"Terapia con miRNAs en la enfermedad cardiovascular y envejecimiento: descubrimiento de nuevos candidatos"

"microRNA therapies in cardiovascular disease and aging: discovery of novel candidates"

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1. ABSTRACT

Cardiovascular diseases are, as of now, the main cause of death in the developed world. **Aging** is a main risk factor determining their development. In most of the cases, pharmacological treatments do not directly asses the problem and have only palliative effects, which decreases quality life of the patient and increase dependence on the treatment to follow a normal lifestyle. Therefore, looking for novel effective therapies to treat the cause of the disease will be crucial in the near future.

microRNAs (miRNA) have been proved to be master regulators of many biological processes that take places in the cell, since they are capable of regulating gene expression. Studies *in vitro* and *in vivo* have associated dysregulation of the miRNAs expression with pathology in cardiovascular disease and aging, thus postulating them as possible therapeutic targets.

As result, **miRNAs-mimics** and **anti-miRs** have emerged as a new promising therapeutic approach to correct gene expression alterations regulated by miRNAs that have been seen in disease. Here, a detailed revision of the application of miRNA-based therapies to treat CVD is made. Although, currently there are only 2 studies that have reached clinical trials, many preclinical studies have published encouraging results.

Continuing previous work of the group BSICoS, a second aim of this master's thesis is to develop tools for the study of the interaction of **age-related miRNAs (BIO-AGEmiRNA)** with their target genes in relation to cardiac function using human iPSC-derived cardiomyocytes. A **inducible expression vector** was created to be expressed **mature miRNAs**, specifically miR-4435, downstream of a fluorescent reporter gene. The vector was tested *in vitro* in HEK293 cells and proper expression of the reporter was observed. However, expression of the mature miR-4435, and therefore proper processing of the pre-miRNA, could not be confirmed due to technical issues. Future work will continue to validate the remaining BIO-AGE-miRNAs *in vitro*, first in HEk393 and then in iPSC-derived cardiomyocytes were the molecular mechanism and functionality of the BIO-AGEmiRNAs will be investigated. The developed vector will be most certainly a useful tool to complete this future work.

2. INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of death worldwide accounting for 31% of total, almost 17.8 million deaths. Specifically, ischaemic heart disease (also called **coronary heart disease, CHD)** and stroke were the top 2 in 2016 [1]. These numbers constitute an increase of 21.1% since 2007 [2]. Furthermore, an age-related increase in the prevalence of CVD (considering CHD, Heart Failure stroke, and hypertension) has been observed in both males and females in the last years (National Health and Nutrition Examination Survey, NHANES). **Heart failure (HF)** is a complex high prevalent age-related clinical syndrome that impairs the ability of the ventricle to fill or eject blood. By 2035, it is predicted that approximately 45.1% of the US citizens would suffer from CVD, which would be associated with an enormous economic burden [2]

By promoting healthy dietary and lifestyle habits in the population, nearly 80% of CVDs could be prevented, since smoking, lack of physical activity or high body mass index are the main risk factors [2], [3] that precipitate the development of defects in mitochondrial functionality, insulin signalling, endothelial homeostasis and redox balance resulting in an early appearance of senescent attributes leading to CVD [4].

Currently, pharmacological treatment is composed principally by antithrombotic agents, antihypertensives, diuretics, β-Blocking agents and agents acting on the renin-angiotensin system, which act mainly on protein targets like enzymes, receptors or transporters [5]. Surgical procedures are also being done and in case of severe damage, a heart transplantation could replace the failing organ [6].

The development of CVD therapeutics have increased median life-expectancy [4]. However, CVD medications are associated with higher side-effects risks [7]. Together with this, patients who had suffered any CVD are polymedicated, which increases the likelihood of side effects due to adverse drug-drug interactions and complicates adherence, especially in older adults [8]. Therefore, there is need for highly specific medicines to ameliorate life quality and health of CVD´s patients.

With life expectancy on the rise, the world population is becoming increasingly aged. By 2050, 16% of the population is expected to be over 65 and people aged over 80 to triple [9]. Aging is a physiological process considered a major risk factor that increases the likelihood of suffering a CVD. A number of age related detrimental transformations occur on the human heart like left ventricular hypertrophy, cardiomyocyte loss, diastolic dysfunction or cardiac fibrosis [10]. Concerning subcellular changes (Figure 1), mitochondrial function [11], calcium handling [11] as well as cell-cell communication structures (intercalated discs and gap junctions) are severely altered with age as seen in animal models [12]. Furthermore, diverse signalling pathways such as FOXOs (cell growth, proliferation,

differentiation and lifespan extension), AMPK (glucose uptake, glycolysis, oxidation of free fatty acids and mitochondrial biogenesis), SIRT1 (gatekeeper against oxidative stress, inflammation and cardiovascular aging), $p66^{Shc}$ (oxidative stress in mitochondria), JunD (oxidative stress) and NF-kB (regulates gene expression of factors that mediate cell adhesion, proliferation, inflammation, redox state, and tissue-specific enzymes) link aging with cardiovascular dysfunction [4]. Gaining knowledge about aging and CVD molecular basis would help researchers into development new curative treatments, instead of palliative ones.

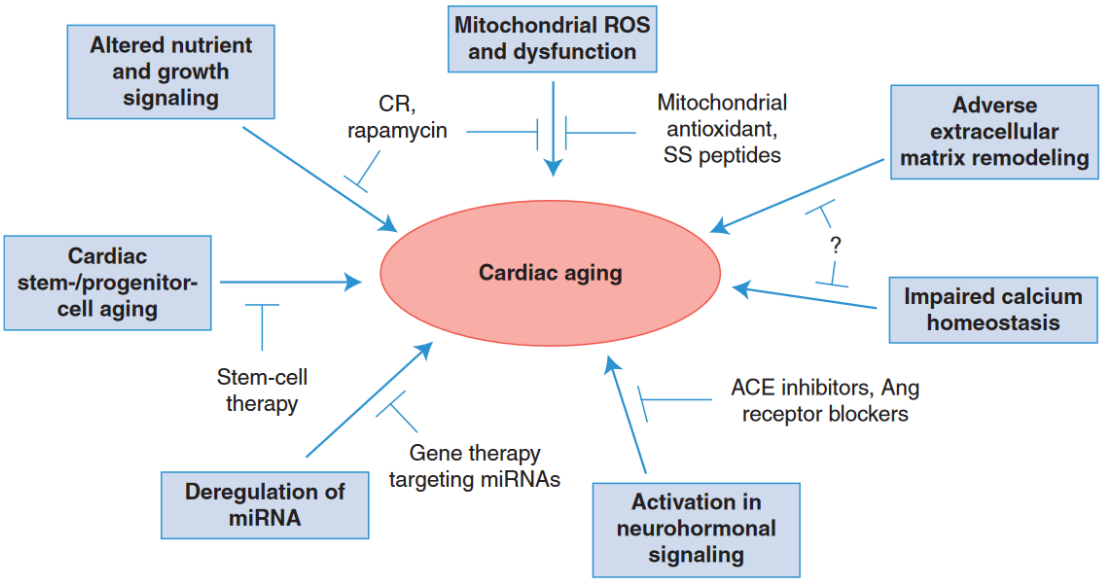

Figure 1. Schematic representation of the molecular mechanisms of cardiac ageing and potential cardiac interventions. ROS, Reactive oxygen species; CR, calorie restriction; SS, Szeto –Schiller; miRNA, microRNA; ACE, angiotensin-converting enzyme; Ang, angiotensin [11].

microRNAs (miRNAs) have been proved to be important master regulators of biological processes, including aging-related modifications in the heart [13]

One of the research lines of the **group BSICoS (Biomedical Signal Interpretation and Computational Simulation)** is discovering miRNAs involved in human cardiac aging. Long-term aim includes development of possible therapies and determination of biomarkers related to heart dysfunction in aging. Up to now, most studies where aging was investigated considered **chronological age (CA)** instead of **biological age (BA)** as the main aging variable. However, CA does not always reflect the health status of an individual since it just accounts for time, not for environmental or genetic factors. In regard to human cardiovascular aging, high BA has been linked with increasing risk of suffering a

CVD [14]. BSICoS analysed the human Left ventricule (LV) miRnome in relation to BA rather than just CA using RNAseq data form the GTEx consortium [15]. A total of 20 BA-related miRNAS (**BIO-AGEmiRNAs)** (14 upregulated and 6 downregulated) were identified [15]. Then, using bioinformatic tools, Ramos-Marquès et al., described a heart-related BIO-AGEmiRNA gene regulation network, that might help to understand how LV functioning is modulated by BA-associated miRNAs at the molecular level. In order to validate the network, some of the putative interactions of BIO-AGEmiRNA with their potential target genes have been evaluated experimentally [16]. These included targets of **MIR24-2 and MIR4435-1HG** and some of the miRNA-mRNA regulatory axes were *in vitro* validated by luciferase expression assays . When miR-24-2 was assayed, 5 out of 7 target genes tested showed a positive interaction: *ADRA1A, ACTN2, ATP2A2 (SERCA2A), POPDC2* and *TMOD1*. In the case of the miR-4435, *DSP* was the only one showing a significant interaction (10% reduction in the luciferase activity in presence of the miRNA).

3. HYPHOTHESIS AND AIMS

Since miRNAs are involved in a variety of biological processes regulating gene expression at the post-transcriptional level, including CVD and aging, it is hypothesized that they have a therapeutic and biomarker potential in such processes.

The aim of the bibliographic revision in this Master's thesis is to summarise all the progress that has been done up to now in the field of miRNA therapeutics related to CVD and aging, considering both miRNA inhibitors and replacement therapies. Therefore, the following partial aims were proposed:

1) Compile methods and approaches used to develop miRNA therapeutics and their most recent advances.

2) Analyse the state of the art of miRNA therapeutics for CVD.

The experimental aim of this Master's thesis is to develop tools for the molecular and functional characterization of the validated interactions of BIO-AGEmiRNA with their target genes [16] in **cardiomyocyte-derived iPSC (CM-iPSC).** Future studies will use recombinase mediated cassette exchange (RMCE) in human induced pluripotent stem cells (hiPSC) [17] to create BIO-AGEmiRNAs inducible expression lines.

In order to achieve this objective, the following partial aims are proposed:

1) Construction of inducible expression vectors of BIO-AGEmiRNA's for RMCE in iPSC.

2) Analysis of the expression of the transgenic miRNA and the correct intracellular processing of the mature miRNA.

4. BIBLIOGRAPHIC REVISION

4.1 microRNA: Biogenesis and function

MicroRNAs (miRNAs) are small endogenous non-coding RNA constructions of 21-25 nucleotides in length found both in animals and plants. They display an essential posttranscriptional regulatory role by generally binding to the 3'untranslated region of a given target mRNA (although, binding to the 5'-untranslated region or to the open reading frame has also been described) which results in degradation or in translational repression of the mRNA [13].

Although, the majority of the miRNAs are located in non-coding intergenic regions, a few are encoded in intronic or exonic regions. When the latter occurs, miRNAs are usually co-transcribed with their host genes and processed afterwards in an independent manner. In other cases, they possess their own promoters and originate monocistronic transcripts. Yet, many miRNAs are found near each other in the genome creating miRNA clusters that are expressed polycistronically.

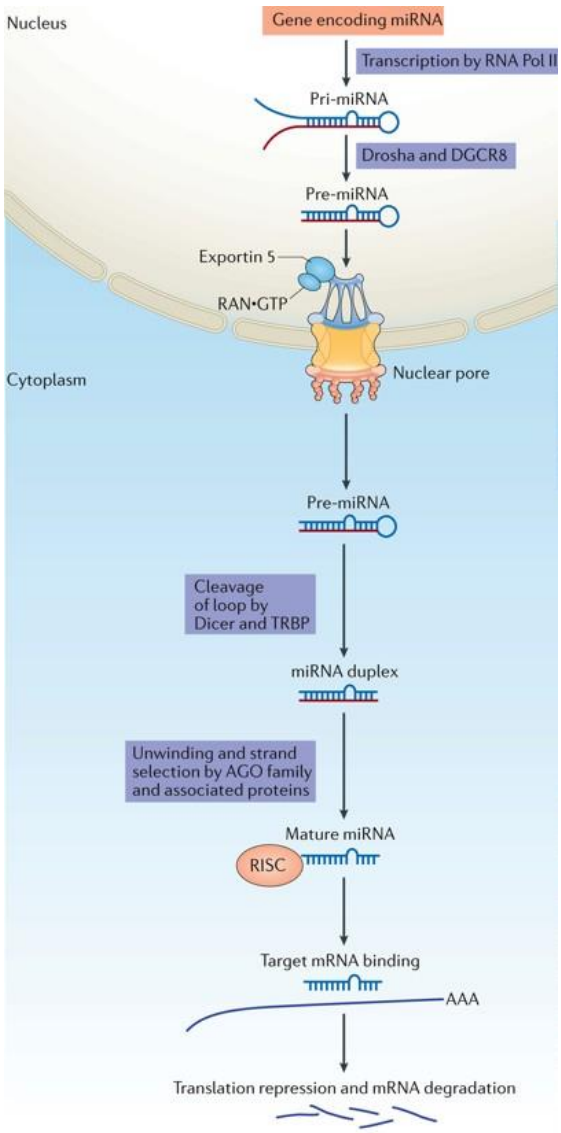

Figure *2.* Overview *of microRNA biogenesis*. [18]

miRNAs are generally transcribed by RNA polymerase II into a **primary miRNA (pri-miRNA)** that folds into its secondary hairpin-structure (Figure 2). The pri-miRNA is then cleaved in the nucleus by the Microprocessor (a complex formed by Drosha, a class 2 RNase III enzyme, and DGCR8, a dsRNA) to obtain the long **precursor miRNA (pre-miRNA)**. The pre-miRNA is exported to the cytoplasm where a highly specific RNAse III cytoplasmic endonuclease (Dicer), creates a 22-nucleotide mature miRNA duplex. Dicer will associate to TRBP, so that the miRNA duplex can be loaded onto Argonaute protein (AGO) forming the RISC-loading complex (RNA-induced silencing complex). The stability of the two ends of the duplex determines which strand remains bound to the AGO protein. Several studies suggest that an RNA Helicase (not identified) mediates the unwinding and removal of the discarded strand of the miRNA duplex. Once

loading is completed, the miRNA guides the RISC complex to its target mRNA, which is silenced through degradation or by blocking its translation [18]

Contrary to siRNAs where one RNA molecule is fully complementary to one particular mRNA, microRNAs target complete genetic pathways which are related in function (one miRNA can target more than 100 genes). This is possible due to the fact that microRNAs have an approximately 7 nucleotide match known as seed sequence that seems to be one of the primary determinants of the pairing specificity between the miRNA and the mRNA [19].

4.2 Basic nomenclature of the miRNA

miRNA nomenclature follows basic rules [20]. Mature miRNAs have the prefix "miR" followed by an identifying number, e.g. miR-499. In the case of Pre-miRNAs this prefix is substituted by a lower-case prefix "mir" followed also by the same identifying number.

The miRNA species can be distinguished by adding three- or four-letter prefixes before the prefix that indicates specie, e.g. hsa-miR-101 in Homo sapiens. An extra lower-case letter is added to differentiate miRNAs with similar sequences that only vary in one or two nucleotides, e.g. miR-123a or miR-123b.

When two pre-miRNAs are located at distinct loci but lead to the same mature miRNA, an additional hyphen and number is added to the miRNA name, e.g. miR-194-1 or miR-194-2.

4.3 micro RNAs in heart physiology and pathogenesis

4.3.1 Developing heart

The role of miRNAs in cardiac development and disease has been deeply studied. Many studies in Dicer knockout mice have shown that miRNAs are essential for both embryonic and postnatal cardiac development. Specific deletion of Dicer in embryonic mouse hearts lead to heart failure and subsequent death in the second half of the pregnancy [21]. Moreover, embryos in which Dicer is inactivated in the developing heart had a poorly developed ventricular myocardium and died soon after birth due to heart failure [21]. In the heart of adult mice, targeted deletion of Dicer causes early death and induces unfavourable cardiac remodelling [22].

miR-1/miR-133 family controls cell proliferation and differentiation, as well as, cardiac development [21]. Being specifically expressed only in cardiac and skeletal muscle, are amongst the most expressed miRNAs in the heart. Overexpression of miR-1 and miR-133a in developing hearts of mice diminish the thickness of the ventricular wall, reduces cardiomyocyte proliferation and induces heart failure leading to mice death [23]. The miR-17–92 cluster (miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a) seems to be indispensable for correct heart development, since loss of function of this cluster in mice

provokes immediate postnatal death due to severe ventricular failure [24]. Another example of a miRNA family essential during postnatal development of the heart are miR-15 family members (miR-15a, miR-15b, miR-16, miR-195 and miR-497), which suffer an upregulation in the new-born's developing heart that appears to mediate in cell survival and cell cycle regulation [25].

4.3.2 Cardiac remodelling after myocardial infarction: Hypertrophy, fibrosis and apoptosis

In the adult heart, in presence of a stress situation (e.g., hypertension, heart attack), cardiac remodelling can be induced. This adaptative response is essential to try to maintain heart function at first, but when prolonged in time hypertrophic growth (massive thickening of the ventricular walls) becomes pathologic, and causes severe fibrosis in the myocardium, reduction of the cardiomyocyte population (apoptosis) and reactivation of the cardiac fetal gene program, leading all to heart failure. Cardiac fibrosis is the excessive build-up of extracellular matrix proteins in activated fibroblasts. This increases stiffness in the heart which reduces heart contractility, augmenting the likelihood of heart failure. Apoptosis due to cardiac stress diminish the number of fully functional cardiomyocytes in the heart [26].

Changes in the abundancy of one or more miRNAs are related to disease onset in humans and other organisms [27]. Modulation of miRNA expression in mice have revealed the great influence of those in multiple aspects of the heart physiology, from controlling myocyte growth to regulating contractility, fibrosis, and even angiogenesis [28] Moreover, unique expression patterns of miRNAs related with pathological cardiac hypertrophy, heart failure, and myocardial infarction in humans and murine models of CVD have been identified [27]. Important miRNAS involved in the development of CVD are summarized in Table 1 and a detailed review about this topic can be found in the Master thesis of Natalia Hernández (2020).

4.4 miRNAs as Biomarkers

The features of a perfect biomarker are: easy traceability, high stability, organ or disease specificity, strong robustness and needing only slightly or non-invasive procedures in order to be measured within the human body. Clinical biomarkers should desirably not only indicate whether a disease is present, but also specify the level of severity and the most suitable therapy [29].

miRNAs meet most of these features and therefore could be postulated as ideal biomarker candidates for both diagnosis and disease prognosis. miRNAs are actively secreted by tissues and are present in most biological fluids which facilitates its detection [30]. Circulating miRNAs present in biofluids are stable molecules avidly investigated in biomarker research [13]. Furthermore, certain miRNAs show higher expression in certain tissues and some are even tissue specific [31]. On top of that, miRNA

expression analysis have been proved useful to discriminate between diseases otherwise difficult to distinguish such as Crohn's disease and ulcerative colitis [32]. Furthermore, different miRNA expression patterns have also been identified in short term vs long term glioblastoma survivors patterns [33], demonstrating miRNA physiological relevance as biomarkers.

Table 1. Summary of the main miRNAs whose dysregulation is linked to CVD.

miRNA	EXPRESSION	PHYSIOLOGICAL FUNCTIONCTION	PHENOTYPIC OUTCOME	REFERENCES
miR-1	Downregulated	Regulates cardiomyocyte hypertrophy through repression of hypertrophy inducing genes: Calmodulin, myocyte enhancer factor 2a and GATA binding protein 4	Increase cardiac hypertrophy	[21]
miR-15/16 family	Upregulated	regulates cardiomyocyte proliferation and survival in response to injury	Increase cardiomyocyte apoptosis	[25]
miR-24	Downregulated	Suppression of the BH3-only domain containing protein BIM (proapoptotic protein)	Increase cardiomyocyte apoptosis	[34]
miR-29	Downregulated	Regulates expression of extracellular matrix proteins: multiple collagens, matrix metalloproteinases, fibrillins and elastin	Increase fibrosis	[35]
miR-34a	Upregulated	Regulates PNUTS and SIRT1 (protect cardiomyocytes from apoptosis)	Increase cardiomyocyte apoptosis	[36]
miR-199	Downregulated	Controls hypoxia-inducible factor-1α (HIF-1α) and sirtuin 1 (Sirt 1), and other hypoxia-triggered pathways	Increase cardiomyocyte apoptosis	[37]
miR-212/132 family	Upregulated	Regulates Forkhead box O3 transcription factor	Increase cardiac hypertrophy	[38]
miR-208a	Upregulated	Targets the Thyroid-hormonereceptor-associated protein 1 (THRAP1) and β-MHC (involved in cardiac remodelling)	Increase cardiac hypertrophy and fibrosis	[28]
miR-214	Upregulated	Control Ca2+/calmodulin-dependent protein kinase IIδ, and the cell death mediators, cyclophilin D and BIM	Diminish cardiomyocyte apoptosis	[39]
miR-320	Downregulated	Regulates heat shock protein 20 (HSP20)	Diminish cardiomyocyte apoptosis	[28]

Since miRNA biomarkers could present a potent breakthrough in early disease detection and response to therapy progression, many companies are developing screening panels. The first miRNA microarray that was approved for use in the clinics was produced in 2012 for cancer detection of uncertain primary origin, it allowed identification of 42 different tumours by quantifying expression levels of 64 miRNAs with a 90% accuracy [40].

However, a number of limitations need to be overcome for miRNA to reach further clinical applications. Isolation from body fluids (peripheral blood, saliva, urine…) gives poor yields for quantification and there is high variability in detection assays due to low inter-platform concordance [41]. Furthermore, the use of non-standardised statistical analyses from miRNA clinical testing also increments the inconsistency when data of different studies have to be compared. Moreover, genetic variability could cause deviations in miRNA expression and function assessment in populations with distinct ethnic backgrounds [29].

Concerning CVD, a new high-throughput miRNA profiling panel that contains clinically relevant miRNAs related to cardiac disease, has identify miR423-5p as a biomarker for heart failure. This microarray aims to diagnose heart pathologies as well as provide a cardiovascular risk profile evaluation, considering that it integrates information from a collection of heart disease related- miRNA [42].

4.5 Models to study cardiovascular disease and develop new therapies

The approval of novel therapies is a long process that starts with drug discovery, including the identification of the molecular target and the finding of effective drugs. Then, once the drug has been formulated and tested *in vitro*, preclinical *in vivo* testing in animal models is the next step before initiating clinical trials in humans. The potential of miRNAs as disease targets has been described in the last years, therefore miRNA therapeutics are mainly in the drug discovery or preclinical model testing (mice and pig essentially) phases. Only a few of them that have successfully entered in clinical trials are linked to CVD, such as LNA antimiR-92a and antimiR-132 (Table 2) [43].

Table 2. Clinical Trials with miRNA-target Therapeutics. [43].

Targeted miRNA	Developmental Drug	Chemistry/ Mechanism	Indication	Sponsor/Collaborators	Clinical Trial Identifier	Phase
miR-92a	MRG-110	LNA antimiR	Wound healing	miRagen Therapeutics, Inc	NCT03603431	Phase I
					NCT03494712	Phase I
miR-16	Mesomir	TargoMir	Malignant pleural mesothelioma	Asbestos Diseases Research Foundation/EnGeneIC Limited	NCT02369198	Phase I
miR-34a	MRX34	miRNA mimic	Cancer/melanoma (advanced)	Mirna Therapeutics	NCT01829971	Phase I
					NCT02862145	Phase II
miR-122	Miravirsen	Various	Hepatitis C	Various	NCT01646489	Phase I
	RG-101				NCT00979927	Phase I
	Other				NCT00688012	Phase I
					NCT01200420	Phase II
miR-155	Cobomarsen (MRG-106)	LNA antimiR	Blood cancer (eg, chronic lymphocytic leukemia)	miRagen Therapeutics, Inc	NCT02580552	Phase I
					NCT03713320	Phase II
					NCT03837457	Phase II
miR-21	RG012	AntimiR	Alport syndrome	Sanofi Genzyme	NCT03373786	Phase I
					NCT02855268	Phase II
miR-29b	Remlarsen (MRG-201)	miRNA mimic	Cutaneous fibrosis	miRagen Therapeutics, Inc	NCT02603224	Phase II
miR-132	CDR132L	AntimiR	Heart failure	Cardior Pharmaceuticals GmbH	NCT04045405	Phase Ib

The ideal model to study any disease before initiating any human trials should not only precisely represent the physiology and biochemistry of such disease in humans but also be inexpensive, easy to handle, highly reproducible and ethically approved [44].

4.5.1 *In vivo* models (preclinical animal models)

Murine models are extensively used in pre-clinical trials. They are cost-effective, have shorter gestation times and are easier to manipulate in a laboratory environment than large animal models. As a result they are more suitable for "high-throughput" studies [45]. Yet, substantial differences in cardiovascular parameters such as heart weight, heart rate, blood pressure and the coronary artery system are found between rodents and those of bigger animals and humans [43] (Table 3). Thus, their response to pharmacological treatments may not be the most reliable outcome predictors and conclusions extracted from those trials should be considered carefully [46]. For instance, the coronary circulatory system of pigs has no anastomoses between distinct vascular branches, whereas in dogs, coronary arteries present several ramifications. Thus, the young human heart may be more similar to the pig one, while the older human heart, in case it has a background of ischemic episodes leading to collateral growth, may resemble more to the dog one [47].

Table 3. Advantages and limitations of the different animal models when compared with humans.

ANIMAL MODEL	ADVANTAGES	LIMITATIONS	REFERENCES
Murine models	• Wide range of transgenic and knockout strains • Easy to genetically modify	• Size and structural differences of the cardiovascular system. • Heart to body mass ratio of 0,55-0.60 (0,4 in humans) • Ten times higher heart rate than humans • Naturally resistant to develop an atherosclerotic lesion • Studies in CVD are based on genetic modifications, pharmacological inducted or surgery procedures. They cannot mimic the real conditions underlying the disease	[48]
Dog	• Electrophysiological system very similar to that of humans. • Prone to develop atrial fibrillation (being the main model in this disease)	• Coronary arteries are massively collateralized (not useful for studying myocardial infarction in humans)	[49], [50]
Pig	• Collateral coronary circulation and arterial anatomy very similar to human (useful model for myocardial infarction) • Parameters like heart weight, blood pressure, and heart rate are comparable to the ones of humans	• Weight and size of the animal • Studies in CVD are based surgery procedures or pharmacologically induced. They cannot mimic the real conditions underlying the disease	[43], [50]

Non-human primate	• Lifespan more similar to humans, would allow for Chronic heart failure research • The most phylogenetically similar model to humans (up to 98.77% of DNA sequence similarities)	• Present ethical and financial issues • Studies in CVD are based surgery procedures or pharmacological inducted. They cannot mimic the real conditions underlying the disease	[51]

A great amount of studies have not been replicated in larger animal models, generally because of the higher associated costs, handling difficulties or limited sampling size [43]. However, data obtained from those studies, despite manifesting certain limitations may be more predictive for human CVD than those obtain with small animal models, due to higher similarities in cardiac physiology with the human body [44].

Nonetheless, translation of toxicity and effectivity results from small to large animals or humans can fail mainly due to physiological differences between models [43] [45] (Table 3). Before initiating a first-in-human trials, "proof of concept" (solid evidence which demonstrates that is feasible) and safety evaluation in large animal models are often mandatory steps [43]. However, several aspects that influence CVD such as age, sex, comorbidities, polypharmacy and genetic background of each patient cannot be precisely replicated in experimental models and can only be considered and tested in human clinical trials with a cohort of patients having the desired characteristics [52].

4.5.2 In vitro and ex vivo models

In vitro culture systems have been largely used in CVD research [45][53]. Monolayer cultures like CM-iPSC, which display most functional characteristics of cardiomyocytes, and are broadly used as human *in vitro* models [54]. However, their immature phenotypes fail to mimic the adult heart functioning and cannot model chronic heart disease phenotypes [55]. New *ex vivo* 3D cultures such as engineered heart tissues (EHT) and living myocardial slices (Figure 3) may reflect with higher accuracy how an adult heart will behave under certain disease conditions or drugs tested. They would allow to detect cardiotoxicity as well as overcome differences in between the different animal species used as animal models, rising as intermediate models between *in vitro* and *in vivo* models [43]

EHTs are created from CM-iPSCs cultivated onto scaffolds to generate beating tissues. As described by Prondzynski et al., EHTs were a reliable representation of the diseased patients heart, for example if the patient has hypertrophic cardiomyopathy, the CM-iPSCs will also share this phenotype [56]. Compared with a 2-dimensional cell culture system, EHTs exhibit a more conserved structure and mature-like phenotypes which are highly similar to those in adult cardiomyocytes.

To prepare living myocardial slices, small or large mammalian hearts from commonly used pre-clinical models or human hearts coming from both diseased and healthy individuals, are explanted (Figure 3).

Watson et al created a protocol for the preparation of adult ventricular myocardial slices with a 97% preserved cardiomyocyte viability and functionality for up to 1 week [57]. The left ventricles or other parts of the heart are then isolated and dissected into small tissue blocks, which are later cut into thin slices by a vibratome. Myocardial slices are sections of approximately 100-400 micrometres thick which form a 3-dimensional structure composed by the original structure of the tissue where the electrical and mechanical connections are conserved. Furthermore, such thickness allows for oxygen flow and small compound diffusion preserving the myocardial slice. Drug treatment assays for drug screening as well as electrophysiological analyses by adjusting the voltage that stimulates the contraction of the myocardial slices, cardiac fibrosis and heart failure trials have been successfully performed in myocardial slices [43].

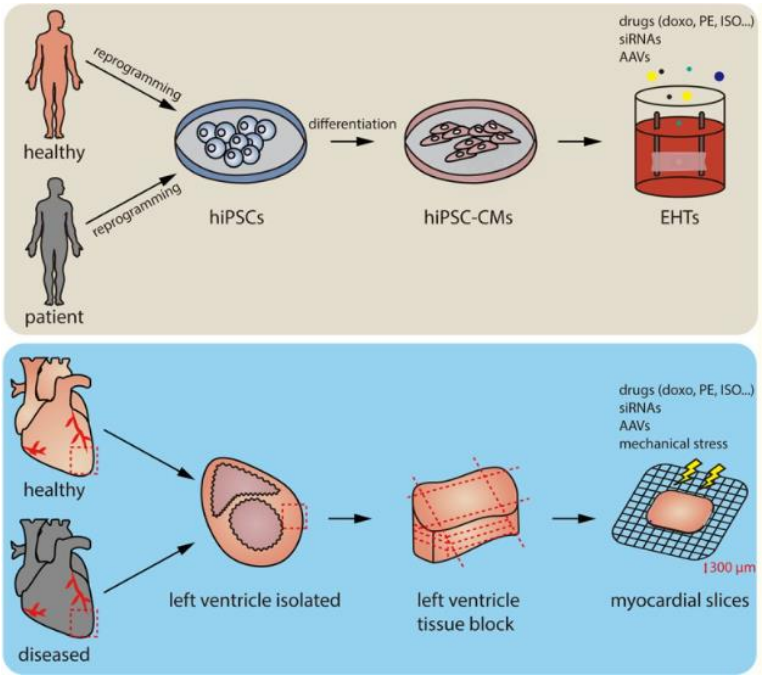

Figure 3. Workflow of 3-dimensional *ex vivo* models: Engineered heart tissues (EHTs), and living myocardial slices. *[43]*

Therefore, the model chosen for drug discovery and testing should be one that best mimics the disease in humans for all the parameters that want to be measured, since none of the existent animal models can completely portrait the multiple pathological outcomes in complex diseases.

4.6 microRNA-based therapies

miRNAs unbalance in disease is either caused by a major miRNA overexpression, which will likely cause the inhibition of a set of genes, or by a largely diminished expression, that will unleash otherwise repressed target genes in the affected cells. In order to control these pathological changes, two

therapeutic approaches are being considered (Figure 4): miRNA replacement therapy, that will consist on the reintroduction of the depleted miRNA in disease, or miRNA silencing, that will lower its abundance to physiological levels [58].

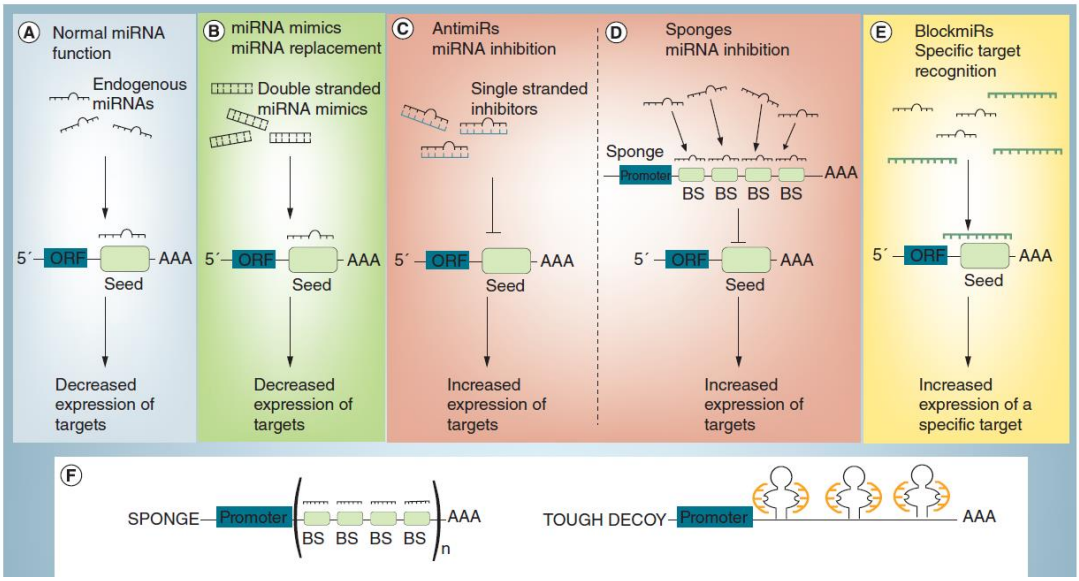

Figure 4. Different therapeutic strategies towards regulating miRNA and mRNA expression. Adapted from Bernardo et al. 2015. *[26]*

In the miRNA replacement treatment, there are two types of miRNA mimics that can be designed to restore normal expression by artificially increasing the endogenous miRNA population [59], [60]:

- Synthetic mature miRNA molecules that mimics the exact sequence of the inhibited endogenous miRNA.
- Short hairpin RNAs (shRNAs) which are synthetic RNA molecules that before reaching the mature miRNA state need to be processed by the endogenous processing machinery of the miRNA pathway of the targeted cell.

In the latter, the fact that an extra step is needed so that the shRNAs would be functional, can minimise potential toxicity, since the amount of active mature miRNA will not only depend on the dose administered and the delivery vehicle but also on the processing rate [29].

However, the world of miRNA inhibitors is much wider [29], going from short, single stranded synthetic antisense-oligonucleotides (ASOs) that have high-affinity binding sites in the selected miRNA, to miRNA target decoys [61] or cirRNA sponges that can target more than one miRNA at a time (it has multiple repetitions of the miRNA binding sites [61]) [62]. The main effect of a miRNA inhibitor is gene expression activation and of a miRNA mimic, gene expression suppression[35].

As it will further discussed below, delivery is a major obstacle for RNA drug development, since achieving effective concentrations on the desired area is challenging [58]. On top of that, the administration method is another factor that needs to be considered in order to decrease toxicity and off-target effects [29].

The high flexibility of the target binding region (seed sequence) in miRNA is a double-sided feature. It allows them to target entire pathways, but, off-target effects can easily take place. Nevertheless, this in an issue shared with traditional medications, since even though they are usually designed to target a specific protein they interact with others causing adverse reactions [63]

Although, no miRNA-based drug has still reach the clinic, there are several Phase I and II ongoing clinical trials [43] (Table 2). Furthermore, other RNA-based drugs targeting CVD or other heart-related pathologies have already been approved by the US Food and Drug Administration (FDA) [64]. The most recent example is, Patisiran, a drug based on a small-interfering RNA to treat peripheral nerve disease has been recently approved.

4.6.1 Development of miRNA-based drugs

4.6.1.1 Bioinformatic analysis for target prediction

Multiple databases are available to predict miRNA-mRNA interactions. One of the most frequently published algorithms or miRNA target detection, TargetScan [63], predicts miRNA gene targets based on seed regions binding to mRNA, by computing almost all identified miRNA sequences displayed in miRbase (http://www.mirbase.org/).

These approaches are mainly focused on detecting complementary regions between the 3'-UTR of the mRNA and the seed sequence of the miRNA [65]. However, miRNA regulatory networks are extremely complex and therefore not completely understood. As an example, high miRNA-mRNA association can occur despite poor binding to the seed region [66] and vice versa, high complementarity of the seed sequence does not always guarantee that a mRNA is regulated by an specific miRNA[67]. Therefore, it is common that predicted interactions have an accuracy rate of approximately 26% [68].

Another plausible explanation as of why miRNA/mRNA interactions cannot be entirely predicted by seed matching *in silico* is than contextual features should be taken into consideration. As an example, mRNA with complex secondary structures may prevent miRNA-mRNA interactions since only a few single-stranded regions are accessible. Zhao et al. showed that miRNAs have a greater likelihood of binding to 3'-UTR which are thermodynamically favourable, meaning that they have a relaxed secondary structure which is highly accessible [69]. In fact, interactions that show low free energy values of around −20 kcal/mol or less, suggest tight binding [63]. This demonstrates that RNA

accessibility is critical for target recognition and that it should also be considered when analysing *in silico* miRNA-mRNA interactions.

4.6.1.1.1 From bioinformatic prediction to the clinic

High-throughput screening together with *in vitro* validation of bioinformatic predictions have accelerated the study of putative therapeutic miRNAs. *In vitro* cultures of primary cells, immortalized cell lines, and iPSC-derived cells are used to validate *in vitro* interactions as well as therapeutic potential of the miRNA mimics and antimirs. Vectors and modified oligonucleotides are employed to overexpress a specific miRNA or to silence a specific gene function, disrupting the biochemical pathways similar to what occurs under disease conditions when miRNAs are dysregulated[43]. Furthermore, an increasing amount of animal models are available to asses *in vivo* validation. Additionally, non-human primate models have been successfully incorporated into preclinical miRNA investigations and supported the initiation of clinical trials, such as for the miR-122 repressor, Miravirsen, in the treatment of Hepatitis C Virus (HCV)[63]

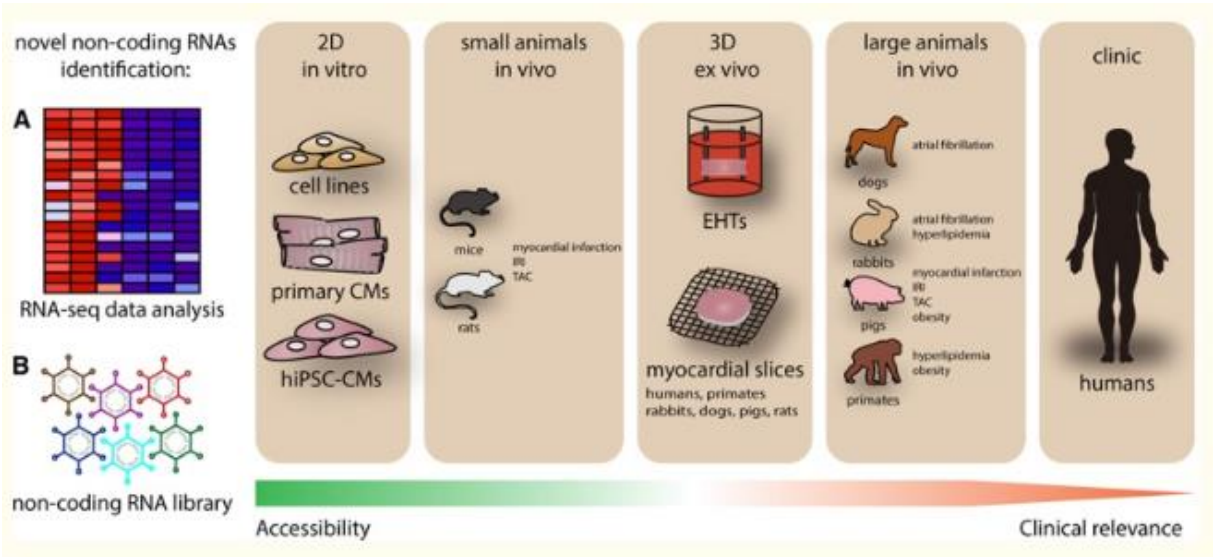

Figure 5. Processes of noncoding RNA (ncRNA)-based drug development[43]

In order to develop a new drug therapeutic, several steps have to be followed to jump from the bioinformatic studies to the human clinical trial (Figure 5). Here, a practical example is described for miR-92a.

MiR-92a overexpression in *in vitro* studies in human endothelial cells inhibited the expression of pro-angiogenic factors and the migration of endothelial cells had been reduced as well as their ability to adhere to fibronectin[70]. Furthermore, forced overexpression in zebrafish blocked blood vessel formation [70].

In vivo studies with miR-92a inhibitors demonstrated pro-angiogenic effects in acute myocardial infarction models in mouse and pig[70], [71] and ameliorated vessel repair after neointimal injury in rat [72]. Additionally, a light-inducible antimiR-92a showed effectiveness in increasing angiogenesis and wound healing in db/db mice (a model of diabetic impaired wound healing)[73]. So, since several studies proved that miR-92a is involved in the regulation, efforts were made into developing an effective antagonist to miR-92a that would serve as a therapy to promote angiogenesis in the heart and skin to accelerate healing.

The pharmaceutical company Miragen proceed with a screening of several locked nucleic acid (LNA)-modified miR-92a inhibitors, the one that obtained better results in *in vitro* and *in vivo* studies turned out to be MRG-110. Treatment with MRG-110 showed an upregulated expression of the proangiogenic gene ITGA5 (miR-92a target) *in vitro* in both HUVEC cells and primary human skin fibroblasts and *in vivo* in mouse skin, exhibiting its on-target effects [74].

In db/db mice with excisional wounds, MRG-110 was injected intradermally which lead to an augment in wound re-epithelialization, a rising in ITGA5 protein expression. Furthermore, no systemic toxicity or distal angiogenesis was detected. In a wound healing study performed in normal pigs, MRG-110 also increased acute excisional wound healing and angiogenesis without appreciable systemic toxicity[74].

Since this studies demonstrates that a novel LNA-modified antimiR-92a, MRG-110, accelerates angiogenesis and wound healing in compromised and acute wounds in both mice and pigs [75]. It was approved for clinical trials in humans, and it is currently in Phase I.

4.6.1.2 Delivery vehicles

Lipid bilayers facilitate the passive diffusion of small neutral or slightly hydrophobic molecules, while impede large, charged molecules penetration [76]. That is why naked miRNAs are defectively taken up by cells due to their negative charge. Furthermore, since the dosage that reaches the target cell is difficult to predict, undesired off-target effects are likely to occur (Figure 6). On top of that, they show, short half-life when presented naked in systemic circulation, due to rapid degradation by circulating nucleases, in contrast with endogenous circulating miRNAs that are transported in exosomes that prevents them from degradation.

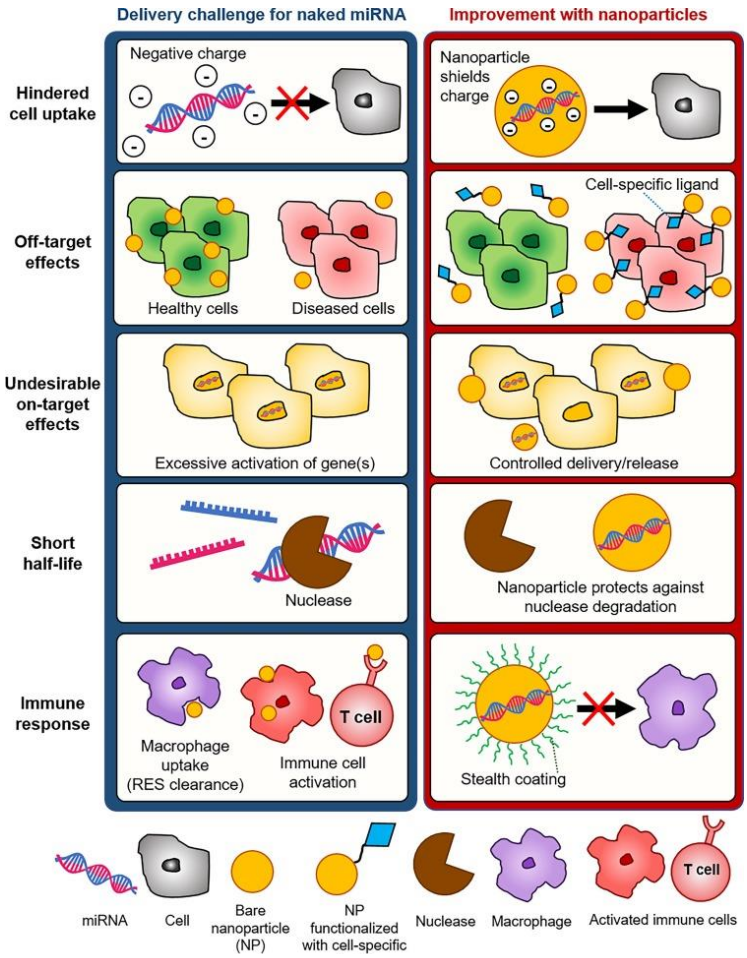

Figure 6. Challenges of miRNA delivery *in vivo*. [77]

In order to stabilize naked miRNAs, their backbone can be modified introducing chemical modifications which will favour carrier-free *in vivo* delivery [77], as it will be explained in 4.6.2. Nanoparticles are one of the most successful approaches used to stabilize the therapeutic miRNA and protect it from nuclease degradation (Figure 6). If tissue-directed, they also provide a more specifically high concentration delivery at the required action site while not affecting healthy tissue, which should increase their therapeutic index [78]. However, one of the main problems that some delivery vehicles pose is low miRNA encapsulation efficiency, since they tend to diffuse in the aqueous solution needed to prepare the nanocarriers [77]. Recently, a wide range of nanoparticle formulations have emerged, being a highly innovative approach that could become an effective option for the therapeutic delivery of miRNAs (Table 4). To name a few of this biodegradable and biocompatible particles: organic-based lipid nanoparticles (LNPs), extracellular vesicles, bacterial minicells, and nanoparticles derived from inorganic materials such as silica, gold and polyamidoamine (PAMAM) dendrimers [79].

Table 4. Summary of the main delivery vehicles used for cardiovascular disease and delivery of miRNA therapeutics

VEHICLE	VEHICLE DESCRIPTION	MAIN CHARACTERISTICS	REFERENCES
Lipid-Based Carriers	• Amphipathic molecules that consist on one or more phospholipid bilayers with an aqueous core • Cationic lipids are commonly used. They have a hydrophilic amino head group (positively charged) that through electrostatic interactions spontaneously interact with the negatively charged phosphate backbone of the miRNAs	• Biocompatible • Great transfection efficiency (cell membranes are negatively charged) • Cell toxicity (strong electrostatic interactions compromise the cell membrane integrity) • Tend to aggregate (interact with negatively charged serum proteins). • Immunogenicity cases have been reported (combination with neutral lipidic molecules is thought to decrease it)	[87], [78], [88], [89]
Polymer-Based Carriers	• Can be composed of either natural (e.g chitosan and hyaluronic acid) or synthetic materials (e.g PEI and PLGA) • All of them contain amine groups that interact with the phosphates present in nucleic acids	• Natural polymers easily and compactly hold nucleic acids. They display high biocompatibility and biodegradability. They can be modified to ameliorate miRNA release efficiency. • PEIs has high transfection efficacy, but also high toxicity (can be avoided by adding chitosan and polyethylene glycol (PEG)). Non-biodegradable. • PLGA is biocompatible and non-cytotoxic. Degradation rate varies depending on composition from several months to years.	[78], [90], [77]
Extracellular vesicles	• Endogenous particles essential for cell-to-cell communication whose content is highly dependent on the cell type.	• Distinct sizes and different biological origin. • The main subtypes are exosomes (30–120 nm), microvesicles (100–1000 nm) and apoptotic bodies (>1000 nm).	[91]
Inorganic Nanoparticles	• Gold, calcium phosphate, silica and iron oxides forming particles	• High biocompatibility • They are usually modified to facilitate miRNA entrapment and prevents nanoparticle aggregation and miRNA degradation • Conjugated with antibodies, or combined with other carriers to increase delivery specificity	[88]
Bacterial Minicells	• Anucleate nanoparticles that come from inhibiting cell division genes in parental bacterial cells at partition time	• Average 400 nm in size • Conjugated with specific antibodies to target a specific organ-tissue	[92]
Microbubbles	• Albumin coated microspheres filled with the miR therapeutic and gas that release their content when ultrasounds are applied at the target site	• Non-invasive technique that allows specific delivery of miRs therapeutics to the heart	[93] [76].
Viral vectors	• Adeno-associated virus (AAV) vectors, which allow encapsulation of nucleic acids up to 4.5-5kb	• Higher safety profile than other virus-based vectors, while maintaining high transduction efficiency on several tissues. • AAV9 is cardiac specific (supposedly has cardiac tropism).	[94]

4.6.1.3 Administration

Different administration routes can be used to efficiently deliver the miRNA therapeutic to the target organ or tissue. Both local and systemic delivery of the miRNA-drugs are considered [76]:

- Systemic delivery: nanoparticles and vesicles are modified with tissue-specific ligands to enhance delivery to target tissue and subsequent uptake by receptor-mediated endocytosis. They are directly injected in the systemic circulation.
- Local delivery: in case of heart-related therapies, intracoronary injection or intramyocardial injection can be used in order to inject miRNA therapeutics locally [80], [81]. Nasal nanoparticle delivery has been also tested in case of brain delivery [82] and heart delivery [83] in mice.

Local injection in the heart produces higher transduction rates and diminish off-target events. However, efficient diffusion of the therapeutic through the myocardium is still a challenge. Furthermore, local injection is coupled with electromechanical mapping and/or positron emission tomography to analyse blood flow in the targeted area, allowing for extremely precise administration and consequently achieving high efficiency administration around the site of injection.

Systemic administration of miRNA mimics and inhibitors, apart from being expensive, since high amounts of the therapeutic agent are needed, it demonstrates low efficacy on *in vivo* trials [76]. Another important drawback is an increasing chance of potential side effects to occur, since one miRNA usually has multiple targets, that might have different or even opposite functions depending on the organ or cell type [76]. For instance hsa-miR-24-3p shows divergent effects when expressed in vascular (promotes endothelial cell apoptosis and inhibits angiogenesis after ischemia) [84] or cardiac cells (apoptosis inhibition of cardiomyocytes in mice) [85]. This suggest that, independently on the administration, a tissue- and/or cell-specific delivery might be desirable.

A strategy to overcome undesired effects in systemic deliveries is the use of tissue-directed approaches. Recently many strategies are being exploited to improve targeting cardiac-specific uptake of the delivery vehicles. Complete antibodies, Antibody Fab fragment, oligonucleotide aptamers or a targeting peptide are amongst the most interesting targeting options [86]. Another one that has previously been treated is the microbubbles mediated by ultrasounds delivery of the therapeutic miRNA. Encouraging results have been also observed with antibodies, cardiac-targeting peptides and pH-sensitive-targeting peptides [77]

4.6.2 AntimiRNAs therapies

4.6.2.1 Antisense oligonucleotides

Antisense oligonucleotides (ASOs) have been used for more than 40 years to silence defective mRNAs [95]. Recently, they have turn out to be an essential tool to target miRNAs. However, the effects observed when targeting miRNA differ from those that take place when targeting mRNAs: ASOs are believed to silence the miRNA in an RNase-independent manner, mainly by steric blocking, while in case of mRNA the degradation is RNase-dependent [96].

ASOs should be designed so that they have a high miRNA binding affinity while efficiently decreasing the amount of the target miRNA. Low or non-toxicity and lack of additional side effects are also required. The binding affinity is a parameter related to the stability of the ASO-miRNA duplex, and therefore is measured by the melting temperature (T_m) of the oligonucleotide. Higher T_m values indicates a stronger duplex (more energy is needed to disrupt the binding) [96].

Non-modified ASOs as composed only of DNA bases are very susceptible to be degraded by nucleases. Therefore, extracellular and intracellular stability (resistant to nucleases) is also essential for the ASOs in order arrive fully functional to its tissue or cell target, where the miRNA that needs to be silence is [97]

Chemical modifications to ameliorate binding affinity and nuclease resistance are mostly introduced in the sugar ring (usually at the C2' position) and/or in the backbone of the ASO (Figure 7). The latter consist on internucleotide linkage adjustments or substituting the entire backbone structures for a construction analogous to the DNA [98]. The properties of each modification have been reviewed by Lima and colleagues [96].

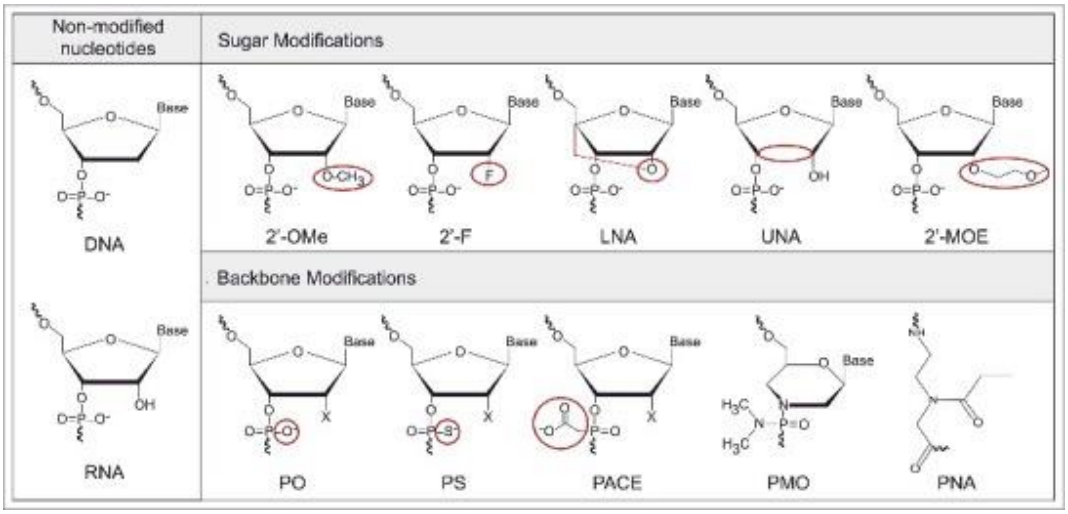

Figure 7. Chemical modifications applied in the ASOs design. Unmodified nucleotides – DNA: deoxyribonucleic acid, RNA: ribonucleic acid; Sugar modifications – 2'-OMe: 2'-O-methyl, 2'-F: 2'-fluoro-RNA, LNA: Locked Nucleic Acid, UNA: Unlocked Nucleic Acid, and 2'-MOE: 2'-O-methoxyethyl; Backbone modifications – PO: phosphodiester, PS: phosphorothioate, PACE: phosphonoacetate, PMO: Phosphorodiamidate Morpholino Oligomers, and PNA: Peptide Nucleic Acid. In red is highlighted the site and the type of modification [96]

ASOs composed by, 2'-MOE and 2'-OMe sequences mixed with PS or PO backbones, were studied to prove to their potency to repress miR-21 during a sustained period [99]. miR-21 is upregulated in hearts from patients that present severe heart failure and in fibroblasts of failing mouse hearts, but its role in cardiac remodelling is still not defined, since genetic suppression does not change the pathological cardiac response to pressure overload [26]. The most frequently used chemical modifications for anti-miRNA therapeutics are LNA, 2'-F-RNA, 2'-OMe, PNA, and PMOs.

Additional molecules can be linked to the ASOs so that uptake, half-live or delivery are improved when administered *in vivo*, since most of these molecules present enormous difficulties to diffuse across cell membranes. One of the most popular organic compounds that is added is a cholesterol molecule at the 3'-end. ASOs with a cholesterol modification are denominated AntagomiRs [100].

Finally, it is important to highlight that ASOs containing unmethylated deoxycytidine-deoxyguanosine (CpG) dinucleotides motifs are likely to activate the immune response in mammalian systems [101]. Therefore, avoidance of this type of construct is recommended, especially when the ASO is being designed for *in vivo* testing.

A few examples of miRNAs that can be targeted by ASOs are summarise in Table 5. miR-15a and miR-15b showed an upregulation in post-ischemic HF models, which was correlated with increasing apoptosis due to hypoxia induced cell death [102]. Zhang et al. showed that cardiac miR-21 is

upregulated with age in a mouse model of aging, furthermore, miR-21 has demonstrated to have profibrotic effects provoked by the activation of the ERK–MAP kinase pathway in cardiac fibroblasts during injury [103]. miR-34a is involved in both cardiac and vascular aging, being a promising target to design therapeutics to treat CV aging [104]. Inhibition of three miR-34 family members (miR-34a, -34b, and -34c) has demonstrated to ameliorate cardiac dysfunction in mouse models of HF [105]. Callis et al., discovered that when miR-208a is upregulated it contributes to cardiac hypertrophy, and disturbs electric conduction leading to arrhythmias in mice [106]. miR-328, is upregulated in AF which is linked with a decrease in L-type Ca^{2+} channel density, inducing atrial electrical remodelling. Delivery of antagomiR against this miRNA was able to revert the arrhythmogenic phenotype [107].

Table 5. Summary of the main ASOs preclinical studies used for cardiovascular disease. i.v (intravenous injection), i.p (intraperitoneal injection), i.c (subcutaneous injection)

miRNA	Model	Type of ASO	Administration	Phenotype effects/Expression modulation of miRNA inhibition	Reference
miR-15 family	Mice and Pigs after Ischemia-Reperfusion Surgery	LNA	i.v	• *in vitro:* cardiomyocytes show resistance to hypoxia-induced cell death • *in vivo* (mice and pig): Effective repression of miR-15 levels • *in vivo* (mice)*:* infarct size reduction and cardiac function improvement	[108]
miR-21	TAC mouse model	Cholesterol-conjugated 2'-O-Me ASO	i.v	• Inhibition of ERK-MAPK signaling pathway • Interstitial fibrosis is reduced, and normal cardiac function restored	[109]
miR-21	Pigs that underwent transient percutaneous occlusion of the left coronary	LNA	intracoronary infusion	• Cardiac fibrosis and hypertrophy are diminished • Overall, improved cardiac function.	[110]
miR-25	Mice in which TAC was active for 3 months	Antagomir	Daily tail vein injection for 3 days and 3 extra injections once a week for 3 weeks	• De-repression of SERCA2a and IP3R1: Restoration of Ca2+ uptake	[111]
miR-34 family	MI and TAC mice	LNA	daily s.c. injection for 3 days	• Upregulation of genes linked to cell survival, contractile functions and angiogenesis. • Pathological left ventricular remodeling is diminished • 95% knockdown in the heart lasted for up to 2 months	[105]

miR-92a	Pigs in which percutaneous ischemia/reperfusion Was performed	LNA	Local (with a catheter) or i.v	• Reduction in miR-92a expression is achieved with both administration methods in the infarct area • Reduction in the infarct size is only seen in local delivery. It led to a boost in the ejection fraction and lower left ventricular end-diastolic pressure.	[112]
miR-92a	mouse models of limb ischemia and MI	Antagomir	i.v	• Blood vessel growth is boosted • Regain of function in damaged tissue	[113]
miR-208a	Hypertension-induced heart failure in Dahl Hypertensive rats	LNA	Subcutaneous injection	• Pathological myosin switching (from Myh6 to Myh7) and cardiac remodeling are inhibited • Enhanced cardiac function • Increased survival after induced HF	[114]
miR-208b	Dilated cardiomyopathy mice	LNA	i.p.	• Transition of adaptive to maladaptive remodeling is repressed	[115]
miR-199b	Mouse model of heart failure (calcineurin transgenic mice and TAC mice)	Cholesterol-conjugated 2'-O-Me antagomiR	i.p on three consecutive days	• Recovery of physiological expression of Dyrk1a • Reduction in nuclear NFAT activity • Inhibition and reversion of cardiac hypertrophy and fibrosis	[116]
miR-328	Mouse model of atrial fibrillation	Cholesterol-conjugated 2'-O-Me antagomiR	i.v	• Atrial fibrillation is converted to sinus rhythm	[117]

4.6.2.2 BlockmiRs

Whereas the rest of the methods to control miRNA expression focus on targeting the dysregulated miRNA, BlockmiRs functions protecting the mRNA from being degraded by miRNAs [26].

BlockmiRs are steric blockers that interact with the mRNA at the area where the miRNA is supposed to bind, therefore, blocking its access and impeding its down-regulatory function. This method of modulating mRNA expression combined with miRNA sponges, increases expression of proteins encoded in the mRNA whose expression was repressed. Nonetheless, it has low gene-specificity because the seed sequence or the 3'UTR target sequence can be common in many genes [96].

The unique and more useful characteristic of the blockmiRs is that while protecting a specific mRNA of being downregulated, the miRNAs targeting this mRNA can still regulate its other mRNA targets [26]. However, this technology has not been applied yet to target miRNAs related to CVD.

4.6.2.3 miRNA-sponges

miRNA-sponges, act as competitive inhibitors, since they are mRNA molecules with multiple binding sites (around 4 to 12) for the target miRNA that will sequester the upregulated miRNA. Thus, the endogenous target mRNA will not be repressed and will execute its function normally [118]. Increasing the number of binding sites augments the sponge activity, this is a crucial step if saturation occurs, the RNA is likely to be degraded. Adding spacers between the binding sites enhances the binding likelihood, and reduces the formation of RNA secondary structures [119].

miR- sponges within the AAV6-CMV cassette are not expected to exhibit a functional effect in other tissues, since they seem to have heart tropism [120]. Hereafter, a few examples of miRNA sponges against miR-21, miR-34a and miR181 are exposed (Table 6).

microRNA-21 (miR-21) controls vascular smooth muscle cell (VSMC) proliferation and phenotype transformation. Therefore, AAV-mediated miR-21 sponge was developed to supress VSMC proliferation *in vitro* and neointimal formation *in vivo* (rats), to diminish the chances of vein graft failure, an important complication of cardiovascular surgery [121].

Inhibition of miR-34a or the miR-34 family with miRNA sponges show cardioprotective results in mice hearts after heart injury. However, this inhibition shows no appreciable phenotypic effect in control mice [118]. Nonetheless, chronic inhibition of miR-34 would enhance tumorigenesis [122]

The AAV6-CMV-miR-34 15-nucleotide sponge *in vitro* exhibit an approximately ∼40–50% knockdown of miR-34a, -b, and -c expression when treated cells were compared to control. However, *in vivo* experiments showed that neither AAV6-miR-34-sponge 15 nucleotide nor its 8 nucleotide version were able to supress miR-34 family in the heart [118].

miR-181 family regulates cytoplasm and mitochondrial genes linked to oxidative stress. For instance, miR-181a/b regulates PTEN expression (cytoplasm) and miR-181c controls mt-COX1 (mitochondria) in cardiomyocytes. Downregulating miR-181 family expression in the heart using AAV6 delivery with a miR-sponge showed how different family members exert its function in distinct cellular compartments. Reduction of miR-181c in the mitochondrial compartment exhibit cardioprotective effects against oxidative stress (aging hallmark), whereas suppressing miR-181a/b in the cytosol has severe effects during ischemia/reperfusion injury due to overproduction of ROS. This miRNA family pose a perfect example of how finely tuned miRNA functions are, and how important is knowing all the functions to adequately target them during dysregulation [123]

Table 6. Summary of the preclinical studies applying miRNA-sponges technology to CVD.

i.v:intravenous injection, nt: nucleotide

miRNA	Model	Type of sponge	Administration	Delivery vehicle	reference
miR-21	*In vitro*: Primary rat VSMCs *In vivo*: Male SD rats	2 tandem repeats of 20 nt, with a mismatch between positions 9-12, separated by a short 4 nt spacer	Vein graft	VSMC cells that were engrafted suffer incubation with Adenovirus encoding miR-21-Sponge for 48 h	[121]
miR-34	*In vitro*: H9c2 cells (cardiomyoblast cell line) *In vivo*: adult mice C57BL/6 or FVB/N background	Four tandem repeats, each of 15 nt long, of either the miR-34a, miR-34b, and miR-34c binding sites.	*In vivo*: i.v	Adeno-associated virus vector 6 (AAV6) under the cytomegalovirus (CMV) promoter	[118]
miR-34	*In vivo*: adult mice C57BL/6 or FVB/N background	Eight tandem repeats of the miR-34 seed region (8 nt long)	*In vivo*: i.v	Adeno-associated virus vector 6 (AAV6) under the cytomegalovirus (CMV) promoter	[118]
miR-181	*In vitro*: H9c2 cells (cardiomyoblast cell line)	10 tandem repeats complementary to the seed sequences of miR-181 family	Transfection	The sponge sequence is cloned into an Enhanced Green Fluorescent Protein plasmid	[123]

4.6.2.4 Tough decoy (TuD)

TuDs are synthetic molecules formed by a long RNA hairpin of approximately 60 base pair with an internal loop that has two miRNA binding sites (Figure 8). It acts as a competitive inhibitor preventing endogenous miRNA binding to target mRNAs[61].

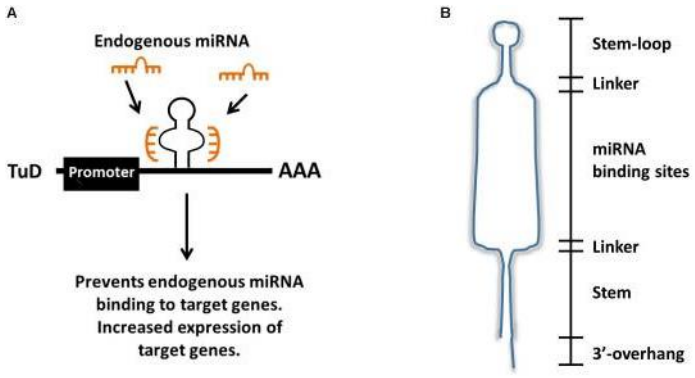

Figure 8. Structure of the tough decoy molecules

As we can observe in the figure 8 ,TuD present several distinct regions: an 18-bp long stem, two miRNA-binding sites, a 26-nt long stem-loop that links the miRNA binding sites and four 3-nucleotide linkers (2 on each chain) defining the two miRNA-binding sites and separating them of the stem-loop and the stem. Usually, in each of the miRNA binding sites, a 4-nt long mismatch is added in between

nucleotides 10 and 11 from the 5' end of the miRNA to avoid perfect-base pairings with miRNAs. Thus for, preventing the cleavage of the miRNA-binding site [118]

A few examples of TuD that target miRNA related to CVD are shown in Table 7. When studying, the effects of TuD-miR-34a in the heart, the results obtained where the same as with the previously exposed miR34-sponge approach: despite being taken up by the cardiomyocytes in the heart, no significant inhibition of miR-34a or any of the family members was achieved. These results are contrary however to the high inhibition was achieved with LNA-ASOs technology [36].

Table 7. Summary of the preclinical studies applying TuD technology to cardiac disease. i.v (intravenous injection)

Target miRNA	Model	Administration route	Delivery vehicle	reference
miR-25	*In vitro*: H9c2 cells *In vivo:* pressure-overload murine HF model	*In vivo*: single i.v dose	AAV9	[61]
miR-34a	*In vivo*: adult mice C57BL/6 or FVB/N background	*In vivo*: single i.v dose	AAV6	[118]
miR-146a	*In vivo*: Wild-type mice subjected to TAC surgery (leads to pressure overload)	*In vivo*: single i.v dose	AAV9	[124]

miR-25 regulates *Serca2a* activity, which is in charge of calcium uptake during excitation-contraction coupling in cardiomyocytes. miR-25 upregulation, disrupts calcium uptake and contributes to heart failure [111]. AntagomiRs, designed to diminish miR-25 have already proven to be effective, however in this case TuDs are studied since they are highly resistant to endonucleolytic degradation and present improved miRNA binding affinity [61]. The AAV9 TuD exhibits selective inhibition of miR-25 both *in vivo* and *in vitro*. In addition, miR-25 TuD expression in the heart lasts longer and therefore has a more powerful inhibitory effect when compared to the antagomir-25, previously created. Furthermore, Western blot analysis shows that miR-25 TuD achieves almost a complete restoration of the physiological Serca2a levels, ameliorating cardiac dysfunction and fibrosis [61].

miR-146a is expressed in fibroblasts after HF and targets SUMO1, which diminishes SERCA2A SUMOylation, inhibiting cardiomyocyte function both in mice and humans failing hearts [124]. mir-146a is secreted from activated fibroblasts inside an extracellular vesicle that is taken up by cardiomyocytes. Surpressing miR-146a activity through TuD improves cardiac contractile function by restoring SUMO1 levels in mice with pathologic cardiac hypertrophy [124].

However, it is important to notice that downregulation of miR-146 might not always be beneficial, since it´s role in endothelial cells aging it is still not well defined. Whereas some studies report that

miR-146 is upregulated in a cellular model of endothelial senescence [125], others affirm the contrary [126]. Furthermore, miRNA-146a has anti-inflammatory properties and it is widely expressed in immune cells. In fact, a protocol to deliver miR-146a directly to the atherogenic lesion to diminish macrophage recruitment and atherosclerotic plaque size in mice was reported [127].

4.6.3 miRNA mimic-based therapies

miRNA mimics are described as oligonucleotides that act in the same way than the endogenous miRNA. Therefore, miRNA mimics bind to target mRNA leading to its translational repression and or degradation suppression [36]. In the table 8, a list of miRNA mimics investigated in CVD is shown.

MiRNA mimics have been tested to restore the correct regulation of cardiac functioning by increasing the amount of a determined miRNA that it is downregulated and causing a heart-related pathology. Among them, miR-19a-mimics are useful to induce regeneration in the myocardium after infarct. Furthermore, taking into account that miRNA-19 is severely downregulated in an aged HF-prone mouse, findings that were corroborated in human samples, this mimic could also help to induce cell renovation and decrease apoptosis associated with aging. miR-199a seems to be extremely downregulated in cardiomyocytes when the amount of oxygen available is reduced. This reduction results in an upregulation of its target, the hypoxia-inducible factor (Hif)-1α. Therefore, by delivering a miR-199a-mimic during hypoxia conditions Hif-1α expression is repressed, and p53 is stabilized, reducing apoptosis [37].

Table 8. Summary of the preclinical studies with miRNA replacement technology in cardiac

miRNA	Target gene and Physiological function of the miRNA	Model	Mimic effects	Administration	References
miR-19a	• Controls PTEN, gene involved in cell proliferation and apoptosis	Mice	• Enhances cardiomyocyte proliferation and stimulates cardiac regeneration right after MI as well as in the long-term.	intra-cardiac injection of AAV9-miR-19a/19b	[128]
miR-21	• Promote cardiac fibrosis by targeting mitogen-activated protein kinase (MAPK) signaling • Regulates VSMC differentiation in response to BMP signaling • Regulates magrophage activation	Mice	• Cardiac macrophages at the infarct zone, switch their phenotype from pro-inflammatory to reparative. • Angiogenesis is enhanced and hypertrophy, fibrosis and cell apoptosis	i.v administration of nanoparticles containing miRNA-21	[129]

			reduced in the myocardium.		
miR-29	• Downregulated in fibrotic disorders • Controls collagen genes (COL1A1, COL1A2, COL3A1) and extracellular matrix proteins expression, such as fibrillin (FBN1) and elastin (ELN1)	Mice	• Decreased collagen expression • Fibrosis is diminished	doxycycline (Dox)-inducible miR-29b transfected in the mouse heart	[130]
miR-126	• Targets the angiogenesis related protein Spred1, a regulator of the MAP kinase signaling downstream of VEGF and of fibroblast growth factor (FGF) signaling, • Regulates phosphoinositide-3-kinase (PI3K)-regulatory subunit 2a (PIK3R2a), (PI3K signaling)	*In vitro*	• Promotes angiogenesis • Attenuation of the infarct size area and inflammation • lower cardiacfibrosis	exosomes enriched with microRNA (miR)-126ko	[131]
MiR-199a	• Downregulatedin cardiac myocytes during oxygen deprivation. This induces its target genes: hypoxia-inducible factor-1α (HIF-1α) and sirtuin (Sirt 1), and subsequent activation of hypoxia-triggered pathways.	Adult Mice	Restoration of physiological miR-199 levels inhibits HIF-1α expression and its stabilization of p53, a tumor supressor responsible for sustaining the genome integrity, which leads to a reduction in apoptosis.	Single dose Intracardiac administration into the infarct border	[132]
miR-302–367 cluster	• Downregulated after birth • Target genes involved in the Hippo signal pathway (Mst1, Lats2, Mob1b) • Mediates in cardiomyocyte proliferation during development	Adult Mice	• Enhanced cardiomyocyte proliferation • Promotes cardiac regeneration and improved function after MI surgery	neutral lipid emulsion	[133]
miR-590	• Associated with heart regeneration through modulation of Homer1 and Clic5	Adult Mice	• Cardiomyocyte proliferation • Improved cardiac function • Reduction of infarct size	Single dose Intracardiac administration into the infarct border	[132]

4.6.4 Current state of miRNA-based drugs and repurposing to treat CV

Over the past few years there has been a great increase in the miRNA research field for both diagnostic and therapeutic purposes. Still, no miRNA therapeutics have been approved by the FDA nor by the EMEA (European medicines agency) for clinical use [134]

One of the most promising miRNA-based drugs was MRX-34 (a liposome-based miR-34 mimic) designed to treat liver cancer, certain solid tumours or hematologic malignancies. It entered clinical trials in 2013 (NCT01829971). However, the study had to be terminated at phase 1 since severe immune-related adverse events occurred [134]. Nonetheless, encapsulating this miRNA in highly specific nanoparticles that would only deliver it to the desired area and adding molecules that would hide the mimic from the immune system could help to increase its therapeutic potential, avoiding adverse reactions.

Thus far, out of all active clinal trials with either miRNA mimics or anti-miRs, only two target the cardiovascular system. One of them, which is now recruiting candidates for a second phase 1 clinical trial (NCT 03603431), consist on MRG 110, a locked nucleic acid (LNA)-modified antisense oligonucleotide that is thought to have a pro-angiogenic effect by directly inhibiting miR-92a. This would accelerate wound healing as a result of increasing blood flow in the affected area (https://clinicaltrials.gov/). The other compound (CDR132L) is an antimir directed against miR-132. This miRNA mediate cardiac hypertrophy via FOXO3 downregulation and autophagy. CDR132L is in Phase Ib.

However, some of the drugs that are in clinical trials could be successfully repurposed to target the cardiovascular system. For example, LNA-antimiR-155 (Cobomarsen) indicated for blood cancer could be very useful to restore cardiac normal functioning since *in vivo* studies have showed that inhibiting miR-155, could ameliorate cardiac inflammation. Another example, could be miRNA-29b mimic (Remelarsen) which targets cutaneous fibrosis, since miR-29 family members (miR-29a, miR-29b, and miR-29c) are downregulated in fibrotic disorders leading to increased levels of essential extracellular matrix components such as collagen and elastin, which will cause severe fibrosis in the heart.

5. MATERIALS AND METHODS

5.1 Sequence Analysis and Bioinformatic tools

The BIO-AGEmiRNAs that have been selected for this study are: miRNA 3916, miRNA 4435 and miRNA 24-2 (Table A1). All of them were upregulated with increasing biological age in human LV [15]

Perl Primer on-line software (Open-source PCR primer design, Sourceforge) was used to design the DNA oligonucleotides used as PCR primers (Table A2). The oligonucleotides were designed to amplify the pri-miRNA DNA sequence plus 250 flanking nucleotides to facilitate folding.

5.2 Bacteria and culture medium

Chemically-competent *Escherichia coli,* strain DH5α, were used for miRNA cloning and recombinant DNA vector amplification purposes.

Bacteria cultures in liquid media utilized Luria Bertani (LB) medium (MP Biomedicals, 3002022) with 100 µg/ml de Ampicilin (Sigma Aldrich, A9518-5G). In case of solid culture, LB-agar plates (Nzytech, MB11801) also supplemented with 100 µg/ml Ampicillin were used. Bacterial cultures were incubated O/N at 37˚C.

5.3 Cloning Vectors

The plasmid used to clone the miRNAs of interest, the **pZ:F3-P TetOn 3f-tdT-F (V5)**[17], was available at BSICoS. V5 is composed by an Ampicillin Resistant gene (allows selection of transformed bacteria), a Puromycin resistance gene (allows selection of transfected iPSCs) and inducible tdTomato fluorescent protein (tdT). TdT expression is controlled by a Tetracyclin Response Element (TRE) in a TetON set up: binding of the reverse tetracycline transactivator (rtTA) protein to tetracycline allows activation of the TRE promoter. We can also find two pairs of cHS4 insulators that isolate the whole transgene expression from the surrounding environment (Figure A1).

5.4 Polymerase chain reaction (PCR)

The miRNAs of interest were amplified by PCR in two different samples of human genomic DNA: D086 (Hek293) and D101 (White blood cells), using a high fidelity Taq DNA polymerase, Phusion Taq (Thermo, F-530L), following the protocol recommended by the manufacturer and specific oligonucleotides and amplification programs for each amplicon (Table A2 and A3). The reactives used for the PCR mixture are shown in Table A4

5.5 Agarose gel electrophoresis

To assess PCR results or restriction patterns, they were run on 1-1,5% agarose in 0.5x TBE buffer (50mM Tris, 45mM Boric Acid and 0.5mM EDTA) (Invitrogen, 15581044). To purify fragments from gel, the bands of the appropriated molecular weight were excised.

In all cases, gels were stained with Green Safe Premium 1:10000 (NZYTech, MB18081). The electrophoresis were performed at 135 volts. The samples were loaded into the wells after being mixed with loading buffer (30% glycerol, 0.25% bromophenol blue) (Invitrogen,10816015), except when digestion buffer (Red Anza buffer(Invitrogen,INVGN02028)) or NZYtaq II 5x gel load reaction buffer (NZYtech,17111) were used.

A molecular weight ladder,1 Kb Plus DNA Ladder (Invitrogen, 10787-018), was loaded to compare with the molecular weight of the samples. The iBright CL1000 (Invitrogen) was used to image the agarose gels.

5.6 Nucleic Acids purification

Purification of DNA fragments derived from excised agarose gel bands or directly from the PCR product was performed using NZYGelPure (NZYtech, MB01102), following the recommended instructions by the manufacturer.

Extraction and purification of plasmids was carried out with NZYSpeedy Miniprep Kit (NZYtech, MB21002) and Purelink HiPure Plasmid Midiprep Kit (Invitrogen, K210004). The first one was used to obtain a small quantity of the vector that could be prepared for sequencing. The second one, to obtain enough amount and of high quality plasmids, for storing and further experiments.

5.7 Cloning

5.7.1 Vector and insert digestion

Digestion mix for cloning purposes contained 2 µg of the DNA insert and 1 µg of the corresponding DNA of vector, as well as 0,5-1 U/µl of each restriction enzyme (Table A6) in the appropriate digestion buffer. The digestion mixes were incubated at 37˚C for 4 hours followed by an inactivation step at 80˚C for 20 minutes and then run on gel and purified according to section 5.6.

5.7.2 Ligation

The digested products were purified as indicated previously and quantified using a spectrophotometer. The ligation mix contains 25 ng of the digested vector, the adequate amount of insert following the molecular ratio 3:1 or 5:1 (insert:vector), 1 µl of T4 DNA ligase buffer (New England

Biolabs, M0202A) and 20 U/µL de T4 DNA ligase (New England Biolabs, M0202L). The total volume of the mix was 10 µl.

The mixture was incubated at 22˚C for 1 hour, followed by an inactivation step of 65˚C for 10 minutes. Each ligation reaction had its negative control sample that included only the vector (without insert), so that the amount of undigested or re-ligated vector could be determined.

5.7.3 Transformation of DH5α competent bacteria

Twenty five µL of DH5α competent bacteria were thawed on ice and incubated on ice for 30 minutes with 3 µL of the ligation reaction. Next, heat shock was induced at 37˚C for 60 seconds, followed by 5 minutes in ice. Subsequently, 500 µL of SOC medium were added and tubes were incubated at 37˚C for 1 hour at 250 rpm. Finally, 200 µL of the transformed bacteria were plated in LB-agar culture plates containing 100 µg/ml ampicillin. After incubation O/N at 37ºC, individual colonies of ampicillin resistant bacteria could be observed and numbers in each condition annotated.

5.7.4 Screening of recombinant colonies

5.7.4.1 Colony PCR

Colonies of interest were replicated on a clean agar plate (control plate) and in parallel, bacterial lysates were prepared by inoculating each of these colonies in 12 µL of miliQ water. Osmotic pressure together with the initial PCR denaturation step releases the bacterial nucleic acid content. Separately, the NZYTaq II DNA Polymerase mixture was prepared (Table A5) using specific primers for each gene (Table A2) and specific amplification programs (Table 3A). Recombinant colonies were determined by the presence of specific size amplification products on agarose gels after electrophoresis.

5.7.4.2 Enzymatic digestion

Verification of colony PCR results or screening without it (when no colonies grew in the ligation negative control plate) was carried out by enzymatic digestion as indicated in section 5.7.1 with approximately 300 ng of plasmid DNA. The enzymes used in this work are included in Table A6.

5.8 Sequencing

One or two recombinant clones were confirmed by sequencing using the Sanger method (STAB VIDA) using three oligonucleotides to cover the whole cloned region (Table A7). The sequences obtained were analysed with "Contig Analysis" program developed by Vector NTI (Invitrogen).

5.9 Cell culture

Hek293 cells culture medium is composed of DMEM (Biowest, S18240L0093), with 10% FBS (Thermo Fisher Scientific, 10270098) and 1% penicillin and streptomycin (Thermo Fisher Scientific, 15140122).

A vial of approximately 10^6 Hek293 cells was thawed by immersion in a water bath at 37˚C. The cells were transferred to a clean 15 mL tube and 9 mL of complete medium were added dropwise. To remove traces of the DMSO (cryoprotectant toxic for the cells at room temperature) cells were centrifuged (300 xg, 5 min) and resuspended in 15 mL of fresh medium so that they could be seeded into a 75 cm^2 culture flask. The medium was refreshed every two days by removing it, washing it with PBS and adding fresh medium. The culture was kept in flasks of 75 cm^2 or 25 cm^2 according to the experimental needs.

When the cell monolayer in the flask reached a confluence close to 100%, the cells were diluted and reseeded in another flask to allow further proliferation. For transfection, cells were counted in a hemocytometer taking 15 µl of the cell suspension and mixed with 15 µL of Trypan Blue (Thermo fisher, 15250061), a viability dye. Cells were seeded at 250000 cells/well in 12 well plate for transfection.

5.10 Transfection

Transfection was performed with Lipofectamine 2000 Reagent (Invitrogen, 11668027) 24h after seeding the cells, following the manufacturer's instructions. Briefly, for each well, 1 µg of the plasmid containing the miRNA to be tested was mixed with 54.7 µl Optimem (Thermo Fisher Scientific, 31985062) (tube A). The same amount of Optimem was mixed with 3,28 µL of lipofectamine (tube B). Subsequently, the contents of tube A were poured into tube B and incubated 5 minutes at room temperature.

109 µL of the tube A + tube B mixture were added per well. Since each condition was tested in triplicate, these quantities were adjusted to create a common mix for all the replicates. Transfection was performed using V39 vector.

5.10.1 Assessment of the transgenic miRNA expression

Doxycycline at 1 µg/ml per well was added to the cultures 24 hours post-transfection. After 24 additional hours, the cultures were visualized and imaged under a fluorescence microscope (Nikon, TS2). Next the cells were lysed for total RNA extraction, including small RNAs, using the RNA lysis buffer of the extraction kit RNA/DNA purification kit (Norgen biotek corp., Cat.48700). Total RNA was isolated according to the manufacturer’s protocol.

Retrotranscription of miRNAs was carried out with qScript microRNA cDNA Synthesis Kit (Quanta Biosciences, 95107-025) using 200 ng of RNA according to the recommendations of the manufacturer.

qPCR was done using Power SYBR Green PCR MM (Thermo, 4368706) and primers for miR-4435 and RNU6 (normalizer gene) (Table A8). An RT negative control (without retrotranscriptase) and a sample without template were used as negative controls in qPCR.

6. RESULTS

6.1 Creation of a miRNA inducible expression vector.

The scheme presented in Figure 9, grafically explains the steps that have been followed in order to create a new vector in which expression of the miRNA of interest can be induced by doxycycline. This process is detailed in the following sections.

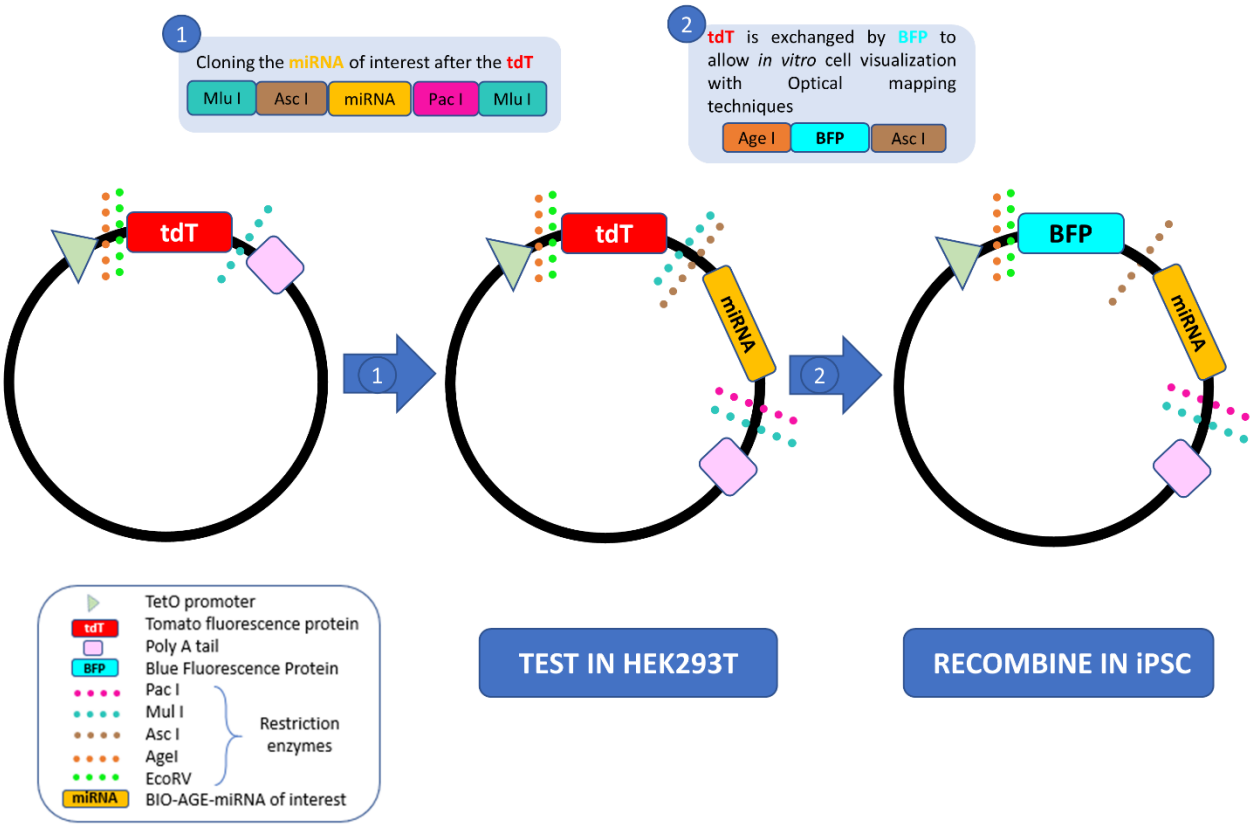

Figure 9. Workflow scheme of the creation of a new vector. The different cloning steps as well as the composition of the different inserts are described.

6.1.1 Cloning the miRNA of interest in V5

6.1.1.1 miRNA amplification

PCR amplification of miR-4435, miR-3916, miR-24-2, was only succesful for miR-4435 (Figure 10). Several modifications were made in the original PCR procol, like decreasing the annealing temperature

or adding DMSO, in order to achieve amplification of miR-24-2 and miR-3916, but they were ineffective. Therefore, in order to proceed with the study of those miRNAs in a future work, they have been synthesized, and during this study only miR-4435 will be analysed.

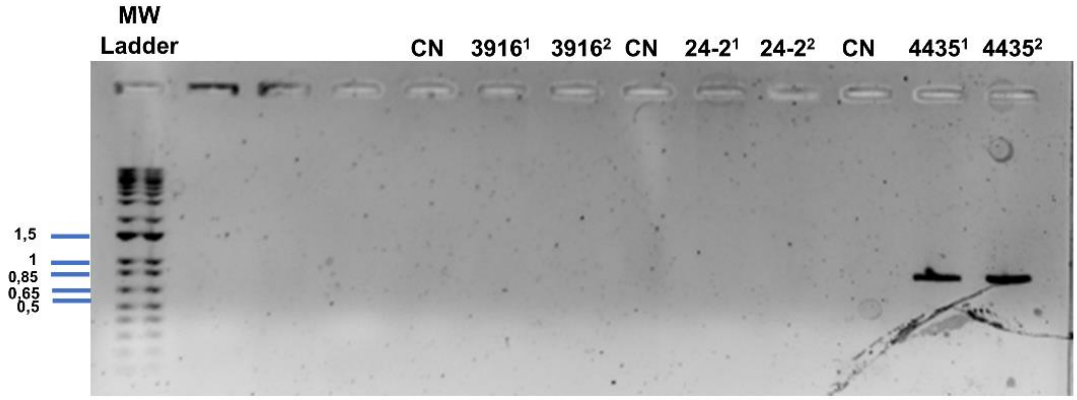

Figure 10. PCR amplification of miRNAs of interest. Amplifications of miR-4435, miR-3916, miR-24-2. The upper index on top of each sample name corresponds to the two different DNA samples used, being 1 referred to D86 and 2 to D101. CN: Negative Control (one for each PCR reaction). The bands observed corresponds to the miR-4435 amplicon at the expected molecular weight (approx. 820pb). MW ladder: Molecular weight ladder.

6.1.1.2 Cloning of mir-4435 in the inducible expression vector

In order to clone the miR-4435 in the destination vector V5, both V5 and the purified PCR product, as well as a positive digestion control (V1) were digested with MluI. Then, V1 digestion product was analysed by gel electrophoresis as a positive control to confirm that MluI succesfully digested the samples, as observed in Figure 11. After digesting with the restriction enzyme, vector V5 had to be dephospohorylated using alkaline phosphatase to avoid religation. Ligation was set up and the resistant colonies counted after overnight culture at 37ºC. (Table 10).

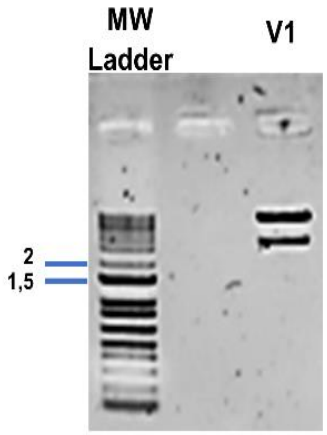

Figure 11. Digestion of vector V1 with MluI. MW ladder: Molecular weight ladder (Kb).

Table 10. Ligation results. Two plate replicates were made of each one of the ligation reactions, the first column corresponds to replicate one in both 3:1 and 5:1 ratios, and the second column to replicate two. Number of colonies in each LB-agar plate are shown.

	Insert:Vector ratios			
	3:1		5:1	
Vector with insert	48	114	52	45
Empty vector (Negative Control)	1		1	

Colony PCR yielded only 12 colonies with the correct fragment size that were considered positive (Figure 12).

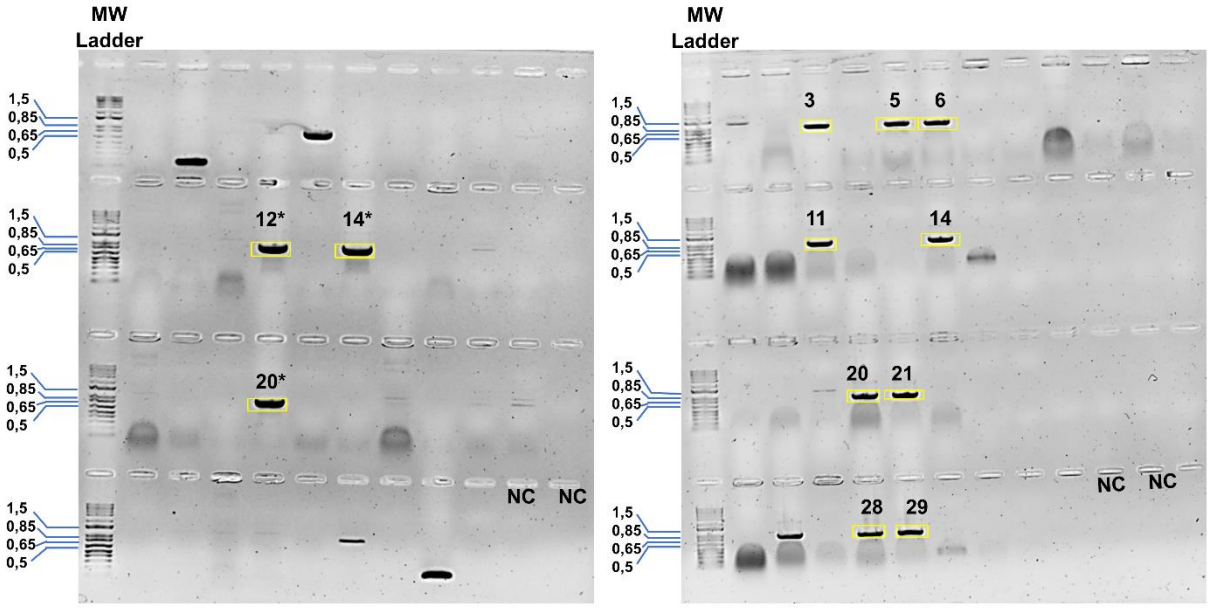

Figure 12. Colony PCR results. Amplification of recombinant plasmids in bacterial lysates resulted in a fragment of approximately 820 bp, highlighted with yellow boxes. MW: molecular weight DNA ladder (Kb).

These positive clones were further confirmed by restriction digestion with NcoI and two were recombinant since their restriction patterns matched those generate *in silico*.

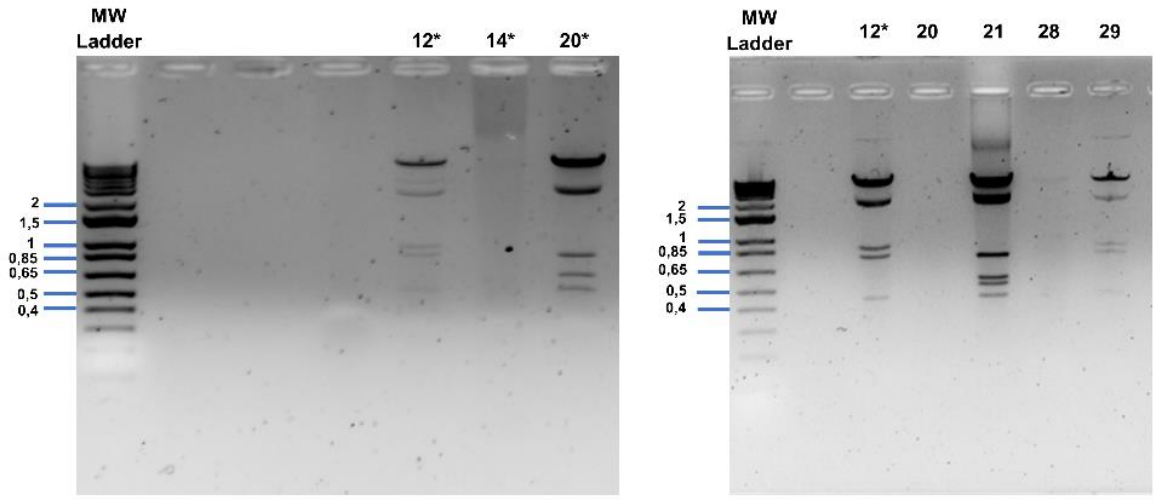

Figure 13. Identification of recombinant colonies by restriction digestion. Numbers on top of the wells correspond to the colony number. MW: molecular weight DNA ladder(Kb).

Clones 12 and 29 were sequenced to definitively confirm the correct insertion of mir-4435.
Once the sequence was verified, the recombinant colonies were cultured in 100 ml of LB-Ampicillin so that enough vector could be prepared. The vector was confirmed one more time by enzymatic digestion with NcoI before proceeding with transfection. The new vector was assigned the code V39.

6.1.1.3 Analysis of the expression of the mir4435 inducible expression vector (V39)

HEK-293 cells were seeded in 6 wells of a 12-well plate. After 24h they were transfected with V39. Induction of mir-4435 expression was performed by adding doxycycline 24h post-transfection. The expression of the tdT reporter upstream of miR-4435 RNA sequence was confirmed by fluorescence microscopy (Figure 14).

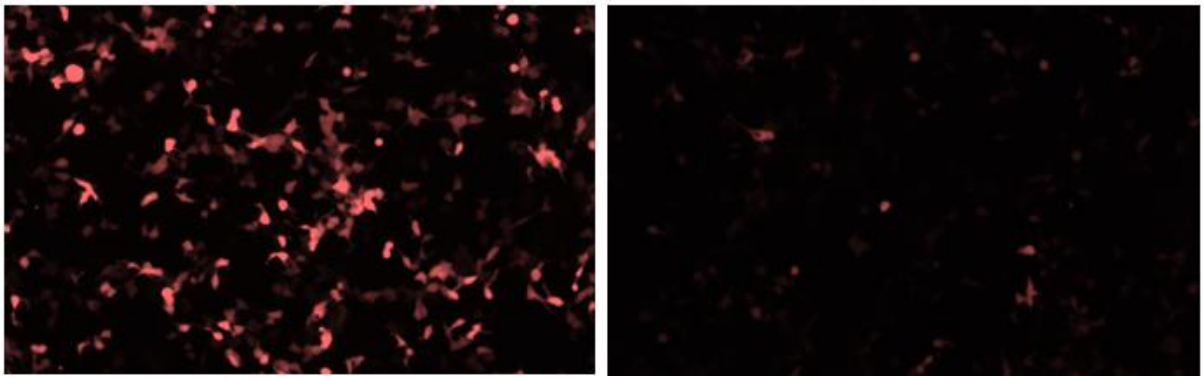

Figure 14. Transfection of Hek293 with V39. Hek293 were transfected with V39 and treated with (left panel) or without (right panel) 1µg/mL of doxycycline for 24 hours. Images were taken using the 10X magnification objective.

As it can be observed in figure 14, where doxycycline was added, tdT reporter expression is strongly induced. A certain amount of leakiness is observed in Hek293, likely due to the high transfection efficiency in these cells that can lead to multiple plasmid copy number per cell. In iPSC, just one copy of the inducible construct will be recombined.

This result allows to continue with the workflow as initially designed since expression of tdT as protein indicates the production of the RNA with tdT and the pre-miRNA.

6.1.1.4 Analysis of miRNA processing to obtain mature mir4435 in Hek293

The transfected cultures with and without induction were processed for total RNA extraction and retrotranscription of microRNA. The correct processing of the miRNA in Hek293 was evaluated by qPCR of the mature miR-4435. Unfortunately, the outcome of this experiment showed no amplification in any of the tested samples for miR-4435, but it was positive for the normalizer miRNA (RNU6). The lack of a positive control within this experiment does not allow to obtain a conclusive result: either the miRNA expression assay for miR-4435 failed or the miRNA is not being processed properly in Hek293 from the transgenic construct. Time constrains did not permit to repeat this experiment.

6.2 Replacement of tdT by BFP for iPSC functional characterization of the mir4435

MiR-4435 overexpression effect at the molecualr and electrophysiological levels will be carried out *in vitro* in CM-iPSCs. For electrophysiological measurementes, optical mapping techniques will be used. Optical mappin uses fluorescente probes to measure calcium concentrations and voltage. Thes probes have emission spectra close to tdT. Therefore, the tdT has to be replaced by tagBFP (blue fluorescent protein). TagBFP will allow to follow miRNA expression in the cell culture, but will not overlap with the Rhod-2 spectrum of the calcium probe.

6.2.1 Cloning of BFP to replace tdT reporter gene

PCR amplification of tagBFP using V15 as templated, was succesfully achieved as reported in figure 15A. tdT was excised out from V39 by digestion with AgeI and AscI (Figure 15B). The BFP fragment, obtained by PCR, was also digested with the same enzyme combination and purified before ligation. Ligation was performed with a 1:3 and 1:5 (vector:insert) molecular ratio.

After bacterial transformation with the ligation product, only two colonies were grown that putatively contained the recombinant vector. However, none of them showed the expected restriction pattern when digested with HindIII (Figure 15C). Further attempts are required to achieve the correct vector.

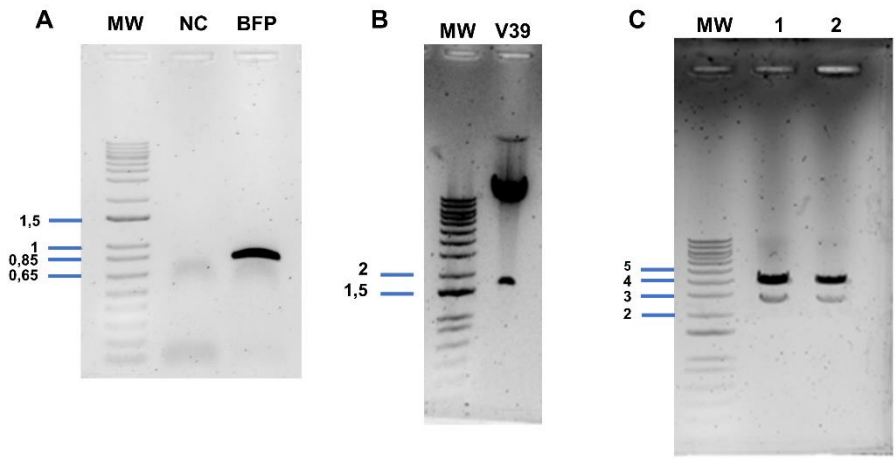

Figure 15. Cloning of BFP into V39 to replace tdT reporter gene. A) PCR amplification of tagBFP (BFP). The band observed corresponds to the BFP amplicon at the expected molecular weight (699 pb).**B)** Enzymatic digestion of V39 to excise out tdT. The high molecular weight bad was purified. The lowest ban corresponds to the tdT at the expected molecular weight (1477 pb).**C)** Restriction digestion of two ampicillin resistant colonies. NC: Negative Control. MW ladder: Molecular weight DNA ladder (Kb).

7. DISCUSSION

CVDs are the most important cause of death in the world and one of the major triggers for CVD is aging, which is concomitantly increasing in the same direction. Due to the increasing prevalence of CVD, new effective therapies are needed. Targeting pathways dysregulated in CVD and with age as well as possible genetic background leading to disease might help to palliate what in the next few years could be considered as a non-infectious pandemic disease, age-related CVD.

Dysregulation of miRNAs have been demonstrated to alter diverse signalling pathways being both the cause and the result of many pathological conditions, including in CVD. Thus, understanding the gene-miRNA interactions that participate in such processes may be translated into putative therapies to prevent CVD or accelerated aging and its detrimental effects in the heart and vasculature. Also to be used as biomarkers. Biomarkers of accelerated aging, would be a huge advance, since it will allow early treatment (prevention) before the onset of CVDs. On top of that, understanding how miRNAs act and the targets they have could be decisive for an extraordinary change in how CVDs have been treated so far, allowing for personalization of the medicine. By performing analysis of the miRNA profiles in an individual, a specific drug to target the miRNAs that are dysregulated could be designed.

miRNA therapeutics studies related to CVD are gaining more relevance since they have showed good results in preclinical studies [135]. However, animal models possess a homogeneous genetic background and they cannot mimic the long-term natural conditions (age, lifestyle, comorbidities) under which the disease is developed in humans, since disease models in animals are commonly abruptly induced either via genetic mutations or surgery. Therefore, despite the fact that animal models of CVD are essential to study its pathophysiological mechanisms as well as to test newly developed drugs before entering into clinical trials, their translational to human value remains limited. As it has been observed by Phase II and Phase III clinical trials failure rates are over 50% of drugs proved to be effective in preclinical models. Novel *in vitro* systems of human origin, like iPSC-CM, EHTs or myocardial slices, might be efficient tools to advance in miRNA therapies development with translational value [136]. In fact when EHT are mixed with fibroblast or endothelial cells, they have improved mimic adult phenotypes, since they show rod-shaped cardiomyocytes with well-organized sarcomere structures, systolic contraction, and inotropic responses to drug stimulation [136].

A major drawback for the successful development of miRNA therapeutics is effective delivery, naked charged RNAs tend to be cleared from the systemic circulation and on top of that, once they reach or are locally introduced in the target tissue or organ they still have to penetrate the lipidic bilayer of the cell.

Another issue that can occur, as it happens with the miR-25 TuD, is that the seed sequence of the antimiR molecule besides binding its original target (miR-25), also binds miR-92a. This is due to the fact that the miR-17-92 family presents the same seed sequence than miR-25. Taking into account that miR-92a is also upregulated in cardiac disease, in this case the off-target interaction could be beneficial. Another example of which off-target interactions could be beneficial is the case of the antagomir-122 (Miravirsen) that has successfully reach phase-II clinical trials offering long-term suppression for the treatment of patients with hepatitis C virus infection. Since miR-122, besides stabilizing RNA of the hepatitis C virus allowing no further replication, it is involved in the metabolism of hepatic cholesterol and fatty acids. Therefore, a reduction in miRNA-122 could have a double beneficial effect both inhibiting the virus replication and decreasing the cholesterol levels in plasma. *In vivo* studies both in mice and non-human primates would support this hypothesis, since miR-122 inhibition resulted in decreased total cholesterol plasma levels[137].

However, if an antimiR interacted with a miRNA that tends to be downregulated in cardiac disease or aging, it would only increase the damaging effects caused by a severe dysregulation.

Tissue and cell-specific delivery might play an important role in miRNA-based therapies, since the same miRNA can have different functions in different organs or within the same tissue. So, in order to avoid unspecific interactions, delivery should be improved. An example, it would be an antimiR against miR-146a, because although its downregulation after heart failure seems to be beneficial, miR-146a function in vascular aging is still controverted [125], [126].

Furthermore, immune adverse response due to the drug itself (it has been mostly reported in the case of miRNA mimics) or the vehicle that contains it are concerning. For instance, liposome linked toxicity, since it is one of the used carriers. Therefore, low immunogenic carriers that are capable of effectively reaching and entering through the lipidic bilayer should be developed to further success in future research.

In the case of the miRNA mimics it is thought that because of its double stranded nature they easily induce the secretion of inflammatory cytokines and type I interferons (IFNs) when they bind to Toll-like receptors (TLRs) [138], and that would be the main cause of the immune rejection of these therapies. Therefore, hiding them from these proteins or encapsulating them in low immunogenic recipients should diminish the adverse reaction and increase their therapeutic effects.

Besides most miRNA therapeutics being still on the preclinical phase, and with only two miRNA based drugs in clinical trials (LNA-anti-miR 92a and antimir-132), miRNAs therapeutics remain promising approaches to target diverse diseases if we are able to fix all the drawbacks that have been mentioned

before (immunogenicity and effective delivery), since miRNAs are key post-transcriptional controllers of gene expression.

So far, only two miRNA therapies to treat CVD are on clinical phase of testing. Therapy repurposing of miRNA therapies in more advanced stages than CVDs' ones, but with putative effect to treat them, could be another approach to accelerate miRNA-based treatment of CVD. Coupling of such therapeutics with highly specialized tissue delivery, like nanocarriers functionalized for specific recognition only in the cardiac tissue, such as RNA aptamers [139], or local delivery by inhaled nanoparticles[83], could allow to apply these therapies.

The experimental work of my Master thesis continues previous research of the BSICoS group were putative interactions of BIO-AGEmiRNA (mir24-2 and mir4435) with target genes were demonstrated *in vitro* using luciferase reporter assays [15], [16] Here, tools to carry out further investigations of the functional effect of such interactions *in vitro* are developed and partly tested. Inducible expression vectors containing the BIO-AGEmiRNAs (mir24-2, miR-4435 and miR-3916) were attempted to develop. These vectors are suitable for RMCE in human iPSC [17]. The future goal is to regulate by inducible expression the BIO-AGEmiRNA in CM-iPSC to observe the molecular and functional effect at electrophysiological level using optical mapping techniques. Unfortunately, only vector containing miR-4435 (V39) was successfully created, since amplification of miR-3916 and miR 24-2 were not successful.

The mir4435 inducible expression vector was tested in Hek293. Three different transfection conditions were designed in order to analyse miR4435 expression: Non-transfected cells and V39 transfected cells with or without induction. The tdT fluorescent protein was used to report for transgene expression. Natural fluorescence of Hek293 cells was set as background for microscopy analysis. Upon induction, clear tdT signal was observed, however the TetO promoter seems to have some leakage as non-induced cells expressed the reporter protein (low red fluorescence). A more sensitive variant of this promoter such as the dox-controlled HIV-1 [140] could be considered in case substitution would be needed in *in vitro* or *in vivo* experiments. This will be extremely beneficial, because lower doses of doxycycline will prevent both promoter leakage and will have a lesser impact disturbing the microbiome of the animal model, which could lead to either disease or antibiotic resistance.

qPCR of the transfected Hek293 was performed to analyse the correct maturation of miR-4435 *in vitro*. However, non-conclusive results were obtained and due time limitations this step could not be properly assessed. Still, according to published works [141], [142, p. 99], it is expected that the DNA fragment cloned (the pre-miRNA DNA sequence flanked by 250 bp) results in correct expression of the mature miR-4435.

Future work will be done so that miR24-2 and miR3916, that are being synthetically synthesized, will be cloned as well to create their inducible expression vectors compatible with optical mapping techniques. Furthermore, if miRNA analysis in iPSC validation of miRNA-target interaction resulted in measurable functional impact in human cardiomyocyte physiology, a direct relationship could be established between BIO-AGEmiRNA and cellular function. In this case, more complex *in vivo* studies might be considered in order to study their significance as possible targets for anti-aging therapeutics.

8. CONCLUSIONS

- miRNA therapies are avidly investigated in cardiovascular research since miRNA have been demonstrated to be involved in CVD and aging.
- Two major challenges for miRNA therapies need to be overcome to fulfil their expected potential in CVD treatment: improved deliveries and reduced immunogenicity.
- As of now, two miRNA-based therapeutics for CVD have reach clinical trials.
- MiRNA therapy repurposing coupled with highly specialized tissue-directed drug delivery could be a significant approach to accelerate miRNA-based treatments in CVD.
- An miRNA inducible expression vector compatible with rapid genome engineering by RMCE has been created.
- This vector has been partially tested. It is expected to become a useful tool for proceeding with the BIO-AGE-miRNA interaction studies in human iPSCs-derived cardiomyocytes.

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10. ANNEX

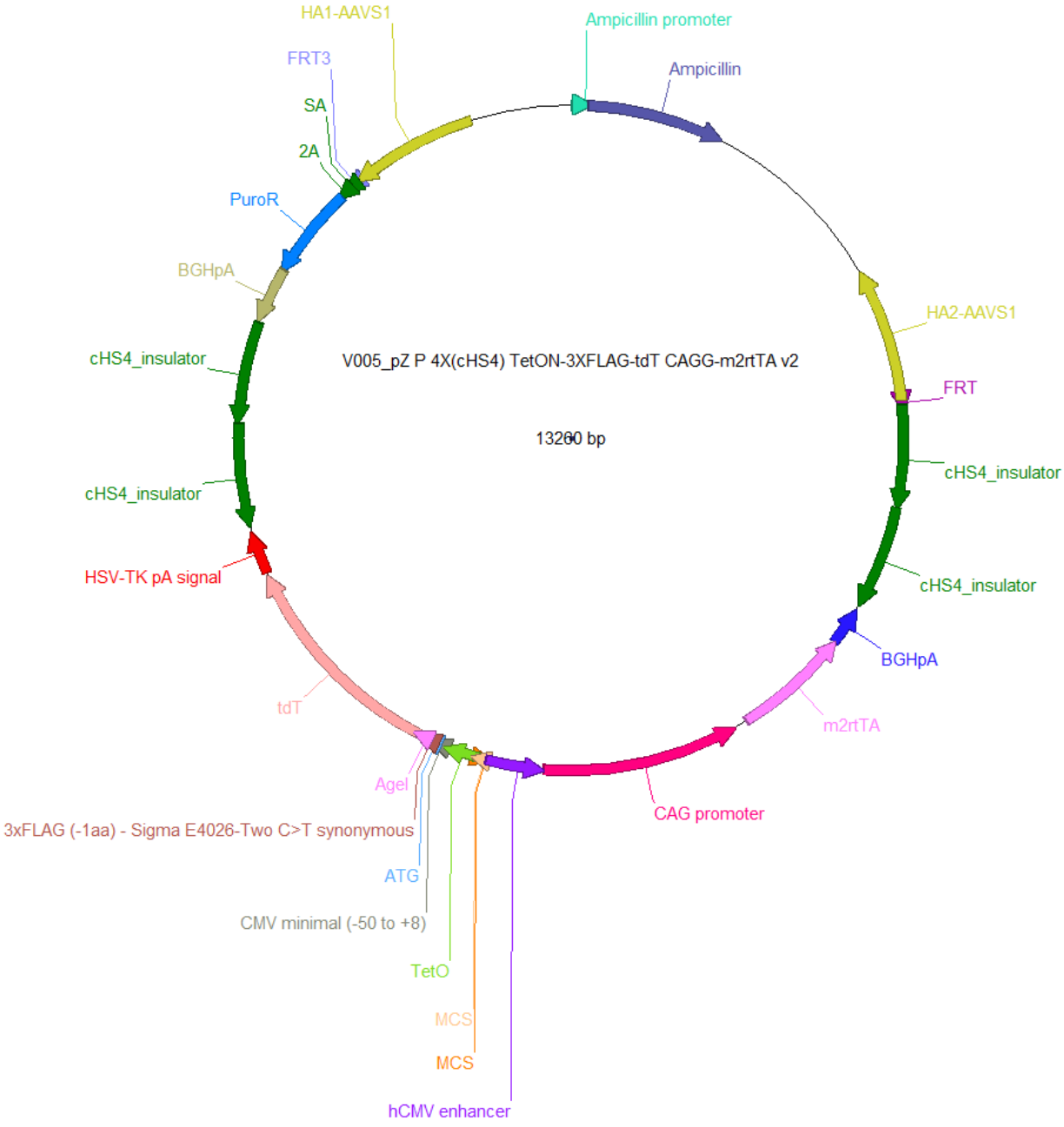

Figure A1. V5 Vector map (Obtained with Ape software)

Table A1. Cloned regions

Region of interest	REFSEQ
Pre-miR-3916	NC_000001.11
Pre-miR-4435	NC_000002.12
Pre-miR-24-2	NC_000019.10
BFP	---

Table A2. Primers for PCR amplification Blue: MluI, Red: AscI, Purple: PacI, Orange: AgeI, Green: EcoRV.

Amplicon	Oligonucleotide	Sequence (5'→3')	Tm (°C)	Tm used for PCR (°C)
Pre-miR-3916	Forward	AAAACGCGTGGCGCGCCTCCTCCCCATTTCTGTACTG	61,5	60,5
	Reverse	ATTACGCGTTTAATTAAGGAACTTCAGACTCATGCC	60	
Pre-miR-4435	Forward	AAAACGCGTGGCGCGCCTCCTCCCCATTTCTGTACTG	59	59
	Reverse	ATTACGCGTTTAATTAAGGAACTTCAGACTCATGCC	60,1	
Pre-miR-24-2	Forward	AAAACGCGTGGCGCGCCGCTGCTTGTGAGCAGGGTCCA	67,8	67
	Reverse	TTTACGCGTTTAATTAAAAGCCTCCAGGCCCGGAAAGG	69,5	
BFP	Forward	AAAACCGGTGATATCATGAGCGAGCTGATTAAGGAG	60,1	60
	Reverse	AATGGCGCGCCTCAATTAAGCTTGTGCCCCAG	61,3	

Table A3. PCR cycling programs

Phusion ™ High – Fidelity DNA Polymerase			
Cycle Step	**Temperature (°C)**	**Time**	**Use**
Initial denaturation	**98**	**30 s**	PCR and Colony PCR
Denaturation	**98**	**10 s**	
Anneling	**X**	**30 s**	
Extension	**72**	**30 s**	
Final extension	**72**	**10 min**	
Hold	**4**	**∞**	

NZYtaq II			
Cycle Step	**Temperature (°C)**	**Time**	**Use**
Initial denaturation	**95**	**3 min**	PCR
Denaturation	**95**	**30 s**	
Anneling	**X**	**30 s**	
Extension	**72**	**30 s**	
Final extension	**72**	**10 min**	
Hold	**4**	**∞**	

Table A4. PCR reactives

Reactive	Final concentration
Nuclease-Free Water	
5X HF Buffer	1X
dNTPs 2 mM	200 µM
Forward primer 10 µM	0.5 µM
Reverse primer 10 µM	0.5 µM
Phusion Taq DNA polymerase	0.02 U/µL
Template (gDNA)	< 30 ng

Table A5. Colony PCR reactives

Reactive	Final concentration
Nuclease-Free Water	
5X Nzytaq II Reaction Buffer (NEB)	1X
$MgCl_2$ Solution, 25 mM	1,5 µM
dNTPs 2 mM	200 µM
Forward primer 10 µM	0.25 µM
Reverse primer 10 µM	0.25 µM
Nzytaq II DNA polymerase	0.02 U/µL
Bacterial lysate	5 µL

Table A6. Restriction enzymes.

Restriction Enzyme	Recognition Sequence	Type	Company	Reference
Nco I	C'CATG,G	ANZA 20 u/µl	Thermo	IVGN0214
Age I	A'CCGG,T	ANZA 20 u/µl	Thermo	IVGN0074
Mlu I	A'CGCG,T	ANZA 20 u/µl	Thermo	IVGN0286
Asc I	GG'C GCG,CC	ANZA 20 u/µl	Thermo	IVGN0214
Hind III	A'AGCT,T	STANDARD 10 u/µl	Thermo	ER0501

Table A7. Oligonucleotide sequence employed for DNA sequencing. Blue: MluI, Red: AscI, Purple: PacI

Oligonucleotide	Sequence (5'→3')
Pre-miR-4435 **(**Forward)	AAAACGCGTGGCGCGCCTCCTCCCCATTTCTGTACTG
Pre-miR-4435 (Reverse)	ATTACGCGTTTAATTAAGGAACTTCAGACTCATGCC
P169 (Reverse)	CTGTCTTTTTATTGCCGTCATA

Table A8. Oligonucleotide sequence employed for qPCR

Oligonucleotide	Regions that the primer amplifies	Sequence (5'→3')
P253 (Forward)	**hsa**-miRNA-4435-5p	CCAGAGCTCACACAGAGGAA
P133 (Universal primer) (Rverse)	RNU6 and **hsa**-miRNA-4435-5p	GCATAGACCTGAATGGCGGTA
P096 (Forward)	RNU6	CGCAAGGATGACACGCAAAT