. This miRNA represses the expression directly, in the case of *CX43* and *KCNJ2*, or indirectly, *RYR2* (trough the phosphorylation mediated by repression of PP2A) and *KCND2 (*trough IRX5 that negative regulates this channel*)*. For this reason, high miR-1 levels are related to the slowdown of cardiac conduction due to depolarization of the cytoplasmic membrane and cardiac remodelling, as a consequence of the down-regulation of proteins involved in the management of intracellular Ca^{2+} levels (*RYR2*), ionic channels (*KCNJ2* and *KCND2*) and in cell-cell communication (*CX43*) [2], [23], [41], [42].

Moreover, low levels of **miR-1** and **miR-133** (both transcribed from the same gene cluster) promote overexpression of *KCNH2* and *KCNH*4 channels, which are located in pacemaker, atrial and ventricular cells. Due to their ability to target these genes, miR-1 and miR-133 can contribute to cardiac electrical activity. In fact, reduced levels of these miRNAs have been associated with aging in guinea pig model triggering abnormal cardiac function [2], [19], [28], [43].

The **miR-17-92** cluster is also up-regulated in arrhythmias. Its target related to arrhythmias is *CX43.* The overexpression of this cluster in a transgenic mouse model leads to aberrantly cardiac conduction [3].

The overexpression of **miR-328** in AF has been related with an increase in the vulnerability of developing this disease since their targets are *CACNA1C* and *CACNB1*, important genes involved in calcium handling [44].

Table 4. *miRNAs, their targets, pathways or processes affected and final effects involved in arrhythmias. Targets coloured in red are down-regulated targets by miRNAs respectively.*

miRNA	***Targets***	***miRNA final effect***	***References***
miR-1	*CX43* *KCNJ2* *PP2A* *IRX5*	Slowdown of cardiac conduction	[2], [23], [41], [42]
miR-1/-133	*KHCN2* *KHCN4*	Abnormal cardiac electricity	[2], [19], [28], [43]
miR-17-92	*CX43*	Aberrant cardiac conduction	[3]
miR-328	*CACNA1C* *CACNB1*	Dysregulation of calcium handling	[44]

4.2.2.3. Myocardial infarction

Myocardial infarction (MI) is a CVD that involves a reduction in blood flow to the heart, often caused by the breakdown of atherosclerotic plaques which occludes coronary arteries. The main consequences include cardiomyocytes death by apoptosis due to hypoxia, ischemic lesions and structural alterations. At midterm, such alterations induce the increase of extracellular matrix proteins and hypertrophy, which can lead to cardiac dysfunction and finally to chronic HF. After MI, processes of cardiac remodelling and cardiomyocyte proliferation take place to restore

cardiac function [3] . In both processes, miRNAs play an important regulatory role (Figure 2, Table 5).

The role of **miR-126** in MI is related to the ability of this miRNA to negatively regulate *SPRED-1* and consequently is involved in vascular integrity and angiogenesis after MI in mouse models [45]. MiR-126 is down-regulated and it has been related to myocardial recovery after MI [46].

Similarly, **miR-214** is up-regulated in mice and human tissue after MI and exerts a cardioprotective effect during ischemia/reperfusion. This miRNA modulates cardiomyocyte survival trough the repression of *NCX1*, a sodium/calcium exchanger, and BIM, a pro-apoptotic protein that participates in intrinsic apoptosis pathways [19]. Besides, **miR-24** and **miR-25** (both down-regulated in MI), regulate processes in mouse myocardium with MI such as contractility through negative regulation of *SERCA2A* and coupling of excitation and contraction through the regulation of calcium homeostasis repressing *JPH2* [23], [47].

Table 5. *miRNAs, their targets, pathways or processes affected and final effects involved in myocardial infarction. Targets coloured in red are down-regulated targets by miRNAs.*

miRNA	*Targets*	*Affected pathway/process*	*miRNA final effect*	*References*
miR-126	*SPRED-1*	Vascular integrity and angiogenesis	Reduce microvasculature density	[45], [46]
miR-214	*Ncx1* *Bim*	Sodium/calcium handling intrinsic apoptosis	Cardioprotective	[19]
miR-24	*Bim*	intrinsic apoptosis	Reduce apoptosis Reduce scar size	[19], [27], [28]
miR-24, miR-25	*SERCA2A* *JPH2*	Contractility function Calcium homeostasis	Dysregulated excitation and contraction	[23], [47]
miR-294	*Wee1*	G2 to M transition	Facilitate remodelling post-MI	[48]
miR-410 ***miR-495***	*Cited2*	TGF-β	Improve cardiomyocyte proliferation	[3]
miR-539	*Oga*	O-GlcNAcylation	Suppression of O-GlcNAcylation	[49]
miR-34a	*Pnuts*	Cardiomyocytes apoptosis Response to DNA damage Shortening of telomeres	Cardiomyocyte death	[3], [27]
miR-433	*Azin1* *Jnk1*	TGF-b ERK/MAPK	Inhibition of fibrosis post-MI	[3]
miR-92a	*Itga5* *Sirt1*	Angiogenic processes	Angiogenesis reduction	[50], [51]

miR-294 is up-regulated in MI and it acts by negatively regulating *Wee1*, a tyrosine kinase that negatively regulates entry into mitosis (G2 to M transition). Thus, this miRNA is involved in

cardiac remodelling post-MI by facilitating cell division in a mouse model of MI [48]. **miR-410** and **miR-495**, up-regulated in MI, regulate the expression of *Cited2* which positively regulates TGF-β, and subsequently improve cardiomyocyte proliferation *in vitro* [3]. **miR-539**, up-regulated in MI, negatively regulates *Oga* in the infarcted zone in a mouse MI model. The suppression of *Oga* leads to the increase of O-GlcNAcylation signalling. This dysregulation has been involved in several diseases like MI [49].

Up-regulation of **miR-34a** has been associated with age-related cardiomyocyte death in left ventricular remodelling after MI by inhibition of *Pnuts*. Pnuts contributes positively to recovery after MI in mice, since this protein regulates cardiomyocytes mortality, the response to DNA damage and shortening of telomeres [3], [27]. Furthermore, the down-regulation of *Azin1* and *Jnk1* by **miR-433**, up-regulated in MI, leads to *TGF-β* and ERK/MAPK pathway activation respectively. This pathway activation during cardiac remodelling after MI, activates fibrotic processes which take place during this stage of the disease [3].

miR-92a, a component of miR-17/92 cluster, is up-regulated in ischemic tissue in mice. Their increased expression negatively regulates angiogenic processes induced by ischemia *in vitro* and *in vivo* in mice model. This performance is due to the inhibition of proteins that promotes the angiogenic processes: *Itga5* and *Sirt1.* In a pig model of I/R, the repression of miR-92a demonstrated desired pro-angiogenic properties after ischemia and MI [50], [51].

4.2.2.4. Dilated cardiomyopathy (DCM)

Abnormalities in heart contractility lead to the development of DCM. In this disease, **LV loses contractile capacity and increases its size**. Chronic inflammation and hypertrophy are also important processes in this disease. These abnormalities are due to genetic mutations in genes that encode for important proteins in contractility, such as sarcomere or cytoskeleton [3]. miRNAs play an important role in the development of contractile function, inflammation processes and hypertrophy, and therefore, in DCM (Table 6).

Table 6. *miRNAs, their targets, pathways or processes affected and final effects involved in DCM. Targets coloured in red are down-regulated targets by miRNAs.*

miRNA	*Targets*	*Affected pathway/process*	*miRNA final effect*	*References*
miR-132	*Pten*	PI3K/AKT	Apoptosis, fibrosis and inflammatory response activation	[3]
miR-133		PI3K/AKT		[3]
miR-148a			Reduction cardiac Wall Chamber dilation Rise LV volume Reduction of ejection fraction	[3]
miR-451a	*Myc*	Inflammation	Activation and proliferation of CD4+ T cells	[3]

Low **miR-132** expression levels lead to high levels of its target *Pten* and subsequently suppression of the PI3K/Akt pathway, which activates apoptosis, cardiac fibrosis and the

inflammatory response. **miR-133a**, also down-regulated in DCM, plays a regulatory role in the same signalling pathway [3].

Low expression levels of **miR-148a** (downregulated in DCM) triggers a reduction of the cardiac wall thickness, dilation of the chamber, rise in LV volume and reduction of the ejection fraction [3].

As previously mentioned, chronic inflammation is crucial for DCM development. **miR-451a** is down-regulated in CD4+ T cells of DCM patients. This miRNA targets *Myc which* is positively regulated in these cells. So, miR-451a down-expression leads to CD4+ T cells activation and proliferation [3].

At last, **miR-19a**, **miR-19b** expression is reduced and **miR-208b** expression is increased in DCM. These miRNAs are related to cardiac hypertrophy and fibrosis during DCM, since the miR-19 family has anti-fibrotic properties (see section 3.2.1.8) and miR-208 have pro-hypertrophic properties (see section 3.2.1.1.) [2], [3], [52], [53].

4.2.2.5. Long QT syndrome

The long QT syndrome (LQTS) is a genetic channelopathy caused by mutations in different ion channels that is defined by the prolongation of ventricular repolarization [3]. For this reason, maintenance of cardiac electrical activity is critical in this pathology. Some miRNAs have been found to contribute to the regulation of targets involved in this process (Table 7).

Up-regulation of **miR-103a-1**, **miR-134**, **miR-3619** and **miR-143** has been observed in LQTS. These miRNAs targets *KCNH2* which encodes for hErg, the most important component of I_{KR} repolarization current [54].

It has been demonstrated that a down-regulation of **miR-1/133a** leads to the development of LQTS in miR-1/133 cluster KO mice. These prolonged action potentials in cardiomyocytes are derived from an alteration of the β-adrenergic signalling pathway and consequently, dysregulation of cardiac channels [55].

Table 7. *miRNAs, their targets, pathways or processes affected and final effects involved in Long QT syndrome. Targets coloured in red are down-regulated targets by miRNAs.*

miRNA	***Targets***	***Affected pathway/process***	***miRNA final effect***	***References***
miR-103a-1 miR-134 miR-3619 miR-143	*KCNH2*	Repolarization	Dysregulation of repolarization	[54]
miR-1/-133a	β-adrenergic effectors	β-adrenergic	Dysregulation of cardiac channels	[55]

4.2.2.6. Cardiac aging

Aging is one of the most predominant risk factor for CVD. Cardiac aging is a physiological process controlled by genetic and environmental factors that involves the progressive degradation of function, morphology and ability to maintain the cell homeostasis in the heart. The number of

myocytes, cardiac output and maximum heart rate generally decrease with age. Conversely, the amount of fibrotic tissue and lipid deposition generally increases [56], [57].

The main molecular mechanisms affected in aging are (Figure 5): (1) Nutrition and growth signalling, including the IGF-1 pathway, the mTOR / eIF4E signalling pathway, and the Sirt family of proteins; (2) Mitochondrial abnormality due to ROS accumulation with age or due to interference with the Pcg-1α protein, which regulates mitochondrial function; (3) Neurohormonal regulation which includes Renin-Angiotensin-Aldosterone system (RAAS) associated with aging, β-adrenergic signalling pathway or Pi3k / Akt signalling pathway [58].

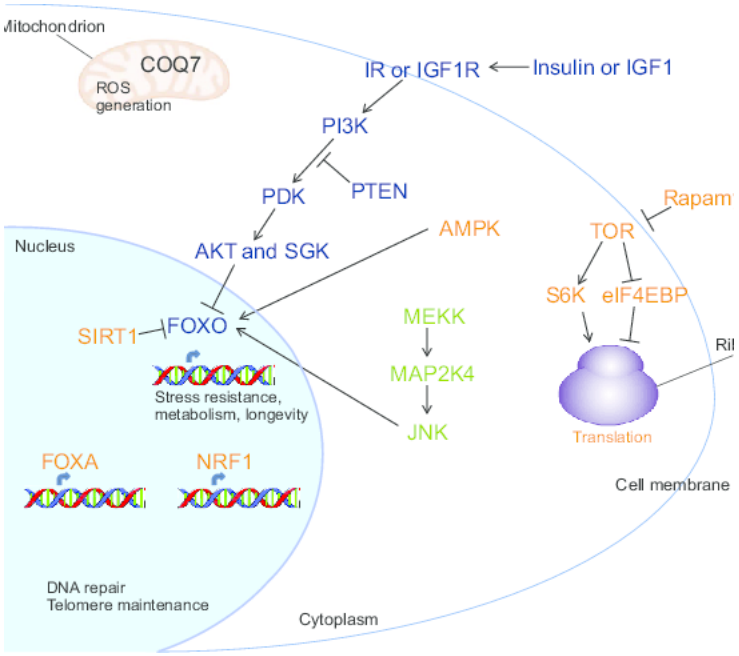

Figure 5. *Aging-involved pathways. Image obtained from* [59]

miRNAs have been identified as regulators of aging processes and modulators of longevity, since their expression has been significantly altered in the non-pathological and pathological aging (Table 8) [56].

miR-22 is expressed at higher levels with aging in human and mouse heart tissue, and negatively regulates *Ogn.* Its downregulation leads to increased cardiac fibroblast senescence, a process closely related to aging. In aged cardiomyocytes, miR-22 overexpression leads to increased hypertrophy and impaired diastolic heart function through repression of *Pparα* and *Sirt1*. miR-22 inhibition reduces hypertrophic processes and increases myocardial autophagy (impaired during aging) [23], [56].

miR-34a has also been found to be overexpressed during aging in mouse, mostly in cardiomyocytes and in human atrial (Boon et al., 2013). This increased expression leads to the promotion of cardiomyocyte death and decreased contractile function and, due to *Pnuts* inhibition, induces other aging related functions such as induction of telomere shortening, inhibition of DNA damage repair, cardiac senescence and destruction of the intrinsic regeneration capacity of cardiomyocytes, as mentioned in section 3.2.1.5. This cellular mortality has also been related to the regulation *of Bcl2, Sirt1* and *Ccnd1* by miR-34a in aging [23], [56]. In fact, miR34a inhibition reduces cell death, fibrosis and cardiac functional deterioration in aging mouse heart [60].

In contrast, the expression of the **miR-17-92** cluster decreases with age in HF-prone mice and **miR-18a**, **miR-19a** and **miR-19b** are only decreased in failing aged heart (not in healthy aged-heart). This miRNAs negatively regulate the genes *CTGF* and *Tsp1* in aged cardiomyocytes, so they promote the reduction of cardiac fibrosis, inhibiting pro-fibrotic genes [57]. **miR-17-3p**, down-regulated with age, targets Par4, a pro-apoptotic protein. So, the role of miR-17-3p in aging is decrease cardiac apoptosis [61].

Table 8. *miRNAs, their targets, pathways or processes affected and final effects involved in cardiac aging. Targets coloured in red are down-regulated targets by miRNAs.*

miRNA	*Targets*	*Affected pathway*	*miRNA final effect*	*References*
miR-22	*Ogn* *Ppar* *Sirt1*	Senescence, hypertrophy Diastolic heart function	Increase cardiomyocyte senescence Increase hypertrophic processes and impaired diastolic heart function	[56], [62]
miR-34a	*Pnuts* *Bcl2* *Sirt1* *Ccnd1*	Senescence Apoptosis DNA damage repair	Telomere shortening induction Inhibition of DNA damage repair Cardiac senescence and apoptosis	[56], [60], [62]
miR-17-92 cluster	*CTGF* *Tsp1*	Fibrosis	Reduction cardiac fibrosis	[57]
miR-17-3p	*Par4*	apoptosis	Decrease fibroblast senescence and apoptosis	[61]

In human, the contribution of miRNA to cardiac aging is poorly understood. The BSICoS group has analysed the relation of miRNAs with cardiac aging in a transcriptional study. In the left ventricle, a total of 20 miRNAs associated to biological aging, BIO-AGEmiRNA, have been described [7].A downstream BIO-AGEmiRNA gene regulation network has been established using bioinformatics tools and some of the miRNA-gene interactions have been validated [63]. In this work, the validation of the network of interactions is expanded and a novel system for the study of interactions based in fluorescence has been created.

5. Models for study the role of miRNAs in cardiac diseases and aging: translation to humans

The study of the phenotype of different heart diseases and the molecular role of miRNAs, requires the use of relevant *in vitro e in vivo* experimental models. These should be representative of the condition to be studied, although in some cases, human features are only partially represented [64], [65]. Animal models can be classified in small, such as rodents (rat or mouse) or large like pigs, dogs, or sheep [22], [64], [65].

Many human cardiomyopathies cannot be properly modelled with animal models because of differences between species. Human models, including cellular systems like primary cells (cardiomyocytes) or derived from induced pluripotent stem cells (iPSC) can be used to carry out

functional and molecular studies. Recent advances in stem cell research and genome engineering allow the creation of versatile *in vitro* models of human conditions [66].

In addition to interspecies differences in physiology, longevity, organism size or genomic homogeneity, it is also important to consider the evolutionary conservation of miRNAs and their target sequences in mRNAs. It is believed that selective pressure to keep miRNA and MRE occurred [67].

In this section, we summarize the main models used in the study of miRNA contribution in CVD and aging and highlight the translation of results to human for each condition (Table 9).

Table 9. *Models used in the study of miRNA function or targets.*

miRNA	**Role/ Influence on or targeting**	**Animal model**		**Human model**		**Ref.**
		In vivo	***In vitro***	***In vivo***	***In vitro***	
Cardiac hypertrophy						
miR-22	Pro-hypertrophic role	miR-22 KO mouse	Mice cardiomyocytes		iPSC-CMs	[68] – [71]
miR-1/-133	Anti-hypertrophic role	TAC mice, transgenic mice and exercised rats		Heart samples		[30]
miR-208a-3p miR-23a-3p miR-22-3p	Pro-hypertrophic role	miRNA KO mouse			iPSC-CMs iPSC-CMs	[23], [26], [69], [72], [73]
miR-29	Differences between human and mouse	Gene expression HCM mouse models	Rat cardiac myocytes/ fibroblasts	mRNA/miRNA expression data HCM patients		[74]
Myocardial infarction						
miR-132	Cardio-protective	LAD ligation in miRNA KO model				[75]
miR-21	Cardio-protective	LAD ligation in murine model		Infarcted area heart tissue		[76] [34]
miR-30e-5p	Role in apoptosis and calcium handling				iPSC-CMs with hypoxia-induced apoptosis	[77]
miR-21 miR-214 miR-223	Up-regulated in infarcted zone	Mouse		Infarcted area heart tissue		[34]
miR-29b mir-149	Down-regulated in infarcted zone	Mouse		Infarcted area heart tissue		[34]
miR-1-2	No agreement	Attenuated in fibrotic process in mouse			Upregulated in human iPSC-CMs	[73], [78]

miR-133 miR-30c	CTGF quantity inversely proportional to fibrosis	Rat model and ventricular cardiomyocytes from rat		Cardiac biopsies with aortic valve stenosis		[35]
miR-24	Down-regulated	MI mouse model, primary cardiomyocytes, rat model				[79], [80]
miR-144-3p	Enhancement fibrosis post MI	Pig MI model, mouse cardiac fibroblasts			Human cardiac fibroblast	[81], [82]
miR-34a	Related to fibrotic processes, protective role	Aged miRNA KO mouse, AMI mouse model, Neonatal and adults rat hearts, Myocardial and heart tissues from neonatal rats				[60], [83], [84]
Heart Failure						
miR-25	Capacity to alter calcium handling repressing SERCA2A		Neonatal rat ventricular cardiomyocytes	Left ventricular samples	HEK293	[85]
miR-24	Target JP2	Mouse and rat bioinformatically		Bioinformatically	HEK293	[47]
miR-24	Up-regulation	Knocking down JP2 mouse	ventricular cardiomyocytes from rat aortic stenosis model			[47]
miR-19b	Target CX43	Transgenic mouse			human iPSC-CMs	[86] [87]
Atrial fibrillation						
miR-223 miR-664	Up-regulated	Canine model of AF				[44]
miR-101 miR-320 miR-499	Down-regulated	Canine model of AF				[44]
miR-328	Target CACNA1C and CACNB1	Canine model of AF, transgenic mouse	Canine atrium samples	Human atrial samples		[44]
Dilated cardiomyopathy						
miR-133a-1 miR-133a-2	HF development	miR-133a-1 / miR-133a-2 KO				[88]

Long QT syndrome						
miR-1/-133	Role in adrenergic control No up-regulation of CX43, IRX5, Cav1.2	miR-1/-133 KO mouse				[55]
miR-1-2	Up-regulation IRX5	miR-1-2 KO mouse				[89]
miR-133a	Prolongation of QT	Ventricle isolated myocytes from mouse and in miR-1/-133 KO mouse				[36]
miR-1	Targets Cv1.2	Rat yes Mouse no			Yes	[55]
miR-134 miR-103a-1 miR-143 miR-3619	miRNAs related to hERG channels regulation				U2OS and HEK293T cells	[54]
Cardiac aging						
miR-34a	Up-regulation of mir-34a in old hearts	Young and old mouse models		Human atrium biopsies		[60]
miR-17-92 cluster	Role in cardiac remodelling	Aged-related HF mice				[57]
miR-17-3p	Senescence and apoptosis	Mouse model	Mouse cardiac fibroblast Mouse samples TAC-induced pathological hypertrophy	Left ventricular tissue DCM patients		[61] [90]

5.1.1.1. *In vitro* and *in vivo* models of CH

During hypertrophic processes, there are several signalling pathways, genes, and proteins involved, as described in section 3.2.1.1. Therefore, genetic engineering to modulate the expression of pro- and/or anti-hypertrophic genes can be used to model this pathologic condition. Furthermore, it is also possible to use chemical compounds to induce hypertrophy in cells and in animal models such as endothelin-1, angiotensin II and leptin [22].

KO mice have been widely used in loss-of-function assays. For instance, **miR-22 KO mouse** served to demonstrate its role in the homeostasis of calcium and in the alteration of myofibrillary proteins amount under stress conditions [68]. In this same model, reduced *SERCA2A* expression was observed in cardiomyocytes [68]. Tu Y et al. demonstrated the pro-hypertrophic role of miR-22. In fact, its upregulation in mouse cardiomyocyte cultures induced hypertrophy *in vitro* [69]. Huang et al. also studied the role of this miRNA *in vitro* with cardiomyocyte cultures from neonatal rats and *in vivo* with miR-22 KO mouse model. After their studies, they determined the importance of this miRNA in cardiac remodelling and cardiomyocyte hypertrophy under stress conditions [70]. Although miR-22 is known to be an evolutionarily highly conserved microRNA from simple animals to humans, since the seed

sequence remain identical between species [71], all the previously mentioned reports were carried out in mouse models. It remains unclear its relevance in humans.

Càre A et al. studied the control that **miR-133** and **miR-1** exert on cardiac hypertrophy in human heart patient samples and in murine models. The **expression pattern discovered in animal models and in human patients were similar**. It was concluded that in both cases, miR-133 and miR-1 maintain an inverse correlation with myocardial hypertrophy [30] .

Aggarwal et al. studied the expression of miRNAs in an *in vitro* model of cardiac hypertrophy based on **cardiomyocytes derived from induced pluripotent stem cells (iPSC-CMs)**. Compared to the control model, 250 mature miRNAs with differential expression were observed. Among them **miR-208a-3p, miR-23a-3p, miR-22-3p** were found up-regulated which is consistent with previous studies where these miRNAs have been related to hypertrophic processes in animal models [23], [26], [69], [72], [73].

The role of **miR-29** in HCM has also been studied by Liu et al. The main goal of this study was to evaluate the expression of this miRNA and pro-fibrotic genes in mouse and human models of HCM. On the one hand, it was suggested that the induction of *ET1* signalling in cardiac myocytes leads to an augment of ROS and secretion of TGF-β. As previously mentioned, *TGF-β* inhibits miR-29a and subsequently, the induction of fibrotic processes takes place. On the other hand, it has been observed that there are **differences in the expression of miR-29 gene and anti-hypertrophic/fibrotic pathways in mouse versus human HCM** [74].

Banal et al. studied different miRNAs in a mouse model of HCM, concluding that down-regulation of **miR-1 and miR-133a** began in pre-disease stages in a way that precede the upregulation of genes involved in the cardiac remodelling. During the initial stages of the disease, up-regulation of **miR-214 and miR-132** was confirmed and finally, in late stages, downregulation of **miR-133 and miR-30** was observed, which may also contribute to the development of cardiac fibrosis in mouse [91].

5.1.1.2. *In vitro* and *in vivo* models of cardiac fibrosis and MI

There are two methods to generate MI animal models. On one hand, a complete or partial narrowing of the coronary artery which usually leads to the development of ischemia, and on the other hand, surgical procedure as LAD (left anterior descending artery) ligation, cryo-injury or balloon occlusion. Moreover, drugs such as isoproterenol or Adriamycin can be used [22]. As previously mentioned, fibrotic processes are one of the most important events characterized after MI.

Chen et al. used **LAD ligation in a miR-132 KO mice** in order to study the role of this miRNA in myocardial remodelling. Their observations showed that miR-132 is up-regulated after MI and promotes tissue remodelling since it can modulate the scar size and the cardiac function [75]. LAD ligation is also use by Gu et al. to study the effect of **miR-21** in a murine model concluding that this miRNA has a cardioprotective role in MI [76].

Mo et al. simulated the hypoxia-induced apoptosis after a MI in human **iPSC-CMs** and studied the role of **miR-30e-5p**. This study demonstrated that miR-30e-5p overexpression mitigates hypoxia-induced apoptosis through Bim downregulation and rescues hypoxia-induced calcium handling defect [77].

Van Rooji E. et al. studied the miRNAs expressed after MI in the infarcted area, where fibrosis is abundant, and non-infarcted border area doing a miRNA profiling in response to MI. They used a **MI mouse model** and **heart tissue** from the infarcted border area of **patients** who had received a heart transplant. They could confirm that the dysregulated miRNAs in the murine MI model are regulated similarly in human hearts. In this way, up-regulation of **miR-21, miR-214 and miR-223** is observed at the edge of the infarcted zone, while **miR-29b and miR-149** were significantly downregulated in this zone after MI [34].

miR-1-2 was upregulated in the expression profile study in human **iPSC-CMs** carried out by Aggarwal et al. although this fact does not agree with previous rat models where this miRNA had been determined to be attenuated during fibrotic processes [73], [78].

Duisters et al. studied the role of **miR-133 and miR-30c** as regulators of *CTGF* (which induces ECM synthesis) and their influence on fibrosis processes and myocardial matrix remodelling *in vivo* and *in vitro*. In this study the authors used **rat animal models**, cardiac **biopsies of patients** with aortic valve stenosis (which causes ventricular hypertrophy) and **myocytes and fibroblasts obtained from rat ventricles**. They revealed that the miRNA-mRNA predicted binding sites are highly conserved in a wide range of species including human, suggesting the potential importance of these binding sites in *CTGF*. Finally, the expression of these two miRNAs showed to be inversely proportional to the amount of *CTGF* mRNA and protein in rat and human models and therefore inversely proportional to fibrosis [35].

Wang et al. demonstrated the relationship of **miR-24** and cardiac fibrosis after MI using a **mouse model** and **primary cardiac fibroblasts** from mouse. They observed that miR-24 was down-regulated and levels of Furin and TGF-β were reduced increased MI, which could improve cardiac function and may reduce fibroblast differentiation in the area of the infarct border [79]. These same results were obtained by Chen et al. who used **adult rat models** [80]. Yet, the role of miR-24 in a human system in relation to cardiac fibrosis has not been investigated.

miR-144-3p was also related to the enhancement of cardiac fibrosis after MI. This miRNA had been shown to be associated with MI in humans [81]. Subsequently, Yuan et al. corroborated this fact using a **pig MI model**. This miRNA was observed to be highly expressed in the infarcted area in MI pigs compared to control pigs. It was also observed that deletion of miR-144-3p *in vitro* could lead to up-regulation of ECM-related genes (*α-SMA*, *Col1A1*, and *Col3A1*) and promote proliferation and migration in human cardiac fibroblasts, playing an important role in remodelling and cardiac fibrosis. Lastly, *PTEN* was identified as a target of **miR-144-3p** thus promoting the proliferation, migration and synthesis of collagen in human cardiac fibroblasts [82]. Previously, Qu et al. observed that **knocking down the expression of *PTEN*** up-regulates the mRNA levels of *COL1A1* and *COL3A1* in mouse cardiac fibroblasts [92].

Finally, an aged **miR-34 KO mouse** (73-83 weeks old miR-34$^{-/-}$) allowed to study its role in fibrosis during aging. Importantly, aging aggravates cardiac dysfunction after MI, therefore the role of miR-34a in a **mouse model of MI** was also studied. Two weeks after MI, a significant improvement was seen in mice in which miR-34a levels had been reduced by using **anti-miR-34a**. The study of miR-34 in angiogenesis and neovascularization *in vivo* and *in vitro*, revealed that its inhibition exerts positive effects on endothelial cells [60]. Yang et al. studied the role of miR-34a in cardiac repair and regeneration after MI in neonatal and adult rat hearts. These authors finally demonstrated the regulatory role of this miRNA in cardiac regeneration through

action on other of its targets: *Sirt1*, *CyclinD1* and *Bcl2*. [84]. Subsequently, Shao et al. also corroborated the protective role of miR-34a in myocardial cells against ischemia-reperfusion injury. In this study, an *in vivo* model was constructed with cardiomyocytes from neonatal rats. Finally, reduced expression was observed after reperfusion of myocardial cells and heart tissues from neonatal rats. miR-34a reduces myocardial ischemia/reperfusion injury due to decreased TNFα expression [83]. In human, the contribution of mir-34 to MI needs to be studied.

5.1.1.3. *In vitro* and *in vivo* models of acute HF

Different methods for the generation of animal models of acute HF can be used such as occlusion (partial or complete) of coronary artery branches, Coronary Artery Ligation, Coronary Artery Embolization and Viral myocarditis [22]. Genetic engineering also can be used to modelling HF.

Wahlquist et al. used neonatal rat ventricular cardiomyocytes, cell cultures (Hek293 cells) and left ventricular samples from human patients in order to demonstrate the role **miR-25** (up-regulated in HF) repressing *SERCA2A* and consequently, altering calcium handling *in vivo and in vitro* [85].

Xu M. et al. studied the regulation that **miR-24** does on *JP2*. Firstly, the potential binding sites of miR-24 to the 3´UTR region of the *JP2* mRNA in mouse, rat and human were predicted bioinformatically and later the interaction was tested in HEK293 cells. The results were similar in rat, mouse and human sequences concluding that the role of miR-24 targeting JP2 have been maintained during evolution. Its up-regulation was demonstrated in ventricular cardiomyocytes derived from a **rat aortic stenosis model** and their role in HF was modelled **knocking down *JP2*** [47].

Lin et al. studied the role of **miR-19b** *in vitro* in viral myocarditis, a life-threatening disease that leads to HF or arrhythmia, using human iPSC-CMs. In this study, it was demonstrated that *CX43* expression levels decrease due to miR-19b and this loss leads to abnormal heart rhythms [86]. This was consistent with another study carried out by Danielson et al. in **transgenic mice** [87]. Therefore, this miRNA is associated with arrhythmogenesis and HF even in human.

5.1.1.4. *In vitro* and *in vivo* of AF

AF in humans is characterized by slow, multifactorial, heterogeneous development of the disease. For this reason, animal models can only partially represent the pathophysiology of AF. Even so, it has been seen that the response to this disease is similar in humans and animals [93].

A canine model of AF established by right atrial tachypacing was used to study the role of miRNAs in this pathology by Lu et al. Significantly up-regulated (**miR-223, miR-328, miR-664**) and down-regulated (**miR-101, miR-320 and miR-499**) miRNAs were identified. Specifically, the greatest change in expression was seen for miR-328. Its endogenous expression in the heart and up-regulation were verified by Northern blot analysis. Later, this fact was verified in humans with AF using **human atrial samples** from patients with rheumatic heart disease. *In vivo* experiments where pre-miR-328 was overexpressed in **dogs** using **adenoviral vectors** showed an increased vulnerability of the animals to develop AF. On the other hand, they also developed a pre-miR-328 transgenic mouse model carrying the cardiac-specific α-MHC promoter in **mice**. As expected, mice develop spontaneous AF. They also demonstrated that by downregulating this miRNA the vulnerability to develop AF was reduced. Besides, *CACNA1C* and *CACNB1* were found as targets of miR-328. Experimentally, the overexpression of this miRNA in canine atrium

and in transgenic mice increased the vulnerability to AF and showed the characteristic phenotype of this disease in animals [44].

5.1.1.5. *In vitro* and *in vivo* models of DCM

There are different methods to establish DCM animal models: immunization model, viral infection model or genetic abnormality models. Surgical intervention is also possible to carry out coronary artery occlusion or Cryo-injury to generate MI or ischemia / reperfusion, which after the infarction will lead to a DCM phenotype [22].

In fact, the role of **miR-133a** by Bagnall et al. in HCM mouse model (see section 4.1.1.1.), is in line with what was shown by Liu N et al. This group carried out the study of miR-133a in the regulation of cardiomyocyte proliferation and suppression of gene expression in smooth muscle. To do this, they constructed single KO mice (**miR-133a-1 KO** or **miR-133a-2 KO**) and double KO mouse (**miR-133a-1/miR-133a-2 KO**) models. Single mutants had a wild-type phenotype, but double mutants that managed to survive until adulthood, developed DCM and HF. These pathological phenotypes were related to the aberrant expression of some genes and abnormal cardiomyocyte proliferation. These abnormalities were attributed, in part, to elevated expression levels of *SRF* and *CyclinD2*, targets of miR-133a [88].

5.1.1.6. *In vitro* and *in vivo* models of LQTS

Despite the high conservation of ion channels between humans and mouse, there are several electrophysiological differences. Using genetic engineering can be established a LQTS phenotype in mouse. The generation of a KO or transgenic mouse model is the tool more extensively used [94]. Another possibility is the use of iPSC to model LQTS *in vitro*.

miR-1 and **miR-133** are the most studied miRNAs involved in LQTS. Besser J. et al carried out the study of miRNA related to the adrenergic control of cardiac repolarization in mouse models. Specifically, they studied the miR-1/133a cluster. They found cell-specific expression since these miRNAs only were detected in cardiomyocytes. The deletion of this cluster led to the development of the LQTS phenotype in cardiomyocytes obtained from the **KO mouse**, although a single target responsible for the modulation of adrenergic signalling was not found in their models [55].

Zhao Y et al. in 2007 studied their influence in cardiac conduction in **miR-1-2 KO** mice model. These mice exhibited abnormalities in the propagation of cardiac electrical activity despite normal heart anatomy and function. In addition, the target gene of miR-1-2, *IRX5*, was studied, observing that in these KO mice there was an increase in *IRX5* mRNA levels that led to downregulation of *KCND2* [89]. Contrary, Besser et al. do not observe a significant up-regulation of *CX43, IRX5* and Cav1.2 in their miR-1/133a KO mouse. In fact, it has been previously described that miR-1 targets Cv1.2. in rat and humans, however the miR-1 target sites are not conserved in the 3´UTR in mouse [55].

Malkovich et al. determined that elevated levels of miR-133a led to a significant prolongation of the action potential in ventricle isolated myocytes and caused QT prolongation in mouse [36]. From a genetic point of view, Hedley et al. hypothesized that mutations in miR-1 and miR-133a could explain the LQTS phenotype. Through the alignment of DNA sequence of miR-1 and miR-133a from LQTS patients, allelic variations in these sequences were found. Although it was determined that these variations do not affect to mature miRNAs and are not responsible for

the development of this syndrome in this cohort. This was the first **study of genetic variations in miRNA** in LQTS patients [95].

On the other hand, as previously mentioned (see section 3.2.1.7), impairments in *hERG* gene function were considered the mechanism that causes this syndrome. Because of this, Lian J et al. studied the modulation of these genes by miRNA in **HEK293T or U2OS cells**. Finally, it was concluded that there are several miRNAs that regulate the expression of hERG channels such as **miR-134, miR-103a-1, miR-143 and miR-3619**. In fact, this authors emphasize the need to verify this in animal models [54].

Some authors propose the use of **patient-specific iPSC-CMs as LQTS model**. For example, Itzhaki et al. obtained iPSC-CMs from a LQT2 patient. Using patch-clamp and extracellular multielectrode recordings, it has been observed that the patient missense mutation in *KCNH2* leads to an elongation of action potential in iPSC-CMs compared with controls. Besides, this cells also showed arrhytmogenic features [96]. Despite human iPSC-CMs have not been used to study the miRNA contribution miR-1 and miR-133 to LQTS, it would be interesting in further studies.

5.1.1.7. *In vitro* and *in vivo* models of aging

Animal models to study aging include those of **natural aging**, **progeroid syndromes** (like the BubR1 progeroid mice [97]) or induced aging like **D-galactose aged mice** [98].

Boon et al. studied the aging process in young (6-8 weeks) and old (18-20 months old) mice models. First, differences in fibrosis and hypertrophic processes, telomeres shortening and cardiomyocyte apoptosis was observed. The old mice present more fibrosis and hypertrophy, shorter telomeres and more cardiomyocyte mortality. In this way, they observed miRNAs differentially expressed comparing young and old mice. Specifically, the **miR-34 family**, consisting of miR-34a, miR-34b and miR-34c, was significantly up-regulated in hearts of aged mice. In fact, the analysis of miR-34a expression in human **atrium biopsies of aged patients** revealed that this miRNA was also overexpressed in humans [60]

The **miR-17-92** cluster has been linked to cardiac aging. Some miRNAs in this cluster may be down-regulated. For example, van Almen et al. demonstrated changes in the expression of this cluster in a model of age-related heart failure in **mice**. From all members of this group, **miR-18a, miR-19a** and **miR-19b** were the most strongly repressed and were associated with cardiac remodelling. In fact, its up-regulation in aged failure-protected heart leads to *TSP-1* and *CTGF* relief to dampen the fibrotic remodelling [57].

On the other hand, Du et al. demonstrated the role of **miR-17-3p** in senescence and apoptosis in the heart in a transgenic expression of this miRNA in **mouse model** and in a **cellular model** of mouse cardiac fibroblasts [61]. Subsequently, Shi et al. studied the expression of this miRNA in **human samples with DCM** and in **mouse** samples with TAC-induced pathological hypertrophy concluding that its overexpression promoted a decrease in cardiomyocyte apoptosis *in vitro* and *in vivo* [90].

The implication of miRNAs in aging of the human heart has to be further studied to understand how these regulators can modulate it.

6. Perspectives of miRNA therapeutics in cardiac diseases

6.1. Use of miRNAs as biomarkers

Although miRNAs are located mainly in the cytoplasm, their presence into the circulatory system has been confirmed, as well as in most body fluids such as saliva or urine, due to cellular lysis, apoptotic bodies or active secretion. Therefore, they could be detected in these fluids and serve as **biomarkers** for the **prognosis** and **diagnosis** of different diseases, including CVDs [2], [13]. It is possible to differentiate several types of extracellular/circulating miRNAs: (1) miRNAs located in **microvesicles**, exosomes or apoptotic bodies; (2) **protein-associated** miRNAs, mainly associated with AGO2, RNA-binding proteins like Npm1 or HDL (3) **free** circulating miRNAs [11], [13], [99], [100].

Their association with proteins or their encapsulation in vesicles, increases stability and resistance to degradation by RNAases in extracellular environments circulating miRNAs [28], [101]. The advantages of their use as biomarkers are diverse: great stability, great sensitivity for detection and the possibility of multiplexing analysis to increase specificity. However, there are limitations for their use as biomarkers such as the lack of normalization of its quantification as compared with tissue miRNA and the lack of consistent and robust protocols for pre-analytical sample handling, miRNA extraction and measurement. These limitations need to be considered to obtain reliable results [2], [11]. Some examples of CVD biomarkers are shown in Table 10.

Table 10. *Examples of CVDs biomarkers in different fluids and cells. Up-regulated miRNAs are marked in green, down-regulated miRNAs are marked in red. Plas: plasma, EPC: Endothelial progenitor cells, Plat: platelets, S: Serum, WB: Whole blood.*

Disease	miRNA	Fluid/ Cell	References	Disease	miRNA	Fluid/ Cell	References
HF	miR-106a-5p	Plas	[102]	**AMI**	miR-1	Plas	[103]
	miR-126	EPC	[46], [104]		miR-133a	Plas	[105]
	miR-18a-5p	Plas	[102]		miR-29b	Plas	[103]
	miR-199a-3p	Plas	[102]		miR-499	S	[106]
	miR-26b-5p	Plas	[102]		miR-132-5p	Plas	[107]
	miR-27a-3p	Plas	[102]	**DCM**	miR-646	S	[108]
	miR-30e-5p	Plas	[102]		miR-639	S	[108]
	miR-508-5p	EPC	[104]		miR-155	S	[108]
	miR-652-3p	Plas	[102]		miR-636	S	[108]
Arrhythmias	miR-21	S	[109]		miR-548c	WB	[110]
	miR-328	Plas	[44]	**HCM**	miR-29a	S	[111]
	miR-150	Plat	[112]		miR-155	S	[111]

For example, Wei et al. performed a study in patients with AF and / or HF to examine the role of **miR-126**. Blood samples were collected and in both diseases, downregulation of this miRNA was found in AF or HF patients compared to controls [46]. Another example is miR-499. **miR-499** serum levels are up-regulated in MI and involved in mitochondrial fission and subsequently,

cardiomyocyte apoptosis in a mouse model of I/R. This miRNA is downregulated by p53 in this model and repress CaN which participate in the CaN/Drp1 apoptosis pathway. These results also have been observed in infarcted human hearts using samples of left ventricle of patients with ischemic MI. So miR-499 exerts a cardioprotective effect after AMI and could be used as biomarker [1], [106], [113].

6.2. Use of miRNAs in specific therapy

Many miRNAs are currently under study to be used as therapies. miRNA expression can be modulated *in vivo* by different strategies including antisense technology or gene therapy. Also, by using antagomiRs, miRNA sponges, target occupiers and "erasers" technology is possible to reduce pathogenic or aberrantly expressed miRNAs and even module the miRNA's target expression levels. In order to increase miRNA expression, miRNA mimics technology is used. To sum up, since most of miRNAs negatively regulate their targets, the biological effect of a miRNA inhibitor is the activation of its target. On the contrary, the final effect of a miRNA mimic is the suppression of gene expression [6].

6.2.1. Antisense miRNA oligonucleotides (AntagomiRs)

Chemically modified single-stranded **RCOs** (reverse complement oligonucleotides) can bind to the mature miRNA within the RISC complex and act as a competitive inhibitor. They can also bind to the pre-miRNA and prevent its processing or entry into the RISC complex. Finally, it can also interfere nuclear exportation of the pre or pri-miRNA. Another **antimiRs** approaches are the use of **2´-MOE** (2´-O-methoxyethyl phosphorothionate modification), in which antagomiR is conjugated to cholesterol in order to facilitate the uptake, and **LNA** (locked nucleic acids) based antimiRs to be directed against the seed region of a miRNA to target multiple miRNA family members (8 nt) or to be directed against single miRNAs (15 nt) [5], [6].

6.2.2. miRNA mimics

This tool consists on the restoration of the effective concentration of a miRNA with a synthetic dsRNA. One of the strands is identical to the native miRNA (guide chain) and the other strand is complementary or partially complementary to this chain (passenger chain). This bicaternary structure is necessary to reach an efficient recognition, Dicer cutting and loading of the guide chain in the RISC complex [6]. Moreover, backbone and ribose modifications in dsRNA are required to raise the stability *in vivo* and improve pharmacodynamics [18].

6.2.3. Sponging, target occupiers and erasers

Some strategies have been tested to decrease miRNA expression. One way to interfere with miRNA function is to remove the miRNA and thereby prevent it from binding to their mRNA targets. An approach to reduce miRNA levels in mammal cells, called "**miRNA sponges**" was reported in 2007 [114]. In this technique, a series of perfectly or imperfectly paired binding sites for a specific miRNA, which serve as competitive inhibitors, are introduced into an expression cassette in the 3´UTR of a reporter gene. This effectively reduces the concentration of the programmed RISC available for binding to its native targets, and thus alleviates the inhibitory effect of specific miRNA on mRNA targets [114]. Rather than inhibiting individual miRNA, this approach is effective in inhibiting miRNA families[6].

Another way to interfere with miRNA function is using **targets occupiers**. For this, an oligonucleotide with perfect complementarity to the miRNA target sequence will mask it,

preventing miRNA's binding. This approach provide a great specificity since targets the mRNA. If it would target the miRNA, this would influence several mRNA targets [6].

A third strategy to inhibit miRNA function is called **erasers**. In this case, the endogenous miRNA inhibition is carried out with the expression of tandem repeat of a sequence perfectly complementary to the target miRNAs [6] that acts as a competitor to the MRE in mRNA.

7. Materials and methods

7.1. Selection of Genes of interest (GOI) and sequence analysis

Targets of the BIO-AGEmiRNA (Figure A1) were chosen based on their functional relevance and/or involvement in CVDs that indicated a direct contribution in cardiac function. For such selection, bibliographic databases such as Pubmed, Uniprot, GeneCards, Web of Science and OMIM were used.

The GOI sequences were obtained from the databases Nucleotide and Ensembl. Later, the identification of the gene regions where the interaction with different miRNAs takes place was obtained from miRWalk 2.0 database. The sequences were analysed with the Ape software to establish the cloning regions taking into account the predicted zones of interaction with the miRNAs (Table A1).

7.2. Bacterial strains and culture media, cloning vectors and miRNAs

The DH5α strain of *Escherichia coli* chemically competent were already available in the laboratory.

Luria Bertani (LB) medium (MP Biomedicals, 3002022) supplemented with 100µg/mL of the antibiotic ampicillin (Sigma Aldrich, A9518-5G) was used to culture bacteria in liquid medium. These were incubated at 37°C O/N (overnight) shaking. LB-agar medium (Nzytech, MB11801) was used for bacterial colony growth, supplemented also with 100µg/mL of ampicillin. Incubation was carried out at 37°C O/N.

The fluorescence reporter vector pmirFLUO MCS (V36) (Figure A2) was built during this Master Thesis (Table A2). V36 was built as follows: *Renill*a luciferase was replaced by *GFP* (from GFP-expressing vector, V2, Figure A3) and Firefly luciferase (Fluc) is replaced by *tdT* (from tdT expressing vector, V3, Figure A4) (Table A.2). Once this vector was generated, *ADRA1A* and *SERCA2A* were cloned from the pmirGLO vectors into the pmirFLUO vector.

The luciferase reporter vector pmir-GLO MCS (V19) (available in the laboratory) (Figure A5) contains an ampicillin resistance gene (Amp^r), MCS and two constitutively expressing luciferases: *Renilla,* under the control of the SV40 virus promoter and *Firefly* (*Fluc*), under the control of the PGK promoter. The latter is co-transcribed with the GOI cloned downstream, separated by the autocatalytic peptide (P2A) and the 3 x FLAG tag. This tag will be interesting for future experiments where the study of protein expression will be carried out. The luciferase reporter vectors for GOIs *ADRA1A, MYBPC3, ACTN2, CASQ2, DSP, SERCA2A (ATP2A2), POPDC2, DMD, TMOD1* (Table A.2) were already available in the laboratory.

The BIO-AGEmiRNAs used in this work were miR-3916, miR-24-2-5p and miR-24-2-3p (Riboxx, M-00202-0002).

7.3. Retrotranscription of RNA to cDNA

Two hundred nanograms of RNA from human left ventricle myocardium already available in the laboratory were retrotranscribed to cDNA using qScript Flex cDNA Kit (Quanta Biosciences, 95049-100). cDNA was diluted 1/10 in RNAase-free water and stored at -20°C.

7.4. Polymerase chain reaction (PCR)

The Pearl Primer program was used to carry out the PCR primers design. This program allows calculate the melting temperature (Tm) and detect the possibility of unwanted amplifiable dimers of primers.

A high fidelity Taq DNA polymerase (PhusionTM High-Fidelity DNA Polymerase, Thermo fisher) was used following the protocol and PCR cycle recommended by the company taking into account each particular GOI to establish the elongation time and annealing temperature (Table A3).

To carry out the construction of the pmirFLUO MCS vector, the genes coding for *GFP* and t*dT* were amplified by PCR from vectors V2 and V3 respectively. Finally, PCR was also performed using cDNA as template in order to amplify 5 GOI: *KCNH2, KCND3, CAMK2D, CACNA1C* and *KCNQ1*. Subsequently, the amplified fragments were purified as described in section 7.6.

7.5. Electrophoresis

1% agarose gels in 50 or 100 mL of 0.5X TBE buffer (50mM Tris, 45mM Boric Acid and 0.5mM EDTA) (Thermo Fisher, 15581044) were used. The gels were stained with Gel Safe Green 1:100 (NZYTECH). Samples were loaded onto the gel using loading buffer, except when Red Anza Buffer (enzymatic digestion) or NZYtaq II 5X gel load reaction buffer (colony PCR) were used. 1Kb Plus DNA Ladder (Invitrogen, 10787-018) was used as the molecular weight marker. The visualization of the electrophoresis results was carried out using the iBright CL1000 (Invitrogen).

7.6. Nucleic acids purification

After the analysis of the electrophoresis results, the gel band corresponding to the appropriate molecular weight was excised for purification. Nucleic acids were also purified directly from the PCR product whenever possible. For both procedures, NZYGelPure (NZYtech, MB01102) was used, following the steps recommended by the manufacturer and eluting in elution buffer provided by the kit.

Extraction and purification of vectors was performed using the commercial NZYSpeedy Miniprep Kit (NZYtech, MB21002).

7.7. Cloning

7.7.1. Digestion

PCR fragments and vectors were digested for at 37°C for 4-6 hours and then at 80°C for 20 minutes for enzymatic inactivation. The digestion products were purified as explained in section 7.6. All the restriction enzymes used are shown in Table A4.

7.7.2. Ligation

Vector and insert concentration were quantified in nano spectrophotometer (Implen). The ligation mix was prepared containing 1:3 molecular ratio (vector:insert) using 25 ng of digested

vector and T4 DNA ligase (NEB, 0202). In addition, a negative ligation control (vector without insert) was included as religation control. The ligation cycle consists on an incubation of 22°C for 2 hours or at 16°C O/N and subsequent inactivation at 65°C for 10 minutes.

7.8. Transformation of DH5α Competent Strains

DH5α bacteria (stored at -80°C) were thawed on ice. 25µL of bacteria were incubated on ice with 2µL of the ligation reactions for 30 minutes, then a heat shock was performed at 37°C for one minute and then chilled on ice for 5 min. 500 µL of SOC medium was added and incubated at 37°C and 250 rpm for one hour. Finally, 200 µL of the culture were seeded in LB-agar plates with 100µg/mL of ampicillin and incubated at 37°C O/N.

After this period, the recombinant colonies were counted. In the case of a large number of colonies in the negative control plate, colony PCR was performed to confirm correct insert introduction. If there were no colonies in the negative control, the bacterial culture and enzymatic digestion were carried out directly for subsequent sequencing.

7.9. Screening of recombinant colonies

7.9.1. PCR colony

Bacterial lysates were prepared as follows: selected colonies were replicated in a fresh LB-Agar plate and then immersed in 12µL of H_2O.

For colony PCR, NZYTaq II DNA Polymerase (Nzytech, MB354) and specific primers (Table A3) were used. Five µl of the bacterial lysates were used as template and PCR performed following manufacturer's recommendations. PCR products were run on agarose gels for verification.

Then, colonies positive in the PCR were grown O/N at 37°C at 250rpm in 5mL of LB-Ampicillin. Purified plasmids were obtained using the commercial Purelink HiPure Plasmid Minikit kit (Invitrogen, K210004) and following manufacturer's recommendations. Plasmid was eluted in 50 µl of elution buffer provided by the kit.

7.9.2. Enzymatic digestion

After measuring the concentration of each vector using the nano spectrophotometer (Implen), enzymatic digestion was carried out. The digestion mix contains the selected restriction enzyme and the plasmid (approximately 300 ng) (Tables A2 and A4). The digestion cycle consists on a first incubation stage at 37°C for 2 hours followed by an enzyme inactivation stage at 80°C for 20 minutes.

7.10. Sequencing

Restriction digested confirmed clones were verified by sequencing by the Sanger method (STAB VIDA). For sequencing, two primers were selected to cover the whole recombined fragment (Table A3).Sequence were analysed using the Contig Analysis software (Invitrogen).

7.11. Cellular culture

The amplified plasmids as described in section 7.6 were transfected into Human Embryonic Kidney 293 cells (HEK293 cells). The culture medium used is composed of MEM (Biowest, S18240L0093), 10% FBS (Thermo Fisher, 10270098) and 1% penicillin and streptomycin (100µg/mL) (Thermo Fisher, 15140122). The cells were incubated at 37°C and 5% CO_2.

7.12. Transfection

HEK293 cells were seeded in a 96-well plate (20.000 cells per well). After 24 hours, cells were transfected. To do so, 2 tubes (tube A and tube B) were used for each well. In tube A, 0.3µL of Lipofectamine 2000 Reagent (Invitrogen, 11668027) and 5µL of Optimem (Termo Fisher, 31985062) were added. In the tube B, 100 ng of vector (final concentration in the well 1ng/ µL), 20ng of miRNA to be tested (final concentration in the well 20ng/µL) "CONmiR mimic target hsa-miR-3916" (Riboxx, M-00202-0002) and 4µL of Optimem were added. The contents of tube B were then poured onto tube A. The mix was incubated for 5 minutes at room temperature. Finally, 10µL of this mixture was added per well. The amounts of reactive shown are calculated for one well, however, at least technical triplicates were included in each experiment.

The plasmids and miRNAs used for co-transfection are listed in Table A5. As a negative interaction control, a commercial miRNA "CONmiR mimic negative control-N1" (N1) (Riboxx, K-01000) was used, which does not interact with any known sequence.

The transfection was controlled by fluorescence microscopy using Nikon TS2 microscopy.

7.13. Fluorescence Reporter Assay

The fluorescence reporter assay (DualFluo Assay) (Figure A6) was performed 48 hours post-transfection. It was carried out as follows: cells were first washed with 100 µL of PBS and then 100 µL of Triton X-100 1 % (PanReac, 142314.1611) diluted in PBS was added in order to lyse the transfected cells. Then, 100 µL of each well were transferred to a black-wall plate (Corning 3631) and centrifuged at 500 x g for 5 minutes after measurement. The measurement was performed in Biotek Synergy HT plate reader. Filters used were, for *GFP* fluorescence measurement, 485/20 excitation and 530/25 emission wavelengths. Fluorescence measurement of *tdT* were performed at an excitation 560/20 and 645/40 emission wavelength. Suboptimal emission filters were used for *tdT* due to technical problems of the instrument (Figure 6).

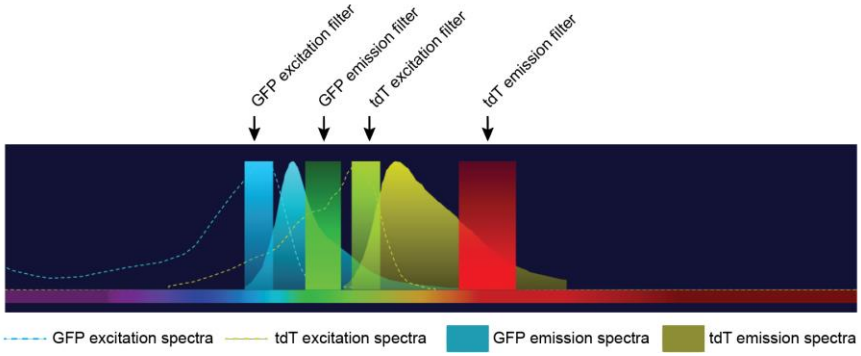

Figure 6. *Filters used to measure tdT and GFP fluorescence. Image obtained from Fluorescence SprectraViewer, ThermoFisher.*

For data analysis, its normalization was carried out by calculating the ratios of *GFP* and *tdT* readings, to later relativize each condition to the condition transfected with the non-targeting miRNA (N1) (100% of the activity). Each condition was performed at least in triplicate.

7.14. Luciferase Reporter Assay

The assay was performed 24h post transfection by using the Dual-Glo Luciferase Assay Kit (Promega, E2940) (Figure A7) following the manufacturer's recommendations with slight

modifications. First, medium was removed from the transfected cells to wash with 50µL of PBS and 50µL of fresh complete medium were added. Next, 50 µL of the reagent was added with the *Fluc* luciferase substrate and centrifugation was carried out at 500 x g for 5 minutes. After 10 minutes of incubation at room temperature, the luminescence was measured on the Biotek Synergy HT plate reader. Once the luminescence of the first substrate was measured, a 1:100 mix of the reagent with *Renilla* substrate and an inhibitor of the first reaction was prepared. 50 µL of this mix were added per well, centrifuged again at 500 x g for 5 minutes and after 10 minutes at room temperature the second luminescence measurement was carried out.

For the data analysis, normalization was carried out by calculating the ratios of *Fluc* and *Renilla* signal, to later relativize each condition to the condition transfected with the non-targeting miRNA (N1) (100% of the activity). Each condition was performed in triplicate.

8. Results

8.1. Construction of pmirFLUO MCS vector for cloning GOIs

pmirFLUO MCS vector was built by modifying the vector pmirGLO MCS (V19) (Figure A8). At the start of this work, V19 had already been modified to replace *Renilla* by *Green Fluorescent Protein* (*GFP)*. The reporter gene *tandem dimer Tomato (tdT)* was amplified by PCR using primers in Table A3. The V19-*GFP* vector and the purified PCR fragment of *tdT* were digested with the restriction enzymes SpeI and SacII. The vector backbone was gel sliced and purified along with the digested *tdT* PCR fragment. The ligation of this fragment with the plasmid and transformation were carried out. A total of 38 positive colonies were obtained, but also 13 colonies corresponding to the negative control for ligation were observed. Therefore, colony PCR was performed to screen possible recombinant colonies (primers used in Table A3). Colonies in which the *tdT* fragment was recombined, the amplified fragment was 1.803 kb (Figure 7). This screening allowed the selection of a number of positive colonies.

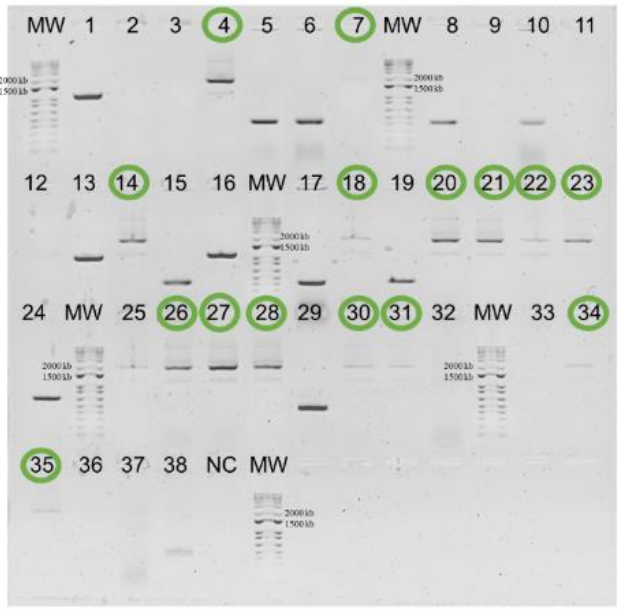

Figure 7. *Colony PCR result. The positive colonies for tdT introduction are highlighted with a green circle. NC: negative control.*

Later, 4mL overnight cultures of some of these colonies were established for the subsequent

purification of the plasmid and its verification by digestion with the restriction enzymes PstI and HindIII. Although all the selected colonies showed the correct restriction pattern (Figure 8), only two were selected for sequencing (colonies 14 and 26). The sequencing primers used are set out in the Table A3. The sequencing analysis revealed no mutations in the *tdT* sequence. The pmirFLUO MCS vector (V36) was then used for *in vitro* testing and as backbone vector to clone GOIs *ADRA1A* and *SERCA2A* from V19-based vectors available at the lab (Table A2).

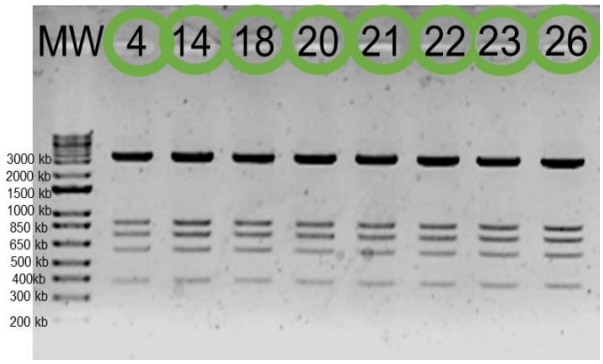

Figure 8. *Result of digestion with PstI and HindIII. MW: molecular weight.*

8.2. Cloning of the GOIs

8.2.1. GOIs obtention

RNAs were retrotranscribed to cDNA, obtaining the following samples c234 (R148), c235 (R150), c236 (R16), c237 (R55) and c354 (R55). Then, the amplification of the 5 GOIs was carried out using the Phusion Taq DNA polymerase (Primers used for PCR amplification included the target sequence of AscI (forward primers) and SalI or XbaI (reverse primers) (Table A3) for subsequent ligation in the backbone. GOIs amplification from some genes was only achieved in cDNAs from the RNA sample R55 (c237 and c354). Only for the *CAMK2D* and *KCNQ1* genes, amplification products of the correct size were observed (Figure 9). Many unsuccessful attempts to amplify the other GOI (*KCHN2, KCND3* and *CACNA1C*) were performed. Although different annealing temperatures or addition of DMSO to the PCR mix were tried, no amplification was achieved.

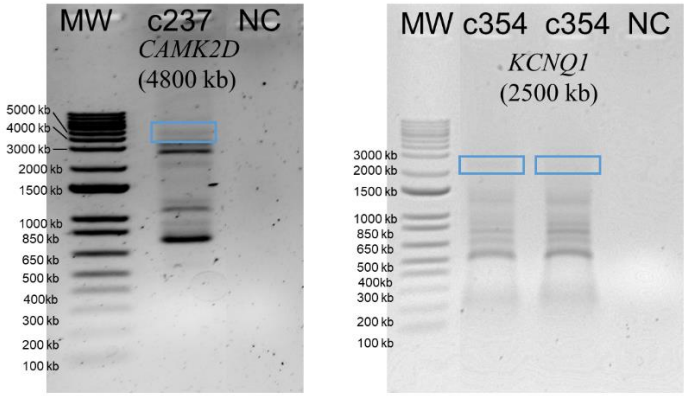

Figure 9. *PCR result of CAMK2D (A) and KCNQ1 (B). The bands indicated with the blue rectangle were excised from the agarose gel. MW: molecular weight, NC: negative control.*

On the other hand, the *SERCA2A* and *ADRA1A* were obtained by digestion with AscI-SalI, from V26, and AscI-XbaI from V21 (Figure 10).

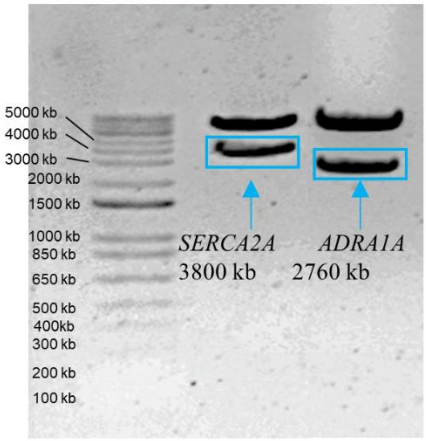

Figure 10. *Result of the digestion of V21 and V26 to obtain the ADRA1A and SERCA2A, respectively. The blue rectangle indicates the band that was sliced from the agarose gel.*

The destination vector, pmirFLUO MCS (V36), was double digested with the corresponding enzymes to ligate *CAMK2D, KCNQ1, SERCA2A* and *ADRA1A* products.

8.2.2. Identification of recombinant colonies and isolation of vectors of interest.

The number of ampicillin resistant colonies obtained from the transformation of each ligation are shown in the Table 11.

Table 21. *Ligation and transformation result for GOIs cloning in pmirFLUO MCS.*

GOI	*Positive control (V36+GOIs)*	*Negative control (only vector)*
SERCA2A	147	21
ADRA1A	3	0
KCNQ1	33	0
CAMK2D	>300	0

In the case of *SERCA2A* and *ADRA1A*, it was not necessary to perform colony PCR screening since no colonies were obtained in the ligation negative control (*ADRA1A*) or the proportion of positive colonies was notably higher with respect to negative colonies (SERCA2A). Therefore, 11 colonies of *SERCA2A* cloning and 3 colonies of *ADRA1A* cloning were grown in LB-Ampicillin. Plasmids of the bacterial cultures were analysed after digestion by determining the restriction patterns (Figure 11). Compared to the *in-silico* restriction patterns, a number of recombinant colonies were obtained for *ADRA1A* and *SERCA2A*. Negative colonies showed the restriction pattern corresponding to the empty vector (pmirFLUO MCS (V36)). Colony 11 for *ADRA1A* and colony 7 for *SERCA2A* were confirmed by sequencing using oligonucleotides form Table A3.

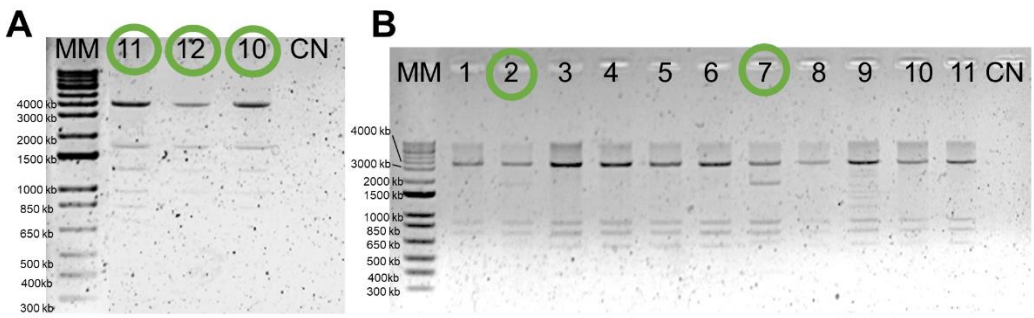

Figure 11. *Result of the enzymatic digestion with PstI and HindIII of pmirFLUO-ADRA1A (A) and pmirFLUO-SERCA2A (B). Recombinant positive colonies are marked with a green circle. MW: molecular weight, NC: negative control.*

For *CAMK2D*, colony PCR was performed using primers from Table A3. Colonies in which the *CAMK2D* was recombined, the amplified fragment was 4,9 kb (Figure 12). Only 3 positive colonies were obtained, but none were confirmed by digestion (Figure 13). On the other hand, for *KCNQ1,* 9 colonies were directly analysed by enzymatic digestion (Figure 13). The results confirmed that no colonies were recombinant neither for *KCNQ1* nor *CAMK2D* genes in pmirFLUO MCS (V36).

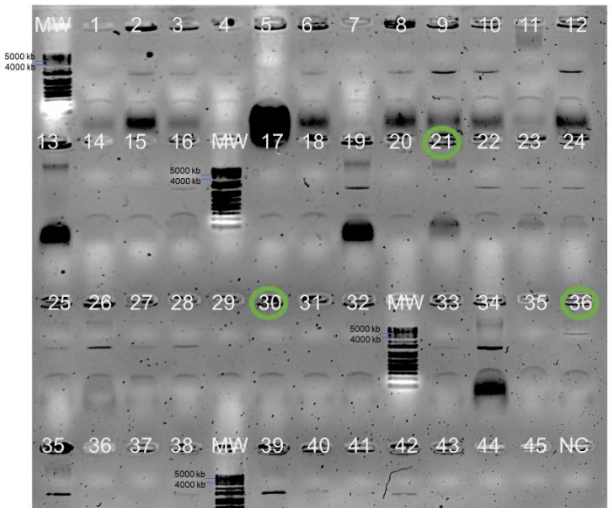

Figure 12. *Colony PCR of CAMK2D cloning. The positive colonies are highlighted with green circles. MW: molecular weight, NC: negative control.*

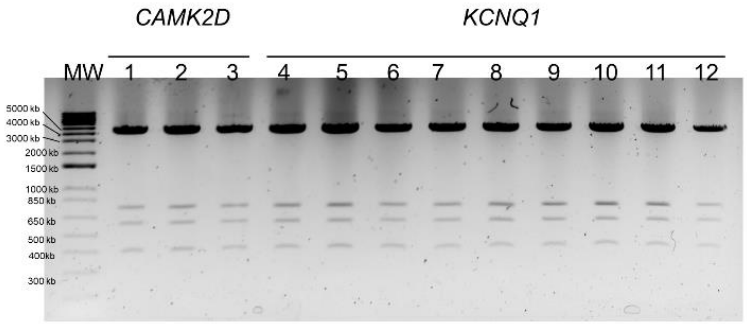

Figure 13*. Result of the restriction digestion of KCNQ1 and CAMK2D cloning in pmirFLUO MCS V36. MW: molecular weight*

8.3. pmirFLUO MCS vector test assay

To assess the fluorescence intensity signal of the newly constructed pmirFLUO MCS (V36), this vector was transfected into HEK293 cells along with a *GFP*-expressing vector (V2) and a *tdT*-expressing vector (V3). Vector V2 and V3 have the strong promoter CAGG while in pmirFLUO MCS V36, *GFP* expression is controlled by the constitutive promoter SV40 and *tdT* by PGK promoter (Figure A2).

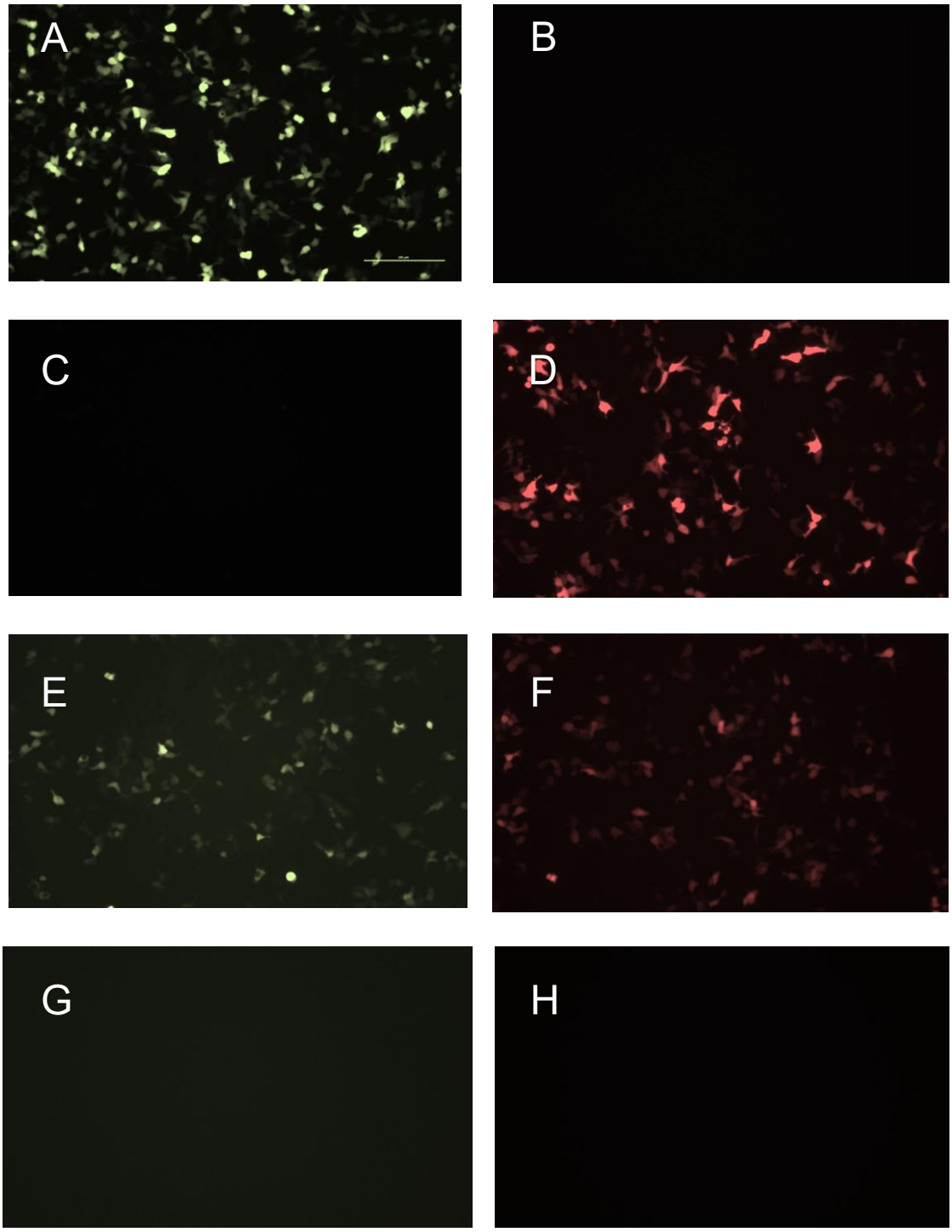

Figure 14. *Fluorescence detection of HEK293 transfected with pmirFLUO. Left column: GFP signal. Right column: tdT signal. A-B: GFP-expressing vector (V2). C-D: tdT-expressing vector (V3). E-F: pmirFLUO MCS vector (V36). G-H: non-transfected cells (NT). Scale bar = 200µm. Objective x10.*

Apart from these three conditions, a control of non-transfected cells (NT) was also performed to observe the background fluorescence of the cells. Moreover, a control of the medium used, without cells was included to observe the possible medium fluorescence background (not shown). The qualitative analysis of the transfection and fluorescence intensity under the different conditions is shown in the Figure 14.

In the case of *GFP* fluorescence channel, an intense signal is observed in the *GFP*-expressing vector as compared to the pmirFLUO MCS vector. The same is observed for the tdT reporter in tdT-expressing vector V3 as compared to pmirFLUO MCS. Transfection efficiency seemed comparable for all reporter-expressing vectors, then, these results suggest that the observed differences can be due to difference in the strength of the promoters under which the fluorescent proteins are expressed. Similar results are observed in the fluorescence quantification of cellular lysates (Figure 15).

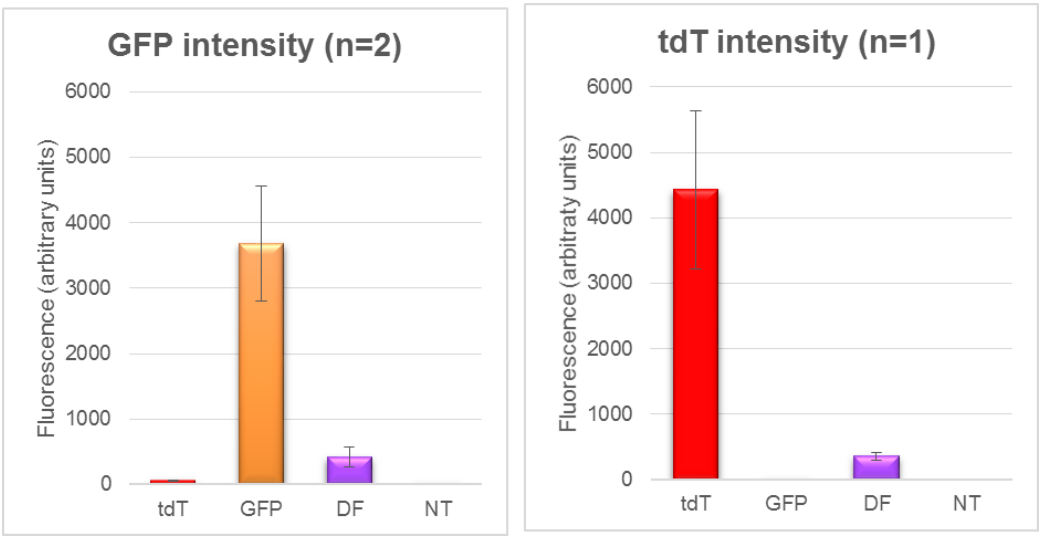

Figure 15. *Fluorescence quantification in HEK293 cell lysates of cells transfected with tdT reporter (V2), GFP reporter (V3) and pmirFLUO (DF) with GFP and tdT reporters (V36). NT corresponds to non-transfected cells. Left panel: Measurement of GFP signal. Right panel: Measurement of tdT signal.*

8.4. Fluorescence reporter assay (Dual Fluo assay)

To validate the pmirFLUO vector, assays of Dual Fluo and Dual Glo (gold standard technique) were performed in parallel for *SERCA2A* and *ADRA1A* genes. The miRNAs used were miR-24-2-5p, miR-24-2-3p and miR-3916. Previous studies in the laboratory, found that miR24-2-5p interacts with and inhibits the expression of *SERCA2A* and *ADRA1A* [63]. Thus, these interactions constituted the positive control of the experiment. The results obtained showed that the low fluorescence levels reached with pmirFLUO MCS were near the plate reader's detection limit. It hardly allowed to discriminate between transfected cells in different conditions (with or without miRNA) and, therefore, precluded the validation of pmirFLUO MCS (data not shown). Again, these results are most likely attributed to the low strength of the promoters in pmirFLUO MCS.

8.5. Luminescence reporter assay (Dual Glo assay)

8.5.1. Analysis of interactions of GOIs with BIO-AGEmiRNA miR-3916.

pmirGLO vectors containing the GOIs *ADRA1A, MYBPC3, ACTN2, CASQ2, DSP, SERCA2A (ATP2A2), POPDC2, DMD, TMOD1* (Table A2) were co-transfected with the BIO-AGEmiRNA miR-3916 or a negative control miRNA (N1) in HEK293 cells.

pmirGLO expressing the GOIs contains two luciferases, *Firefly* (*Fluc*) (transcribed in the same mRNA than the GOI) and *Renilla* (Figure A5). The activity of Firefly luciferase depends on the stability of mRNA which can be compromised because of miRNA interaction. Reduced expression leads to lower luciferase activity and thus, luminescence. Renilla luciferase does not depend on the interaction between miRNA and GOI, so its activity is considered 100% and is used for normalization.

The results obtained from these tests can be seen in the Figure 16. Of the genes tested *ACTN2, CASQ2* and *DSP* show a tendency of a positive interaction with miR-3916. Instead, miR-3916 seems to enhance Fluc activity in *SERCA2A and POPDC2 pmirGLO* vectors, which would be contrary to the predicted activity in the BIO-AGEmiRNA network.

No statistical analysis were performed since only two experimental replicates were completed during this master thesis.

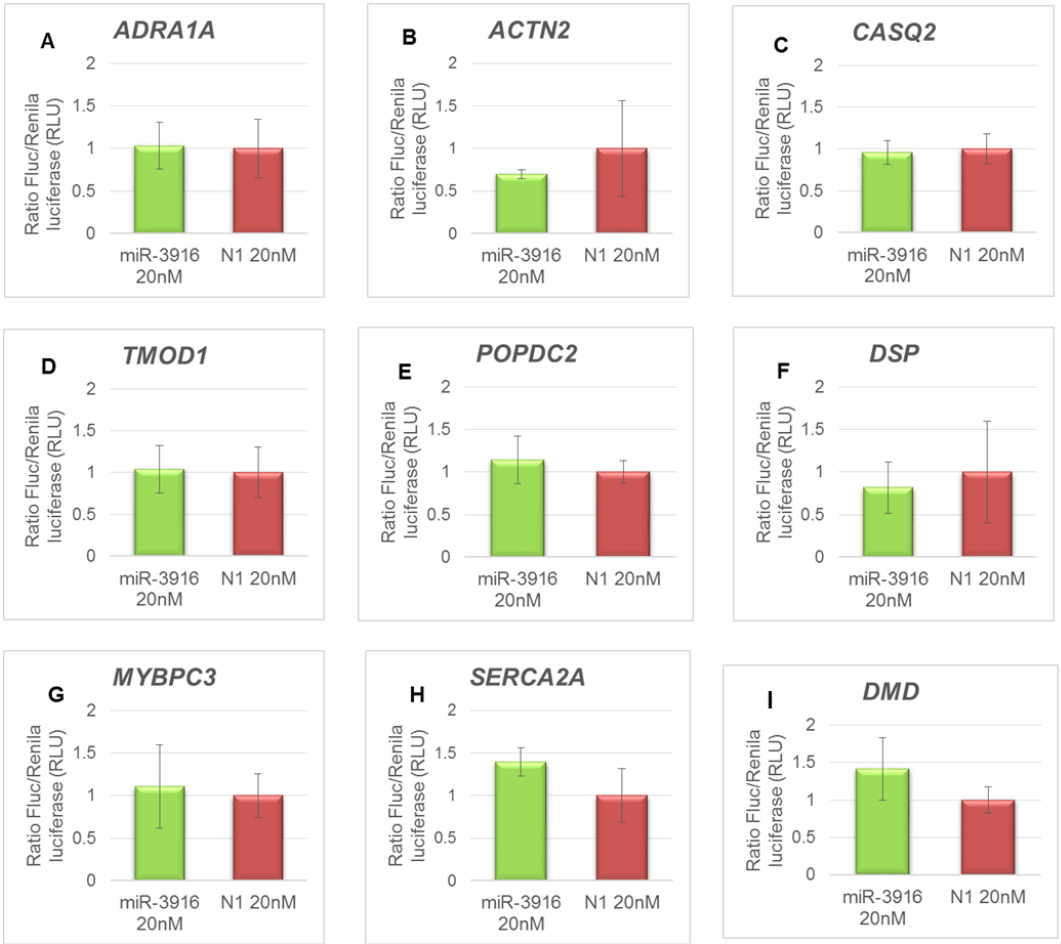

Figure 16. *Analysis of miR-3916 interaction with its putative target genes by luciferase reporter assays. The experiments are performed in triplicate with two biological replicates (n = 2). RLU: Relative light units.*

9. Discussion

CVDs are currently one of the leading causes of death worldwide. In addition, cardiac aging is one of the major risk factor for these diseases.

miRNAs modulate the expression of genes at the post-transcriptional level, so changes in their expression could lead to the development of disease. So far, many miRNAs have been related to different pathological conditions like cancer. Therefore, there is a need to understand the correct spatial-temporal expression of these miRNAs in order to comprehend their contribution to pathophysiology.

There are many studies that reveal important miRNA targets in each of the CVD reviewed in this work. But most of them are carried out in animal models, fundamentally mouse, rat and pig. Studies in human samples or cells of human origin that validate findings in animal species are limited. For instance, the miR-1-2 up-regulation reported by Ikeda et al. in MI mouse model is contradictory to the down-regulation of this miRNA observed in a miRNA profile of human iPSC-CMs in this disease by Aggarwal et al. [73], [78].

Pczynska et al. aligned sequences encoding for mature miRNAs in pig (*Sus scrofa*), mouse (*Mus musculus*) and human (*Homo sapiens*). The sequence alignment revealed that interspecies identity was 97-98%, but the miRNA loci were not maintained between species. The greatest differences between human and pig found in this study were due to the existence of a large specific group of miRNAs sequences for pigs (53% of swine miRNAs) [115]. These observations point to a global miRNA sequence similarity that should ideally ease translation to humans.

Despite the described genomic conservation of miRNAs and binding sites between miRNA-target genes [9], [115], interspecies differences occur at many levels such as cardiac physiology or lifespan. Importantly animal models generally have homogeneous interindividual genetic background, unlike the genetic diversity in the human population, and a controlled environment [64], [65]. Physiological features are more accentuated when comparing small animals and humans. Many studies in mouse have validated the involvement of miRNAs in specific pathologies, like miR-499 in MI [1], [113], miR-1 and miR-133 in CH [30] or miR-24 in HF [47]. Even in pig, as a much closer model to human genetics and physiology [116], the role of miRNAs in CVDs has also been studied. For example, the proangiogenic properties of miR-92a, previously observed in *in vitro* and *in vivo* mice models, were confirmed in a pig model [50], [51]. Also the pro-fibrotic role of miR-144-3p was corroborated using pig models [82].

Although there are several studies in small and large animal models, the contribution of the vast majority of miRNA in human CVD or cardiac aging remains to be demonstrated. Many drawbacks exist to study CVD in human samples. First, obtaining heart tissue samples is difficult since it has to be either post-mortem, from rejected transplants or small biopsies taken in living donors and second, longitudinal studies are not feasible in humans. To fill this gap, human iPSCs have recently emerged as a relevant model for the study of the molecular basis in human pathophysiology or development. Human iPSC-CMs have been used to demonstrate the role miRNAs in CVDs. For example the role of miR-19b in HF and arrhythmias previously demonstrated in mice [86], [87] or the role of miR-208a-3p, miR-23a-3p and miR-22-3p in CH also demonstrated in animal models [23], [26], [69], [72], [73] were confirmed in iPSC-CM.

miRNAs are also dysregulated in genetic diseases, such as HCM, DCM or LQTS, that are caused by mutations in protein-coding genes. For instance, miR-204 in HCM [3], miR-451a in DCM

(Colpaert & Calore, 2019) or miR-1/133a in LQTS [36], [89]. This fact highlights the critical role that miRNAs play in the maintenance of the cardiac homeostasis and functionality. Meola et al. reported that genetic mutations affecting miRNA functions can occur in different ways: either in the miRNA sequence itself, the MRE in the target mRNA or in proteins involved in miRNA processing [117]. It would be interesting to assess possible mutations at these three levels, in CVD where the origin is not clear yet. For this aim, iPSC-CMs in combination with advances in genetic engineering could be used in order to demonstrate the effect of such mutations in miRNAs, as it is done for mutations in protein-coding genes.

Moreover, miRNAs can act like multigenic regulators. For example, miR-133a targets several genes *CTGF, RhoA, Cdc42, TGF-β, COL1α1, KHCN2, KHCN4* [2], [19], [28], [30], [35]–[37] involved in diverse cardiac functions and it has been found altered in different CVD: hypertrophy [28], [30] , fibrosis [2], DCM [3] or LQTS (Shan et al., 2013). miR-21 has been also associated several diseases such as hypertrophy and fibrosis [2], [19], [24]. Therefore, by modulating a single miRNA expression or activity, a whole function can be altered.

Interestingly, miRNAs could become useful biomarkers or be targeted by therapies, however, from all miRNAs associated with dysfunctional situations, only some have been studied as possible prognostic and/or diagnostic biomarkers (O'Brien et al., 2018) or as therapy [6]. This is clearly a research field which is expanding, so this type of study would still be necessary.

For both therapy design and biomarkers discovery, although research in animal models or humans is essential, the first steps in the process require *in silico* and *in vitro* experiments that will determine good candidates to fulfil the therapy/biomarker pipeline. *In silico* studies have shown different strategies to bioinformatically predict interactions between miRNAs and their targets [118], [119]. Previous work of the BISCoS group, aimed at establishing a miRNA-target regulation network of age-related cardiac dysfunction based on bioinformatics [7]. The interaction of miR-24-2 and miR-4435 with target genes were tested and 6 out of 10 tested targets were indeed modulated by these BIO-AGEmiRNAs [63]. In this work we aimed to extend the network validation *in vitro* using simultaneous approaches.

First, we aimed to create a fast and cost-effective reporter system for *in vitro* testing of interactions based in fluorescence and validate it with previously proved interactions with pmirGLO MCS. pmirFLUO MCS vector was developed by modification of the standard pmirGLO MCS however, to exchange luminescence reporters by fluorescence ones. The use of fluorescence for miRNA-target interaction analysis has been already commercialized (miRNASelect pMIR-GFP Reporter System, Cell Biolabs). In this case, the vector consists of a CMV promoter controlled GFP reporter vector and a β-Gal plasmid control for transfection normalization. Our pmirFLUO vector is an all-in-one option since it contains both the reporter for miRNA interaction and the normalizer.

Unfortunately, basal levels of GFP/tdT signal in DualFLUO vector built in this Master Thesis were too low for reliable detection of differences between different conditions. This is likely due to the strength of the promoters driving GFP (SV40 promoter) and tdT (PGK promoter), which have moderate strength in HEK293 as compared to CAGG promoter, used in control transfections (Figure A6 and A7), or CMV, in the commercial vector from Cell Biolabs [120]. Despite the results obtained so far are not satisfactory, the modified pmirFLUO MCS is proposed as a tool for testing miRNA-GOI interactions *in vitro* in the future. The first advantage is that the addition of

substrates and inhibitors during the measurement which can lead to technical errors, are not needed. Second, this new tool would be less time-consuming, since no time gap between both measurements (Renilla and Fluc luciferases activity) is required. Contrary to that, the use of fluorescence allows measurement of both fluorescent proteins simultaneously. And last but not least, the economic cost of this new tool is notoriously lower compared to Dual Glo assay.

To overcome the low fluorescence intensity of Dual Fluo assay, different possible solutions are proposed. First, it would be possible to achieve greater expression levels of the reporters by changing the promoters under which the expression of the two fluorescent proteins is controlled. Substitution with stronger promoters like EF1A or CAGG may overcome this major pitfall [120].

Second, the measurement of fluorescent proteins should be carried out with optimal filters. In these experiments, a suboptimal filter for tdT detection was used due to a technical problem in the detection instrument (Figure 6). tdT fluorescence was read in the emission tail instead of determining it in a wavelength range nearer to its emission peak (620/40). If this would not be feasible, tdT and / or GFP could be changed for another other fluorescent proteins, like BFP (emitting at 457nm).

Another aim of this work was to assess miRNA-gene interactions not tested so far, form the miRNA-gene regulation network described by BSICoS (Figure A1). First, cloning of new GOIs was attempted. Since the experiments were unsuccessful, new attempts to improve the PCR results (Figure 7) are required. Importantly, the genes that were tried to amplify are down-regulated with age. Therefore, could be that since cDNA samples used were of old donors they did express this genes at low levels. To overcome this hurdle, these PCRs could be performed on younger donors' samples.

To keep validating the miRNA-target network, the putative interactions of miR-3916 with 9 GOIs previously cloned in the lab in pmirGLO MCS were tested. Of these, six of nine GOIs tested, seemed not to interact (n=2). On the contrary, *ACNT2*, *CASQ2* and *DSP* showed a slight positive tendency to interact with miR-3916. The percentages of validated interactions (33%) are in disagreement with previous results of the group were 60% of the tested interactions with miR-24-2 or miR-4435 showed positive interaction [63]. This indicates that miR-3916 might not contribute in the same extent as miR-24-2 or miR-4435 to cardiac aging. Although the results obtained, we cannot discard that these genes may be targets of other miRNAs in the bioinformatics network, since one miRNA can regulate several genes, and the same gene can be regulated by several miRNAs [11]. In fact, *ACTN2* and *DSP* genes, which seem to interact with miR-3916, had already demonstrated *in vitro* positive interactions with miR-24-2-5p and miR-24-2-3p, for *ACTN2*, and miR-4435 for *DSP* [63].

What is more, in the case of *SERCA2A* and *DMD*, Dual Glo assay raise the possibility that miR-3916 stabilize their target gene. As previously mentioned, this phenomenon has already been proposed by some authors. Two possible mechanism could describe this possible miRNA-mediated target stabilization: (1) Induction of transcription trough miRNA binding to promoter regions [17] or (2) Enhancement of target translation [10]. Despite this, many studies are necessary to verify this miRNA-mediated up-regulation target. Using human iPSC or tissue samples from patients, the colocalization of miRNA and its target genes could be evaluated in

order to study these two possible mechanisms in human models. Again, human translation would also be important for these studies.

Although further *in vitro* testing is required to confirm these miR-3916 interactions, the results obtained so far, allow to hypothesize that miR-3916 could have a role in dysregulating *in vivo* functions related to these genes such as cytoskeleton (*ACTN2*), cell-cell communication and cardiac conduction (*DSP*) or calcium handling (*CASQ2*).

This work has further demonstrated that the bioinformatics network of BIO-AGEmiRNA-target genes established by the BSICoS group contains positive interactions or a tendency to positive interactions according to the *in vitro* tests carried out in this study. However, testing the physiological role of such interactions would be necessary. *In vivo* tests on myocardial tissue or the use of iPSC-derived cardiomyocytes (as stated above) would be a tool for these future tests, where electrophysiological parameters would be analysed upon overexpression of BIO-AGEmiRNAs.

10. Conclusions

- miRNAs can be a cause for CVD development but their expression can also be modified as a consequence of CVDs.
- There are miRNAs that are involved in several aspects of CVDs and aging. miRNA are multigenic modulators and this needs to be considered when designing miRNA-based therapies
- The great majority of the currently described miRNA related to CVD or aging are not validated in human.
- iPSC emerged as a powerful tool for studying the role of miRNAs in CVDs in human.
- pmirFLUO MCS vector could be an advantageous tool to assess miRNA-target interactions using fluorescence.
- miR-3916 could negatively regulate *ACTN2*, *DSP* and *CASQ2* (n=2) and could promote the *SERCA2A* and *DMD* mRNA stabilization (n=2).
- Cardiac functions related to cytoskeleton, cell-cell communication or calcium handling could be modulated by miR-3916.
- miR-3916 seems to contribute to cardiac aging, although to a lesser extent than miR-24-2 and miR-4435.

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12. Annexes

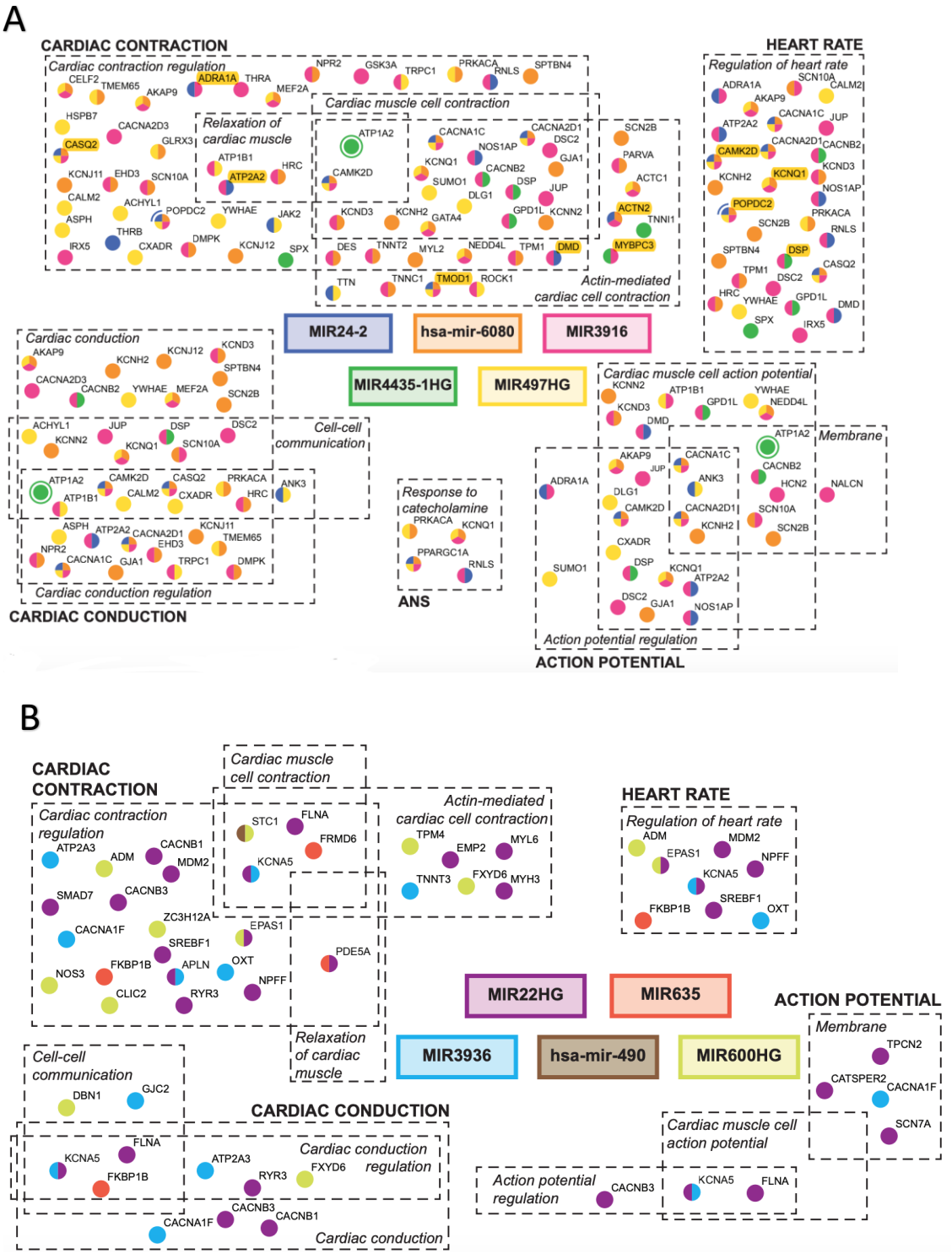

Figure A1. *Predicted BIO-AGEmiRNA gene regulatory networks of cardiac related functions. Network for the top 5 upregulated (A) or downregulated (B) BIO-AGEmiRNAs. Mirror targets are shown as colour coded dots according to their putative regulating miRNA. Sections of cardiac GOs are delimited by dashed-lines and functional categories are shown in capital letters. Image obtained from* [7]

Figure A2. *pmirFLUO MCS vector map (V36). Image obtained from Ape software.*

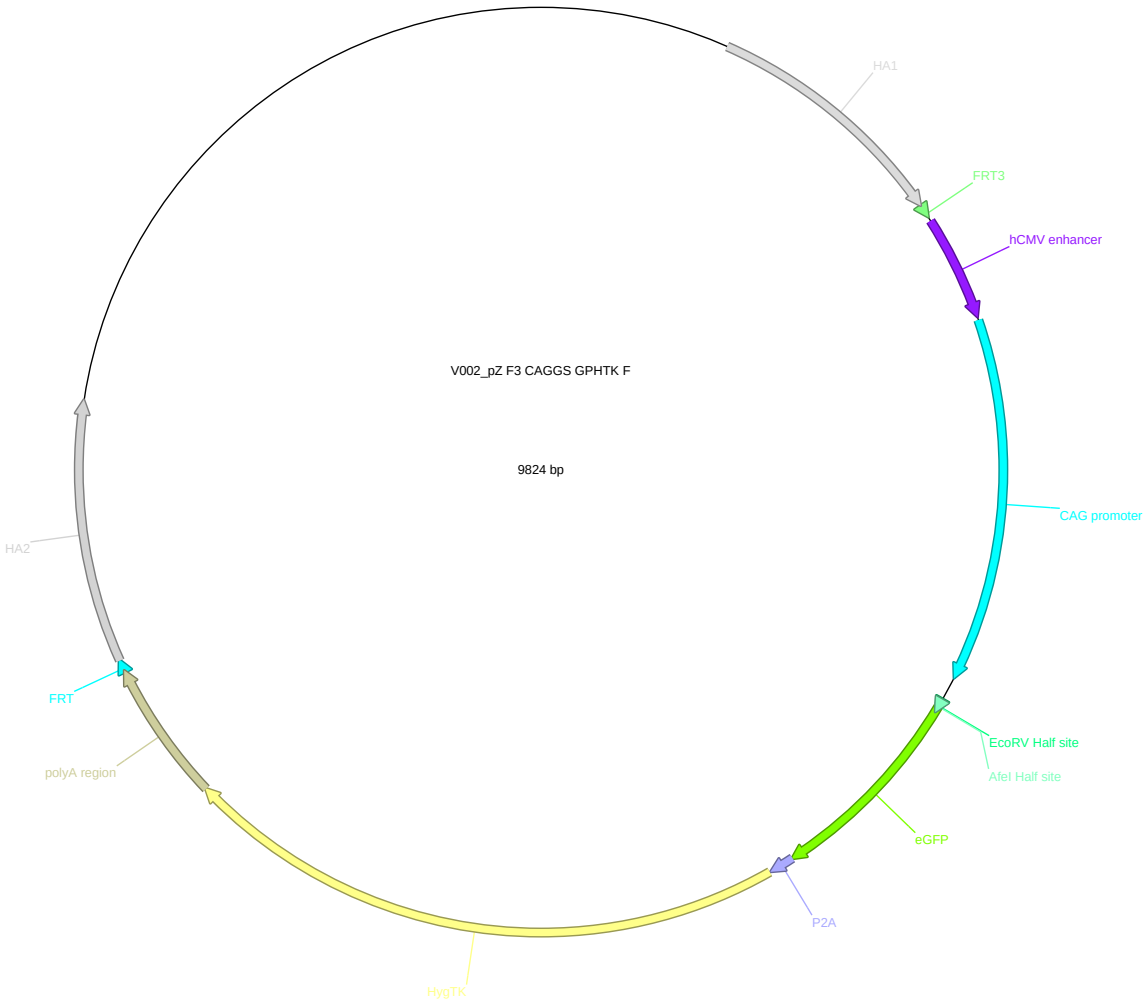

Figure A3. *GFP-expressing vector map (V2). Image obtained from Ape software.*

***Figure A4**. tdT-expressing vector map (V3). Image obtained from Ape software.*

***Figure A5**. pmirGLO MCS vector map (V19). Image obtained from Ape software.*

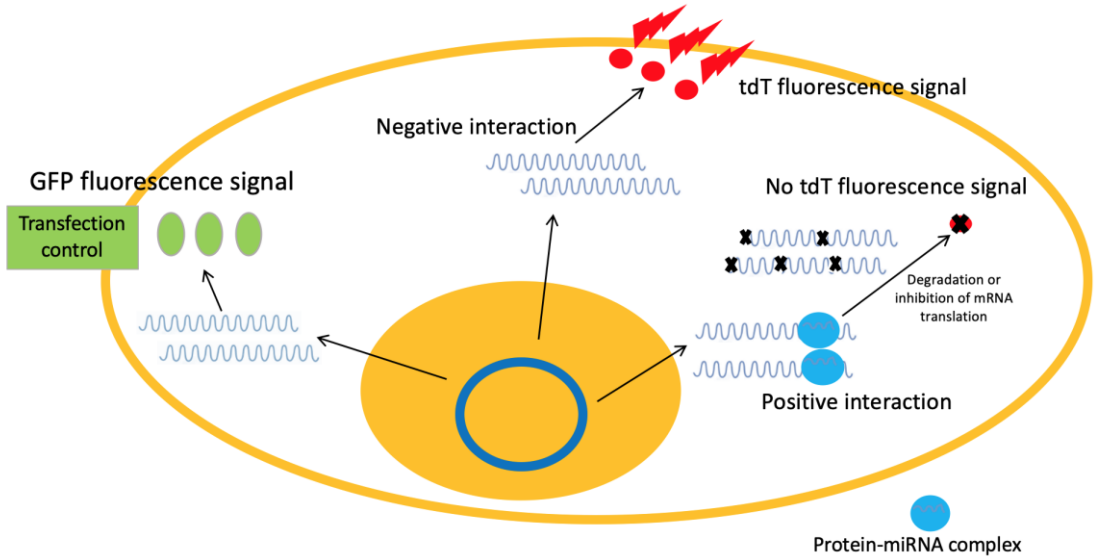

Figure A6. *Fluorescence Reporter Assay.*

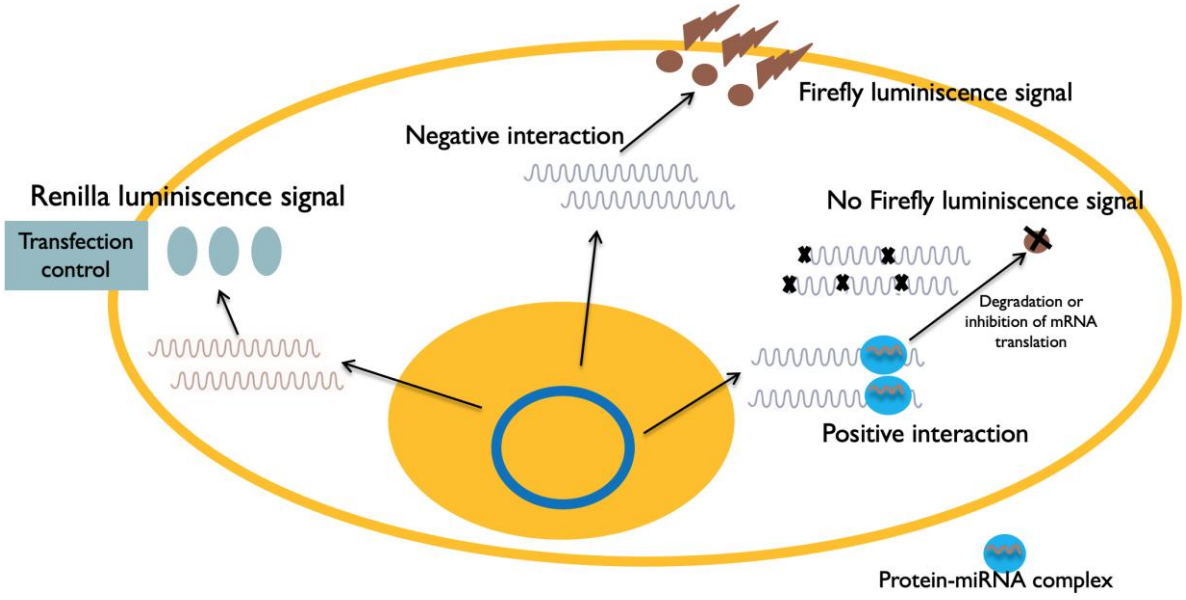

Figure A7. *Luminescence Reporter Assay*

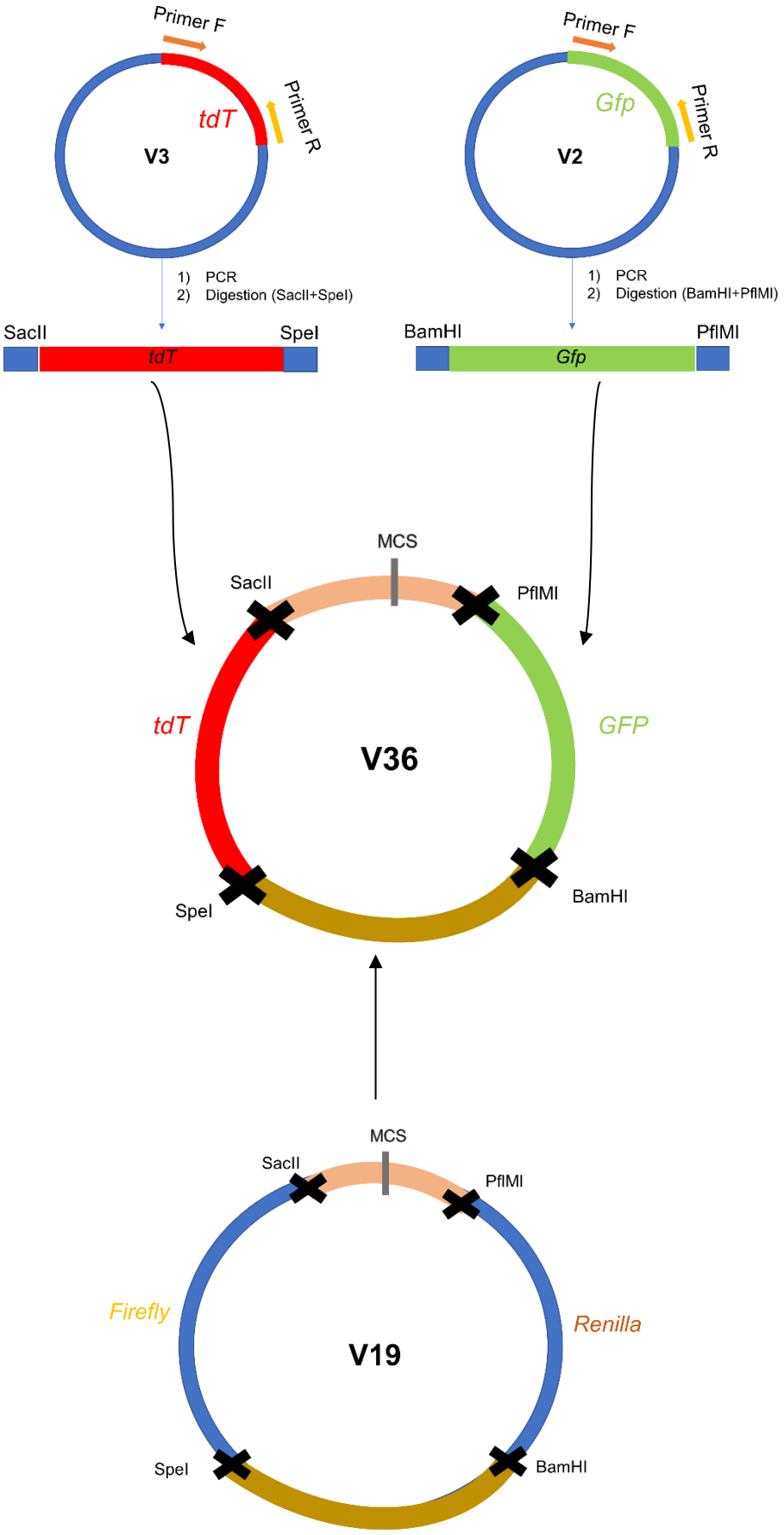

***Figure A8**. pmirFLUO MCS construction (V36).*

Table A1. Genes of interest and cloning regions.

Gen	Amplicon size (kb)	Cloning region	Refseq
ADRA1A	2,76	5´UTR-CDS-3´UTR	NM_000680.3
MYBPC3	4,1	5´UTR-CDS-3´UTR	NM_000256
ACTN2	4,89	5´UTR-CDS-3´UTR	NM_001103.3
CASQ2	2,4	5´UTR-CDS-3´UTR	NM_001232
DSP	1,34	CDS	NM_001008844
SERCA2A	3,8	5´UTR-CDS-3´UTR	NM_001681
POPDC2	1,5	5´UTR-CDS-3´UTR	NM_001308333
DMD	0,69	CDS	NM_004006
TMOD1	3,1	5´UTR-CDS-3´UTR	NM_003275
KCNH2	2,78	CDS	NM_172056
KCND3	1,91	CDS	NM_172198
CAMK2D	4,8	CDS-3´UTR	NM_001321574
CACNA1C	6,8	CDS	NM_001129841
KCNQ1	2,54	CDS-3´UTR	NM_000218

Table A2. Vectors used.

Vector	Size (kb)	Name
V2	9,82	*GFP-expressing vector*
V3	10,92	*tdT-expressing vector*
V21	10,3	*pmirGLO-ADRA1A*
V22	8,9	*pmirGLO-MYBPC3*
V23	12,4	*pmirGLO-ACTN2*
V24	10	*pmirGLO-CASQ2*
V25	8,8	*pmirGLO-DSP*
V26	11,3	*pmirGLO-SERCA2A*
V28	9	*pmirGLO-POPDC2*
V29	8,2	*pmirGLO DMD*
V30	10,6	*pmirGLO TMOD1*
V36	6,3	pmirFLUO MCS
V37	8,97	pmirFLUO *ADRA1A*
V38	9,99	pmirFLUO *SERCA2A*

***Table A3**. Primers for each gene. The sequences have restriction sites for different restriction enzymes: AscI (blue), SalI (green), XbaI (pink). Nucleotides in red were added to maintain the reading frame. Ta: Annealing temperature, Th: Hybridization temperature.*

Amplicon	Sense	Sequence (5´→ 3´)	Use	Ta (°C)	Th (°C)
KCNH2	F	AAGGCGCGCCAATGCCGGTGCGGAGGGGCCA	PCR	74	72
	R	AATCTAGAGGGCCAGGCATGAGGCTGCAGG		72,7	
CAMK2D	F	AACGGCGCGCCA ATGGCTTCGACCACAACCT	PCR and PCR colony	62	62
	R	AAAGTCGACCTCAGCCTTCTGCTGAGACA		62	
CACNA1C	F	AAAGGCGCGCCGAAAGGAGCAGTTTTTGGGGT	PCR	61,5	62
	R	AAATCTAGAGTTCCGGTTAACTCCAGGTCA		62,2	
KCNQ1	F	ATAGGCGCGCCCCATTTCCATCATCGACCTCATC	PCR and PCR colony	61,6	60
	R	ATTTCTAGATTCCTATTCCACAGCCACCT		61	
KCND3	F	AAGGCGCGCCAATGGCGGCCGGAGTTGCG	PCR	68,9	67
	R	AATCTAGATTACAAGGCGGAGACCTTGACAACA		66,7	
PGK promoter	F	CTCACTAGTCTCGTGCAG	PCR colony	57	
P2A	F	TCGGCGCGCCAGGCTCGGGGGCGACAAACTTTAGCTTGCT	PCR colony	60	
P2A	R	TTTGAATTCGAATCCTGGTCCGGGATTCTC	PCR colony	62,6	
tdT	F	TGTTCCTGGGGCATGGCA	Seq	64,5	
tdT	R	TTTGATGACGGCCATGTTGT	Seq	61	
pmirGLO	R	TCGGGCTTTGTTAGCAGC	Seq	60,8	

Table A4. Restriction enzymes used.

Enzyme	Recognition sequence	Type	Company	Reference
AscI (SgsI)	GG'C GCG,CC	ANZA 20U/µL	Thermo	IVGN0214
BamHI	G'GATC,C	ANZA 20U/µL	Thermo	IVGN0056
PstI	C,TGCA'G	ANZA 20U/µL	Thermo	IVGN0236
Van91I (PflMI)	C A N ,N N'N T G	STANDARD 10U/µL	Thermo	ER0711
SalI	G'TCGA,C	STANDARD 10U/µL	Thermo	ER0641
SacII (Cfr42I)	CC,GC'GG	STANDARD 10U/µL	Thermo	ER0201
SpeI (BcuI)	A'CTAG,T	STANDARD 10U/µL	Thermo	ER1251
XbaI	T'CTAG,A	ANZA 20U/µL	Thermo	IVGN0126

Table A5. Vectors and miRNAs tested in DualGlo assays.

Assay	Vectors	miRNAs
DualGlo	○ V21 ○ V22 ○ V23 ○ V24 ○ V25 ○ V26 ○ V28 ○ V29 ○ V30	○ CONmiR mimic target hsa-miR-3916 ○ CONmiR mimic negative control-N1