1. INTRODUCTION

Cardiovascular diseases (CVDs) are one of the leading causes of death worldwide, accounting for 16.7 million deaths per year (the World Health Organization- http://www.who.int/dietphysicalactivity/publications/facts/cvd/en/).
CVDs encompass a wide spectrum of cardiac pathologies including 5.3 million people living with heart failure in the USA alone (the American Heart Association- http://www.americanheart.org/presenter. jhtml?identifier=4478).
Similarly, every year more than 4 million Europeans die from heart and blood vessel diseases (the European Society of Cardiology- http://www.escardio.org/Pages/index.aspx.) On average, CVDs are responsible for almost half of the total mortality.
Cardiac pathologies are not only cause of mortality, but also of morbidity. More than 1 in 3 U.S. adults currently live with one or more types of CVDs, and nearly one million people are disabled from stroke alone. Consequently, CVDs are becoming an important social and economical issue and their burden cannot be measured by death statistics alone. In the EU, CVDs were estimated to cost annually €169 billion, with healthcare accounting for 62% of costs in 2003 [1]. In the U.S., the cost of heart disease and stroke in term of health care expenditure and loss of productivity from deaths and disability was reported to be more than 475$ billion in 2009, and with aging of the population the economic impact will become even greater. In terms of life expectancy, it was estimated that if all major types of CVDs were eliminated, it would increase by nearly 7 years [2].

1.1. Cardiovascular pathology

1.1.1. Heart failure

Heart failure (HF), with an estimated prevalence of 1-2% in the Western world and an annual incidence of 5-10 per 1000, is a clinical syndrome resulting from impaired ability of the heart to supply sufficient blood flow to meet the oxygen needs of body tissues. Importantly, in people older than 50 years, the prevalence of HF increases progressively with age, making HF the leading cause of hospitalization in the elderly. Although HF management has been improved over the last decades, it still remains one of the leading causes of mortality. Not only HF results with a poor quality of life and reduced longevity [3] but its prognosis has been described to be even more malignant than cancer [4]. HF may have several etiologies either primary, starting from the myocardial tissue, or secondary and may be present under different clinical setting. Development of HF generally proceeds in stages, from risk factors to end-stage or refractory disease. Among the causes that lead to HF are coronary artery disease, hypertension, cardiomyopathies, valvular diseases, congenital defects, infectious diseases, diabetes, and cardiotoxic substances (such as alcohol). Though no single causative mechanism or sequence of events has been found for HF, this syndrome is in general

preceded by an initial insult (e.g., cardiomyocyte (CMC) loss, persistently increased workload) responsible for impairing ventricular function in some way (Figure 1.1.).

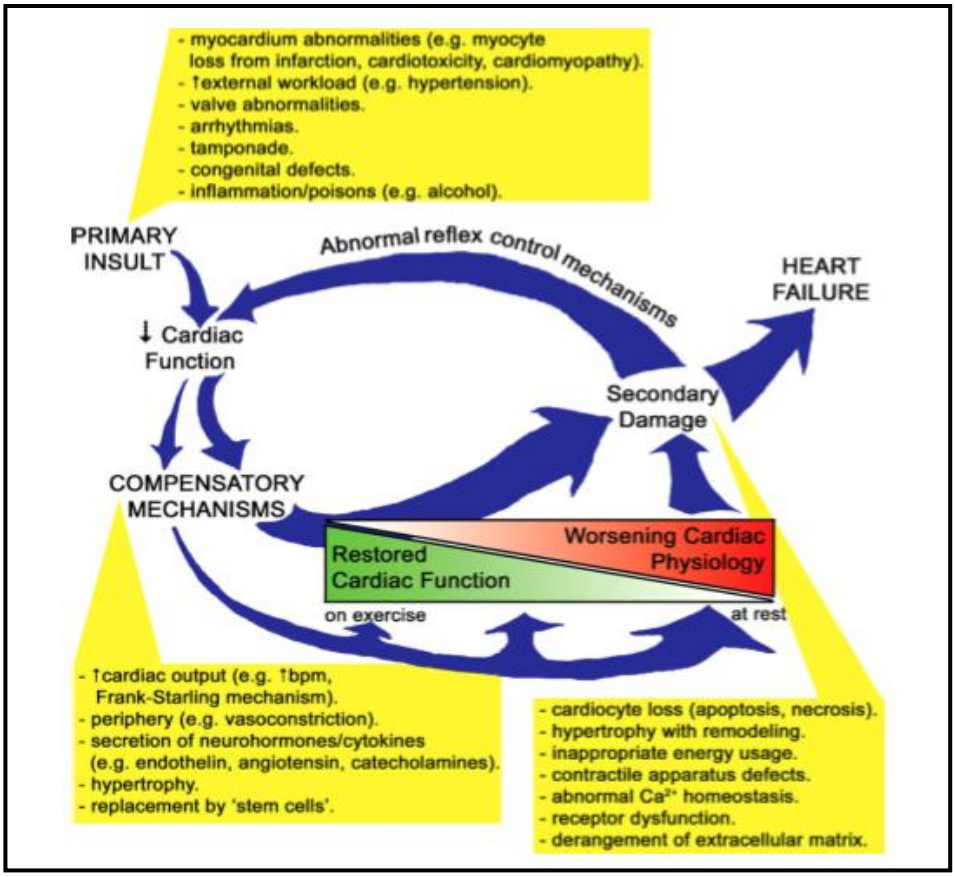

Figure 1.1 *Schematic representation of the main events of heart failure. The sustained activation of compensatory mechanisms leads to worsening in cardiac structure and function, culminating in heart failure (from [5]).*

The initial consequence of left ventricle (LV) dysfunction is the simultaneous activation of several compensatory mechanisms, such as activation of the renin-angiotensin-aldosterone system (RAAS), of the sympathetic adrenergic nervous system and the increase of cytokine production. As a result, the enhanced circulation of neurohormonal factors and stimulation of stretch receptors induce the enlargement of individual CMC (hypertrophy), finally leading to an increased heart size (cardiomegaly). Altogether, these biological events sign the establishment of pathological cardiac hypertrophy (CH), largely recognized for playing a crucial role in the onset of many forms of HF [6].

1.1.2. Cardiac hypertrophy

Physiological CH is defined by augmentation of ventricular mass as a result of increased CMC size and is the adaptive response of the heart to enhanced hemodynamic loads due to physiological stimuli such as postnatal developmental growth, training and pregnancy. In these cases, hypertrophy is characterized by enhanced cardiac function, normal sarcomere organization, and a normal pattern of cardiac gene expression [7] without interstitial fibrosis or increased cell death. Importantly, physical training protects against CVDs and the resultant CH is usually beneficial [8]. Indeed, exercise training in HF after myocardial infarction (MI) enhances cardiac performance and aerobic exercise capacity and reverses pathological hypertrophy and remodeling both in experimental models and in patients [9]. On the other hand, CH is also observed in patients with pathological conditions such as hypertension, MI, and valvular heart diseases. This type of cardiac growth is called pathological hypertrophy and is frequently associated with contractile dysfunction, interstitial fibrosis and re-expression of fetal-type cardiac genes such as atrial natriuretic factor (ANF) and α-myosin heavy chain [10, 11]. While in the short term, the adaptive response of the heart to enhanced hemodynamic loads apparently restores cardiovascular function (i.e., hypertrophy is adaptive), in the long run, the sustained activation of compensatory mechanisms can lead to secondary heart damage evidenced by worsening of LV remodeling (i.e., deleterious alterations in ventricular mass, chamber size and shape). When hypertrophy becomes incapable of sustaining the hemodynamic burden, patients undergo a transition from asymptomatic to symptomatic HF [12]. This maladaptive hypertrophy has become clinically recognized as a harmful reaction when generated by pathological stresses. and it is considered a major predictor for progressive HF and associate with an adverse prognosis [13]. Consequently, pathological CH is the main target of some of the current therapies for curing HF. In fact, the inhibition of its development has been shown to have beneficial effects in animal models of pressure overload, preventing the progression of HF and other related CVDs.

1.1.3. Cardiac fibrosis

Cardiac fibrosis is an important contributor to the development of cardiac dysfunction in diverse pathological conditions, such as MI, ischemia, dilated and hypertrophic cardiomyopathies, and HF. It can be defined as an inappropriate accumulation of extracellular matrix (ECM) proteins in the heart [14-19] due to abnormal proliferation of cardiac fibroblasts (cFIBROs). The increased ECM and fibroblast volume has several important consequences on cardiac functionality. Firstly, it enhances ventricular wall stiffness and consequently impairs cardiac compliance, contributing to impaired diastolic function. Secondly, since neither the ECM nor the fibroblasts contribute to systolic contraction, all the systolic work is being performed by a smaller proportion of the cardiac mass, thus leading to systolic dysfunction. Thirdly, interstitial fibrosis leads to increased distance that oxygen must diffuse negatively affecting the myocardial balance between energy

demand supply [16, 17]. Finally, electrical coupling of the CMC may be impaired by the accumulation of ECM proteins and fibroblasts since such accumulation causes morphologic separation of myocytes [20], signal transmission interference, and hence increases the chance of arrhythmias.
Therefore, factors that contribute to interstitial fibrosis should be recognized, and investigations should address whether approaches that attenuate fibrosis improve ventricular function and remodeling.

1.1.4. Cardiac physiopathology: neurohormonal systems

Although LV heart dysfunction is a key element in the syndrome of HF, the neuroendocrine response to diminishing cardiac function most likely plays an integral role in the progression of congestive HF. The progression of HF first starts with neurohormonal response due to LV dysfunction. The body needs to compensate for decreased blood volume, cardiac output and workload by increasing the volume through the activation of neurohormonal systems [21]. In particular, cardiovascular risk factors are associated with activation of the tissue renin–angiotensin and the endothelin systems [22, 23].

1.1.4.1. The renin angiotensin system

The Renin-Angiotensin System (RAS) is a peptidergic system that primarily regulates the homeostasis of the cardiovascular system, thus playing a vital role in the body. It modulates the physiological processes either by working as an endocrine system, or by exerting local paracrine and autocrine functions in tissues and organs [22].
The primary effector molecule, angiotensin II (ANG II), and the basic components of this intricate regulatory system are summarized in Figure 1.2. ANG II has emerged as a critical hormone that affects the function of virtually all organs, including heart, kidney, vasculature, as wells as brain, and it has both physiological and pathophysiological actions.

1.1.4.1.1. Production

The mechanisms controlling the formation and degradation of ANG II are important in determining its final physiological effects. ANG II, an octapeptide, is formed from enzymatic cleavage of angiotensinogen (AGT) to angiotensin I (ANG I) by the aspartyl protease renin, with subsequent conversion of ANG I to ANG II by the angiotensin converting enzyme (ACE).
Renin is a proteolytic enzyme that is released into the circulation primarily by kidneys, from the renal cortical juxtaglomerular apparatus and also released locally in other tissues (e.g., brain, heart, blood vessels). Its release is stimulated by sympathetic nerve activation (acting via β1-adrenoceptors), renal artery hypotension (caused by systemic hypotension or renal artery stenosis) and decreased sodium delivery to the distal tubules of the kidney.

When renin is released into the blood, it acts upon AGT, a circulating substrate released from the liver, that undergoes proteolytic cleavage to form the decapeptide ANG I.
ACE1, a membrane-bound metalloproteinase predominantly expressed on the surface of endothelial cells in the pulmonary circulation, cleaves off two amino acids to form the octapeptide ANG II. On the contrary, ACE2, a recently identified carboxypeptidase, cleaves one amino acid from either ANG I or ANG II [24], decreasing ANG II levels and increasing the metabolite ANG 1–7, which has vasodilator properties. Since ACE is the primary enzyme leading to ANG II generation ,the balance between its two isoforms, ACE1 and ACE2, is an important factor controlling ANG II levels [25]. However, ANG II can also be produced by ACE-independent pathways, especially in the heart, where the majority of ANG I is converted by chymase [26].

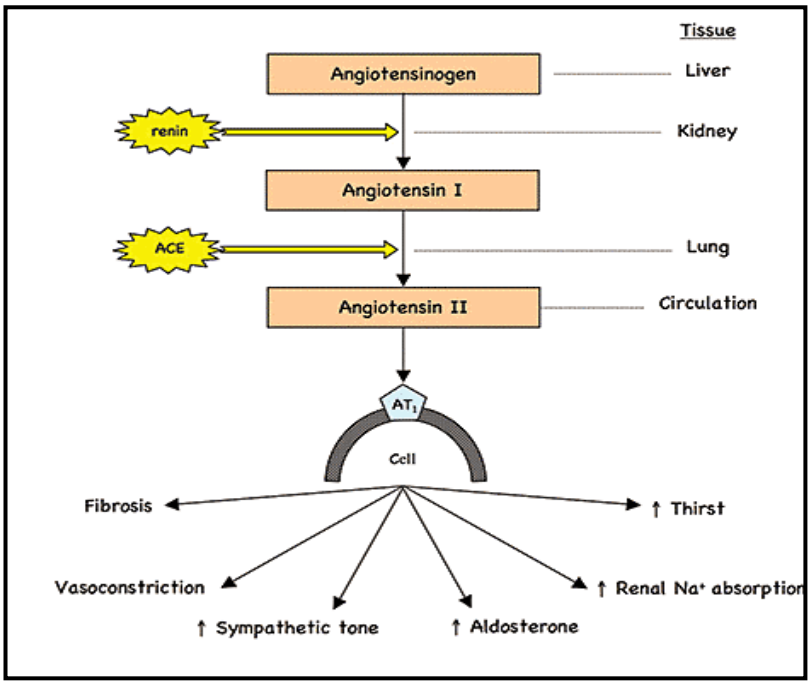

Figure 1.2 *The renin-angiotensin system (RAS).*
Schematic representation of the RAS: a linear cascade starting from angiotensinogen leads to the generation of angiotensin II (ANG II) through the enzymatic action of rennin first, and of angiotensin converting enzyme (ACE) on angiotensin I. Two different ANG II receptors exist: angiotensin II type-1 receptor (AT1) and angiotensin II type-2 receptor (AT2). The here represented AT1 receptor mediates the main effects of ANG II in the cardiovascular system and it is thought to play a more important role than the AT2 receptor in cardiovascular disease (from [27]).

1.1.4.1.2. Receptors

ANG II exerts its biological actions through the activation of two receptor subtypes, ATR1 and ATR2, which are seven transmembrane glycoproteins with 30% sequence similarity belonging to the G-protein-coupled receptor (GPCR) superfamily. Most species express a single autosomal AT1 gene, but two related AT1A and AT1B receptor genes are expressed in rodents. They share 95% homology in their aminoacid sequence. The tissue-specific effect of increased ANG II levels and enhanced RAS activity depends on the cellular expression and activation of ATR1s, critical receptors in cardiovascular and renal pathophysiology.
ATR1s are ubiquitously distributed (blood vessels, heart, kidney, liver, brain, lung) and both ATR1 and ATR2 are localized in CMCs, cFIBROs and in vascular smooth muscle cells (VSMCs). ATR1 mediates virtually all of the known physiological actions of ANG II not just in cardiovascular, but also in renal, neuronal, endocrine, hepatic setting, as well as in other target cells.
The major physiological actions of ANG II relevant to CVDs are summarized in Table 1-1 and include: regulation of arterial blood pressure (BP), electrolyte and water balance, thirst, hormone secretion, and renal function [28]

AT_1	AT_2
• Vasoconstriction (via ↓ NO, ↑ intracellular Ca^{2+}, and ↑ superoxide) and ↑ BP	• Foetal tissue development
• Inflammation (via ↑ NFκB)	• Vasodilatation (via ↑ bradykinin and NO) and ↓ BP
• Cell growth and proliferation (via *c-fos, c-myc, c-jun*)	• Inflammation (via ↑ NFκB)
• Anti natriuresis	• Growth inhibition (VSMC, endothelial cell, cardiomyocyte, cardiac fibroblast, via ↓MAP kinases)
• Increased atherogenicity (via ↑ OxLDL)	
• Modulation of sympathetic nervous system activity	
• ↓ renal blood flow	• Improvement in cardiac function (LVEDV, LVESV and EF) and decrease in chronotropic effect
• ↑ myocardial contractility	
• ↑ arrhythmias	• Vascular cell differentiation
• ↑ PAI-1	• Extracellular matrix composition
• ↑ endothelin release	• Apoptosis (via ↓MAP kinases)
• ↑ sympathetic activity	

Table 1-1 *Key pharmacological effects of angiotensin II (ANG II mediated by activation of AT1 or AT2 receptors (from [22]).*

1.1.4.1.3. Actions

In the cardiovascular system, ANG II has several very important functions that exerts via AT1 receptor. Basically, ANG II activity produces: 1) a rapid pressor response; 2) a slow pressor response; 3) vascular and cardiac hypertrophy and remodeling. In addition, long-term exposure to ANG II also plays a vital role in stent restenosis, reduced fibrinolysis, and renal fibrosis.
ANG II is an important growth factor, causing cell proliferation, cell hypertrophy, cell differentiation, and apoptosis. Depending on the cell type, ANG II appears to have different growth effects (Figure 1.3).

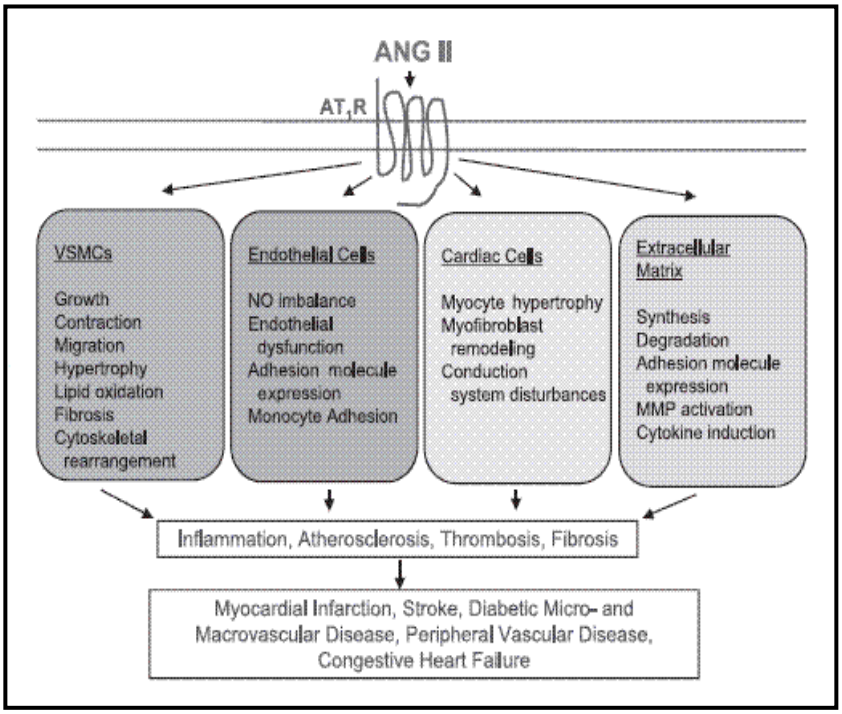

Figure 1.3 *Angiotensin II (ANG II) modulates the cardiovascular physiology and pathology by inducing signaling pathways in vascular smooth muscle cells (VSMCs), endothelial cells, and cardiac myocyte and fibroblasts, and by affecting their interaction with the extracellular matrix (from [29]).*

Vascular actions

In the vasculature, ANG II plays its role by both modulating BP and the morphology of VSMCs. In particular, acute stimulation with ANG II regulates salt/water homeostasis and vasoconstriction, thus increasing BP, while chronic stimulation promotes cell growth, resulting in VSMC hyperplasia and hypertrophy [30, 31].

Cardiac actions

In the heart, ANG II has both direct and indirect actions, which in turn affects contractility by inducing both a positive inotropic effect as well as an increase of the heart rate by inducing a chronotropic effect, and finally cell growth.
ANG II works through the ATR1 to increase cFIBRO concentration and expression and to promote CMC hypertrophy, by increasing proto-oncogene and growth factors expression. Several lines of evidence support a role for ANG II as a critical factor responsible for myocardial fibrosis. ANG II induces fibroblast proliferation, alteration of fibrillar collagen turnover and stimulation of aldosterone, leading to accumulation of collagen type I and III fibers and fibrosis [32]. The consequential increase in volume of the non-myocyte compartment of the heart, results in a distortion of tissue structure that promotes the progression of LV hypertrophy (LVH) and the globular heart formation with post MI remodeling and HF (for review, of see Ref. [33]).
ANG II has both remodeling effects on the heart that are mediated through its ATR1 and possible anti-remodeling effects mediated by activation of the ATR2. ATR2 counter-balances the actions of ATR1: vasodilatation, inhibition of cell growth, apoptosis etc. Although the concentrations of ATR2 are normally less than that of ATR1, during HF the levels of ATR2 increase. It is widely reported the hypothesis that such an increased expression, represents a compensatory mechanism to protect the heart from ANG II overproduction and to limit the negative impacts of the ANG II-induced cardiac remodeling [34].

1.1.4.1.4. Signal transduction

Once ANG II binds to the ATR1, it activates a series of signaling cascades, which in turn regulate the various physiological effects of ANG II.
Traditionally, the pathways induced by ANG II have been divided into two classifications: G protein- and non-G protein-related signaling. In addition, to activate the G protein-dependent pathways, ANG II also cross-talks with several tyrosine kinases via ATR1, including receptor tyrosine kinases [EGFR, PDGF, insulin receptor and nonreceptor tyrosine kinases [c-Src family kinases, Ca^{2+}-dependent proline-rich tyrosine kinase 2 (Pyk2), focal adhesion kinase (FAK), and Janus kinases (JAK)]. In addition, activation of NAD(P)H oxidases and generation of reactive oxygen species (ROS) [35] also contributes to the ANG II pathological effect on the vasculature Protein kinase C (PKC) and mitogen activated kinases (MAPK) [including ERK1/2, p38, MAPK, and c-Jun NH2-terminal kinase (JNK)] are among the serine/threonine kinases activated through ATR1 and mainly implicated in cell growth and hypertrophy.

The induction of the above pathways is tightly regulated and their overactivation in patients with overstimulated RAS or enhanced responsiveness to ANG II, is responsible for the initiation and propagation of the main pathological events promoting CVDs [36, 37].
The temporal and spatial patterns of signaling pathway activation are the most likely determinants of a particular functional response. Multiple studies show that the activation of different pathways by ANG II is time dependent.

G- Protein-Coupled Pathways

One of the major effects of ANG II is vasoconstriction, which is mediated by "classical" G protein-dependent signaling pathways. When activated by the binding of its agonists, ATR1 couples to G-protein subunits, which activate downstream effectors including phospholipase C (PLC), phospholipase A2 (PLA2), and phospholipase D (PLD) [38]. Within few seconds, activation of PLC produces inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG).
These are second messengers, whose function is to activate the downstream effectors molecules involved in several pathways.
IP3 binds to its receptor on sarcoplasmic reticulum, opening a channel that allows both Ca^{2+} efflux into the cytoplasm and Ca^{2+} binding to calmodulin. The consequential activation of myosin light chain kinase (MLCK), which in turn phosphorylates the myosin light chain, enhances the interaction between actin and myosin, causing smooth muscle cell contraction [39]. In addition, DAG activates PKC, which participates as an effector in the Ras/Raf/MEK/ERK pathway. These downstream molecules contribute to the vasoconstrictive properties of ATR1 activation and lead to ANG II growth promoting effects, through the activation of immediate early genes [40].

Mitogen-Activated Protein Kinases

MAPK pathways regulate the most important cellular processes, such as protein synthesis and metabolism, transport, volume regulation, gene expression, and growth. ANG II has been shown to activate signaling cascades that activate MAPK, including extracellular signal regulated kinase (ERK1/2), JNK, and p38MAPK, which are implicated in VSMC differentiation, proliferation, migration, and fibrosis [41, 42]. The ERK pathway is the best characterized of the MAPK pathways and it has been reported that ERK1/2 activation occurs within 5 minutes after ANG II binding to ATR1. Moreover, the mechanism of ERK1/2 activation by ANG II potentially involves multiple signaling mechanisms, including EGFR transactivation, PKCs, and intracellular Ca^{2+}. In VSMCs, ATR1–induced ERK1/2 activation occurs via Ca^{2+} and Src-dependent transactivation of the EGFR [43-45]. Src and Ca^{2+}-dependent kinase Pyk2 phosphorylate EGFR on tyrosine, leading to formation of the Shc/Grb2 complex and ERK activation. This scaffold permits activation of Raf, which in turn phosphorylates the MAPK/ERK kinase (MEK). Raf associates with the small G protein Ras, leading to MEK activation, and subsequent phosphorylation of ERK1/2 on threonine/tyrosine residues [41]; recently, PKC-ζ has also been shown to associate with Ras and activate ERK1/2

[46, 47]. Recent data implicates ERK (p42/44 kinase) in ANG II mediated VSMC contraction.
In neonatal cFIBROs, phorbol ester–sensitive PKCs and intracellular Ca^{2+} have been shown to mediate MAPK signaling in response to ANG II [48]. The EGFR also mediates β_2-adrenergic receptor–induced ERK1/2 activation in adult cFIBROs [48].
An alternative Ca^{2+} independent mechanism by which the ATR1 induces ERK1/2 activation is through phosphatidylinositide 3-kinase (PI3K) and subsequent activation of atypical PKCζ [46, 49].
Thus, activation of ERK ½ is the main event occurring just after the ATR1 activation by ANG II and leading to the consequent modulation of cellular phenotype.

1.1.4.2. The endothelin system

Endothelins are a family of peptides, which comprises endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3), each containing 21 amino-acids, out of which ET-1 is the principal cardiovascular isoform.
Endothelin (ET) is a potent vasoconstrictor, that also exhibits inotropic, chemotactic and mitogenic properties, influences salt and water homeostasis through its effects on the RAAS, vasopressin and ANF and stimulates the sympathetic nervous system [50-52].

1.1.4.2.1. Production

Each member of the ET family is represented by a separate gene that encodes a specific precursor for the mature isoform. ET-1 generation is increased by many stimuli, including vasoactive hormones, growth factors, hypoxia, shear stress, lipoproteins, free radicals, endotoxin and cyclosporine Production of ET-1 is inhibited by endothelium-derived nitric oxide (NO), nitrovasodilators, natriuretic peptides, heparin and prostaglandins (for review, of see Ref.[23]). The major site of generation of ET-1 is in endothelial cells [53-55]. ET-1 is also produced in the heart, kidney, central nervous system and posterior pituitary, by vascular endothelial and smooth muscle cells as well as cFIBROs and CMCs.
The initial product of the human ET-1 gene is a 212 amino-acid peptide called pre-proendothelin-1, which is converted to proendothelin-1 after removal of a short secretory sequence. Proendothelin-1 is then cleaved by furin to generate a biologically-inactive, 38 amino-acid precursor, big endothelin-1 (big-ET-1) [56] (Figure 1.4). The formation of mature ET-1 requires cleavage of big-ET-1 by a family of membrane bound zinc metallo-proteases, termed endothelin converting enzyme (ECE). ECE-1 is the physiologically active ECE [57], which is relatively selective for big-ET.1. There are two splice variants, ECE-1a and ECE-1b, that have functionally distinct roles and tissue distributions [58]. ECE-1a is expressed in the Golgi network of ET-producing cells, such as endothelial cells, and cleaves big-ET.1 to form ET-1. ECE-1b is localized at the plasma membrane where it cleaves extracellular big-ET.1, present at low concentrations.

1.1.4.2.2. Receptors

ET-1 exerts its biological actions through the activation of two receptor subtypes, EDNRA (ET-A) and EDNRB (ET-B). Both receptors belong to a large family of transmembrane GPCR and are expressed in a variety of tissue.
EDNRA receptors are highly expressed in VSMCs but not in endothelial cells. They are also found in CMCs, fibroblasts, hepatocytes, adipocytes, osteoblasts and brain neurons [59, 60] and exhibit higher affinities for ET-1 and ET-2 than for ET-3 [60]. EDNRB receptors exist predominantly in endothelial cells and smooth muscle cells, but are also found in CMCs, hepatocytes, fibroblasts, osteoblasts, different epithelial cells and neurons and have equal subnanomolar affinities for all ET iso-peptides [59].
The EDNRA is the primary vasoconstrictor and growth promoting receptor, while EDNRB receptor primarily inhibits vasoconstriction and cell growth, but it can also mediates vasoconstriction. Vasoconstriction to EDNRB receptor agonists varies with species, vessel type and vessel size.

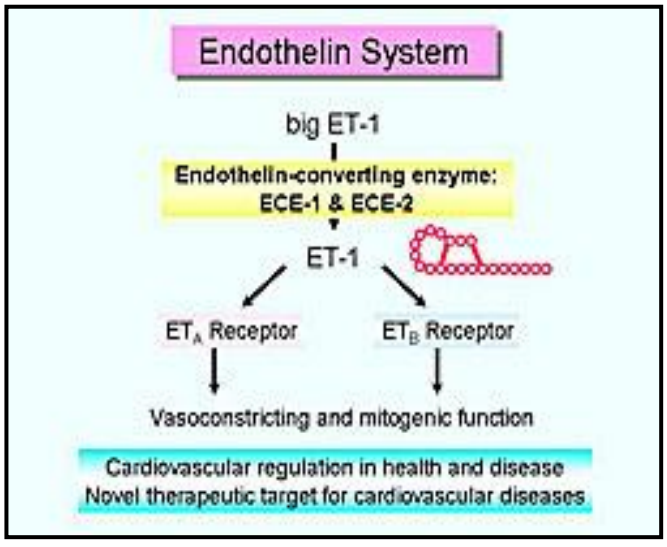

Figure 1.4 *The endothelin (ET) system.*
Schematic representation of the ET system: a linear cascade starting from ET precursor (big-ET-1) leads to the generation of endothelin-1 (ET-1) through the enzymatic action of endothelin converting enzyme (ECE). Two ET-1 receptors exist: endothelin type-A receptor (ET-A) and endothelin type-B receptor (ET-B). ET-A receptor mediates the main effects of ET-A in the cardiovascular system and it is thought to play a more important role than the ET-B receptor in cardiovascular disease (from http://www.j-circ.or.jp/english/sessions/reports/69th/sym01.htm).

1.1.4.2.3. Actions

Similarly to ANG II, ET-1 is considered to play a major role in the pathogenesis of various CVDs by its direct effects on CMCs, cFIBROs, and VSMCs and indirectly by stimulating the expression of different proteins that in order leads to CMC hypertrophy. The major physiological actions of ET-1 relevant to CVDs are summarized in Table 1-2.
In summary, the overall physiological effect of ET-1 is to increase BP. The cardiovascular effect of endogenous ET-1 depends on the balance between EDNRA- and EDNRB-mediated effects. Therefore, the cardiovascular effects of endogenous ET-1 generation may change in disease states, if the number or function of EDNRA- and EDNRB-receptors are altered.

Vascular actions

ET-1 is the most potent known vasoconstrictor agent of conduit arteries *in vitro*[56]. ET-1, ET-2 and ET-3 induce transient vasodilatation due to NO and prostacyclin release, before the development of sustained vasoconstriction [61]. Vasoconstriction to ET-1 is mediated by VSMC EDNRA and EDNRB receptors. Endothelial cell ETB-receptors mediate vasodilatation through production of endothelium-derived vasodilators.
Concentrations of ET-1 in blood are lower than those that cause vascular contraction *in vitro* or *in vivo*. Cultured endothelial cells secrete substantially more ET-1 towards the adjacent VSMCs than into the lumen. Therefore, ET-1 is thought to be a locally-acting paracrine substance, rather than a circulating endocrine hormone. ET is believed to play an important role in vascular remodeling associated with experimental and human hypertension [54, 56] Increased VSMC hypertrophy, migration and proliferation are among the key events that contribute to remodeling of the vasculature associated with CVDs.

Cardiac actions

ET-1 has positive chronotropic and inotropic effects *in vitro [62]*. Intracoronary administration of ET-1 causes coronary vasoconstriction, resulting in MI and lethal ventricular arrhythmias [63]. In animals, low doses of ET-1 have a positive inotropic effect *in vivo*, whereas higher doses have a negative inotropic effect [64], possibly due to myocardial ischemia from coronary vasoconstriction and high afterload.
Systemic administration of ET-1 in humans decreases cardiac output. Indeed, plasma ET-1 levels increase in various models of heart disease.
In cardiac tissue, ET-1 is synthesized and secreted from both CMCs and cFIBROs. As a potent vasoconstrictor and mitogen, increased circulating ET-1 levels would be deleterious to the myocardium by restricting coronary flow, by promoting hypertrophy of CMC, by stimulating proliferation of cFIBROs [65].

Organs and organ systems	Effects of endothelin-1
Systemic vascular bed	Causes vasoconstriction through vascular smooth muscle cell ET_A- and ET_B-receptors. Causes vasodilatation through ET_B-receptors located on endothelial cells. Mitogenic effect on vascular smooth muscle cells
Pulmonary vascular bed	Causes vasoconstriction through vascular smooth muscle cell ET_A- and ET_B-receptors
Heart	Positive chronotropic and inotropic effects *in vitro*. Decreases cardiac output *in vivo*, due to increased afterload and a baroreceptor mediated decrease in heart rate. Mitogenic effect on cardiac myocytes and coronary vascular smooth muscle cells
Kidney	Constriction of afferent and efferent arterioles, decrease in renal plasma flow and glomerular filtration rate through ET_A-receptors. Preventing tubular reabsorbtion of sodium and water through ET_B-receptors. Mitogenic effect on human mesangial cells.
Endocrine	Stimulates ACE and aldosterone release

ET= endothelin; ACE = angiotensin-converting enzyme

Table 1-2 *Endothelin-1 (ET-1) effects on cardiovascular physiopathology (from [23]).*

1.1.4.2.4. Signal transduction

ET-1 has been shown to activate several signal transduction pathways linked to cellular hypertrophy, growth, migration and proliferation in several cell types including cardiac tissues and in CMCs [55] as well as in kidney mesangial cells, and more recently in the vascular system [66]. Therefore, the ET-1 system contributes to the pathogenesis of CVDs by activating signaling pathways that are capable of modifying hypertrophic and proliferative responses.
These pathways may or may not involve PKC depending on the cell type.

PKC dependent pathways

The binding of ET-1 to its EDNRA receptor leads to activation of phosphoinositide-specific phospholipase Cβ (PLCβ), which then hydrolyzes the membrane phospholipid, phosphatidylinositol-4',5'-bisphosphate (IP2) to two second messengers: hydrophobic DAG, which remains in the membrane, and soluble IP3 . IP3 diffuses into the cytoplasm and activates calcium channels of the sarcoplasmic reticulum, which leads to an increase in Ca^{2+} levels in the sarcoplasm and cell contraction. DAG together with Ca^{2+} activates the PKC. ET-1 has been shown to activate PKC in CMCs and in other cells, including adult and neonatal cFIBROs. PKC has been shown to be involved in ET-1-induced signaling in VSMCs. Moreover, growing evidence suggests that PKC activity modulates proliferation and contraction of VSMCs evoked by ET-1. Furthermore, in cFIBROs the ET-1 induced mitogenesis is PKC-dependent. Thus, ET-1- induced activation of PKC and its downstream effects appear to be important in regulating cardiovascular functions (for review, of see Ref [23]) .

Mitogen-Activated Protein Kinases

Another key signaling event evoked by ET-1 receptor activation consists of the MAPK cascade. The first component of this cascade is Ras, a member of the small GTP-binding protein family. Ras cycles between an active GTP-bound conformation and an inactive GDP bound form. Once activated, Ras, bound to membrane, recruits Raf, also known as mitogen activated protein kinase kinase MAPKK. Raf phosphorylates MEK or MAPKK at specific serine/threonine residues, which in turn, phosphorylates MAPK, such as ERK1/2 on threonine and tyrosine residues. MAPK are serine/threonine protein kinases, which are activated in response to a variety of external stimuli such as growth factors, hormones and stress. Several reports have demonstrated that ET-1 activates ERK1/2 signaling pathway in many cell types including CMC, fibroblasts, and VSMCs. ERK1/2, p38MAPK and JNK are the principal MAPK. In VSMCs ET-1-induced activation of MAPK pathway may be mediated through several intermediate signaling components including Ca^{2+}-dependant and -independent serine/threonine/tyrosine kinases. The above described cascade appears to be the main pathways mediating the ET-1 effects in VSMCs (for review, of see Ref [23]).

1.1.4.3. The renin-angiotensin-system and the endothelin system

There is inter-dependency between endogenous ET system and the RAS, as ANG II increases tissue ET-1 and induces vascular hypertrophy, while endogenous ET-1 contributes to the cardiovascular and renal effects of ANG II.
ANG II stimulates the endothelial production of ET-1 in situ and thereby potentiates contractions to noradrenalin (NA) in mesenteric resistance arteries. This suggests that vascular ET-1 production acts as an amplifier of the pressure effects of the RAS that may play an important role in hypertension. In addition, ANG II mediates CH indirectly by stimulating release of NA from cardiac nerve endings end ET-1 from endothelial cells [67].

1.1.4.4. The apelin system

Apelin (AP) is the endogenous ligand for the previously orphaned GPCR (APLNR) This novel peptidic signalling pathway is widely represented in the heart and vasculature, and it has wide representation in the central nervous system and a variety of peripheral tissues [68].
On vasculature, AP could exert either a vasocontractive or a vasodilated action, depending on the cell type and signaling pathways activated [69].
On heart, AP has been shown to increases contractility at sub-nanomolar concentrations [70]. Although these studies all support a positive inotropic role for AP, some discrepancies in the reported findings has been observed.
Since its discovery, the AP system has emerged as an important regulator of cardiovascular homeostasis that may play a role in the pathophysiology of HF and represents an exciting target for the development of new therapies [71].

1.2. Study models for cardiovascular disease

In order to study cardiovascular pathologies and the efficacy of appropriate therapies, it is necessary to mimic cardiac events. To this attempt, investigators have developed many animal and *in vitro* models of cardiopathies. Many transgenic mice are available, and also many surgical techniques have been developed to reproduce the effects of major cardiac events. In addition, researchers also take advantage of many neurohormonal factors that generate cardiac damage *in vivo* as well as *in vitro* by using different cellular models, like primary vascular or cardiac cell lines.

1.2.1. Animal models of cardiac disease

Animal models of CVDs have been proved to be critically important for the discovery of pathophysiological mechanisms and for the advancement of diagnosis and therapy. They offer a number of advantages, principally the availability of adequate healthy controls and the absence of confounding factors such as marked differences in age, concomitant pathologies and pharmacological treatments.

1.2.1.1. Genetically modified animal models

Genetically modified animals are becoming of primary importance to the discovery and development of cures and treatments for several CVDs.
Animals whose genetic composition has been altered by the addition of foreign DNA is said to be transgenic. Transgenic mice overexpressing a gene can be generated in order to investigate the role of a molecule of interest for a cardiovascular phatophysiology. As shown in this study, mice overexpressing a microRNA can be useful to investigate their role in CVDs.

1.2.1.2. Surgical techniques

Mouse models mimicking human diseases are important tools for understanding the underlying mechanisms of many disease states.
Several surgical models have been described that mimic human neurohormonal-induced hypertension and pressure-overload-induced CH.
Aortic banding is a surgical protocol that produces LV pressure overload and causes myocardial hypertrophy [72]. This model produces marked LVH, but does not reproduce neurohormonal activation or left ventricular systolic dysfunction [73].

1.3. Treatments for cardiovascular disease

There are many ways to reduce the incidence of cardiac disease outcomes. Conventional methods include the prevention of heart disease by controlled lifestyle and physical exercise and the use of drugs that improve cardiac

functionality. Some examples of conventional treatments, focusing on the above mentioned neurohormonal systems, are reported in this section.

1.3.1. Conventional treatments for cardiovascular disease

1.3.1.1. Lifestyle

Physical exercise and controlled diet is the easiest way to reduce the incidence of cardiac events. Findings of several controlled studies of both aerobic and resistance exercise regimens have indicated improvements in surrogate measures of ventricular function and patient's wellbeing. Also salt restriction has been recommended even for patients without overt clinical signs of salt and water overload. Other guideline recommendations, sometimes without a strong evidence base, include alcohol restriction (especially with alcohol abuse), smoking cessation, vaccination against influenza and pneumococcus, avoidance of high-altitude destinations, and bed rest for individuals who are acutely decompensated.

1.3.1.2. Drugs

Many pharmaceutical treatments have been used in order to improve condition of patients after a MI event or in HF, but also to prevent these pathologies in patients with high risk of major cardiac events.
Considering the involvement of elevated levels of ANG II and ET-1 in the cardiovascular pathophysiology, a wide range of molecules that antagonize their effects have been developed for the treatment of various CVDs.
RAS may be blocked at various levels. ACE inhibitors (ACEi) and ANG II receptor antagonists (ARA) have been shown to be effective in the treatment of hypertension and HF. Renin inhibitors have the potential to be beneficial in the treatment of the same diseases, but it seems that they are not associated with any side effects that have been seen with ACEi (cough, angioneurotic edema).
ACEi decrease the conversion of ANG I to ANG II, thereby minimizing the multiple pathophysiological effects of ANG II, and decrease the degradation of bradykinin. Bradykinin promotes vasodilatation in the vascular endothelium and causes natriuresis in the kidney. The beneficial effects of ACEi in HF and MI include improvements in survival and cardiac performance as well as reduction of rate of hospitalization, symptoms, neurohormonal levels, and reverse remodeling [74]. However, ACEi show some adverse effects that appear to be unrelated to ANG II blockade. In fact, i) competitive inhibition of ACE results in a reactive increase in renin and ANG I levels, which may overcome the blockade effect; ii) ACE is a relatively nonspecific enzyme that, in addition to ANG I, includes other substrates such as bradykinin and other tachykinins. Thus, inhibition of ACE may result in accumulation of these substrates; iii) production of ANG II can occur through non-ACE pathways as well as through the primary ACE pathway, and these alternative pathways are unaffected by ACE inhibition; iv) specific adverse effects are associated with ACEi effects on the enzyme.

ARA represent the newest class of drugs for the treatment of hypertension. In fact, since their initial introduction, ARA have demonstrated clinical efficacy in treating LVH, HF, diabetic nephropathy, and post-MI [75]. ARAs were developed to overcome several of the deficiencies of ACEi. Their mechanism of action differs from that of the ACEi, which also affect the RAS, and offer more complete ANG II inhibition by interacting selectively with the receptor site [76].
Similarly, various reports have shown beneficial effects of different ET antagonists in CVDs [77]. The effects of ET-1 on renal function, cardiac and vascular growth indicate the potential of anti-endothelin therapy in preventing complications of hypertension, such as vascular remodeling, LVH, hypertensive kidney damage, and atherosclerosis. ET-1 also appears to be involved in the pathophysiology of HF. Long-term anti-endothelin therapy may improve symptoms and favorably alter the progression of HF [78]. Anti-endothelin therapy might offer additional benefits in the prevention of progression of cardiovascular pathology in addition to the known benefits of RAAS inhibition. Therefore, further studies are necessary to determine the role of anti-endothelin therapy in the treatment of CVDs and to determine the different roles of selective ENDRA- or ENDRB-receptor antagonism versus mixed ENDRA/B-receptor antagonism in human diseases.

1.3.2. Innovative treatments for cardiovascular disease

Traditional therapies for CVDs, in particular for HF, have involved the use of multiple drugs to improve cardiac contractile function by modifying neurohumoral signaling (e.g., β blockers and ACEi) or normalizing Ca^{2+} handling by CMCs [79]. While these strategies promote short-term improvement in cardiac function, the 5-year mortality rate for HF patients remains close to 50%. Currently, heart transplantation represents the most effective therapy for end-stage HF, but this approach obviously cannot be applied to the millions of affected individuals worldwide and is not suitable for patients with milder forms of the disease Thus, there is a great need for the development of novel therapeutics, preferably new drugs, that will improve the quality of life and prolong survival of HF patients.
An understanding of the mechanistic underpinnings of CVDs represents an essential step toward this goal.

1.3.2.1. Modulation of gene expression

Cardiac and vascular remodeling is associated with the activation of a pathological gene program that weakens cardiac performance. In particular, HF and associated pathological CH are accompanied by a reprogramming of cardiac gene expression and activation of “fetal” cardiac genes. This transcriptional reprogramming has been shown to correlate with loss of cardiac function. On the other hand, improvement in cardiac function in response to drug therapy or implantation of LV assist device, is accompanied by normalization of cardiac gene expression [80]. Strategies to control cardiac gene expression, therefore, represent attractive, albeit challenging, approaches for HF therapy. Much of our current understanding of cardiac gene expression indicates that it is controlled at the level of transcriptional regulation, in which transcription factors associate with their regulatory enhancer/

promoter sequences to activate the expression of a specific set of genes [81]. The regulation of cardiac gene expression is complex, with individual genes controlled by multiple enhancers that direct very specific expression patterns in the heart.
The recent discovery of microRNAs have reshaped our view of how cardiac gene expression is regulated by adding another level of regulation at the post-transcriptional level. Technological advances and development of new applicative tools of functional RNA molecules for medicine provide important insights into molecular mechanisms affecting human health and disease and could eventually lead to the discovery of diagnostic biomarkers and the development of novel gene therapies [82].

1.4. MicroRNA

Since their discovery, microRNAs (miRNAs), a novel class of small noncoding RNA, captured the interest of many scientists thanks to their versatile role in many biological and metabolic processes. Before the revolutionary discovery of these small RNAs, it was thought that only proteins could regulate translation. This common knowledge was proved to be wrong in 1993, when a miRNA was shown to regulate a gene product in an unconventional way. The biology of miRNA networks is not yet completely understood, but its study is full of promises. So far, more than 4000 miRNAs in 45 animal species, animal and virus, have been identified, and it seems clear that every multicellular eukaryotic organism uses these RNAs as regulators of gene expression. The current release (miRBase 18- http://www.mirbase.org/) contains over 18 000 miRNA gene loci in over 160 species, and over 21 000 distinct mature miRNA sequences. As in November 2011, the miRBASE database contains 1424 miRNAs in Homo sapiens and 720 in Mus Musculus, but it is likely that the human genome encodes for more than a thousand miRNAs. MiRNAs are proven to interact with many and different biological processes, like embryonal development, cell cycle, cell identity, organogenesis, stress-response, cell proliferation and in the pathology of some diseases, among which there are CVDs.

1.4.1. Discovery

In 1993, two independent research groups noticed that in Caenorhabditis elegans the deletion of a gene, lin-4, was necessary for the correct postembryonal development [83]. Surprisingly, this gene did not code for a protein, but for a small RNA 22-nucleotide long, later called miRNA. Studying this RNA fragment, researchers found also that lin-4 was antisense complementary to the lin-14 gene and negatively regulated its post-trascriptional expression level. However, the importance of these discoveries was not clear until 2001, when a great number of small RNAs was found both in vertebrates and invertebrates.

1.4.2. Biogenesis and structure

MiRNAs are small, non-coding endogenous and conserved single strand RNA molecules of 21 to 26 nucleotides, produced from an hairpin-shaped transcripts. In mammals, a large fraction of these transcripts derive from miRNA genes that are found as part of introns of either protein-coding or noncoding genes. A small number are also found within exons, whereas many others are present in genomic repeats or have an unknown origin. Moreover, a significant number of miRNAs are expressed in clusters in which 2 or 3 miRNAs are generated from a common parent miRNA. The genes of miRNAs differ from other genes in that they do not have the canonical TATA box and they do not contain introns.
A schematic representation of miRNA biogenesis is represented in Figure 1.5. Transcription of miRNA genes by RNA polymerase II produces a primary transcript, referred to as the primary miRNA precursor (pri-miR), which is several hundred or thousands of nucleotides long and has a 33-nt stem–loop configuration comprising a 5' end cap structure and a polyadenylated 3' tail sequence. Within the nucleus, pri-miRNA is converted into a 60- to 70-nt transcript, termed pre-miR by the action of a nuclear ribonuclease III, named Drosha. The pre-miR is exported out of the nucleus by exportin-5 (Exp5)/RanGTP, and hydrolysis of RanGTP to RanGDP releases the pre-miR in the cytoplasm. The pre-miR is then processed into a 18- to 22-nt miRNA duplex by another RNase III, called Dicer which is associated to another dsRBP. The duplex is probably unwound by a helicase activity, and one strand, the so called "passenger" strand (or miR*), is degraded whereas the other strand, called the "guide" strand, accumulates as a mature miRNA. The miRNA is then handed over to Argonaute, which binds to the 3' end of the miRNA. The association of Dicer, Argonaute, and a miRNA forms a ribonucleoprotein (RNP) called the miRNA-induced silencing complex (miRISC), which, after binding with a messenger RNA (mRNA) target, accumulates in cytoplasmic foci known as processing bodies (P-bodies) [84] and stress granules (reviewed elsewhere [85]). Once within the P-bodies, translationally repressed mRNA is either sequestered in storage structures or can be processed for degradation. Thanks to this process, the mRNA is no more available for translation into protein and the expression of the corresponding gene is therefore downregulated [86].

1.4.3. Mechanism of action

MiRNAs negatively regulate gene expression at the post-transcriptional level by base-pairing with complementary sequences in the 3' untranslated regions (UTRs) of protein coding transcripts [87]. The binding specificity of individual miRNA for their target mRNAs has been presumed to be dictated by only 6 to 7 of the 22 to 26 nt that compose a miR. This sequence, located at the 5'end of the miRNA molecule is called the "seed" sequence. The perfect pairing of the seed with the correspondent sequence on 3' UTR represents a necessary condition for the downregulation of the of the mRNA target, while for other positions toward the 3' of the miRNA, mismatch or loops are accepted and it probably changes the power of action. The exact mechanisms through which miRNAs regulate gene expression

depends on the overall degree of complementarity with the target, miRNAs will either inhibit translation or induce degradation of mRNA. Usually, the interaction of a miRNA and its target mRNA is characterized by extensive mismatches and bulges, which result in a reduced efficiency of translation rather than a decrease in mRNA abundance [86].

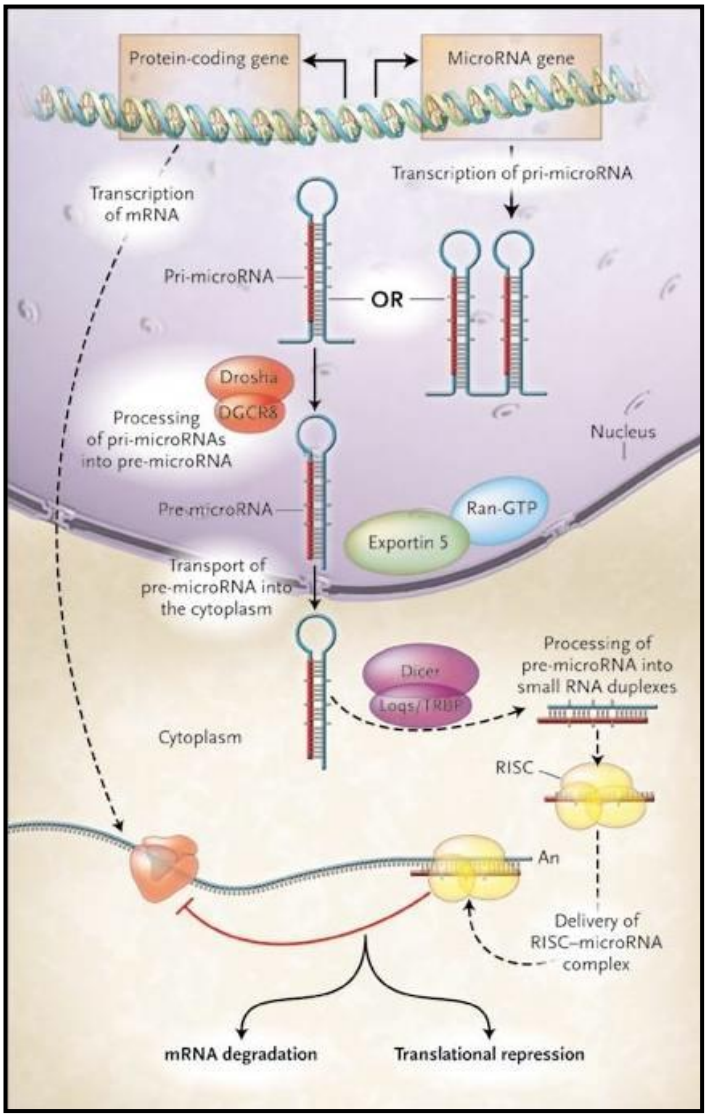

Figure 1.5 *MicroRNA biogenesis and mechanism of action (from[88]).*

1.4.4. Target prediction and validation

Based on the existence of 5' end-restricted complementarity to mRNA targets, it has been predicted that miRNAs regulate a large number of genes. Several algorithms based on different criteria have been developed, such us Diana-MicroT [89], microrna.org [90], PicTar [91] and TargetScan [92]. Most of these algorithms predict a large number of targets, not all of which are necessarily true [93]. Reliability in the identification of animal targets seems to have been improved by the additional evaluation of the energy states of sequences flanking the miRNA target (ΔG) and the presence or absence of stabilizing/destabilizing elements in the target mRNA. It has been reported, in fact, that virtually all miRNA-binding sites are located in "unstable" regions and hypothesized that miRNAs target 3' UTR regions with a less complex secondary structure because more accessible. Bioinformatic analysis predicts that up to one-third of a complete genome may be regulated by miRNAs, thus suggesting that they may play a role in almost every biological aspect. However, the number of targets verified to have biological relevance is still very small in animals compared with plants, because of their small size and the tolerance for mismatches of the animal miRNAs. For this reasons, molecular biology methods have been developed in order to validate at biological level those targets that are identified by a bioinformatic approach.

To date, luciferase assay is considered the main *in vitro* methodology to demonstrate whether a molecule is a real target of a miRNA. Briefly, the 3' UTR of the gene of interest is cloned into the multiple coding region downstream to the Renilla luciferase gene. After cloning, the vector is co-transfected into the mammalian cell line of choice together with the miRNA. If the miRNA binds to the seed sequence on the 3'UTR, the translation of fused Renilla luciferase:gene of interest mRNA will be blocked, decreasing the Renilla luciferase signal (Figure 1.6.). In order to confirm the specific action of the miRNA, a construct containing mutated 3'UTR sequences could be generated and used as further control in the Luciferase assay.

The following step for target validation is the evaluation of target protein levels by Western Blot analysis. This assay has to be performed on protein lysates of cells and/or tissues where the miRNA of interest has been previously over- or down-expressed.

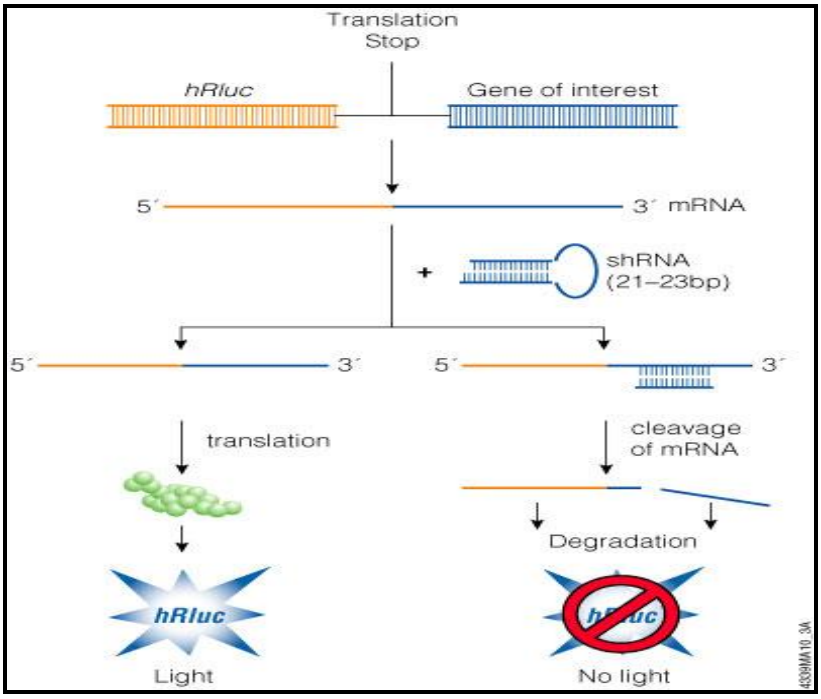

Figure 1.6 *Luciferase assay with short RNAs (shRNA). Renilla luciferase is used as the primary reporter gene, and the gene of interest is cloned into a multiple cloning region located downstream of the Renilla translational stop codon. Initiation of the RNA intereference process by synthetic or in vivo-expressed shRNA toward a gene of interest results in cleavage and subsequent degradation of the fusion luciferase:gene of interest mRNA decreasing the Renilla luciferase signal.*

1.4.5. MicroRNA in cardiovascular disease

The implications of miRNAs in the pathological process of the cardiovascular system and research on miRNAs in relation to CVDs has now become a rapidly evolving field. In the literature there are many examples of cardiac pathologies that show abnormalities in miRNAs expression [94-96] and their role has been proposed as potential targets for CVDs prevention, diagnostics, and therapy [97-102]. A growing body of data indicates that miRNAs are key regulators of cardiac development, contraction, and conduction. Moreover, a first extensive genome-wide profiling of miRNAs expression reported that the expression profile of many miRNAs is altered in human heart diseases and shows a typical signature for each disease etiology. Surprisingly, nearly all of the 18 most enriched miRNAs within the heart have an altered expression during cardiac disease, indicating an extremely dynamic regulation of miRNAs in the adult heart and pointing toward the importance of miRNAs as modifiers of gene expression programs in CVDs.

Several studies have revealed signature patterns of miRNAs that are up- and downregulated during pathological cardiac remodeling in rodents and humans (Table 1-3). Although miR-1, miR-29, miR-30, miR-133, and miR-150 have often been found to be downregulated, miR-21, miR-23a, miR-125, miR-195, miR-214, and miR-199 are upregulated with hypertrophy [103].

Downregulated MiRs	Upregulated MiRs	No Change	Species/Model or Disease
1, 7d*, 10a/b, 26a/b, 29a/c, 30a-3p/a-5p/b/c/d/e/e*, 139, 149, 150, 151, 155, 185, 194, 218, 378	15b, **21**, 23a/b, 24, 27a/b, 31, 103, 107, 125b, 127, 140/*, 195, 199a/a*/b, 214, 221, 222, 351, let-7b/c	133a/b	mouse/TAC[91]
29c, 30e, **93**, 133a/b, **150, 181b**	10b, 19a, **21, 23a**/b, **24**, 25, 27a/b, 125b, 126, 154, **195**, **199a**/a*, 210, **214**, 217, 218, 330, 351		mouse/TAC and CnA Tg[88]
29a/b/c, 30e, 126-5p, 133a/b, 149, **150, 185, 451, 486**	**21, 27a/b**, 146, **214**, **341, 424**		mouse/TAC[89]
30b/c, 150	17-5p, 18b, 19b, 20b, **21, 23a, 25, 29a,** 106a, **125b**, 140, 142-3p, 153, 184, 200a, 208, 210, 211, 221, 222		mouse/TAC[90]
187, 292-5p, 373, 466	*18b*, 20b, **21, 23a**, 106a, **125b**, 133a	**25, 29a**	rCM/PHE[90]
	23†, 24, 125b, 195, 199a, 214	21, 27, 29c, 93, 150, 181b	Human/HF[88]
16, 17-5p, 19b, 22, 23b, 24, 27a, 30a-5p/b/c/e-5p, 107, 126, 130b, 135a, 136, 148a, 150, 182, 186, 192, 199a*, 218, 299-5p, 302b*, 302c*, 325, 339, 342, 452/*, 494, 495, 497, 499, 507, 512-5p, 515-5p, 520d*/h, 520, 523, 526b/b*	*1*, 7a/b/c/d/e/f, 10b, 106b, 17-3p, *21*, 26a, 28, 29a/b/c, 32, 34b, 98, 125a, 126*, *129*/-3p, 130a, 132, 196a, 199b, 200c, 204, 205, 208, 210, 211, *212*, 213, 215, 292-3p, 294, 295, 296, 297, 300, 302a, *320*, 322, 330, 331, 333, 340, 341, 343, 365, 367, 372, *373*, 377, 381, 382, *423*, 424, 429, 432, 500, 520c, 525*		Human/HF[92]

Bold indicates miRs reported validated by Northern blots; italics, miR reported validated by PCR; CnA Tg, calcineurin A transgenic mice; rCM, neonatal rat cardiomyocytes; PHE, phenylephrine; HF, end-stage heart failure. †Expression found variable.

Table 1-3 *MiRNA reported dysregulated in array analysis (from [86]). MiRNA-199a/a* are up-regulated in human and mouse models of CH and HF.*

1.4.5.1. MicroRNA-199

The miR-199 family contains 3 miRNA genes: miR-199a-1, miR-199a-2, and miR-199b, that are all encoded by the antisense strand of an intron of the dynamin gene (Dnm2, Dnm3, and Dnm1, respectively). Furthermore, miR-199a-2 is cotranscribed with miR- 214 (Table 1-4).
MiR-199 genes chromosomal location is different between mice and human as reported in Table 1.4. Each gene is coding for a miR-199 hairpin precursor molecule, that is processed in two different mature isoforms: 5p and 3p, also referred to as miR-199a/b and miR-199a*/b*, respectively. They have a different mature sequence of 23 and 22 nucleotides, thus they can potentially target different gene molecules responsible for adverse cardiovascular remodeling.
Olson was the first author showing that some interesting correlations could exist between miR-199 expression and HF. In particular, he showed that miR-199a is upregulated in mouse models of CH and that its overexpression in CMCs is sufficient to induce cell size hypertrophy [104]. Growing evidence indicates that miR-199 is also involved in oncogenesis and in different cardiac diseases.

MiR-199 has been proved to play a crucial role in the regulation of cell cycle, cell proliferation, apoptosis and cardiac cellular signalling, by affecting gene expression at post-transcriptional level [105-107].

GENE / SPECIES	Dnm1 *neuronal*	Dnm2 *ubiquitary*	Dnm3 *testis, brain, lung*
mmu-miR	miR-199b Ch2 intron 1, 14, 15	miR-199a1 Ch9 intron 14, 15,16	miR-199a2 Ch1 intron 3, 14 *(~ 10 kb miR214)*
hsa-miR	miR-199b Ch9 intron 2, 5, 14, 15	miR-199a1 Ch19 intron 14, 15	miR-199a2 Ch1 intron 14, 15 *(~ 10 kb miR214)*

Table 1-4 *MiRNA-199 genome location.*
MiR-199 genes (miR-199a1,miR-199a2 and miR-199b) are located in the antisense strand within the introns of three dynamin genes in mammals: Dnm 1 is expressed in neurons, Dnm 2 is the ubiquitary isoform and Dnm 3 is expressed in testis, brain and lung.

2. RESULTS

2.1. MiRNA-199a/a expression and molecular targets in the cardiovascular system*

Our first aim was to determine miR-199a/a* expression profile and their potential molecular targets relevant for the cardiovascular system.
To this attempt, we first determined miR-199a/a* expression profile in physiological condition, focusing on cardiac cell populations. We then assessed the miR-199a/a* expression pattern in pathological conditions, by determining their levels in both cardiac tissues and cell populations.
Finally, we identified the molecular targets whose expression can be potentially regulated by miR-199a/a*.

2.1.1. MiRNA-199a/a* are physiologically expressed in the cardiovascular system

As widely reported in literature, a proliferating cell line with no endogenous expression of the miRNA of interest represents the best tool for validating the molecular targets of the miRNA *in vitro*.
Based on this, we first analyzed miR-199a/a* expression in HEK293 (Human Embryonic Kidney), an immortalized cell line where the miRNA resulted to be virtually absent (Figure 2.1). Consistently, a recent study demonstrated that miR-199a/a* is one of the several highly expressed miRNA in connective tissues and in normal skin fibroblasts, whereas its expression is absent in other proliferating cell lines [105]. According to this, data from the microrna.org database shows the highest levels of miR-199a/a* expressions in fibroblasts when compared to those observed in the other cell populations.
It has been recently reported that miR-199a is also expressed in CMCs from rat heart, but barely detectable in fibroblasts [108]. This apparent controversy suggested us to perform a quantitative comparison analysis of miR-199a/a* in cFIBROs and CMCs isolated from mouse heart (Figure 2.1).
Since we confirmed that miR-199a/a* are highly expressed in fibroblasts, we decided to set this as a reference value (=1). MiR-199a/a* resulted physiologically express in both neonatal myocyte and non-myocyte primary cultures, whereas their levels were significantly lower in adult CMCs. Interestingly, the highest miR-199a/a* expression levels were found in AoSMCs (Figure 2.1), suggesting a possible role for miR-199a/a* in smooth vasculature.
This data indicate that miR-199a/a* are expressed in the three main cardiac cell populations: CMCs, cFIBROs, and AoSMCs.

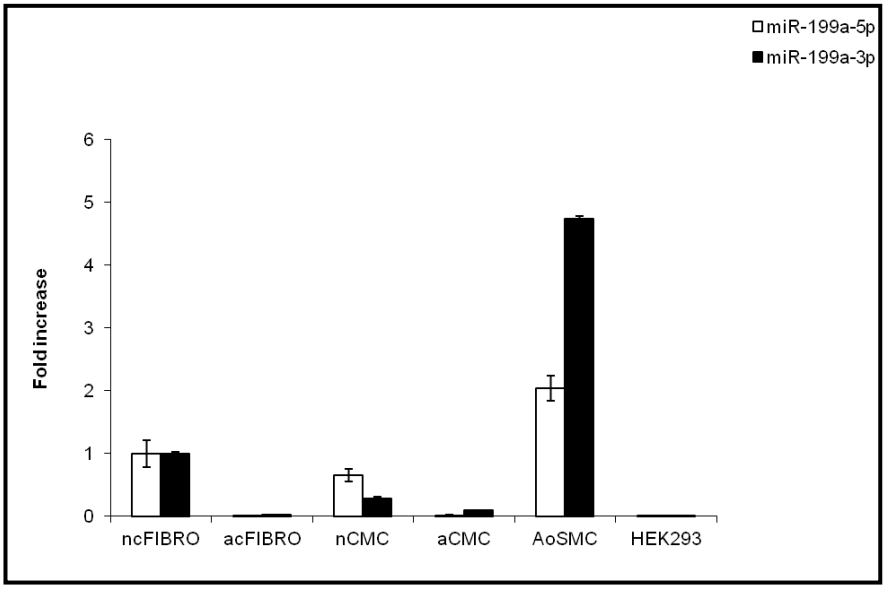

Figure 2.1 *MiRNA-199 expression profile: cell pattern. Real time PCR analysis of miR-199a/a* expression in different cell populations: murine neonatal cardiac fibroblasts (ncFIBRO), murine adult cardiac fibroblasts (acFIBRO), murine neonatal cardiomyocytes (nCMC), murine adult cardiomyocytes (aCMC), aortic smooth muscle cells (AoSMC), human embryonic kidney (HEK293). ncFIBRO was used as reference value (control=1). P < 0.01 vs control.*

Then, we determined the miR-199a/a* expression profile in murine adult tissue. All the results were expressed as percentage of miR-199a/a* levels measured in LV, which were set to 1. Our analysis performed by real-time PCR showed that miR-199a/a* were expressed in LV, RV, atria, and aorta. In particular, miR-199a was predominantly expressed in atria and LV, whereas high levels of miR-199a* were measured in RV and aorta. Interestingly, modest levels of expression were also observed in lung, probably due to the high content of fibroblasts in the connective tissue (Figure 2.2).
This data reveals that miR-199a/a* have a unique tissue specific expression pattern and that both isoforms are expressed in the cardiovascular system in physiological condition.

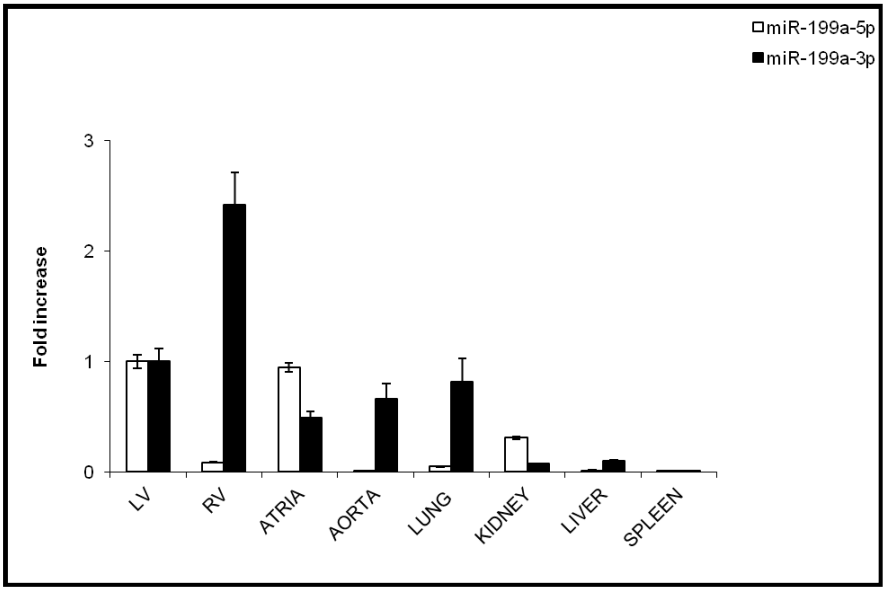

Figure 2.2 *MiRNA-199 expression profile: tissue pattern.*
Real time PCR analysis of miR-199a/a expression in different mice tissues: left ventricle (LV), right ventricle (RV), atria, aorta, lung, kidney, liver and spleen. LV was used as reference value (control=1). P < 0.01 vs control.*

2.1.2. MiRNA-199a/a* expression increase during cardiac hypertrophy

We then assessed whether the cardiac miR-199a/a* expression levels changes under pathological condition.
It has been recently reported, that miR-199a is upregulated in different models of CH, and also in idiopathic end-stage failing human hearts [104, 108]. In particular, miR-199a has been shown to be up-regulated by 10-fold in rat hypertrophic hearts after 12 weeks of abdominal aorta constriction [108].
To determine whether miR-199a/a* is dynamically modulated during CH, we performed a time-course study in a murine CH model that is the transverse aortic arch–constriction (TAC). In this model, multiple signal transduction pathways are induced simultaneously by pressure overload, leading to CMC hypertrophy. At one, four, and eight weeks after TAC, hearts were weighed and separated in LV, RV and atria. Mice operated-without the banding, referred to as sham, were included as controls. As expected, TAC mice showed a significant myocardial hypertrophy that was associated with an increased expression of cardiac fetal genes (ANF, α-skeletal actin (SkA), and β-myosin heavy chain (β-MHC)) (results not shown) [109]. As shown in Figure 2.3, both miR-199a/a* expression in LV significantly increased at four weeks after banding. Notably, while miR-199a levels stayed high up to eight weeks, miR-199a* levels reached a peak at four and returned to basal level at eight weeks after banding.

Consistent with the literature, these results indicate that miR-199a/a* are up-regulated in LV of mice with pathological CH, in a time-dependent and isoform-specific manner.

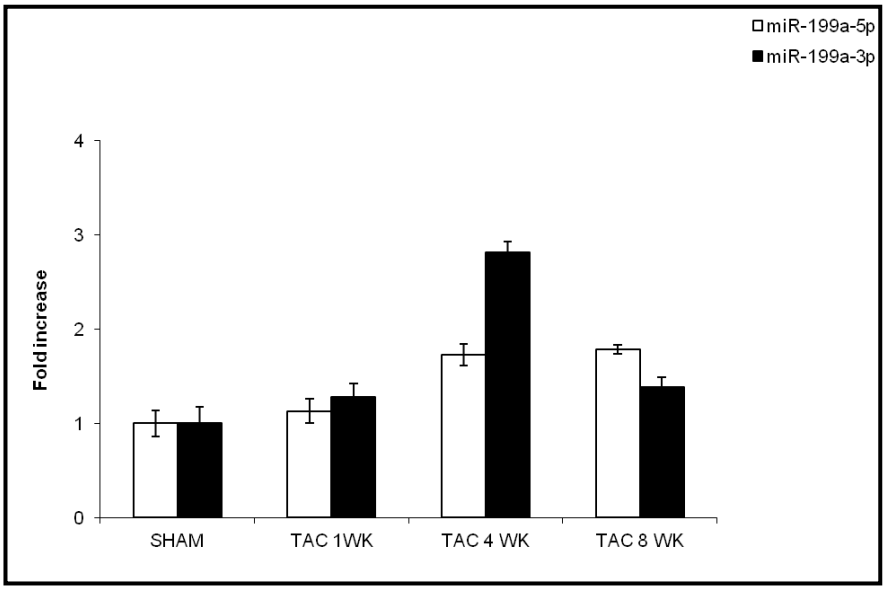

Figure 2.3 *MiRNA-199 expression profile: in vivo cardiac hypertrophy model. Real time PCR analysis of miR-199a/a* expression in left ventricles (LV) of sham-operated without the banding (control=1) and transverse aortic arch-constricted (TAC) mice. Mice were subjected to pressure overload for different time: 1, 4, 8 weeks (representative results, mean ± s.d. of a minimum of n = 3 per group). P < 0.05 vs control.*

2.1.3. ANG-II and ET-1 increase miRNA-199a/a* expression in cardiac fibroblasts and aortic smooth muscle cells

To determine whether miR-199a/a* expression is modulated in cardiac cells in response to pathological stimuli, we analyzed two *in vitro* primary culture cell models. cFIBROs and aortic smooth muscle cells (AoSMCs) were subjected to a time-course agonist treatments and miR-199a/a* levels were determined by a relative quantitative analysis. In this model, pathological concentrations of ANG II and ET-1 are used to reproduce the neurohormonal response normally associated to CH and myocardial fibrosis. Indeed, multiple signalling pathways are activated, ultimately leading to gene expression modulation. Preliminary studies allowed us to establish the ANG II and ET-1 concentrations that significantly affect miR-199a/a* expression. We found that miR-199a/a*a expression in cFIBROs increased either after 30' or 24 h exposure to 10^{-6} M ANG II (Figure 2.4).

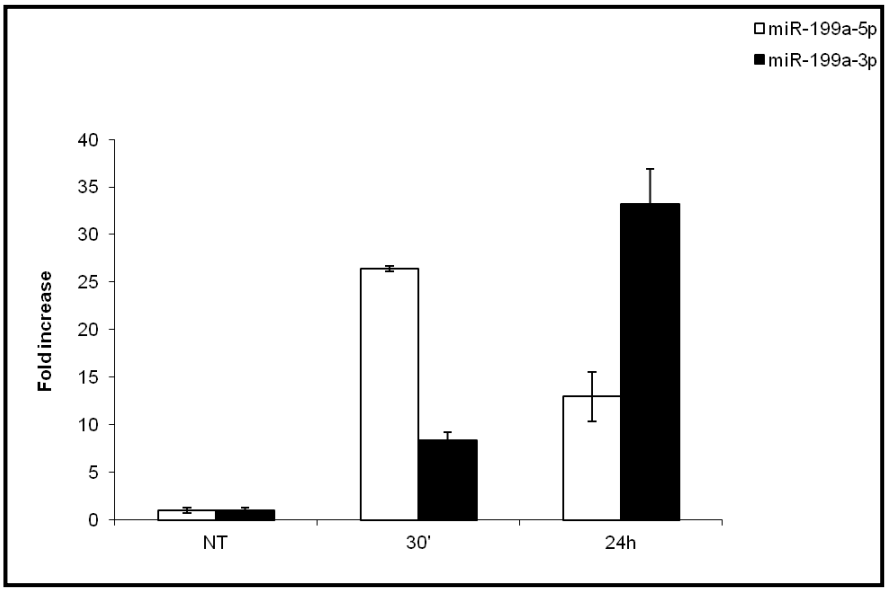

Figure 2.4 *MiRNA-199 expression profile: in vitro angiotensin II (ANG II) treatment. Real time PCR analysis of miR-199a/a* expression in cardiac fibroblasts after 30' and 24 h exposure to 10^{-6} M ANG II.*
Not-treated cells were used as control (=1). P < 0.01 vs control.

Notably, miR-199a levels increased more at 30' then at 24 h ANG II treatment, whereas miR-199a* expression progressively increased with time, reaching a peak at 24 h. Similarly, cFIBROs exposure to 100 nM ET-1 induced a marked time-progressive increase in miR-199a levels, as shown in Figure 2.5. On the contrary, no change in miR-199a* expression was detected even after prolonged exposure to higher ET-1 concentrations.
As represented in Figure 2.6, these results show that ANG II induced a significantly higher increase in miR-199a/a* levels compared to that due to ET-1.

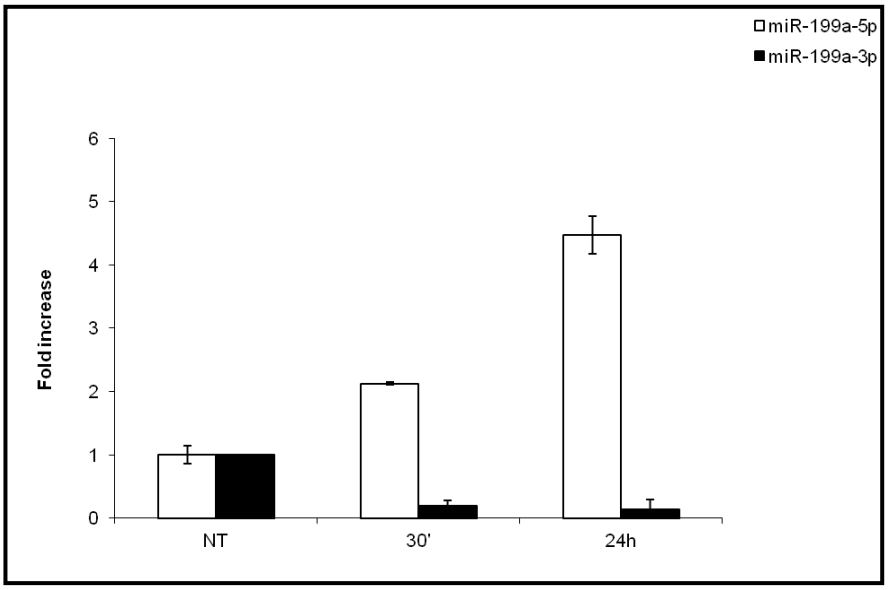

Figure 2.5 *MiRNA-199 expression profile: in vitro endothelin-1 (ET-1) treatment. Real time PCR analysis of miR-199a/a* expression in cardiac fibroblasts after 30' and 24 h exposure to 100 nM ET-1. Not-treated cells were used as control (=1). P < 0.01 vs control.*

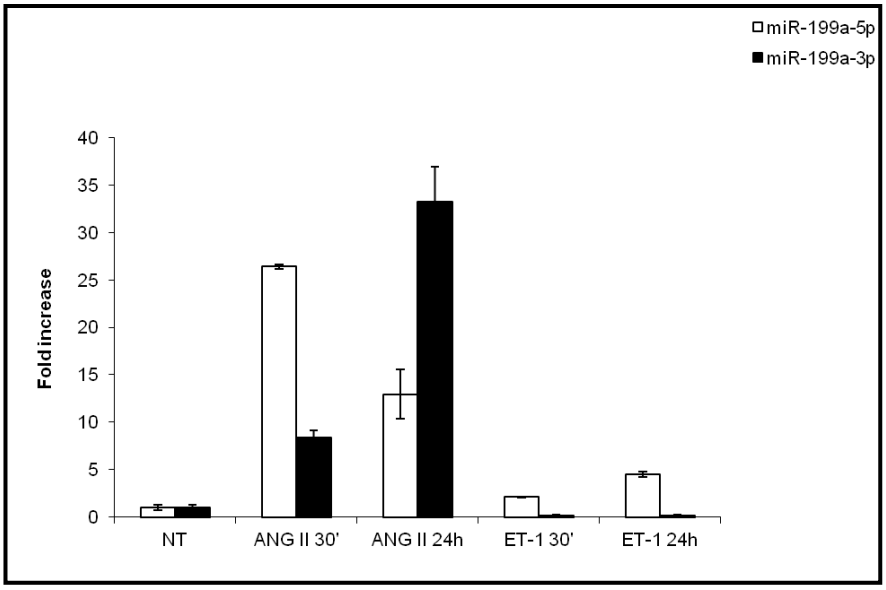

Figure 2.6 *MiRNA-199 expression profile: in vitro angiotensin II (ANG II) and endothelin-1 (ET-1) treatment (comparison analysis graph). Real time PCR analysis of miR-199a/a* expression in cardiac fibroblasts (cFIBRO) after 30' and 24 h exposure to 10-6 M ANG II and 100 Nm ET-1. Not-treated cells were used as control (=1). P < 0.01 vs control.*

We then asked whether the effects of ANG II and ET-1 on miR-199a/a* expression is receptor-type mediated. After an overnight starvation, cells were pre-treated with specific and commercially available receptor antagonists for 30' and then exposed to relative agonist for 24 h. As shown in Figure 2.7, ANG II-induced miR-199a/a* up-regulation markedly decreased in cells pretreated with 50 μM ATR1 antagonist (EMD66684), whereas any change occurred when ATR2 receptors were blocked by the same concentration of its relative antagonist compound (PP123819).

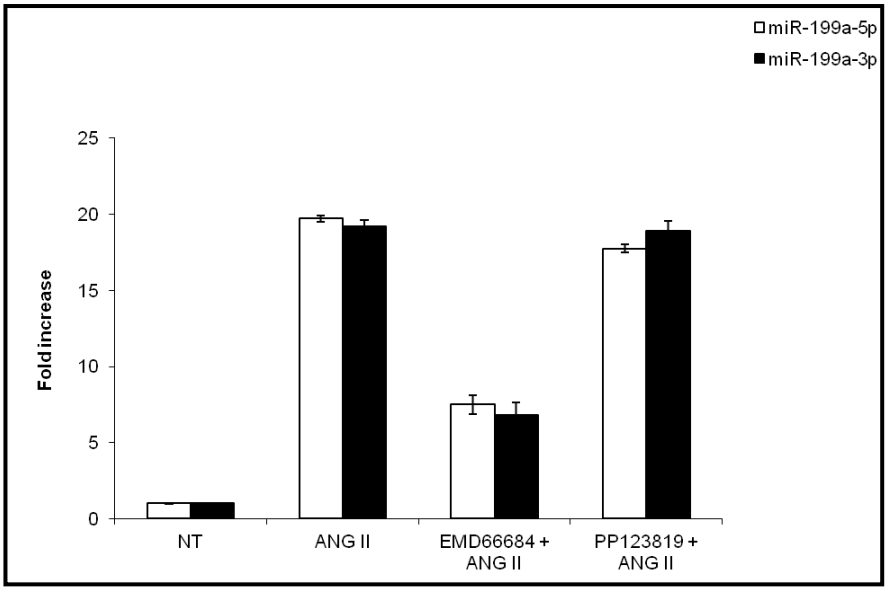

Figure 2.7 *MiRNA-199 expression profile: in vitro angiotensin II receptor (ATR) blockage. Real time PCR analysis of angiotensin II (ANG II)-induced miR-199a/a* expression in cardiac fibroblasts after 30' exposure to 50 mM AT-1 antagonist (EMD66684) and AT-2 antagonist (PP123819).*
Not-treated cells were used as control (=1). P < 0.01 vs control.

Similarly, EDNRA and EDNRB receptors in cFIBROs were blocked by pre-treating cells for 30' with 50 μM BQ123 and BQ788, respectively. Figure 2.8 shows that both ETR antagonists completely abolished ET-1-induced miR-199a up-regulation occurring after 24 h exposure.
This data indicates that while ANG II exerts its effect on miR-199a/a* expression through AT-1, ET-1 can work through both ETRs.

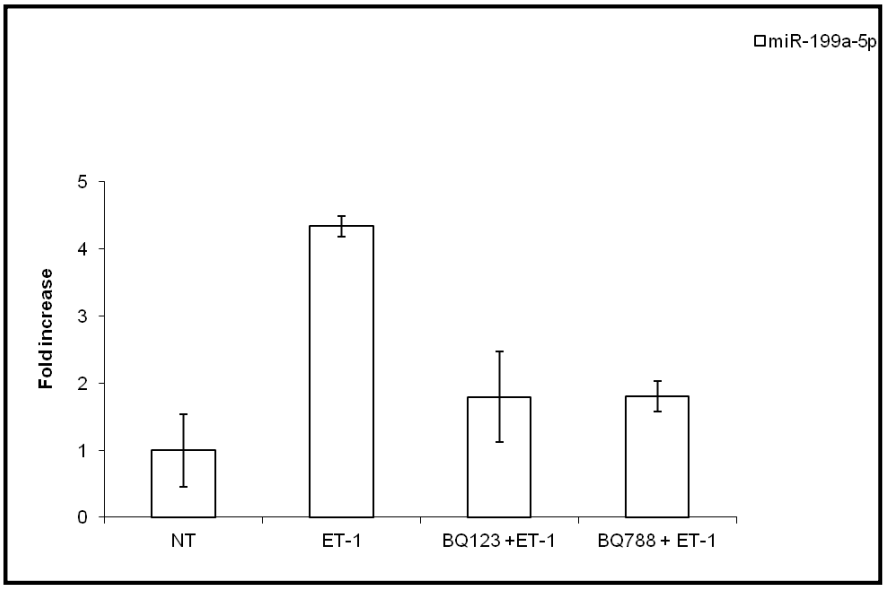

Figure 2.8 *MiRNA-199 expression profile: in vitro endothelin-1 receptor (ETR) blockage. Real time PCR analysis of endothelin-1 (ET-1)-induced miR-199a-5p expression in cardiac fibroblasts after 30' exposure to 50 μM ET-A (BQ123) and ET-B antagonist (BQ788).*
Not-treated cells were used as control (=1). P < 0.01 vs control.

We performed the same time-course studies also on AoSMCs, where we had previously detected a modest expression level of endogenous miR-199a/a*. In this case, both ET-1 and ANG II significantly increased miR-199a expression at two different early exposure stage times: 30' for ANG II and only 5' for ET-1. No significant changes were measured after prolonged treatments. Similarly, we did not observe any increase in miR-199a* levels either after ANG II or ET-1 time-course treatments.
This data demonstrates that ANG II and ET-1 are able to induce miR-199a expression in AoSMCs.
With these studies, we demonstrated that miR-199a/a* expression in cFIBROs and AoSMCs is induced in response to both acute and prolonged stimulation by ANG II and ET-1. Such regulation can vary according to both cell model and miR-199 isoform considered.

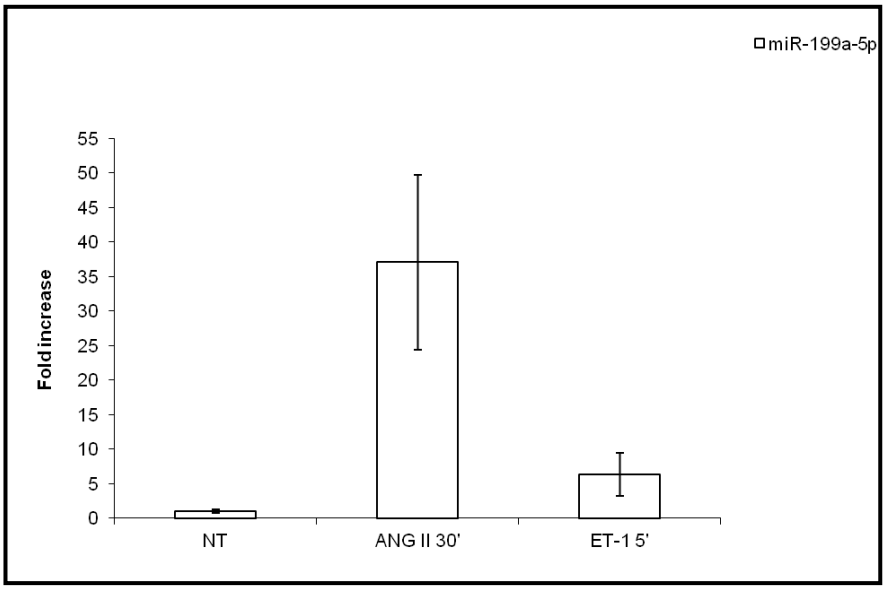

Figure 2.9 *MiRNA-199 expression profile: in vitro angiotensin II (ANG II) and endothelin-1 (ET-1)treatment. Real time PCR analysis of miR-199a-5p expression in aortic smooth muscle cells after 30' exposure to 10^{-6} M ANG II and 5' exposure to 100 nM ET-1. Not-treated cells were used as control (=1). P < 0.01 vs control.*

2.1.4. MiRNA-199a/a* target molecules belonging to neurohormonal systems

According to the main aim of this study, our research continued with the identification of miR-199a/a* targets. The potential miR-199a/a* targets were identified by a bioinformatic approach focused on four databases (Target Scan, PicTar, microrna.org, miRanda). These were found to be the best methods, showing sensitivity values ranging between 65% and 68%. PITA database of predicted miRNA targets is a further and more exhaustive bioinformatic tool used to identify miRNA potential binding sites on our UTR of choice. Indeed, PITA algorithm uses different seed matching tools in order to scan the 3' UTR for potential miRNA targets, and then scores each site using the method recently described [110].
The scope of these computational approaches was to locate the miRNA seed sequences on the 3' UTR (PITA) and, in the case of microrna.org, also to provide the specific tissue expression pattern of the studied miRNA. Altogether, the databases adopted for this computational search were easy to use, but had the disadvantage of predicting a large number of targets.
Accordingly to previous results, we focused the miR-199a/a* targets search on ANG II/ET-1 related molecules. Among the potential targets of miR-199a/a* we found some components of the RAS that we subsequently evaluated for their validation:

1) ACE1, is the main enzyme responsible for ANG II production. Figure 2.10b shows a 40 % reduction in the luciferase signal due to miR-199a seed sequence specific binding on the 3'UTR (Figure 2.10c). Such a decrease was not observed when the miRNA was transfected together with the plasmid containing the mutated binding site. These data were confirmed by Western Blot analysis, showing a significant decrease of ACE1 protein levels induced both by miR-199a and miR-199a* (Figure 2.10c). However, no miR-199a* seed sequences were predicted, so far.

2) AGTR1B, is a GPCR that mediates the main ANG II effects on cardiac and vascular cell populations. As predicted by the bioinformatic analysis, miR-199a* is the isoform responsible for AGTR1B downregulation. In fact, we observed a 40 % reduction in the luciferase signal due to miR-199a* seed sequence specific binding on the 3'UTR (Figure 2.11a), as demonstrated by the mutated control (Figure 2.11b). These results were confirmed by Western Blot analysis, showing a significant decrease of AGTR1B protein levels induced both by miR-199a and miR-199a* (Figure 2.11c). According to these results, PITA tool predictions also identified miR-199a seed sequences on AGTR1B. However, we did not observe a significant decrease in luciferase signal after miR-199a transfection.

3) ECE1, is the main enzyme responsible for ET-1 production. Figure 2.12b shows a 70% reduction in the luciferase signal due to both the miR-199a and miR-199a* seed sequences specific binding (Figure 2.12a), as demonstrated by the mutated controls. Western Blot analysis confirmed that both miR-199 isoforms significantly decreased ECE1 protein levels (Figure 2.12c).

4) EDNRA, is a GPCR that mediates the main ET-1 effects on cardiovascular system. Figure 2.13b shows a 70% reduction in the luciferase signal due to both the miR-199a and miR-199a* seed sequences specific binding (Figure 2.13a), as demonstrated by the mutated controls. These results were confirmed by Western Blot analysis, showing a significant decrease of AGTR1B protein levels induced both by miR-199a and miR-199a* (Figure 2.13c).

5) ET-1, a neurohormonal factor whose functions has been deeply described, also resulted as potential target from our bioinformatic research. The relative luciferase assay showed a 80% decrease in the signal (Figure 2.14b), in presence of both miR-199a and miR-199a*. Figure 2.14 did not show Western Blot analysis, because of some technical problems we encountered in detecting the mature peptide. However, also mutated controls demonstrated that the observed decrease in ET-1 3' UTR-luciferase signal is due to the specific seed sequences binding of miR-199a/a* (Figure 2.14a), thus validating it as miR-199a/a* biomolecular target. Furthermore, these data agree with a recent study showing miR-199a ability to decrease ET-1 mRNA levels [106].

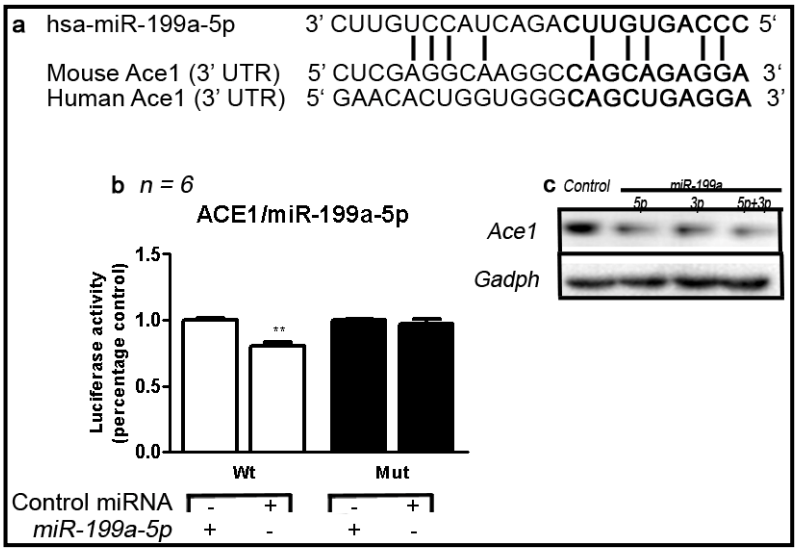

Figure 2.10 *ACE1 miR-199a (miR-199a-5p) target validation. a) Location of miR-199a-5p seed sequence on ACE1 3' UTR. b) ACE1 3' UTR dual luciferase reporter assay performed by cotransfection of miR-199a-5p oligonucleotide with a luciferase reporter gene linked to the ACE1 3' UTR, containing wild-type (Wt) (left panel) or mutated (right panel) miR-199a-5p complementary sites; a nontargeting scramble oligonucleotide was also included (control miR). c) Western blot analysis performed with specific antibodies for ACE1 and GADPH on total protein extracted from HEK293 transfected with miR-199a-5p oligonucleotide; a nontargeting scramble oligonucleotide was also included (control). ** P < 0.01 vs control.*

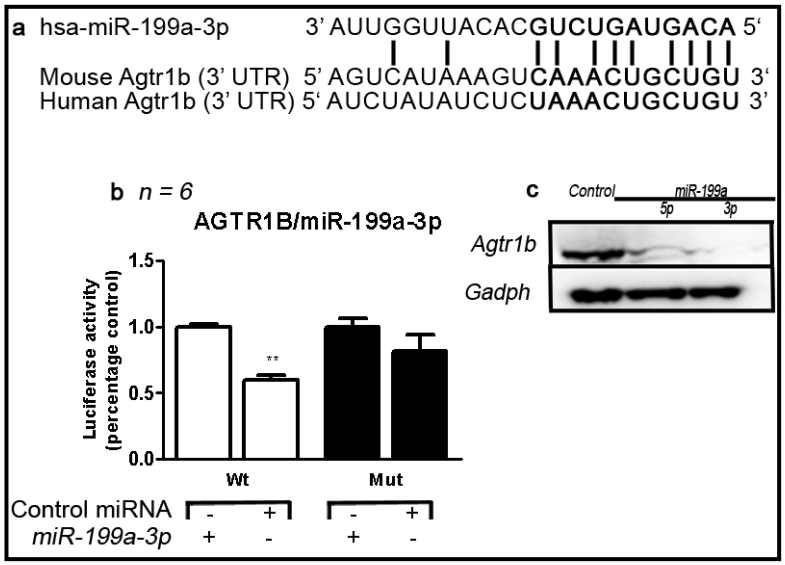

Figure 2.11 *AGTR1B miR-199a* (miR-199a-3p) target validation.*
a) *Location of miR-199a-3p seed sequence on AGTR1B 3' UTR. b) AGTR1B1 3' UTR dual luciferase reporter assay performed by cotransfection of miR-199a-3p oligonucleotide with a luciferase reporter gene linked to the AGTR1B 3' UTR, containing wild-type (Wt) (left panel) or mutated (right panel) miR-199a-3p complementary sites; a nontargeting scramble oligonucleotide was also included (control miR). c) Western blot analysis performed with specific antibodies for AGTR1B and GADPH on total protein extracted from HEK293 transfected with miR-199a/a* oligonucleotides; a nontargeting scramble oligonucleotide was also included (control). ** P < 0.01 vs control.*

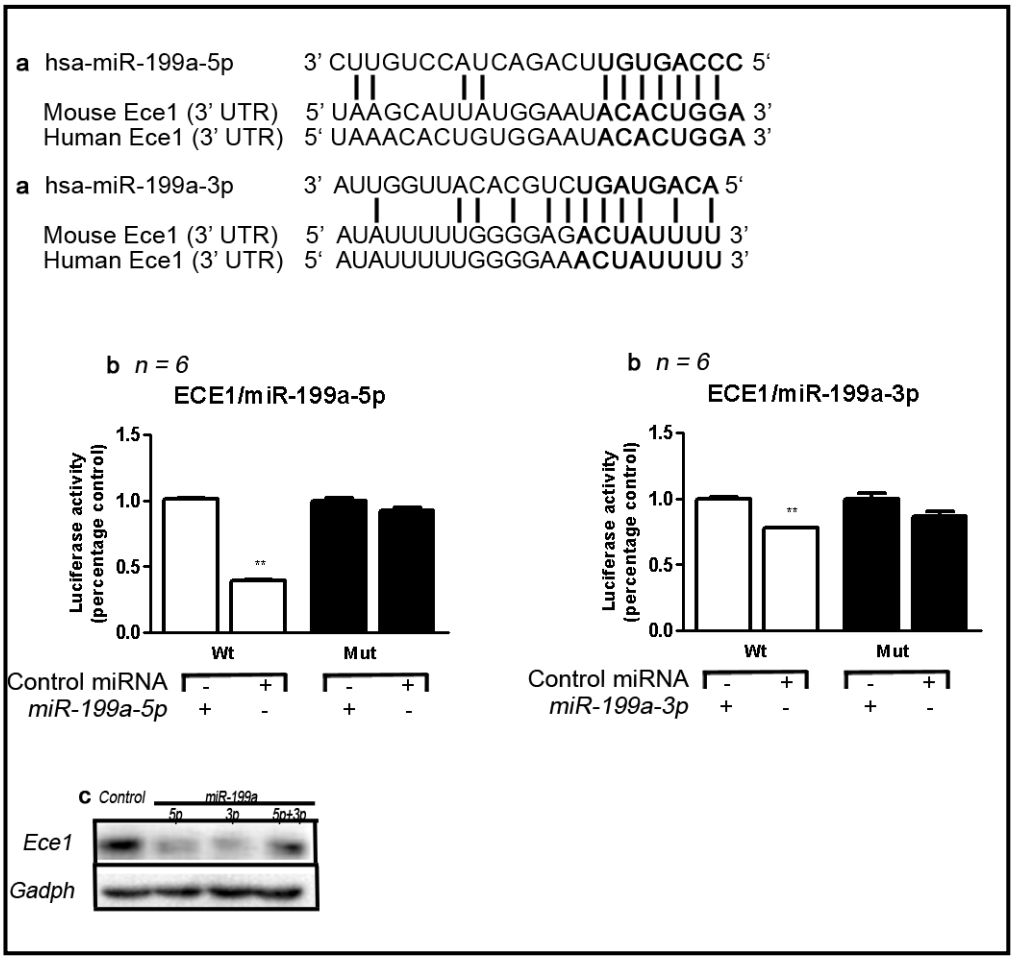

Figure 2.12 *ECE1 miR-199a/a* (miR-199a-5p/3p) target validation. Location of miR-199a/a* seed sequence on ECE1 3' UTR. b) ECE1 3' UTR dual luciferase reporter assay performed by cotransfection of miR-199a/a* oligonucleotides with a luciferase reporter gene linked to the ECE1 3' UTR, containing wild-type (Wt) (left panel) or mutated (right panel) miR-199a/a* complementary sites; a nontargeting scramble oligonucleotide was also included (control miR). c) Western blot analysis performed with specific antibodies for ECE1 and GADPH on total protein extracted from HEK293 transfected with miR-199a/a* oligonucleotides; a nontargeting scramble oligonucleotide was also included (control). ** P < 0.01 vs control.*

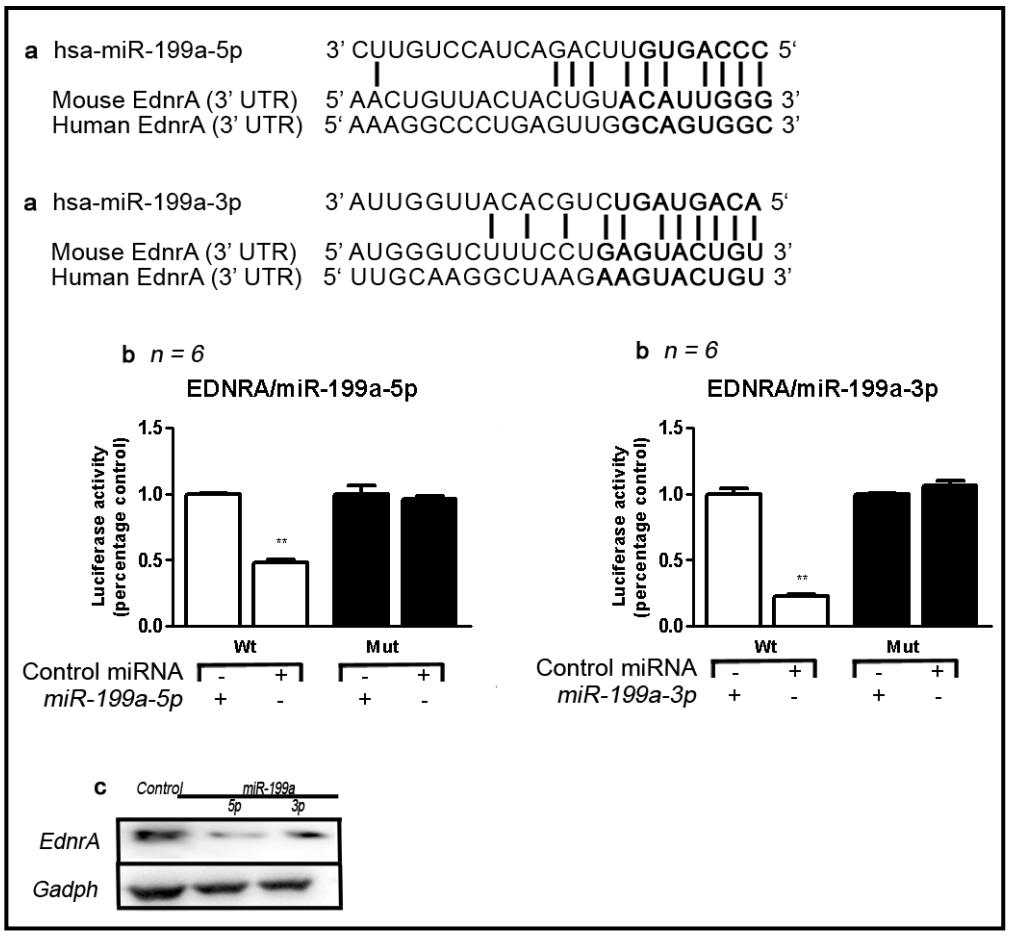

Figure 2.13 *EDNRA miR-199a/a* (miR-199a-5p/3p) target validation. Location of miR-199a/a* seed sequence on EDNRA 3' UTR. b) EDNRA 3' UTR dual luciferase reporter assay performed by cotransfection of miR-199a/a* oligonucleotides with a luciferase reporter gene linked to the EDNRA 3' UTR, containing wild-type (Wt) (left panel) or mutated (right panel) miR-199a-3p complementary sites; a nontargeting scramble oligonucleotide was also included (control miR). c) Western blot analysis performed with specific antibodies for EDNRA and GADPH on total protein extracted from HEK293 transfected with miR-199a/a* oligonucleotides; a nontargeting scramble oligonucleotide was also included (control). ** P < 0.01 vs control.*

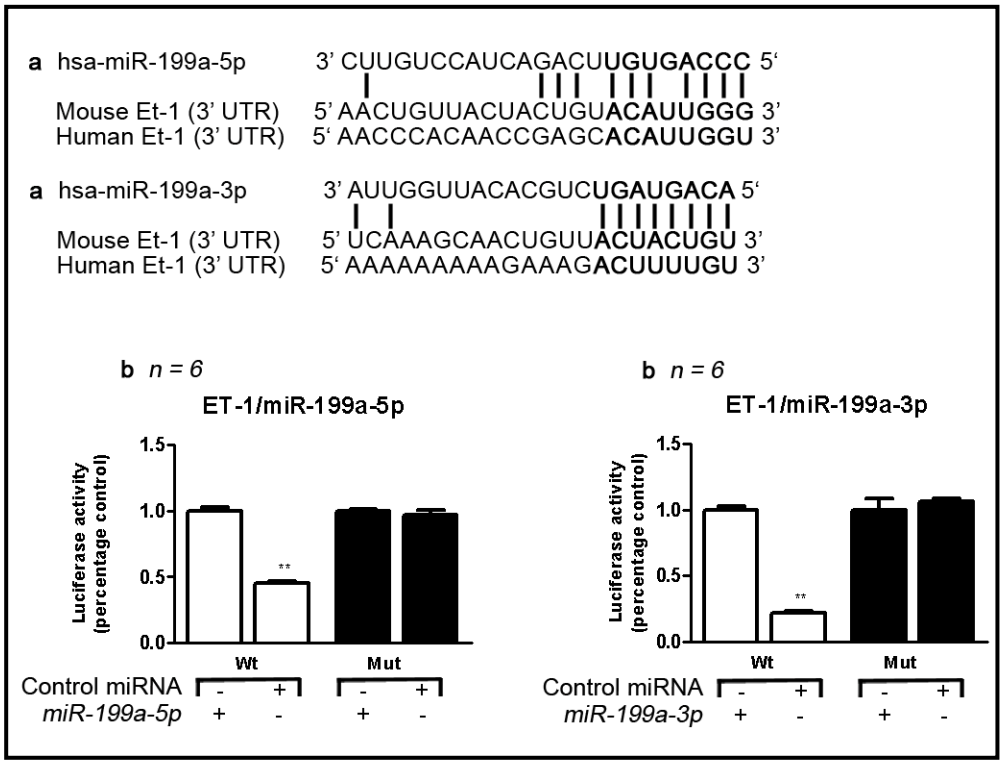

Figure 2.14 *ET-1 miR-199a/a* (miR-199a-5p/3p) target validation. Location of miR-199a/a* seed sequence on ET-1 3' UTR. b) ET-1 3' UTR dual luciferase reporter assay performed by cotransfection of miR-199a/a* oligonucleotides with a luciferase reporter gene linked to the ET-1 3' UTR, containing wild-type (Wt) (left panel) or mutated (right panel) miR-199a-3p complementary sites; a nontargeting scramble oligonucleotide was also included (control). ** P < 0.01 vs control.*

Another gene molecule, whose function is strictly related to the cardiovascular action of the RAS, was identified as miR-199a/a* potential targets:

6) APLNR, is a GPCR binding AP. AP is a molecule showing some important similarities with ANG II and playing a role in cardiac tissues remodelling. Figure 2.15b shows a 70% reduction in the luciferase signal due to both miR-199a/a* seed sequences specific binding (Figure 2.15a). Western Blot analysis confirmed that both miR-199a/a* significantly decreased APLNR protein levels (Figure 2.15c).

These results revealed that miR-199a/a* targets gene molecules belonging to neurohormonal systems ((RAS) and ET system), normally activated after an initial stimulus responsible for impairing cardiac function by promoting vascular and cardiac remodelling.

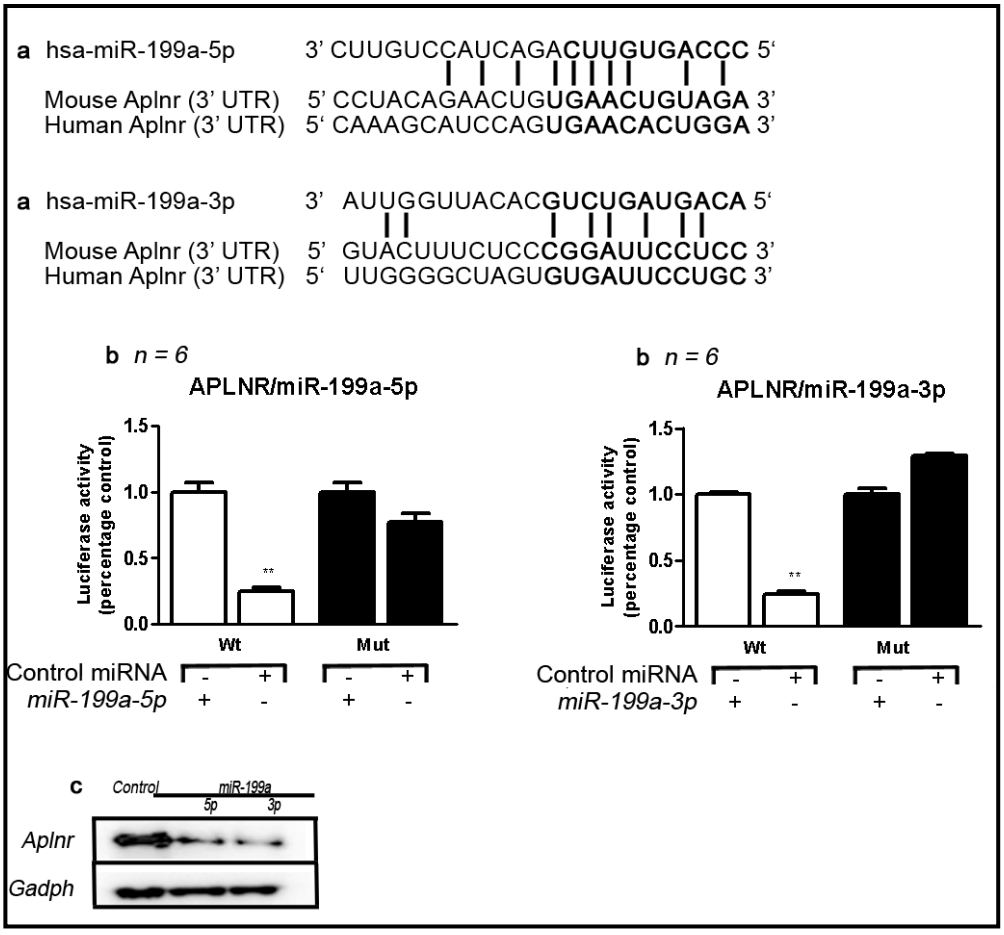

Figure 2.15 *APLNR miR-199a/a* (miR-199a-5p/3p) target validation. Location of miR-199a/a* seed sequence on APLNR 3' UTR. b) APLNR 3' UTR dual luciferase reporter assay performed by cotransfection of miR-199a/a* oligonucleotides with a luciferase reporter gene linked to the APLNR 3' UTR, containing wild-type (Wt) (left panel) or mutated (right panel) miR-199a-3p complementary sites; a nontargeting scramble oligonucleotide was also included (control miR). c) Western blot analysis performed with specific antibodies for APLNR and GADPH on total protein extracted from HEK293 transfected with miR-199a/a* oligonucleotides; a nontargeting scramble oligonucleotide was also included (control). ** P < 0.01 vs control.*

2.2. MicroRNA-199a/a effects on cardiovascular functions*

The second part of this research, was focused on the study of the effects of miR-199a/a* on cardiac and vascular functionality.
To this attempt, we modulated miR-199a/a* expression *in vivo* by both overexpression and silencing approaches. We then performed a series of functional studies both *in vitro* and *in vivo* to determine the role of miR-199a/a* either under normal unstressed conditions or following biomechanical/pathological stress, induced by different kind of stimuli.
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2.2.1. Micro-RNA-199a/a* overexpression

2.2.1.1. Transgenic mice overexpressing miR-199a/a have no gross phenotype*

To gain insight into the mechanism of action by which miR-199a/a* regulate cardiovascular pathophysiology, we investigated the effects of their overexpression *in vivo* by analyzing a transgenic mouse line (Tg). In Tg mice, the mmu-miR-199a gene was located under the control of a constitutive promoter that allows for continual transcription of the associated miR-199a transgene.
We started the mouse line characterization with three different founders whose genotype was determined by PCR amplification of a 300 hundreds mmu-miR-199a gene specific fragment. Tg mice were bred to wild-type (Wt) C57BL_B6 mice for genome background stabilization. Northern blot (results not shown) and real-time PCR analysis performed on total RNA extracted from Tg and Wt LV, showed that miR-199a/a* levels in Tg mice derived from the strain three were up-regulated by 6-folds compared to the Wt controls. Although genotype analysis established that Tg mice derived from strain one and two also expressed the transgene, miR-199a/a* expression levels did not result up-regulated in Tg mice compared to Wt controls.Therefore, we selected strain three as the one expressing the highest miR-199a/a* levels (Figure 2.16).
Tg mice and littermate controls were characterized at basal conditions. We performed a survival growth and a gender-base analysis to investigate whether miR-199a/a* *in vivo* forced overexpression could produce any apparent phenotype.
Overall, our characterization assessed that Tg mice have no gross phenotype and follow Mendelian rules.

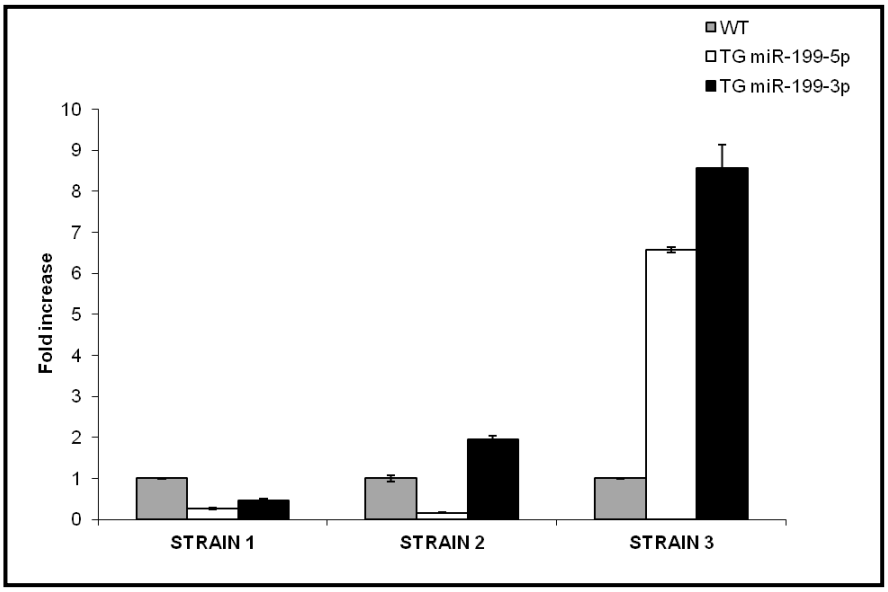


Figure 2.16 *In vivo miR-199a/a* overexpression: a transgenic mice model. Real time PCR analysis of miR-199a/a* expression in left ventricles (LV) of three different strain of wild type (Wt) and transgenic (Tg) mice. Wt mice were used as control (=1). P < 0.01 vs control.*

2.2.1.2. In vivo overexpression of microRNA-199a/a contributes to cardiac dysfunction in pathological cardiac hypertrophy*

To investigate whether overexpression of miR-199a/a* *in vivo* affects heart physiology and cardiac function, Tg mice were characterized as described.
We first analyzed Wt and Tg animals at basal condition by gravitometric, ecochardiographic, and haemodynamic measurements. As reported in Table 2-1, these analyses revealed no differences between Wt and Tg mice under basal condition. Afterwards, mice were subjected to chronic pressure overload through TAC and analyzed at four weeks after banding. After TAC, echocardiographic analysis revealed that cardiac function significantly changed in Tg mice compared to Wt littermates. In fact, we showed increased left ventricular end-diastolic internal dimensions (LVEDD) and decreased fractional shortening (FS) in Tg mice when compared to Wt littermates. In addition, stroke volume (ST) and cardiac output (CO) were significantly decreased in Tg compared to Wt mice (Table 2-1).
This data revealed that at four weeks after TAC, mice overexpressing miR-199a/a* show depressed systolic function and increased cardiac dilatation when compared to Wt animals. In agreement with the above described cardiac dysfunction, LV and atria weight were increased in Tg mice after banding, compared to Wt controls. Notably, gravitometric analysis revealed that also lung mass significantly increased in Tg TAC-mice.

	WT n=18	TG n=12	WT n=10	TG n=9
	BASAL		TAC 4wks	
BW, mg	26 ± 0.7	26 ± 1	27 ± 0.7	28 ± 0.7
LV/BW	3.8 ± 0.1	4.1 ± 0.2	6.0 ± 0.4$^{\zeta}$	5.6 ± 0.6$^{*,\zeta}$
RV/BW	0.9 ± 0.01	0.8 ± 0.01	1.0 ± 0.01$^{\zeta}$	0.9 ± 0.01
Atria/BW	0.039 ± 0.01	0.045 ± 0.01*	0.045 ± 0.01	0.073 ± 0.01$^{*,\zeta}$
Lung/BW	7.1 ± 0.01	7.1 ± 0.01	8.2 ± 0.03$^{\zeta}$	10.4 ± 0.06$^{*,\zeta}$
Liver/BW	4.9 ± 0.01	4.9 ± 0.02	3.9 ± 0.01	4.3 ± 0.01
Kidney/BW	7.3 ± 0.8	7.2 ± 0.9	6.8 ± 0.7	6.9 ± 0.9
Tibia, cm	1.9 ± 0.1	2.0 ± 0.1	2.0 ± 0.7	2.4 ± 0.9$^{*,\zeta}$
IVSd, mm	0.93 ± 0.01	0.93 ± 0.01	0.87 ± 0.01$^{\zeta}$	0.87 ± 0.02$^{\zeta}$
IVSs, mm	1.30 ± 0.01	1.32 ± 0.02	1.34 ± 0.01	1.30 ± 0.03
LVPWd, mm	0.95 ± 0.01	1.02 ± 0.06	0.88 ± 0.02$^{\zeta}$	0.70 ± 0.01$^{*,\zeta}$
LVPWs, mm	1.19 ± 0.01	1.21 ± 0.02	1.23 ± 0.01	1.24 ± 0.01
LVIDd, mm	2.83 ± 0.01	2.84 ± 0.01	3.86 ± 0.15$^{\zeta}$	4.46 ± 0.10$^{*,\zeta}$
LVIDs, mm	1.84 ± 0.01	1.82 ± 0.01	2.89 ± 0.15$^{\zeta}$	3.63 ± 0.05$^{*,\zeta}$
FS, %	34 ± 1	35 ± 1	25 ± 2$^{\zeta}$	18 ± 1$^{*,\zeta}$
ST, µl	9.91 ± 0.16	10.15 ± 0.15	8.29 ± 0.75$^{\zeta}$	6.45 ± 0.41$^{*,\zeta}$
CO, µl/min	4914 ± 89	5022 ± 66	4259 ± 364$^{\zeta}$	3316 ± 217$^{*,\zeta}$
+dP/dT, mmHg/min	6465 ± 417	6605 ± 639	6372 ± 544	10476 ± 839$^{*,\zeta}$
-dP/dT, mmHg/min	4771 ± 310	5105 ± 473	6165 ± 495$^{\zeta}$	9647 ± 807$^{*,\zeta}$
Max. Pres., mmHg	106 ± 4	99 ± 6	162 ± 8$^{\zeta}$	152 ± 3$^{*,\zeta}$
Contractility index	132 ± 6	136 ± 6	139 ± 16	242 ± 18$^{*,\zeta}$
HR, bpm	550 ± 10	540 ± 10	530 ± 25	550 ± 10

Table 2-1 *Echocardiographic and haemodynamic analysis of wild-type (Wt) and transgenic (Tg) basal (control) and TAC mice.*
Echocardiographic parameters of cardiac hypertrophy were evaluated: BW, body weight; (IVSd, diastolic interventricular septum thickness (mm); (IVSs, systolic interventricular septum thickness (mm);LVPWd, diastolic left ventricular posterior wall thickness (mm); LVPWs, systolic left ventricular posterior wall thickness (mm); LVIDd, diastolic left ventricular internal dimension; LVIDs, systolic left ventricular internal dimension; FS, fractional shortening (%); ST, stroke volume (µl); CO, cardiac output (µl/min).
Haemodynamic parameters of cardiac hypertrophy were evaluated: +dP/dT, maximal rate of pressure (mmHg/min); +dP/dT, minimal rate of pressure (mmHg/min); Max,Pres, maximal pressure (mmHg); HR, heart rate (bpm).
** P<0.05 wild-type (Wt) vs transgenic (Tg) mice within the study group.*
ζ P<0.05 basal vs transverse aortic constricted (TAC) mice with the same genotype.

Invasive hemodynamic measurements revealed significant differences in maximal pressure of Tg and Wt mice after TAC (Table 2-1). As reported in Table 2-1,

the maximal and minimal pressure over time (dP/dtMax and dP/dtMIN) were significantly increased in Tg mice compared to the Wt after TAC, whereas no differences were observed at basal condition.
To study the underlying mechanisms of the cardiac dysfunction, morphological features of CMCs, interstitial matrix and vessels, were examined under basal conditions and after pressure overload.
In agreement with the functional analysis, CMC cross-sectional area was significantly increased in miR-199a/a* overexpressing hearts compared with Wt littermates after TAC (Table 2-3), whereas no difference in CMC size were observed at basal condition.
To assess whether miR-199a/a* overexpression affects physiological CMC function, we additionally performed contractility studies on cells isolated from Tg and Wt mice in normal unstressed condition. Data (Table 2-2) showed that the overexpressed miR-199a/a* has no effect on CMC contractility/relaxation at different pacings.

	WT			TG		
HZ	**MEAN**	**SD**	**N**	**MEAN**	**SD**	**N**
0.5	3.601	0.987	21	4.611	1.908	16
1	3.431	1.061	18	4.201	1.746	16
2	4.188	1.253	16	4.484	1.840	14
3	4.808	1.711	17	6.232	1.889	15
4	5.078	1.956	18	5.971	1.986	15

Table 2-2 *Cardiomyocyte (CMC) contractility analysis.*
Contractility of CMC of wild type (Wt) and transgenic (Tg) mice was measured at different pacings: 0.5,1, 2, 3 and 4 hertz (HZ).

Histopathological analysis revealed that mice overexpressing miR-199a/a* showed increased cardiac fibrosis after TAC, whereas no differences could be detected under basal conditions, as indicated both by the percentage of collagen deposition (Table 2-3) and perivascular fibrosis (results not shown). Consistent with this data, we found that Tg mice exhibited increased immunopositivity for CD45 compared to Wt animals after TAC (Table 2-3), indicating more leukocyte infiltration. On the contrary, no differences were distinguished in cardiac inflammation and vascular density between miR-199a/a* overexpressed- and Wt hearts in basal condition (Table 2-3).

	WT n=18	TG n=12	WT n=10	TG n=9
	BASAL		TAC 4wks	
Collagen deposition, %	1.9 ± 0.9	2.1 ± 1.0	4.0 ± 1.0	$20 \pm 2.0^{*,\zeta}$
Cardiomyocyte area, μm^2	221 ± 21	219 ± 35	$257 \pm 28^{\zeta}$	$320 \pm 14^{*,\zeta}$
Coronary density, $\#/mm^2$	42 ± 3	43 ± 4	40 ± 3	42± 3
Capillary density, $\#/mm^2$	89 ± 10	95 ± 12	92 ± 17	110 ± 11
CD45, $\#/mm^2$ (%)	220 ± 20	308 ± 46	$778 \pm 42^{\zeta}$	$1049 \pm 59^{*,\zeta}$
Myeloperoxidase, $\#/mm^2$	1.6 ± 0.26	1.6 ± 0.31	1.8 ± 0.2	1.7 ±0.25

Table 2-3 *Hystopathological analysis of wild-type (Wt) and transgenic (Tg) basal (control) and TAC mice.*
** P<0.05 wild-type (Wt) vs transgenic (Tg) mice within the study group.*
ζ P<0.05 basal vs transverse aortic constricted (TAC) mice with the same genotype.

Altogether these results indicate that miR-199a/a* overexpression *in vivo* has no effect on heart physiology under normal unstressed condition, but it contributes to the worsening of cardiac function during pathological CH.
Finally, we investigated the cardiac response to stress, by subjecting Wt and Tg mice to a dobutamine test. Dobutamine is a sympathomimetic drug used in the treatment of HF and cardiogenic shock [111]. Dobutamine primary activity results from stimulation of the β_1-adrenoceptors of the heart, increasing heart contractility, heart rate and blood pressure [112].
Dobutamine is predominantly a β_1-adrenergic agonist, with weak β_2 activity, and α_1 selective activity, although it is used clinically in cases of cardiogenic shock for its β_1 inotropic effect in increasing heart contractility and CO. Because of these effects, *in vivo* administration of increasing dose of dobutamine, allows to simulate an exercise-induced stress in the heart muscle of animals tested. Maximal pressure seemed to be lower in Tg compared to Wt mice, but no significant differences were observed in the maximal and minimal pressure over time (results not shown).
Contrary to previous results, these data demonstrated the miR-199a/a* overexpression *in vivo* does not affect cardiac response to dobutamine-induced stress, thus suggesting that miR-199a/a* does not interfere with β1-adrenergic-dependent inotropic effect on heart.

2.2.1.3. In vivo overexpression of microRNA-199a/a inhibits the contractile response to neurohormonal stimulation*

In order to investigate the role of miR-199a/a* in cardiovascular remodeling *in vivo*, we additionally studied the effects of miR-199a/a* overexpression on vascular functionality.
Noradrenalin (NA), also known as norepirephrine (NE), is a neurotransmitter released from sympathetic neurons and affecting the heart. As ET-1, NA is able to increases BP by stimulating the vascular tone through α-adrenergic receptor activation. In this *in vitro* study we examined the effects of miR-199a/a* overexpression on NA and ET-1-induced contraction. Aorta from Wt and Tg mice were isolated and vessel rings stimulated by increasing doses of both NA and ET-1. The concentration-response curves for each of these hormones were first generated in the Wt aorta, before proceeding with the analysis of Tg ones.

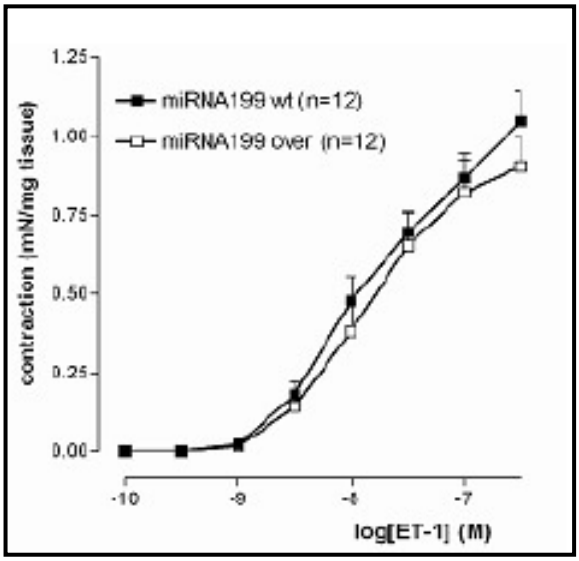


Figure 2.17 *In vitro aortic endothelin-1 (ET-1)-induced contraction analysis. Aorta from wild-type (Wt) and transgenic (over) mice were injected with increasing doses of ET-1 and aortic contraction was measured (mN/mg).*

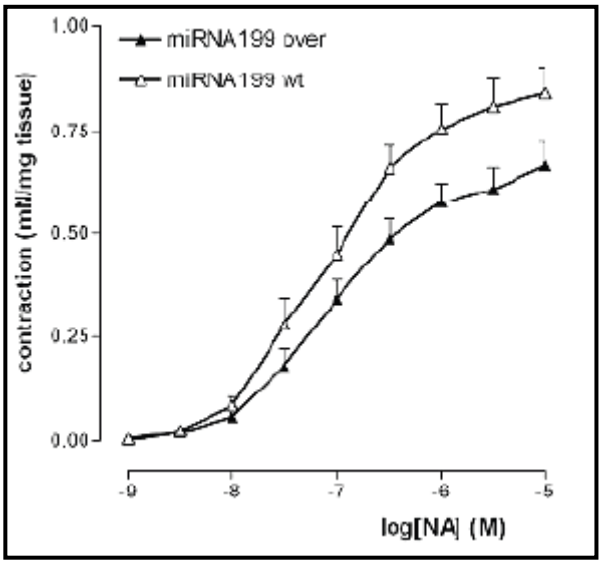


Figure 2.18 *In vitro aortic noradrenalin (NA)-induced contraction analysis. Aorta from wild-type (Wt) and transgenic (over) mice were injected with increasing doses of NA and aortic contraction was measured (mN/mg).*

Similarly, we examined the response of aorta rings to acetylcholine (ACH)-induced vasodilation. As shown in Figure 2.17, ET-1-induced vasoconstriction resulted less pronounced in Tg mice compared to Wt animals. Notably, the same difference between Wt and Tg mice vasocontraction was even more pronounced in response to NA stimulation (Figure 2.18). On the contrary, no differences were observed in vasodilation in Tg and Wt mice (Figure 2.19).
These data indicate that miR-199a/a* *in vivo* overexpression attenuates NA and ET-1 induced vasoconstriction in a dose-dependent manner.

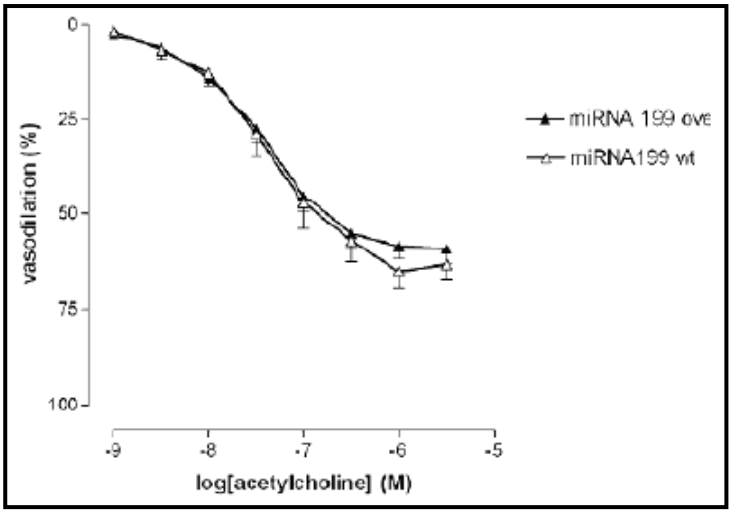


Figure 2.19 *In vitro aortic acetylcholine (ACH)-induced contraction analysis. Aorta from wild-type (Wt) and transgenic (over) mice were injected with increasing doses of ACH and aortic contraction was measured (mN/mg).*

2.2.1.4. In vivo overexpression of miRNA-199a/a decreases serum ANG II levels*

We previously showed that miR-199a/a* is able to down-regulate some of the main components of the RAS. To confirm the hypothesis that miR-199a/a* could exerts its action on this neurohormonal system, we determined whether miR-199a/a* overexpression *in vivo* affects the circulating levels of the main effector of RAS. We detected circulating levels of ANG II by performing a peptide-specific ELISA on serum extracted from Wt and Tg mice. Figure 2.20 shows that ANG II levels under normal unstressed condition were significantly decreased in mice overexpressing miR-199a/a* compared to the Wt littermates.

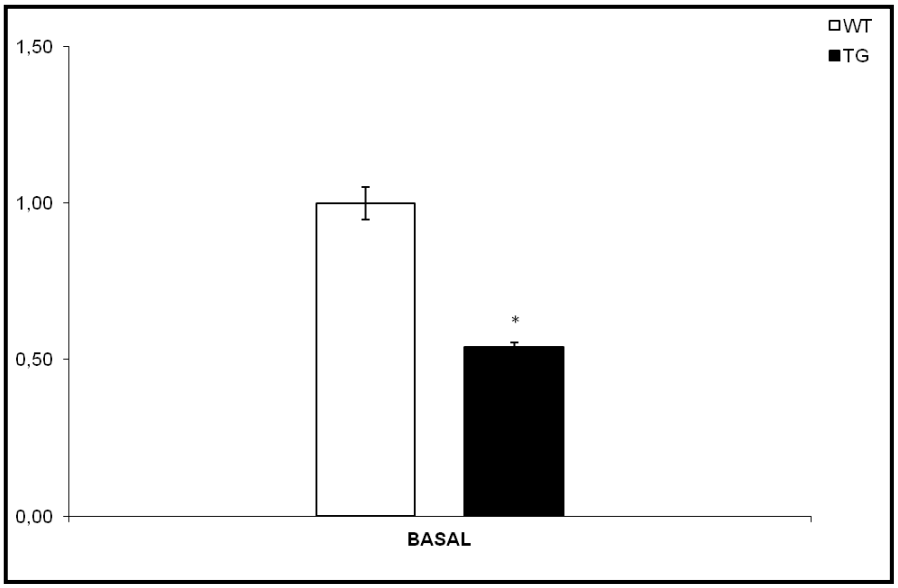


Figure 2.20 *ELISA on angiotensin II (ANG II) circulating level. Serum from wild-type (Wt) and transgenic (Tg) mice were extracted and ANG II levels were measured. Wt mice were used as control (=1). * P < 0.05 vs control.*

Consistent with the observed miR-199a/a* effect on the modulation of vascular tone, this data demonstrates that miR-199a/a* expression is inversely correlated to the levels of one of the main vasoconstrictor peptides known, ANG II.

2.2.2. Micro-RNA-199a/a* silencing

2.2.2.1. Antagomir-199a/a-treated mice have no gross phenotype*

To improve our understanding of miR-199a/a* molecular function in cardiovascular pathophysiology, *in vivo* loss-of-function studies were required. Accordingly, we investigated the effects of miR-199a/a* silencing *in vivo* by analyzing Wt mice treated with chemically engineered antisense RNA oligonucleotides, termed 'antagomir' [113]. Antagomir can efficiently and stably knockdown endogenous miRNAs in living cells [114]. The silencing of endogenous miRNAs by this novel method is specific, efficient, and long-lasting. In order to determine whether the selective silencing of miR-199-a and miR-199a* has some isoform-dependent effect on heart physiology, mice were selectively treated with isoform-specific antagomiR-199a/a*. Antagomirs were delivered by tail vein injection on three consecutive days in C57B6 male mice, at the age of eight weeks. 30 days after the last injection, we analyzed the gross morphology of the hearts and found that mice treated with antagomiR-199a/a* had near normalized heart size compared with vehicle-treated littermates. Cardiac tissues from antagomiR-199a/a*a-treated mice resulted in an almost complete inhibition of miR-199a/a* levels, indicating the

effectiveness of antagomiR-199a/a*. However, the treatment did not appear to be isoform-specific. Indeed, as shown in Figure 2.21 and Figure 2.22, both miR-199a and miR-199a* levels were markedly decreased in LV and atria of both antagomir-199a/a* mice groups, in an aspecific manner. On the contrary, a more isoform-specific effect was observed in the other tissues analyzed, as shown for kidney (Figure 2.23) and lung (Figure 2.24).

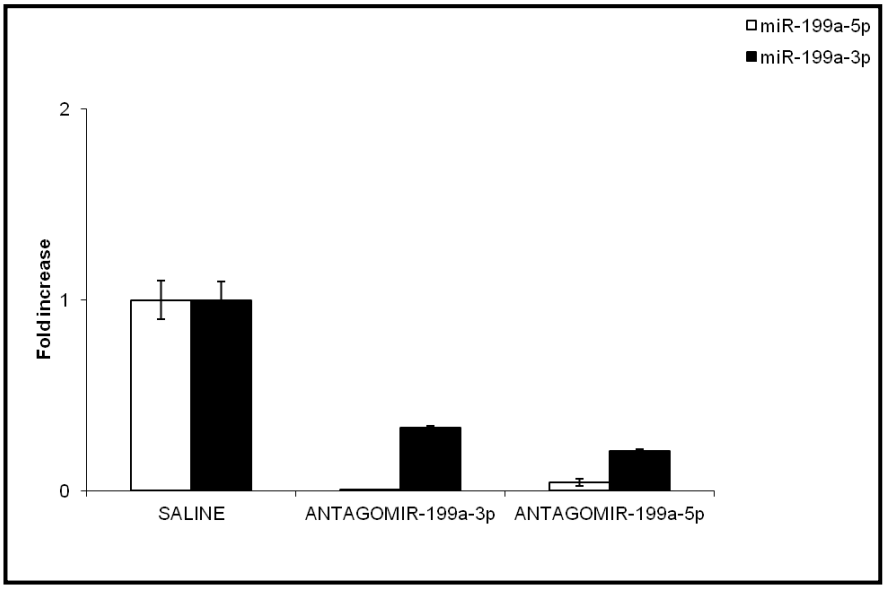


Figure 2.21 *In vivo miR-199 silencing: antagomir-199a/a* injection. Real time PCR analysis of miR-199a/a* expression in left ventricles of antagomir-199a/a*-injected mice. Mice injected with saline solution were used as control (=1). P < 0.01 vs control.*

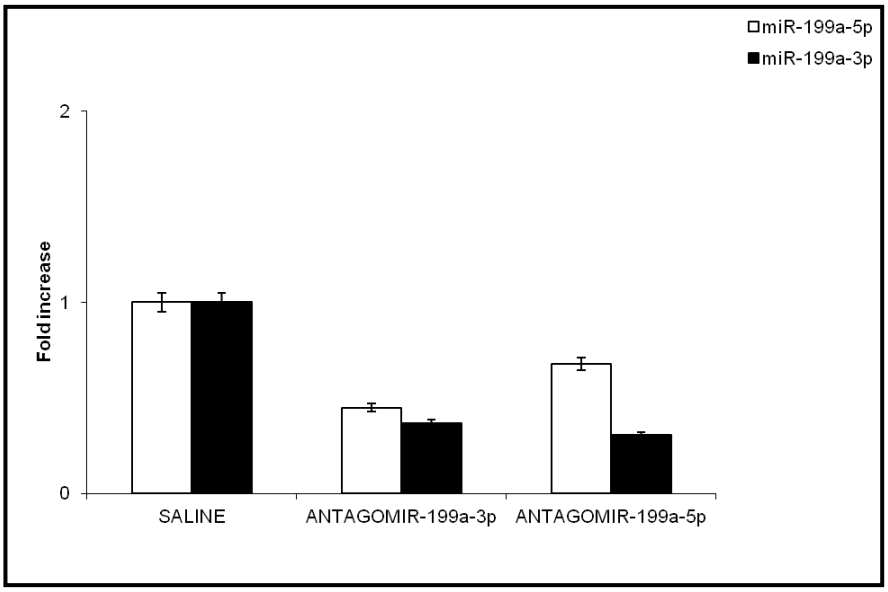


Figure 2.22 *In vivo miR-199 silencing: antagomir-199a/a* injection. Real time PCR analysis of miR-199a/a* expression in atria of antagomir-199a/a*-injected mice. Mice injected with saline solution were used as control (=1). P < 0.01 vs control.*

Antagomir-treated mice tolerated antagomiR-199a/a* well, without signs of illness or discomfort. Furthermore, they did not show any altered phenotype at basal condition, as demonstrated by the gravitometric analysis reported in Table 2-4. In contrast, changes were found when ecocardiographic and haemodynamic analyses were performed. In fact, results showed that antagomir-199a/a* treatment can affect cardiac function at basal condition.

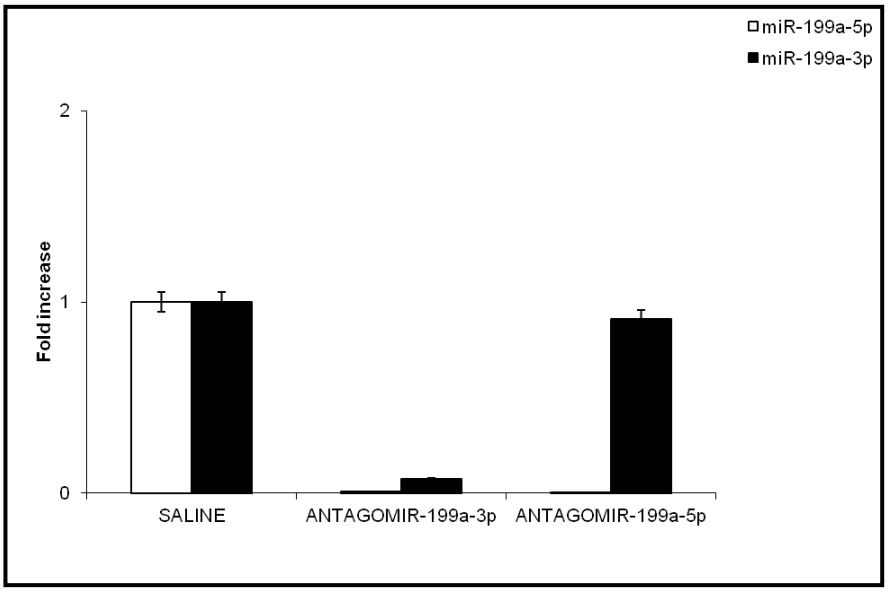


Figure 2.23 *In vivo miR-199 silencing: antagomir-199a/a* injection. Real time PCR analysis of miR-199a/a* expression in kidney of antagomir-199a/a*-injected mice. Mice injected with saline solution were used as control (=1). P < 0.01 vs control.*

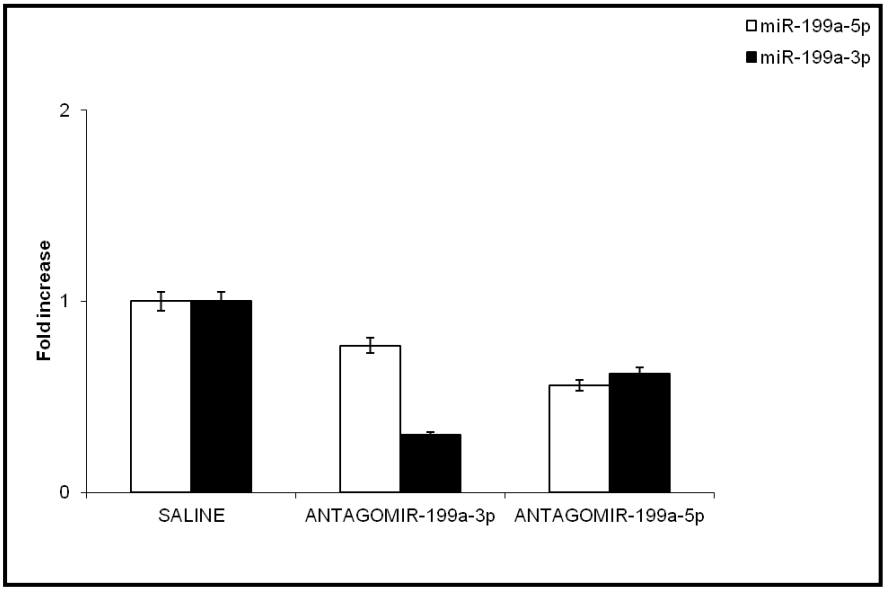


Figure 2.24 *In vivo miR-199 silencing: antagomir-199a/a* injection. Real time PCR analysis of miR-199a/a* expression in lung of antagomir-199a/a*-injected mice. Mice injected with saline solution were used as control (=1). P < 0.01 vs control.*

2.2.2.2. In vivo silencing of miR-199a/a protects against cardiac dysfunction in pathological cardiac hypertrophy*

To investigate the effects of miR-199a/a* silencing *in vivo*, Wt mice were analyzed at four weeks after TAC. Left ventricular weight to body weight ratio was significantly increased in the vehicle-treated group compared with the antagomir-treated mice (Table 2-4). Echocardiographic analysis revealed significantly decreased septal wall thickness, increased cardiac dilation and depressed systolic function in the control group compared to the antagomir-treated mice. This analysis of cardiac function revealed that antagomiR-199a/a* treatment prevented LV dilatation and normalized fractional shortening (FS) and systolic and diastolic contractile defects. In line with this, invasive heamodynamic measurements revealed increased contractility and relaxation in the antagomir-treated mice compared with the vehicle-treated controls.
Histopathological analysis revealed that antagomiR-199a/a* treatment also reduced the increased collagene deposition normally occurring in veichle-mice after banding (Table 2-5). However, antagomir-199a/a* apparently did not prevent perivascular fibrosis in TAC-mice (results not shown).
Taken together, this data indicated that antagomiR-199a/a* prevent the development of CH and associated myocardial fibrosis in TAC-induced pressure overloaded hearts, thus suggesting that antagomiR-199a/a* treatment clearly protects against cardiac dysfunction.

	Vehicle n=10	3p AntagomiR n=10	5p AntagomiR n=10	Vehicle n=9	3p AntagomiR n=6	5p AntagomiR n=7
	BASAL			TAC 4wks		
BW, mg	27 ± 0.5	27 ± 01	26 ± 0.2	23 ± 0.1	23 ± 0.4	24 ± 0.2
LV/BW	4.4 ± 0.04	4.1 ± 0.01	3.9 ± 0.02	6.2 ± 0.05$^{\zeta}$	5.6 ± 0.02$^{\zeta}$	5.2 ± 0.04*,$^{\zeta}$
RV/BW	0.7 ± 0.01	0.6 ± 0.01	0.7 ± 0.01	0.8± 0.01	0.6 ± 0.01	0.9 ± 0.01
Atria/BW	0.031 ± 0.001	0.029 ± 0.001	0.030 ± 0.001	0.025 ± 0.010	0.023 ± 0.001	0.022 ± 0.001
Lung/BW	6.2 ± 0.01	6.4 ± 0.02	7.1 ± 0.01*	7.9 ± 0.04$^{\zeta}$	7.8 ± 0.04$^{\zeta}$	8.0 ± 0.04$^{\zeta}$
Lung Dry/Wet	0.61 ± 0.01	0.63 ± 0.01	0.69 ± 0.01*	0.99 ± 0.01	0.96 ± 0.01*	0.97 ± 0.01*
Liver/BW	4.0 ± 0.06	4.0 ± 0.02	4.0 ± 0.01	4.3 ± 0.01	4.4 ± 0.01	4.2 ± 0.02
Kidney/BW	6.4 ± 0.01	7.6 ± 0.01*	7.3 ± 0.01*	6.8 ± 0.01	8.0 ± 0.01*,$^{\zeta}$	7.0 ± 0.01f
Tibia, cm	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	2.4 ± 0.1$^{\zeta}$	2.4 ± 0.1$^{\zeta}$	2.3 ± 0.2$^{\zeta}$
IVSd, mm	0.99 ± 0.01	0.98 ± 0.02*	0.97 ± 0.02*	1.00 ± 0.01	0.99 ± 0.01$^{\zeta}$	1.01 ± 0.01$^{\zeta}$
IVSs, mm	1.24 ± 0.02	1.23 ± 0.02	1.24 ± 0.01	1.23 ± 0.02	1.24 ± 0.01	1.24 ± 0.01
LVPWd, mm	0.99 ± 0.01	0.98 ± 0.02	0.98 ± 0.02	0.91 ± 0.01$^{\zeta}$	0.98 ± 0.01*	0.98 ± 0.01*
LVPWs, mm	1.32 ± 0.02	1.32 ± 0.02	1.33 ± 0.01	1.32 ± 0.02	1.31 ± 0.03	1.32 ± 0.01
LVIDd, mm	2.93 ± 0.01	3.29 ± 0.04*	3.21 ± 0.05*	3.61 ± 0.05$^{\zeta}$	2.96 ± 0.02*	2.98 ± 0.01*
LVIDs, mm	1.93 ± 0.01	2.32 ± 0.05*	2.29 ± 0.6*	2.51 ± 0.02$^{\zeta}$	1.98 ± 0.01*,$^{\zeta}$	1.97 ± 0.01*,$^{\zeta}$
FS, %	34 ± 1	29 ± 1*	29 ± 1*	31 ± 2	33 ± 3	34 ± 1
ST, µl	9.47 ± 0.13	12.05 ± 0.41*	11.95 ± 0.73*	16.31 ± 0.12$^{\zeta}$	9.51 ± 0.22*	9.93 ± 0.10*
CO, µl/min	4849 ± 47	6319 ± 101*	6204 ± 223*	8326 ± 42$^{\zeta}$	4837 ± 152*,$^{\zeta}$	4902 ± 210*,$^{\zeta}$
+dP/dt, mmHg/min	5393 ± 435	4805 ± 355*	4842 ± 400*	3963 ± 237$^{\zeta}$	5141 ± 49*,$^{\zeta}$	4881 ± 264*,$^{\zeta}$
+dP/dt, mmHg/min	4552 ± 370	3836 ± 309*	4024 ± 364*	4220 ± 372	6061 ± 540*,$^{\zeta}$	5527 ± 531*,$^{\zeta}$
Max. Pres., mmHg	88 ± 6	90 ± 4*	86 ± 5*	124 ± 6$^{\zeta}$	163 ± 6*,$^{\zeta}$	144 ± 11*,$^{\zeta}$
HR, bpm	520 ± 7	525 ± 7	520 ± 7	520 ± 5	525 ± 10	520 ± 5

Table 2-4 *Echocardiographic and haemodynamic analysis of antagomir-199a/a* basal (control) and TAC mice. Mice injected with veichle-oligonucleotide were also included (control mice). Echocardiographic parameters of cardiac hypertrophy were evaluated: BW, body weight; (IVSd, diastolic interventricular septum thickness (mm); (IVSs, systolic interventricular septum thickness (mm);LVPWd, diastolic left ventricular posterior wall thickness (mm); LVPWs, systolic left ventricular posterior wall thickness (mm); LVIDd, diastolic left ventricular internal dimension; LVIDs, systolic left ventricular internal dimension; FS, fractional shortening (%); ST, stroke volume (μl); CO, cardiac output (μl/min). Haemodynamic parameters of cardiac hypertrophy were evaluated: +dP/dT, maximal rate of pressure (mmHg/min); +dP/dT, minimal rate of pressure (mmHg/min); Max,Pres, maximal pressure (mmHg); HR, heart rate (bpm). * P<0.05 antagomir199a/a*- vs veichle-treated mice within the study group. ζ P<0.05 basal vs transverse aortic constricted (TAC) mice within the same treatment group. ∫ P<0.05 antagomir-199a- vs antagomir-199a*-treated mice within the same study group.*

	Vehicle n=10	3p AntagomiR n=10	5p AntagomiR n=10	Vehicle n=9	3p AntagomiR n=6	5p AntagomiR n=7
	BASAL			TAC 4 wks		
Collagen deposition, %	2.0 ± 0.7	1.9 ± 0.8	1.9 ± 0.9	5.4 ± 0.9$^{\zeta}$	2.3 ± 0.7*	2.5 ± 0.8*
CD45 #/mm2 (%)	180 ± 32	174 ± 43	198 ± 53	540 ± 56$^{\zeta}$	326 ± 85$^{\zeta}$	390 ± 95*,$^{\zeta}$

Table 2-5 *Hystopathological analysis of antagomir-199a/a* basal and TAC mice. Mice injected with veichle-oligonucleotide were also included (control mice). * P<0.05 antagomir199a/a*- vs veichle-treated mice within the study group. ζ P<0.05 basal vs transverse aortic constricted (TAC) mice within the same treatment group.*

2.2.2.3. In vivo silencing of miRNA-199a/a increases the contractile response to neurohormonal stimulation*

In order to investigate the *in vivo* role of miR-199a/a* in the cardiovascular remodeling, we additionally studied the effects of miR-199a/a* silencing on vascular functionality. As previously described, we performed an *ex vivo* study aimed at evaluating the effects of miR-199a/a* silencing on NA and ET-1-induced contraction in mice aorta. The concentration-response curves for NA and ET-1 were generated first in the veichle-treated mice, before proceeding with the analysis of antagomiR-199a/a* injected mice. As shown in Figure 2.25, for the same ET-1 dose, vasoconstriction resulted more pronounced in antagomir-treated mice compared to control animals. In particular, miR-199a* antagomir-treated mice were more responsive to induced vasoconstriction compared to miR-199a antagomir-treated mice. Similar effects were also obtained after NA administration (Figure 2.26). This data indicates that miR-199a/a* *in vivo* silencing is responsible for an increased NA and ET-1 induced vasoconstriction in a dose-dependent manner, thus confirming the results obtained in miR-199a/a* overexpression vascular functionality studies. Furthermore, our analysis reveals that antagomiR-199a* has a stronger effect on vascular contractility compared to antagomiR-199a.

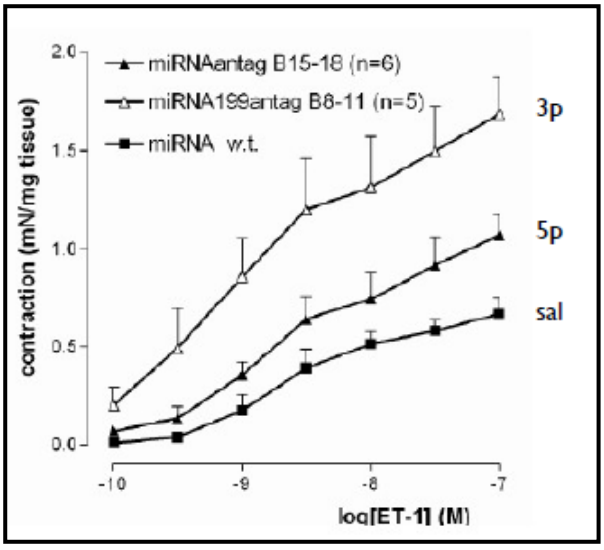


Figure 2.25 *In vitro aortic endothelin-1 (ET-1)-induced contraction analysis. Aorta from wild-type (Wt) antagomir-199a/a* mice were injected with increasing doses of ET-1 and aortic contraction was measured (mN/mg). Mice injected with veichle-oligonucleotide were also included (control mice).*

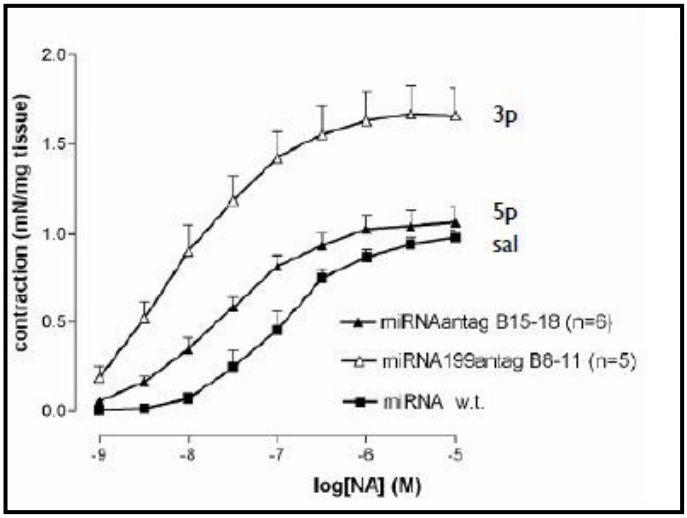


Figure 2.26 *In vitro aortic noradrenalin (NA)-induced contraction analysis. Aorta from antagomir-199a/a* mice were injected with increasing doses of NA and aortic contraction was measured (mN/mg). Mice injected with veichle-oligonucleotide were also included (control mice).*

2.2.2.4. In vivo microRNA-199a/a silencing increases serum ANG II levels*

According to our previous analysis on mice overexpressing miR-199a/a*, we detected the circulating levels of ANG II by performing a peptide-specific ELISA on serum extracted from antagomir-treated mice both under normal unstressed condition and after four weeks TAC. Consistent with our data, ANG II levels were significantly increased in antagomiR-199a/a* injected mice compared to their controls (Figure 2.27). Notably, such an increase was more pronounced in mice subjected to TAC-induced pressure overload compared to that observed in unstressed mice. In particular, the highest increase was detected in antagomiR-199a treated mice after banding.
These results indicate that miR-199a/a* silencing *in vivo* leads to increased ANG II levels in serum, thus supporting the hypothesis that miR-199a/a* affects ANG II circulating levels *in vivo*.

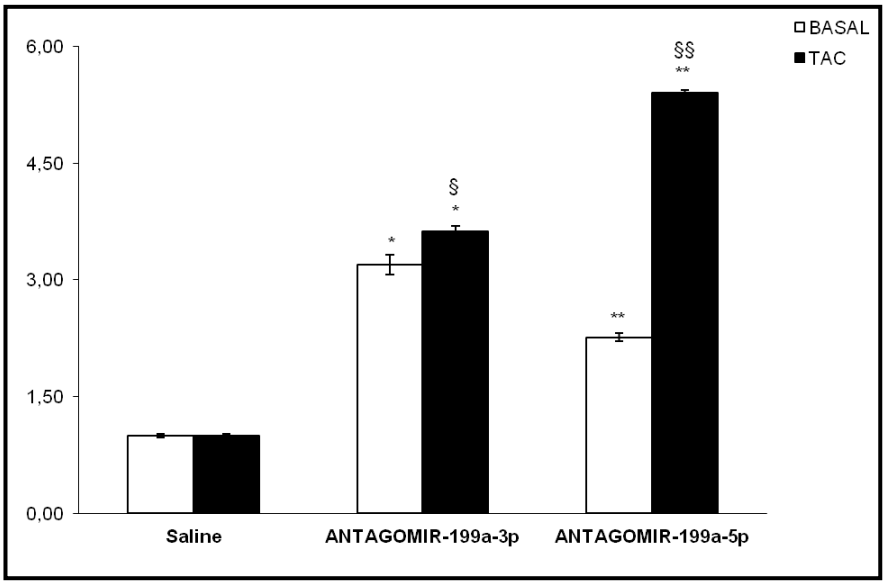


Figure 2.27 *ELISA on angiotensin II (ANG II) circulating level. Serum from antagomir-199a/a* mice were extracted and ANG II levels were measured. Mice injected with saline solution were used as control (=1). * P < 0.05 and ** P < 0.01 vs control mice in basal condition. §P < 0.05 and §§P < 0.01 vs control mice after TAC.*

2.3. MicroRNA-199a/a effects on cardiac and vascular cell signalling*

The third part of this research was aimed to fully describe the molecular mechanisms that correlate miR-199a/a* and the identified targets with the above described effects on cardiac and vascular function.
To this attempt, we modulated miR-199a/a* expression *in vitro* by both overexpression and silencing methods. We then performed a series of *in vitro* specific assays to study the miR-199a/a* effects on different cell types and to analyze the signalling pathways responsible for mediating some of the main cellular processes occurring during cardiovascular remodelling.

2.3.1. MicroRNA-199a/a* affect ANG-II and ET-1-induced calcium release in cardiac fibroblasts and aortic smooth muscle cells

To investigate whether miR-199a/a* acts as a modulator of ANG II and ET-1-induced G-proteins signalling pathways, we studied how it affects the agonist-induced Ca^{2+} release in cFIBROs.

In order to modulate miR-199a/a* expression we first transfected cells with both specific miR-199a/a* isoform mimics and inhibitors for 48 h. In this way, we obtained a full overexpression and silencing of miR-199a/a*, respectively.
We used a fluorescent-based assay for detecting changes in intracellular Ca^{2+} either before or after cell treatment with the ANG II and ET-1 concentrations.
As shown in Figure 2.28, Ca^{2+} release from cFIBROs did not undergo any significant change in not-treated cells, either after miR-199a/a* overexpression or silencing. As expected, ANG II-stimulated Ca^{2+} release from cells.
By monitoring the kinetic over 5 minute, we observed that such an increase kept high up to 2' and 30'' and it slightly decreased starting from 3 minutes after treatment. Notably, miR-199a/a* attenuated ANG-II induced Ca2+ release when overexpressed, whereas no effect was observed in cells not expressing the miRNA.
As shown in Figure 2.29, the same effect on Ca^{2+} release described for ANG II was also observed in cFIBROs overexpressing miR-199a/a* and exposed to ET-1.
These results demonstrate that *in vitro* miR-199a/a* overexpression partially inhibits ANG II and ET-1-induced Ca^{2+} release from cFIBROs.

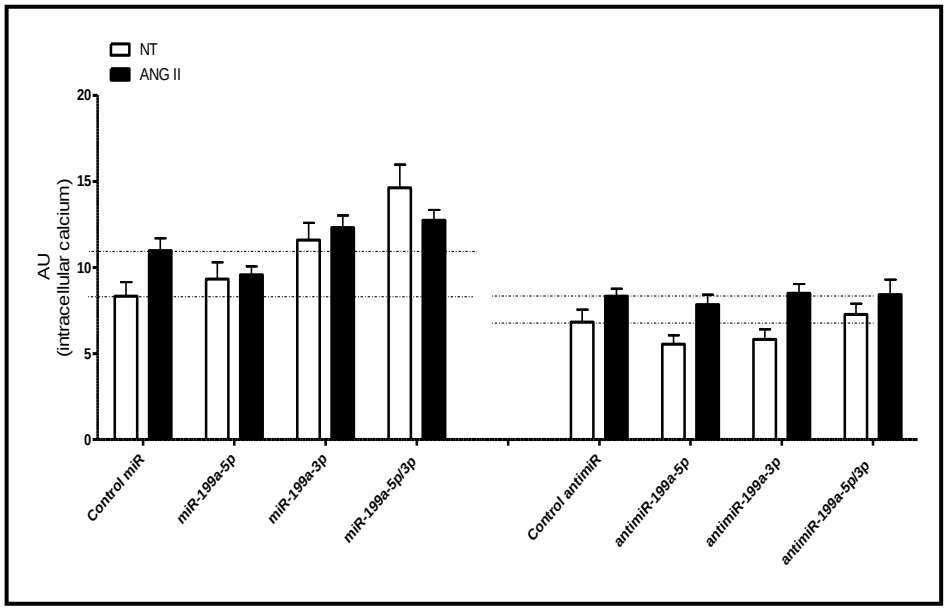


Figure 2.28 *In vitro angiotensin II (ANG II)-induced calcium (Ca^{2+})release from cardiac fibroblasts (cFIBRO). MiR199a/a* was overexpressed (left panel) and silenced (right panel) in cFIBRO, before stimulation with 10^{-6} M of ANG II. Ca^{2+} release was measured for 5' and data were collected at different kinetics (k1,k2,k3 etc..). Data from k3 (1.30') were represented. Not treated cells were used as control.*

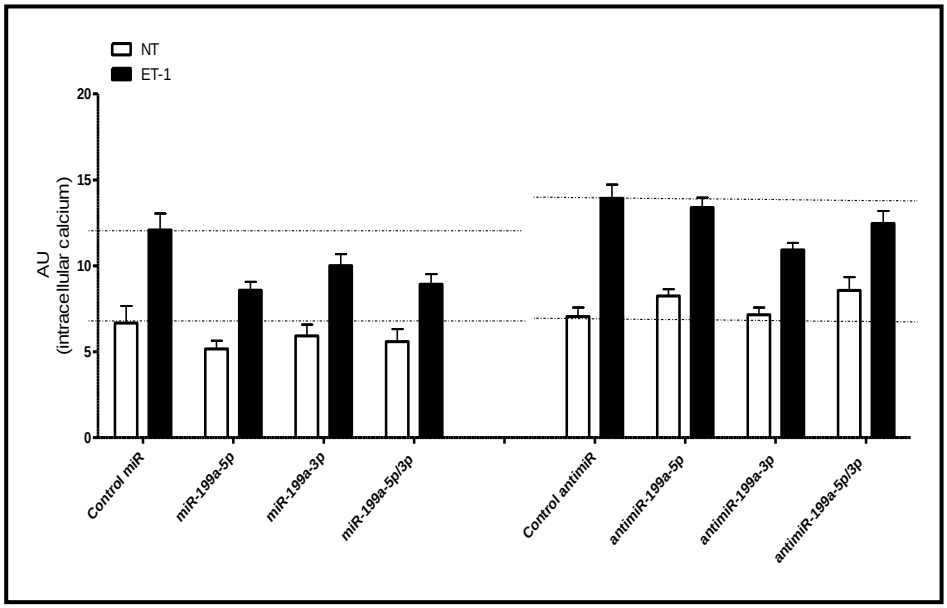


Figure 2.29 *In vitro endothelin-1(ET-1)-induced calcium (Ca^{2+})release from cardiac fibroblasts (cFIBRO). MiR199a/a* was overexpressed (left panel) and silenced (right panel) in cFIBRO, before stimulation with 100 nM of ET-1. Ca^{2+} release was measured for 5' and data were collected at different kinetics (k1,k2,k3 etc..). Data from k3 (1.30') were represented. Not treated cells were used as control.*

We then performed the same analysis in AoSMCs. Figure 2.30 shows that both ANG II and ET-1 led to an increased Ca^{2+} levels, that stayed high up to 2' and 30'', as shown in cFIBROs. As opposed to what we observed in cFIBROs, *in vitro* miR-199a/a* overexpression had no detectable effects on ANG II and ET-1 Ca^{2+} release in AoSMCs. However, we measured a significant decrease in ANG-II-induced Ca^{2+} release from cells not expressing the miRNA. As shown in Figure 2.30, no changes in ET-1-induced Ca^{2+} release occurred among differentially transfected AoSMCs,

These results demonstrate that miR-199a/a* *in vitro* silencing partially inhibits ANG-II-induced Ca^{2+} release from AoSMCs.

All together, this data indicates that miR-199a/a* affects ANG-II and ET-1-induced Ca^{2+} release in a different way, depending both on neurohormonal stimulation and cell type.

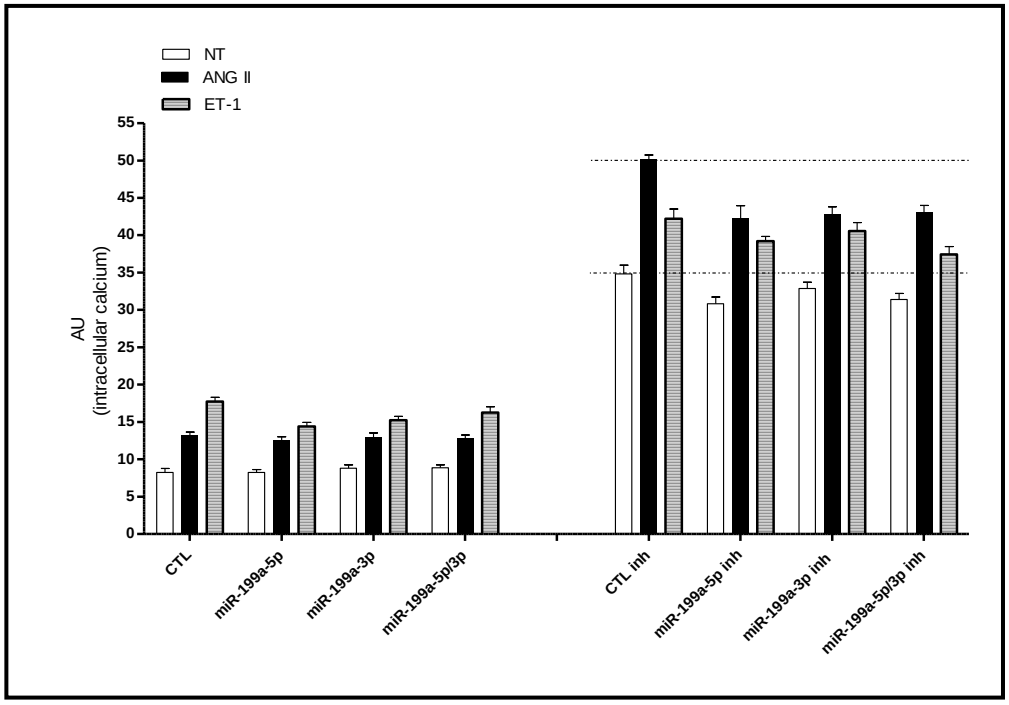


Figure 2.30 *In vitro angiotensin II (ANG II)- and endothelin-1(ET-1)-induced calcium (Ca^{2+}) release from aortic smooth muscle cells (AoSMC). MiR199a/a* was overexpressed (left panel) and silenced (right panel) in AoSMC, before stimulation with 10^{-6} M of ANG II and 100 nM of ET-1. Ca^{2+} release was measured for 5' and data were collected at different kinetics (k1,k2,k3 etc..). Data from k3 (1.30') were represented. Not treated cells were used as control.*

2.3.2. MicroRNA-199a/a* affect ET-1 but not ANG II-induced PLC activation in cardiac fibroblasts and aortic smooth muscle cells

To gain further insight into miR-199a/a* ability to modulate the ANG-II and ET-1 induced G-protein signal transduction, we additionally studied the activity of another key molecule normally downstream activated after neurohormonal stimulation.

As described for the Ca^{2+} assay, we first transfected cells, before proceeding to agonists stimulation and PLC activity detection. Then , we used an ELISA specific assay for measuring PLC activity both in cFIBROs and AoSMCs, either before and after cell treatment with ANG II and ET-1 concentrations.

We assessed that PLC significantly increased its activity after treatment and it lingered on in this state for up to 5 minutes.

As represented in Figure 2.31, no significant changes occurred among cells differently expressing miR-199a/a*, both after ANG-II and ET-1 treatment, a partial attenuation in PLC activity was measured in cells overexpressing miR-199a/a* after ET-1 stimulation.

This data indicates that miR-199a/a* *in vitro* overexpression inhibits ET-1-induced PLC activation in cFIBROs, whereas it does not affect PLC activity neither in basal condition nor after ANG-II stimulation.

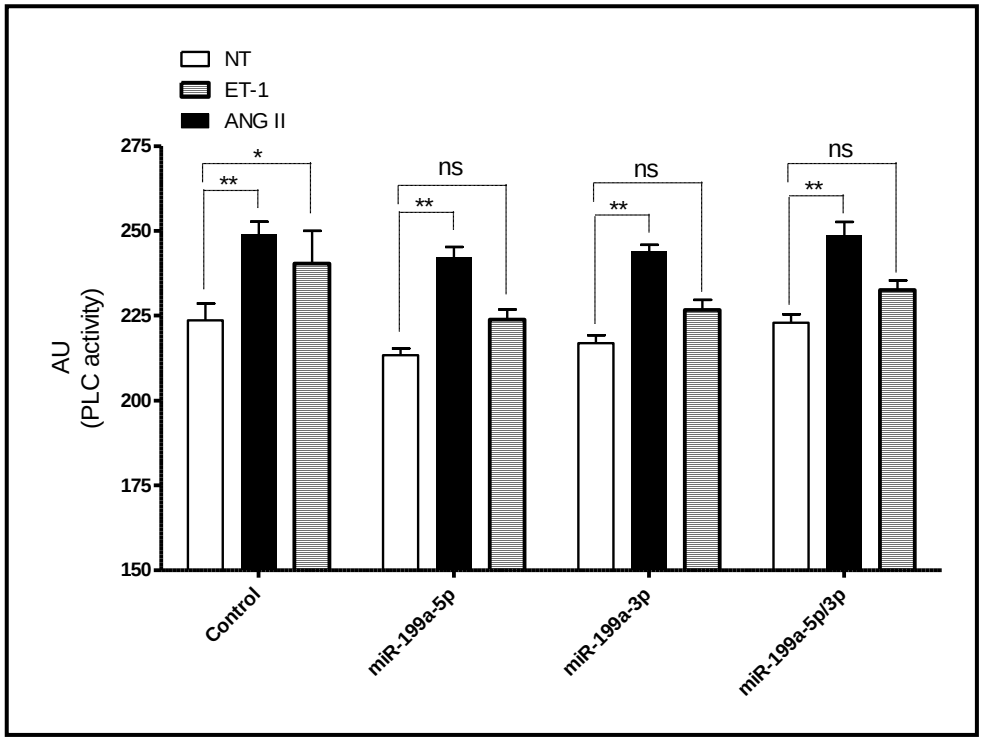


Figure 2.31 *In vitro angiotensin II (ANG II)- and endothelin-1(ET-1)-induced phospholipase C (PLC) activity in cardiac fibroblasts (cFIBRO). MiR199a/a* was overexpressed in cFIBRO, before stimulation with 10^{-6} M of ANG II and 100 nM ET-1. PLC activity was measured for 5' and data were collected at different kinetics (k1,k2,k3 etc..). Data from k3 (1.30') were represented. Not treated cells were used as control. * P < 0.05, ** P < 0.01 vs control.*

As shown in Figure 2.32, agonist stimulation of AoSMCs barely increased PLC activity. Such an increase did not vary among cells overexpressing miR-199a/a*, thus suggesting that it doesn't modulate the pathways at this level.

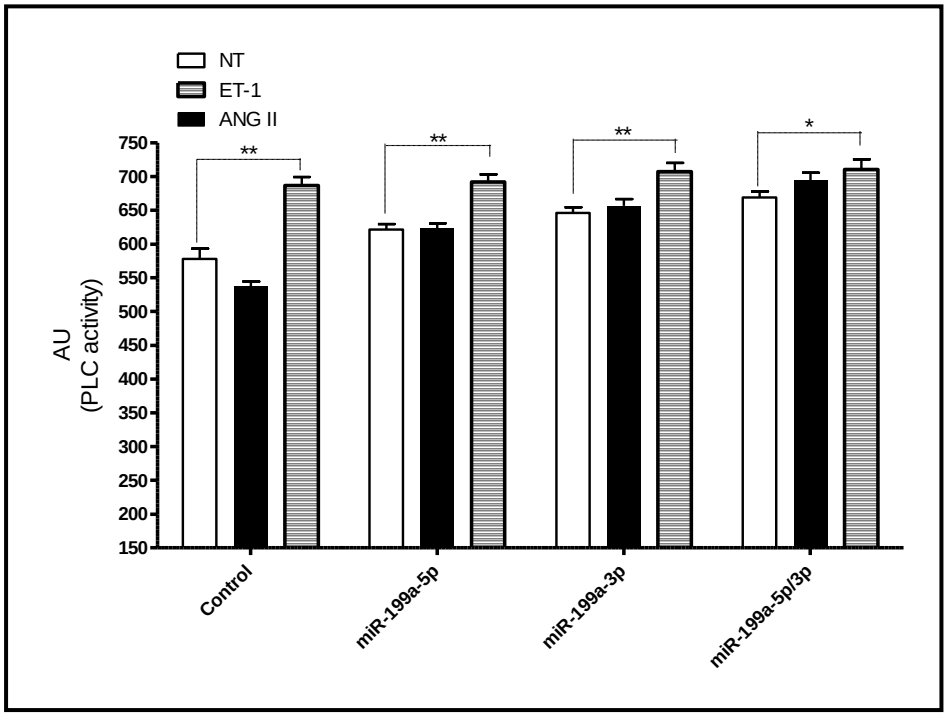


Figure 2.32 *In vitro angiotensin II (ANG II)- and endothelin-1(ET-1)-induced phospholipase C (PLC) activity in aortic smooth muscle cells (AoSMC). MiR199a/a* was overexpressed in AoSMC, before stimulation with 10^{-6} M of ANG II and 100 nM ET-1. PLC activity was measured for 5' and data were collected at different kinetics (k1,k2,k3 etc..). Data from k3 (1.30') were represented. Not treated cells were used as control. ** P < 0.01 vs control.*

3. DISCUSSION

3.1. MiRNA-199a/a expression and molecular targets in the cardiovascular system*

MiRNAs can have a unique-tissue and stage-developmental specific expression pattern, that represents the starting point to discover their biological functions. According to the main aim of this research, the miR-199a/a* distribution in the cardiovascular system has been analyzed. The two miR-199 mature isoforms resulted to be differentially expressed among cardiac cell types suggesting that they can be independently modulated either at basal or pathological conditions.
The investigation on miR-199a/a* expression in a pathological model of CH (TAC) established that both miR-199 isoforms are upregulated during CH. This data confirms the miR-199a up-regulation already reported by others [104] and provides a new evidence about miR-199a* dysregulation in CH.
In addition, we found an additional cells-specific expression of the miRNAs. In fact, we found that cFIBROs and AoSMCs are the cell types with higher endogenous miR-199a/a* levels in the cardiovascular system. Furthermore, we showed that such an expression markedly increases when cells are exposed to pathological ANG II and ET-1 concentrations. These short peptides are known to be the main effectors of two of the most important neurohormonal systems, the RAS and the ET system respectively, and to increase in response to an initial stimulus responsible for impairing cardiovascular function. Based on this evidence, it is likely to speculate that the miR-199a/a* up-regulation occurring during CH is not strictly confined to myocyte cells, but it is also related to an ANG II/ET-1-dependent mechanism on cFIBROs. Furthermore, we determined that such a mechanism is mediated by ATR1 and both EDNRA and EDNRB receptors in cFIBROs. This data represents the starting point for a detailed characterization of the cellular pathway leading to the described miR-199a/a* up-regulation.
Several studies have described the multiple effects of ANG II and ET-1 on cardiac cells. In particular, high levels of these peptides promote cFIBRO survival and proliferation [20, 115]. The result of fibroblast growth is myocardial fibrosis, characterized by an abnormal accumulation of fibrillar collagen within the interstitial space, that affects ventricular function and can ultimately lead to HF. Accordingly, we infer that miR-199a/a* up-regulation occurring in hypertrophic hearts is determined by both the increased miR-199a/a* expression occurring at cellular level and the growth of the cell population responsible for its production.
Recent findings have shown the importance of miR-199 dysregulation in different models of cardiac disease [104, 108, 116, 117]. All these studies were focused on miR-199a/b-5p role in CMC population of failing hearts, by showing its ability to affects CMC signaling and gene expression. However, the expression and functional role of both miR-199 isoforms in other cardiac cell populations and their possible involvement in CVDs, have not been investigated.
Compared to previous studies, the results obtained in the first part of the present research provide a new insight into the molecular role of miR-199a/a* in

pathological CH, by showing that their up-regulation could be related to ANG II/ET-1-induced myocardial fibrosis, normally associated to LV dysfunction.
A similar cellular modulation of miR-199a expression was also observed in AoSMCs, in response to an *in vitro* acute stimulation by both ANG II and ET-1, However, the receptor molecules mediating this effect have not been determined. Increased VSMC hypertrophy, migration, and proliferation are among the key events that contribute to vasculature remodeling associated with CVDs. ANG II and ET-1 have been shown to induce some key signal transduction pathways [29] modulating these cellular processes in AoSMCs [118]. Therefore, miR-199a/a* role in AoSMCs also still needs to be investigated, in order to understand whether the observed increase could play a role in CH or other CVDs and to eventually characterize the ANGII/ET-1 related molecular mechanism responsible for miR-199a up-regulation.
These findings corroborate the idea that miR-199a/a* role in cardiovascular system is determined by different cellular mechanisms in which it is involved, occurring not only in CMCs, but also in cFIBROs and AoSMCs.
The importance of unravelling miR-199a/a* function in the cardiovascular system is highlighted by the identification of the gene network targeted by these miRNAs. Indeed, our studies validated the components of the RAS and the ET systems as miR-199a/a* targets, suggesting us to investigate the effects of miR-199a/a* modulation in the neurohormonal response.

3.2. MicroRNA-199a/a effects on cardiovascular functions*

It has been recently reported, that *in vivo* miR-199 modulation can promote or reverse pathological myocardial hypertrophy and ischemia [116, 119]. In line with this, our *in vivo* analysis on the functional role of miR-199a/a* in heart physiology, supports the evidence that miR-199a/a* overexpression in pathological CH contributes to a more sustained cardiac remodeling that do not occurs when miR-199a/a* is down-regulated. This study consolidates previous discoveries and implicates both miR-199 isoforms as potential therapeutic targets in HF. Interestingly, we showed that *in vivo* miR-199a/a* overexpression does not affect neither cardiac function nor CMC morphological and functional features, in absence of any pathological stimulus. These results disagree with the published *in vitro* data, showing that miR-199a *in vitro* overexpression was sufficient to induce CMC hypertrophy.
As the *in vitro* data demonstrated that miR-199a/a* is implicated with two of the most potent vasoconstrictor peptides known (ANG II and ET-1), we investigated its role in the modulation of vascular tone. Predictably, miR-199a/a* *in vivo* affects the vascular function by either attenuating the contractile response to neurohormonal stimulation when it is overexpressed or enhancing it when it is silenced. In line with this, we showed that miR-199a/a* overexpression markedly decreases ANG II circulating levels, whereas they increase when the miRNAs are silenced.
Based on this evidence, it is tempting to speculate that miR-199a/a* may function as novel negative regulators to control ANG II circulating levels and, thus, modulate the contractile response to neurohormonal stimulation.

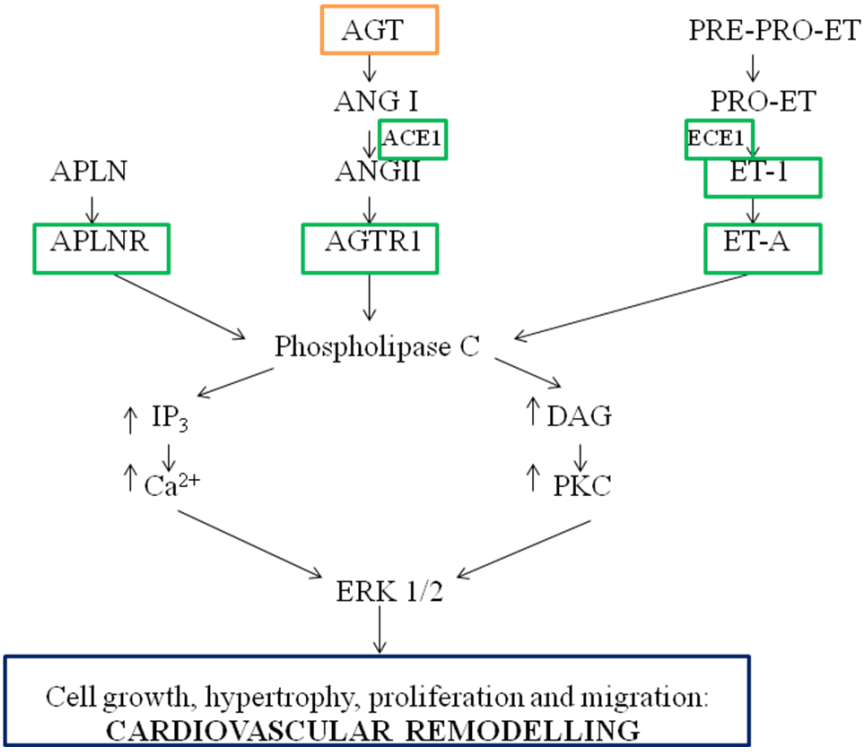
In support of this hypothesis, the identified targets of miR-199a/a* relevant to neurohormonal response: AGTR1B and ACE1 are associated with the RAS, whereas ET-1, EDNRA and ECE1 are associated with the ET system. APLNR, as well targeted by miR-199a/a*, is also playing a role in neuroendocrine activation state. Alltogether, their biological functions are essential for the modulation of the vascular tone. In particular, increased levels of these molecules promote several cellular processes ultimately leading to vasoconstriction, cardiac hypertrophy and remodelling.

3.3. MicroRNA-199a/a effects on cardiac and vascular cell signalling*

ANG II, AP and ET-1 participate to the pathogenesis of CVDs by activating signalling pathways that are capable of modifying cellular hypertrophy, growth, migration and proliferation in several cell types [51, 52, 120]. The molecular mechanisms responsible for mediating these cellular processes work via the induction of immediate early genes through a MAPK-dependent cascade activated by G-proteins signalling transduction, thus suggesting that miR-199a/a* could act as a modulator of these pathways. In Scheme 3.1 an important pathway common to the three peptides is shown.
Our investigation on microRNA-199a/a* effects on cardiac and vascular cellular transduction, revealed that miR-199a/a* are able to differentially affect agonist-induced Ca^{2+} release in cFIBROs and AoSMCs. However, further studies are needed in order to fully understand the molecular mechanism that correlates miR-199a/a* and the identified targets with the described effects on vascular tone and cardiac function. To this attempt, an *in vitro* analysis of the activity and/or the phosphorilation state of several kinases and transcription factors involved the pathway itself has already been started at the time of this study. Our preliminary data allow us to gain insight into the molecular mechanism involving miR-199a/a* with the RAS and the ET system, and ultimately leading to the regulation of the neurohormonal response.

Altogether, the results obtained throughout our study demonstrated that miR-199a/a* play a key role in the cardiovascular system, being able to specifically modulate the vascular function and the cardiac response to stress. The evaluation of miR-199a/a* expression, their targets, as well as their functional role in different cardiac and vascular cell populations, corroborates the idea that the role of the miRNAs can vary according to both the tissue and pathophysiological condition considered. Finally, the demonstration of a link between miR-199a/a* and at least two of the most important neurohormonal systems, lead us to hypothesize the existence of a loop mechanism regulating both miR-199a/a* expression and the neurohormonal response. In particular, we hypothesize that neuroendocrine activation normally associated to various pathological states and induced by different stimuli, is responsible for modulating miR-199a/a* expression in CVDs. On the other hand, the control by the endogenous miR-199a/a* of the expression

levels of some of the main components of neurohormonal systems, contributes to the maintenance of homeostatic balance under basal conditions.



Scheme 3.1 *A signalling pathway common to Angiotensin I (ANG II), endothelin-1 (ET-1) and apelin (APLN). In green, molecules validated as target; in orange, molecule not yet validated. These peptides bind to their receptors on cardiac and vascular cells and cause hydrolysis of phospholipase C (PLC) to inositol triphosphate (IP3) and diacylglycerol (DAG). DAG causes translocation of protein kinase C (PKC) which results in activation of extracellular signal regulated kinase ½ (ERK ½). IP3 causes increase in the intracellular calcium concentration. These changes result in the modulation of several cellular processes, thus leading to cardiovascular remodelling.*

4. CONCLUSIONS AND PERSPECTIVES

In conclusion, results from this doctoral thesis lead to further insight into the role of miR-199a/a* in cardiovascular pathophysiology, suggesting some of the mechanisms through which it can be modulated both in physiological and pathological conditions and eventually affects either the cardiac or the vascular functionality.

During recent years, the recognition of miRNAs as essential regulators of heart and vessel morphology and function, fundamentally changed our view of the cardiovascular system in physiology and pathology.

As shown in this study, the physiological expression of miR-199a/a* in both cardiac tissues and vessels raises the possibility that it may play a key role in CVDs. In pathological condition, miR-199a/a* expression resulted to be directly related to CH both *in vivo* in an murine model of pressure overload and *in vitro* in two different cell models subjected to neurohormonal response.

This finding, might be of relevance for the unraveling of miR-199a/a* function in cardiovascular pathophysiology, considering the fact that aberrant expression of miRNAs is causally related to a variety of disease states, like angiogenesis, vascular diseases, CH, fibrosis and HF.

Although miRNAs are moderate regulators of gene expression in normal unstressed condition, chances in expression and function of miRNAs in response to stress can induce great modifications of overall gene expression, causing the onset of diseases. The complexity of regulation of gene expression by miRNAs is highlighted by the fact that multiple cardiovascular miRNAs have been identified, each of which regulating several hundred target genes. MiRNAs target not only single genes but often functionally related gene networks. In line with this, we showed that miR-199a/a* target gene molecules belonging to neurohormonal systems. Although other miRNAs may also be involved, our results indicate that miR-199a/a* function as critical not-coding RNA molecules for establishing and sustaining the vascular and the cardiac functions, by modulating the neuroendocrine activation according to a regulatory loop correlating miR-199a/a* and the identified targets, as suggested for miR-199a and ET-1 [106].

Recently, several strategies for normalizing gene expression in the failing heart with small molecules that control signal transduction pathways directed at transcription factors has been investigated [121]. Among these, miRNAs have recently emerged as important regulators of gene expression at the posttranscriptional and translational levels. Modern medicine has developed several effective treatments for CVDs, ranging from targeting drugs, lifestyle improvements to surgery. However, one caveat of the current therapeutic regimens is that the active principles can have collateral effects on the circulatory system and thus do not act specifically at cellular level. Therefore, more target-oriented drugs are needed. Given the emerging roles of miRNAs in the modulation of cellular phenotypes, it could be interested to determine whether miRNAs can be used as a new specific therapeutic tool for curing CVDs.

Some of the existing antisense technology and gene therapy approaches can be adapted to manipulate miRNA levels *in vivo*. Modified antisense oligonucleotides

targeting the mature miRNA sequence, (e.g.: antagomiR), can reduce the levels of pathogenic or aberrantly expressed miRNAs [113]. Conversely, miRNA mimics can serve to elevate the levels of miRNAs with beneficial functions.
The functional studies performed on transgenic mice showed that *in vivo* overexpression of miR-199a/a* contributed to cardiac dysfunction in pathological CH, to the increase in CMC size, and other hallmarks of hypertrophy. This model is in line with the *in vivo* loss-of-function studies on miR-199 reported by others [116] and by us in this current research. Altogether, antagomir-199a/a* treatment *in vivo* can restore the cardiac function in pathological CH, thus suggesting miR-199a/a* silencing as innovative therapeutical strategy for curing LV dysfunction, thus preventing HF. On the other hand, we have found that, *in vivo* miR-199a/a* overexpression under normal unstressed condition, not only does not affect heart physiology and cardiac function, but it apparently exerts a beneficial effect on the vascular tone, by inhibiting vasoconstriction induced by neurohormonal stimulation.
Once again, our finding assigns to miR-199a/a* a role in the neurohormonal response and opens up a new perspective from which investigating miR-199a/a* function in cardiovascular pathophysiology. Nevertheless the cardiac and the vascular functions are consistently correlated to each other, our results demonstrated that miRNAs can differentially affect them, depending on the initial stimulus and the considered disease model. More importantly, they imply that modulation of miR-199a/a* expression by either oligonucleotides or mimics administration may have future therapeutic application in the clinical setting.
At the time of this doctoral thesis, a deeper understanding of miR-199a/a* role *in vivo* has already started focusing on a detailed the characterization of cellular signal transduction either modulated by miR-199a/a* or leading to their modulation. Furthermore, the evaluation of miR-199a/a* ability to modulate the vascular tone over time, is currently being performed by haemodynamic monitoring after chronic infusion of both ANG II and ET-1. Such an analysis, will eventually lead us to determine how *in vivo* miR-199a/a* modulation affects the state-of-neuroendocrine activation and consequent vascular and cardiac remodeling in the long-term.
Considering the involvement of elevated levels of ANG II and ET-1 in the cardiovascular pathophysiology, a wide range of molecules that antagonize their effects have been developed for the treatment of various CVDs [122-124]. Among these, specific ANG II and ET-1 receptor antagonists known for their pharmacological action, might be used in the above described chronic infusion studies, in order to evaluate any possible synergetic beneficial action between them and miR-199a/a*.
All together, these studies will hopefully revealed the molecular role of miR-199a/a* in cardiovascular phatophysiology, suggesting possible innovative therapeutical approaches for the treatment of various CVDs.

5. ACKNOWLEDGMENTS

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