Humboldt Universität zu Berlin

DISSERTATION

Sex- and oestrogen-dependent regulation of miRNAs in cardiac hypertrophy

Zur Erlangung des Doktorgrades
d o c t o r r e r u m n a t u r a l i u m (Dr. rer. nat.)
im Fach Biologie

eingereicht an der
Lebenswissenschaftliche Fakultät
der Humboldt-Universität zu Berlin
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eingereicht: 11.06.2014

Datum der Promotion: 15.10.2014

Pedras no caminho

Posso ter defeitos, viver ansioso e ficar irritado algumas vezes,
Mas não esqueço de que minha vida
É a maior empresa do mundo…
E que posso evitar que ela vá à falência.
Ser feliz é reconhecer que vale a pena viver
Apesar de todos os desafios, incompreensões e períodos de crise.
Ser feliz é deixar de ser vítima dos problemas e
Se tornar um autor da própria história…
É atravessar desertos fora de si, mas ser capaz de encontrar
Um oásis no recôndito da sua alma…
É agradecer a Deus a cada manhã pelo milagre da vida.
Ser feliz é não ter medo dos próprios sentimentos.
É saber falar de si mesmo.
É ter coragem para ouvir um "Não"!
É ter segurança para receber uma crítica,
Mesmo que injusta…
Pedras no caminho?
Guardo todas, um dia vou construir um castelo…

Fernando Pessoa

Stones in the road

I may have flaws, live in anxiety, even get angry sometimes
But I do not forget that my life
Is the biggest company in the world...
And I can avoid its failure.
To be happy is to recognise that life is worth living
Even with all its challenges, misunderstandings, and its periods of crisis.
To be happy is to stop being victim of problems
And being the author of your own story.
It's to cross deserts outside of yourself
And to find an oasis inside your soul...
It's to thank God each morning for the miracle of life.
To be happy it is not to be afraid of your own emotions.
It is knowing how to speak about yourself.
It is to have courage to listen to a "no"!
To have the strength to receive a criticism
Even when unjust...
Stones in the road?
I save every single one, one day I'll build a castle....

Fernando Pessoa

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Zusammenfassung

Das Ziel der vorliegenden Arbeit war die Identifizierung von Geschlechterunterschieden in der Expression von miRNAs im späten Stadium der Myokardhypertrophie, sowie der möglichen Rolle von ERβ bei der Regulierung dieser Unterschiede. Geschlechterunterschiede bei kardiovaskulären Erkrankungen und speziell bei Myokardhypertrophie sind weithin bekannt und publiziert. Die zugrunde liegenden Mechanismen sind jedoch nahezu unbekannt. Unsere früheren Studien identifizierten ERβ als determinierenden Faktor für die beobachteten Geschlechterunterschiede bei Druckbelastung mit unterschiedlichen Effekten bei Männchen und Weibchen. Unter anderem führte eine Deletion des Rezeptors zur Aufhebung der zuvor beobachteten Geschlechterunterschiede auf physiologischer und fibrotischer Ebene, sowie in der Genexpression. Es wurde erwartet, dass miRNAs, als Regulatoren der Genexpression, bei Hypertrophie ein dimorphes Expressionsmuster aufweisen.

In dieser Studie wurden insgesamt 30 miRNAs mit Geschlechter- und/oder Geschlecht*Operation-Interaktionseffekten 9 Wochen nach TAC in WT-Mäusen identifiziert. Die gleichen Effekte waren in ERβ$^{-/-}$-Tieren nicht zu beobachten, teilweise aufgrund einer höheren Expression dieser miRNAs in ERβ$^{-/-}$-Weibchen als bei den Männchen. Eine Unterdrückung der miRNA-Expression durch Östrogen wurde bereits in verschiedenen Modellen beschrieben, aber die meisten der bekannten Untersuchungen wurden - aufgrund der bedeutenden Rolle des Hormons bei Brustkrebs - in MCF-7-Zellen durchgeführt; einer Zelllinie, der endogenes ERβ fehlt. Die vorliegende Studie zeigt eine Hemmung vieler miRNAs durch Östrogen und seine Rezeptoren α und β in weiblichen Kardiomyozyten, welches somit die *in vivo*-Ergebnisse bestätigt und die protektive Rolle von Östrogen und ERβ im weiblichen Herzen unterstreicht.

Sechs der miRNAs mit Geschlechterunterschieden in WT-, aber nicht in ERβ$^{-/-}$-Hypertrophie-Modellen wurden als mögliche Fibroseregulatoren identifiziert, da ihnen gemeinsame Inhibitoren des ERK-MAPK-Signalwegs als Zielgene vorhergesagt wurden (Spry1, Spry2, Rasa1 und Rasa2). Die Expression dieser miRNAs, miR-106a, miR-106b, miR-21, miR-24, miR-27a und miR-27b, war in kardialen Fibroblasten durch Östrogen geschlechterabhängig reguliert. In weiblichen Fibroblasten hemmte Östrogen ihre Expression, während es die Expression in den männlichen Zellen induzierte. Darüber hinaus wurden diese miRNAs in weiblichen Fibroblasten in der Regel durch einen oder beide ER-spezifischen Agonisten gehemmt, während bei den männlichen Fibroblasten die Mehrheit dieser miRNAs in Anwesenheit des ERα-spezifischen Agonisten heraufreguliert wurde. Dieses interessante Ergebnis konnte *in vivo* teilweise bestätigt werden, da eine Deletion von ERα die Expression dieser miRNAs (mit Ausnahme von miR-106a) bei Männchen jedoch

nicht bei Weibchen veränderte. Ein fibrotischer Reiz (AngII) verursachte eine Heraufregulation der Expression von miR-106b, miR-24, miR-27a und miR-27b in männlichen Fibroblasten, welche durch gleichzeitige Behandlung mit Östrogen kompensiert wurde. In weiblichen Fibroblasten induzierte AngII keine der miRNAs, führte jedoch zu einer Herabregulation von miR-24, -27a und -27b sowie von miR-106a, -106b, -21 und -24 bei gleichzeitiger Behandlung mit Östrogen.

Zusammengefasst bestätigt diese Arbeit die schützende Rolle von Östrogen und ERβ im weiblichen Herzen. Östrogen und seine Rezeptoren hemmen die Expression vieler miRNAs in weiblichen Kardiomyozyten und kardialen Fibroblasten, sowie *in vivo*. In männlichen Herzen und kardialen Fibroblasten scheint ERα der Hauptakteur zu sein, welcher insbesondere mögliche Fibrose-bezogene miRNAs reguliert. Die verschiedenen Rollen der ERs in weiblichen und männlichen Herzen sind ein bestimmender Faktor der beobachteten Geschlechterunterschiede bei Myokardhypertrophie.

Summary

The present study aimed to identify sex-differently expressed miRNAs in a late stage of hypertrophy (9 weeks) and the possible role of ERs in the regulation of these differences. Sex differences in cardiovascular diseases and particularly in cardiac hypertrophy are known and described. The underlying mechanisms are, however, far from being clear. Our previous studies identified ERβ as an important determinant factor of the observed sex differences in pressure overload, playing different roles in males and females. Among other effects, the deletion of the receptor abolished sex differences observed at physiological, gene expression and fibrosis level. MiRNAs, as gene expression regulators, were expected to present dimorphic expression in hypertrophy.

This report identified a total of 30 miRNAs with sex and/or sex*surgery interaction effect 9 weeks after TAC in WT mice. The same effects were not observed in ERβ$^{-/-}$ animals partially due to the higher expression of these miRNAs in ERβ$^{-/-}$ females than in their WT counterparts. The repression of miRNA expression by oestrogen was previously described in several different models, but due to the hormone role in breast cancer, most of what is known was described in MCF-7 cells, a cell line that lacks endogenous ERβ. This study reveals a repression of a number of miRNAs by estradiol and its receptors α and β in female cardiomyocytes, confirming the *in vivo* results and accentuating the important protective role of oestrogen and ERβ in the female heart.

Six of the miRNAs with sex differences in WT but not in ERβ$^{-/-}$ hypertrophy models were found to be possible fibrosis regulators by putatively targeting common ERK/MAPK pathway inhibitors (Spry1, Spry2, Rasa1 and Rasa2). MiR-106a, miR-106b, miR-21, miR-24, miR-27a and miR-27b were subjected to a different regulation by estradiol in cardiac fibroblasts in a sex-dependent manner. Estradiol represses their expression in female cardiac fibroblasts, whereas in male cells it induces its expression. Moreover, in female fibroblasts these miRNAs are generally repressed by one or both ER-specific agonists, while in male fibroblasts the majority of these miRNAs are up-regulated in the presence of ERα-specific agonist. The interesting result was partially confirmed *in vivo*, where ERα deletion affected the expression of these miRNAs in males but not in females, with the exception of miR-106a that was not affected. A fibrotic stimulus (AngII) caused an up-regulation of miR-106b, miR-24, miR-27a and miR-27b in male fibroblasts that was compensated when co-treated with estradiol. In female fibroblasts AngII did not induce any of the miRNAs, down-regulating instead miR-24, miR-27a and miR-27b and the co-treatment caused a down-regulation of miR-106a, miR-106b, miR-21 and miR-24.

In conclusion, this study reinforces the oestrogen and ERβ protective roles in the female hearts. Estradiol and ERs repress many miRNAs' expression in both female

cardiomyocytes and cardiac fibroblasts, as well as *in vivo*. In male hearts and cardiac fibroblasts, ERα is apparently the major player, regulating in particular potential fibrosis – related miRNAs. The different roles of ERs in male and female hearts are a determinant factor of the observed sex differences in cardiac hypertrophy.

List of Abbreviations

Ang II – Angiotensin II

Col1 – Collagen 1

Col3 – Collagen 3

E2 – Estradiol

ER – Oestrogen Receptor

ERα – Oestrogen Receptor alpha

ERβ – Oestrogen Receptor beta

FCS – Fetal Calf Serum

FS – Female Sham

FT – Female TAC

LV – Left ventricle

LVH – Left ventricle hypertrophy

mL – milliliters

min – minutes

miRNA – micro Ribonucleic acid

MS – Male Sham

MT – Male TAC

O.N. – overnight

OVX – Ovariectomised

P0 – Rplp0, ribosomal protein (http://www.ncbi.nlm.nih.gov/gene/11837)

PBS – Phosphate buffered saline

PCR – Polymerase Chain Reaction

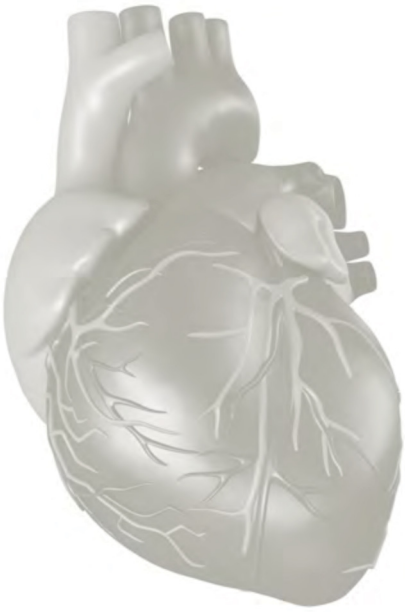
RNA – Ribonucleic acid

qRT-PCR – quantitative Real Time Polymerase Chain Reaction

TAC – Transverse Aortic Constriction

µL – microliters

WT – Wild Type



1. Introduction

1.1. Cardiac hypertrophy

Cardiac hypertrophy can generally be defined as an increase in heart mass as a response to a pressure or volume overload. However, the increase in heart mass, mostly due to an increase in ventricular weight, can be divided in different types: pathological and physiological.

1.1.1. Physiological and pathological hypertrophy

Physiological hypertrophy includes normal postnatal growth, pregnancy-induced growth and exercise-induced cardiac hypertrophy.

This type of hypertrophy is associated with a normal cardiac structure, normal or improved cardiac function and in the case of exercise or pregnancy-induced it is reversible.[1-3] The efficient cardiac pump function happens due to the fibrillar collagen network that provides structural integrity of adjacent myocytes, facilitating myocyte shortening.[4] Exercise-induced hypertrophy is generally considered to be protective and it does not progress to heart failure.[5]

Pathological hypertrophy occurs in response to a pressure or volume overload that can occur in hypertension, valvular heart disease, myocardial infarction or ischemia, associated with coronary heart disease, or abnormalities/conditions that can lead to cardiomyopathy (e.g. inherited genetic mutations, diabetes).[5] Pathological hypertrophy, as physiological hypertrophy, is associated with an increase of heart size. However, it is also associated to myocyte apoptosis, fibrosis and cardiac dysfunction, having an increased risk of heart failure and sudden death.[6-8] The loss of myocytes, is replaced with excessive collagen (fibrosis), mainly type 1 collagen (Col1), and its excessive accumulation stiffens the ventricles. This stiffness impairs contraction and relaxation, impairs the electrical coupling of cardiac myocytes with extracellular matrix proteins, and reduces capillary density. Fibrosis and reduced capillary density increase oxygen diffusion distances, leading to myocardial ischemia and contributes to the transition from hypertrophy to failure.[4]

1.1.2. Ventricular remodelling

Cardiac hypertrophy is associated with a structural remodelling, a concept that arose in 1985 from a study of causes and patterns of increased left-ventricular function after coronary artery ligation in rats.[9]

Remodelling implies changes that result in rearrangement of normally existing structures. Histopathologically, cardiac remodelling is characterised by a structural rearrangement involving cardiomyocyte hypertrophy, cardiac fibroblast proliferation, fibrosis and cell death.[10]

The different patterns of remodelling vary according to the type of mechanical stress, pressure or volume. The classification is based on changes in shape, which is depending on the initial stimulus. Volume overload (e.g. mitral regurgitation) produces myocyte lengthening and leads to an eccentric hypertrophy. Pressure overload (e.g. aortic stenosis) produces a growth in myocyte thickness and leads to a concentric hypertrophy. Finally, the post-infarct remodelling is a combination of infarct expansion, where the stretched and the dilated infarcted tissues increase ventricular volume with a combined pressure and volume overload on the non-infracted areas (Figure 1).[11]

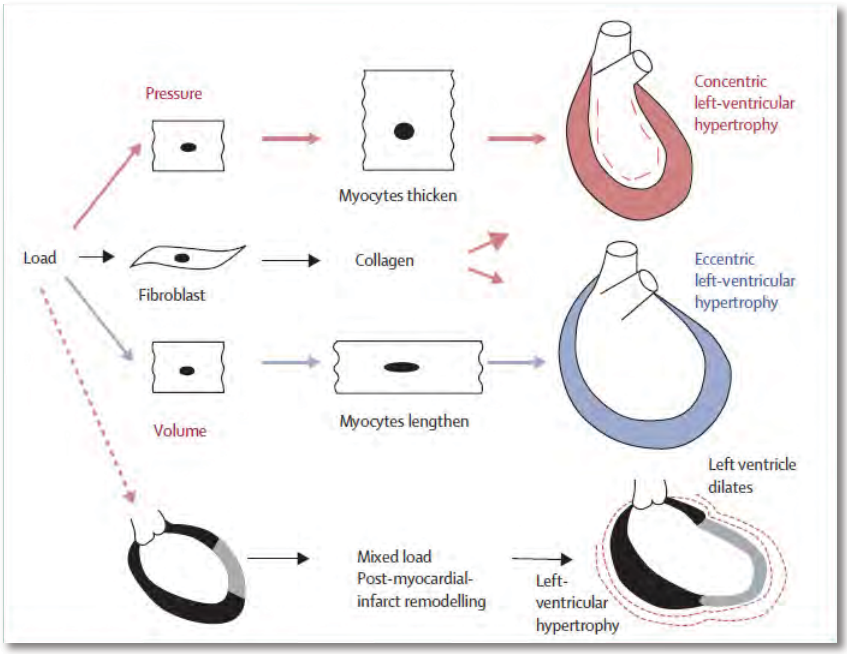


Figure 1. The three major patterns of ventricular remodelling (figure from reference [11]).

1.1.3. Cardiac fibroblasts and fibrosis

The structural remodelling of the heart involves a series of cellular responses in both cardiomyocytes and non-muscle cells. The heart is composed of cardiac myocytes (muscle cells), non-myocytes (fibroblasts, endothelial cells, mast cells, vascular smooth muscle cells) and the surrounding extracellular matrix. Muscle cells represent only 30-35% of the ventricular cell suspension, while non-muscle cells account for approximately 65-70%.[5, 12]

As described above, pathological hypertrophy is typically associated with loss of myocytes and excessive collagen replacement, known as fibrosis. Cardiac fibroblast activation is responsible for the accumulation of type I and III collagens, the major fibrillar proteins of the myocardial collagen matrix, accounting for 90% of total collagen.[13] The alterations in the heart correlate with the collagen matrix remodelling.[14] Cardiac fibroblasts and extracellular matrix proteins accumulate disproportionately and excessively as a

response to a pathological insult, leading to mechanical stiffness. This will contribute to diastolic dysfunction and can progress to systolic dysfunction.[15]

There are two types of fibrosis described, namely reparative fibrosis and reactive fibrosis. The first is described as occurring as a reaction to loss of myocardial material, being mainly interstitial, and reactive fibrosis in the absence of cell loss as a reaction to changes in myocardial load or inflammation, being primarily perivascular. Reactive and reparative fibrosis, usually coexist during ventricular remodelling.[16] However, whether they truly represent different entities or not remains under discussion.[13]

Fibroblast stimulation is essential for reactive and reparative fibrosis. Several humoral factors are believed to be responsible for fibrosis, affecting fibroblast phenotype and function (angiotensin II, Ang II; basic fibroblast growth factor, bFGF/FGF-2; transforming growth factor-β, TGFβ; catecholamines; insulin growth factor-1, IGF-1).[17-21] However Ang II appears to be one of the most important factors in regulation of cardiac fibrosis and remodelling, inhibiting collagen degradation.[22]

1.1.4. Transverse aortic constriction as a validated hypertrophy model

Transverse aortic constriction (TAC) is a validated, reproducible and low mortality model for hypertrophy study. Described in 1991[23], it uses microsurgery techniques to produce a stable pressure gradient across the aorta, by banding the ascending aortic arch. It is characterised by a first phase of compensated hypertrophy followed by a transition to heart failure and mimics human pressure overload-induced heart failure in a number of aspects.

1.2. Heart and sex differences

1.2.1. Sex differences in the healthy heart

It has been widely described that hearts of men and women are not similar. Several studies compare young hearts, adult heart and the ageing effect.

During infancy and childhood, left ventricle (LV) mass is not significantly different, which suggests that the initial number of cardiomyocytes is the same in males and females. However, after puberty, when sex-specific hormonal influences affect the body, the LV mass shows clear sex-differences, being 15-30% larger in males than in females. This indicates that a state of relative cardiac hypertrophy exists in apparently normal adult men.[24]

Aging leads to an increase in septal and wall thickness in both males and females and in LV diameter only in males, resulting in a more pronounced progressive increase of LV in males than in females. Moreover, a progressive slowing of relaxation in females and of both contraction and relaxation in males is described, although not being related to changes in LV mass.[25] Aging was also associated with a preservation of ventricular myocardial mass in females, in contrast to the 1 g/year of myocardium lost in males.[26]

Sex differences exist as well in heart function. Young women have better diastolic function when compared to men, but both show a decrease with aging. Postmenopausal women, however, show a diastolic function similar to age-matched men (Figure 2).

Finally, male and female hearts differ largely in the presence of sex hormones. Men have higher level of testosterone and women of estradiol (E2), the most abundant form of oestrogen, but both hormones are present in both sexes. Yet, after menopause, the level of E2 in women decreases to levels compared to the existent in men's heart. Androgen and oestrogen receptors are present in both sexes' hearts as well.[27, 28]

	Men	Premenopausal women	Postmenopausal women
Cardiac myocyte size	++	+	+
Systolic function	+	+	+
Diastolic function	+	++	+
Estrogen	+	10+	+
Testosterone	10+	+	+
ERα	+	+	+
ERβ	++	+	+

Figure 2. Summary of sex differences in the heart (figure from reference [29])

1.2.2. Sex differences in the hypertrophic heart

It is well known that women typically develop heart diseases in a later time-point than men. Sex differences were also reported in manifestation and transition to heart failure in patients with aortic stenosis.[30] In similar degrees of stenosis, elderly female patients also preserve more frequently the cardiac performance than male.[31] Among heart failure patients, women tend to develop a more concentric hypertrophy and heart failure with preserved systolic function, whereas men show a loss of systolic function.[32-35]

In rodents, significant sex differences were described in the adaptation of the LV to pressure overload, despite a similar degree of hypertrophy and systolic wall stress.[36] At 20 weeks of TAC, male but not female rats, showed an early transition to heart failure, loss of concentric remodelling, elevated wall stress and diastolic dysfunction.[37]

A study from our group showed sex differences in gene expression in an early response to pressure overload in mice. Female-specific regulated genes were related to mitochondria and metabolism and male-specific related to matrix and biosynthesis.[38]

In physiological hypertrophy, despite similar skeletal muscle adaptations, hearts of male rats adapt to physical training by running with improved intrinsic performance, whereas hearts of female rats do not.[39]

Overall, the relationship between sex and hypertrophy is very complex and appears to depend on many different factors like age and model/aetiology and stage of hypertrophy.

1.2.3. Oestrogen protective role in the heart

Sex hormones have often been connected to sex differences in cardiovascular diseases and the disappearing of sex differences with aging has been correlated with the loss of E2 in postmenopausal women. Both oestrogen and androgen receptors are expressed in males and female hearts[27, 28, 40] supporting a role for oestrogen and testosterone in cardiac physiology (Figure 2). The majority of the studies concentrate on oestrogen, as it is considered to be a cardio-protective agent, whereas testosterone role is more as detrimental to heart function.[29]

In our group, intact human heart tissue was used to show that women have an increase of progesterone receptor mRNA expression and protein level in response to E2 treatment, while men do not. The study is a demonstration that oestrogen acts in the heart in a sex-specific manner in humans.[41]

Although certainly not the only factor influencing them, sex hormones have also been continuously under study in rodents and *in vitro*.

Different studies in rodents have shown the E2 effect in both left and right ventricle development, as well as LV hypertrophy (LVH)[42-44]. Others described abnormal cardiac

function and biochemistry resulting from gonadectomy[45], that were prevented in males by replacement with testosterone and in females by replacement with oestrogen or testosterone.[46] A recent study showed that the loss of oestrogen signalling in females, but not males, impairs cardiac function and sensitizes the heart to pathological stimuli, up-regulating numerous hypertrophic pathways.[47]

Cardiac myocytes and fibroblasts, when exposed to E2, are able to respond directly, through the induction of known oestrogen-responsive genes like ESR1 (ERα), ESR2 (ERβ), PGR and Cx43.[40] Oestrogen protective role in hypertrophy was also demonstrated *in vitro*, when it inhibited Ang II and endothelin-1 induced hypertrophy in cardiomyocytes. Mean cell area and hypertrophic mechanisms were substantially stimulated by the hypertrophic peptides and was reduced in E2-treated cells.[48, 49]

In isolated cardiomyocytes, E2 was shown to have an anti-apoptotic effect in a mechanism that involves NF-kB and in primary cardiac fibroblasts E2 was described as having an anti-fibrotic effect, through limiting cardiac fibroblast proliferation and differentiation.[50, 51] Moreover, E2 can also regulate the remodelling of the extracellular matrix, modulating fibroblast protein and gene expression, as well as signalling pathways.[52]

1.2.4. Oestrogen receptors in the heart

Oestrogen genomic action is mediated by nuclear oestrogen receptors (ERs). The oestrogen-ER complex directly binds to a specific DNA sequence or ERE (oestrogen responsive element), acting as a transcription factor. Another possibility is that it acts on transcription indirectly tethering with other transcription activators.[29, 53]

ERα and ERβ are two ER subtypes, expressed from different genes and have distinct tissue distribution.[53] Both ERs are expressed in males and females cardiac tissue, but even though they have equal ERα expression values, males have higher levels of ERβ (Figure 2).[28]

In the sick heart, we showed before that in end-stage dilated cardiomyopathy ERα is up-regulated in both males and females[54] and in aortic stenosis the same happens for both receptors.[28] However, in none of the studies the expression of ERα was different between males and females, either in basal level or diseased heart.

In ovariectomised rats, a selective ERα agonist was sufficient to attenuate cardiac hypertrophy and to improve hemodynamic function.[55]

Nonetheless, both ERα and ERβ are shown to protect the cardiovascular remodelling, for example against aldosterone salt treatment, and they confer redundant, and specific, effects on cardiac protein expression.[56]

1.2.5. ERβ plays a determinant role in sex differences in cardiac hypertrophy

ER knock-out models have been widely used to study cardiac diseases and especially ERβ has been shown to be a determinant factor in sex differences in hypertrophy. Oestrogen protective role in cardiac hypertrophy has often been showed to be mediated by ERβ.

In animal experiments, after 2 weeks TAC, wild type (WT) females presented a significantly less hypertrophy than males. In the same study, ERα$^{-/-}$ animals presented identical levels of hypertrophy than WT, meaning that ERα was not found essential for hypertrophy attenuation. However, ERβ$^{-/-}$ females exhibited an increased degree of hypertrophy, comparable to WT males, indicating a role for ERβ in mediating an attenuated response to pressure overload. This study established the importance of the direct action of ERs in myocardial response to pressure overload.[57]

Another relevant study used ERα$^{-/-}$ and ERβ$^{-/-}$, ovariectomised mice, treated with E2 and subjected to 4 weeks of TAC, confirmed this result. While in sham females E2 treatment did not have any effect in WT or knock-out animals, E2 effect in TAC differed between genotypes. In WT and ERα$^{-/-}$ TAC females, E2 reduced ventricular hypertrophy, whilst in ERβ$^{-/-}$ the same was not observed.[58]

Our own studies also demonstrated the important role of ERβ in the protective mechanism to hypertrophy. ERβ showed to be crucial and necessary for the strict control of cardiac gene expression in this disease, acquiring the role of gatekeeper of the genomic response of the heart to pressure overload.[59] Finally, ERβ was shown to have a determinant role in sex differences in a late hypertrophy stage, 9 weeks after TAC. Males and females WT and ERβ$^{-/-}$ mice showed significant differences in response to hypertrophy. In this study, important sex differences were observed in terms of type of hypertrophy, apoptosis and fibrosis. WT females developed a more concentric hypertrophy in contrast to the eccentric form present in males. Concerning cardiomyocyte diameter, ERβ deletion caused a stronger TAC effect. Gene expression profiling revealed sex differences in mitochondrial genes, stronger repressed in WT males than in females. In ERβ$^{-/-}$ mice, TAC surgery induced proapoptotic genes in both sexes, being higher in males. The results show also a more pronounced cardiac fibrosis after TAC in WT males than in females. This difference disappeared in the absence of ERβ. The authors conclude that sex and ERβ attenuate the development of fibrosis and apoptosis, therefore slowing the progression to heart failure.[60]

1.3. miRNAs

MicroRNAs (miRNAs) are a large family of endogenous, single-stranded, small, noncoding RNAs with ~22 nucleotides (nt) in length, that have emerged in the past years as key regulators of gene expression.

In humans, more than 1000 miRNAs are encoded by as much as 5% of the genome and they regulate around 30% of our genes. A single miRNA can regulate numerous different genes and each gene can be regulated by several miRNAs. Functional studies indicate that miRNAs participate in almost every cellular process investigated. They are currently known to control vital processes such as cell growth, proliferation and differentiation, apoptosis, tissue differentiation, heterochromatin formation and cell proliferation, among others. Furthermore, miRNA dysregulation is linked to cancer, neurological disorders, several types of cancer and cardiovascular disorders.[61]

1.3.1. The discovery of miRNAs – a brief story

The first description of a small endogenous regulatory RNA occurred in 1993, when *lin-4*, a gene known to control the timing of larval development in *C. elegans*, was found to code for a pair of miRNAs, one with 22 nt and the other with 61 nt, instead of coding for a protein. These RNAs had antisense complementarity to multiple sites in the 3'UTR of the *lin-14* gene, located in a region previously proposed to mediate the repression of *lin-14* by the *lin-4* gene product. Once later confirmed on the regulation of *lin-14* by *lin-4*, these discoveries supported a model of translational repression as part of the regulatory pathway that triggers the transition from cell divisions of the first larval stage to those of the second.[62-64] The shorter lin-4 is now recognized as the founding member of the miRNA family.

This discovery opened the path for the finding of a large family of molecules, eventually found to be widespread, being described and highly conserved in animals, plants, fungi and some viruses and revolutionising the comprehension of gene expression regulation.[65-69]

1.3.2. miRNAs as part of a bigger family - the RNAi

MiRNAs belong to a bigger family of small regulatory RNAs called RNA interference (RNAi) that comprises miRNAs, short interfering RNAs (siRNAs) and PIWI-interacting RNA (piRNAs). The three pathways of RNAi share a common mode of action but differ in the mechanism and biogenesis.

For all three classes, the minimal effector is a ribonucleoprotein complex, comprising an Argonaute family protein member bound to a single stranded ~20 to ~30nt RNA. The complex grants the specificity of the base-pairing interactions with the target gene. However,

miRNAs are derived from the genome, whereas siRNAs may be endogenous or introduced in the organism via viral infection or other exogenous sources.[70] siRNAs and miRNAs are generated from double-stranded RNA but while siRNAs duplexes feature perfect base-pairing, miRNAs helices contain mismatches and more extended terminal loops. Both classes, despite their different origins, have converging pathways once they are assembled into the RISC complex (RNA-induced silencing complex). In addition, siRNAs and miRNAs are characterised by the double-strand of their precursors, while piRNAs derive from precursors that appear to be single stranded. The three classes are also associated with distinct subsets of effector proteins: si- and miRNAs bind to members of the Argonaute clade, while piRNAs bind to members of the Piwi clade.[61, 70-73]

The complete understanding of the functions and targets of each class of RNAi is far from being done. Particularly in the case of miRNAs, here in focus, it is now clear that they play key roles in many organisms' development, as well as in diseases, thus became interesting objects of study in many different fields.

1.3.3. miRNA biogenesis

Mammalian miRNA biogenesis can be divided in two broad classes, canonical and non-canonical, based on how pri-miRNAs are processed leading to the production on mature miRNAs.

In the canonical pathway (diagrammed in Figure 3) miRNAs are processed in the nucleus from precursor molecules, known as pri-miRNAs (~1000nt) and transcribed by RNA polymerase II from independent genes or from introns of protein coding genes. These pri-miRNas fold into hairpins and are subjected to an enzymatic cleavage by Drosha and Dicer, two members of the RNase III enzyme family. Drosha acts as first, in the nucleus, together with DGCR8 (DiGeorge syndrome critical region gene 8).[74, 75] The resulting ~65-70 nt precursor miRNA (pre-miRNA) is then exported to the cytoplasm, via Exportin-5 and RanGTP, two transport facilitators.[61]

Once in the cytoplasm, the GTP is replaced by GDP, inducing Exportin-5 to release its pre-miRNA cargo. Then, another endoribonucleolytic reaction occurs catalysed by Dicer, yielding a ~22 bp miRNA duplex. The duplex is incorporated onto an Argonaute protein, where one or occasionally both strands are incorporated into the RISC complex and functions as mature miRNAs, leading to translational repression or mRNA degradation. In mammals, four different Argonaute proteins (AGO1-4) can be involved in the miRNA-mediated repression, but only AGO2 functions with siRNAs.[76, 77]

The non-functional strand, if it is the case, is released and degraded.[72] Which strand is retained depends on the relative thermodynamic stability of the two ends of the duplex intermediate (Figure 3).[78, 79]

The non-canonical pathway, on its turn, does not require all the protein factors mentioned. Mirtrons, an alternate source of miRNA-type regulatory RNAs that derive from short intronic hairpins, have a nuclear biogenesis that appears to bypass Drosha cleavage. While this is essential for miRNA biogenesis, mirtrons are produced instead by splicing. [79-82]

The mirtron pathway merges with the canonical miRNA pathway during hairpin export by Exportin-5, and both types of hairpins are subsequently processed by Dicer. Canonical and non-canonical miRNAs can be distinguished by changes in their expression when one of the processing factors is absent. The loss of Drosha, DGCR8 or Dicer would reduce the expression of canonical miRNAs while the non-canonical would have variable responses, depending on the absent protein. However, most ~22 nucleotides long RNA species in mammals are canonical miRNAs.[79, 81, 83]

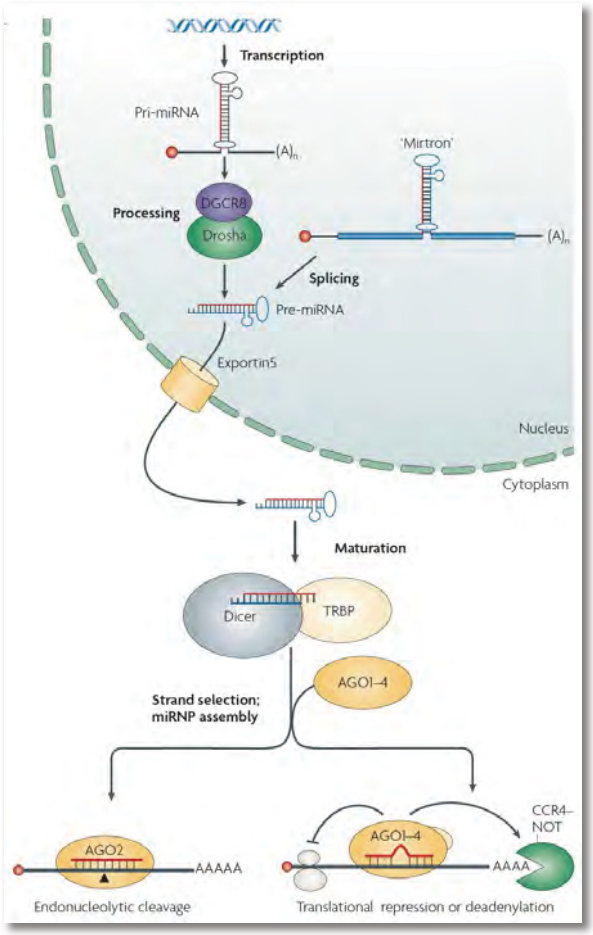


Figure 3. miRNA biogenesis (figure from reference [76])

In the heart, miRNA processing is crucial for cardiac development and homeostasis. The knock-out of DGCR8 develops lethal heart failure due to impaired miRNA synthesis whereas Dicer heart selective knock-outs lead to dilated cardiomyopathy and heart failure.[84-86]

1.3.4. miRNA target recognition and regulatory functions

The complete mechanism through which miRNAs regulate gene expression is still not totally understood. However, it is currently known that, in mammals, miRNAs do this regulation through the inhibition of mRNA translation or its degradation, in both cases leading to repression of protein synthesis.

Typically, miRNA-binding sites are located in the 3'UTRs (3' untranslated regions) of target mRNAs. The recognition of these binding sites occurs via base-pairing of the 'seed' region, which is the sequence between nucleotides 2-8 of a miRNA. However, this is not the only determining factor. Among other factors, for a stable interaction it is also necessary to exist a reasonable complementarity to the 3' half of the miRNA. The imperfect nature of the miRNA:mRNA interaction helps to the understanding of the fact that a single miRNA can potentially target hundreds of mRNAs.[87-89]

Through the miRNA-mRNA sequence complementarity, miRNA-RISC mediated inhibition is commonly divided in three processes: 1) site-specific cleavage, 2) enhanced mRNA degradation, 3) translational inhibition. The first is restricted to small RNAs with a perfect or almost perfect match to the target, but it is commonly referred to as RNAi. The other two processes, in contrast, are normally associated to mismatched miRNA-target sequences, the most common scenario in mammals. The combination of the latter, is usually referred to as non-cleavage repression and can be carried out by any of the four AGO proteins.[90, 91] (Figure 3).

Target sites for animal miRNAs are not equally distributed throughout the mRNA 3' UTR, but rather located in its both ends. The number and the arrangement of these binding sites can influence the degree and specificity of miRNA mediated gene expression. Furthermore, many mRNAs can have several potential different sites for the same miRNA and its proximity can enhance the down-regulation.[92, 93] Moreover, alternative transcripts with different 3' UTR lengths can be targeted by different sets of miRNAs.[94] Despite these and other studies, there is no single model that can depict all miRNA:mRNA interactions.

Besides all miRNA:mRNA interactions, other mechanisms were show to modulate miRNA function. For example, a naturally occurring miRNA sequence variation outside the 'seed' sequence can modify mRNA targeting and end-organ function. This was shown *in vivo*, with miR-499, and supports studies of individual phenotypes or disease modification conferred by miRNA mutations.[95]

An interesting recent study, showed another side of miRNA action and regulation in mouse hearts. Using transgenic expression of pre-miRNAs in mouse hearts, miR-378 and miR-499 were shown to indirectly regulate, 15 to 30 cardiac miRNAs, besides some hundreds of cardiac mRNAs, in a stimulus specific way. This miRNA-mediated miRNA

regulation helps to explain how small direct effects of miRNAs are amplified to generate surprising phenotypes.[96]

1.3.5. Computational prediction of miRNA targets by TargetScan

The prediction of miRNA targets and miRNAs:mRNAs interaction in animal systems is yet a challenge, due to the complexity and the limited knowledge of the rules and regulatory mechanisms of this interaction. MiRNA targets can be predicted above the background of false positives by requiring conserved base-pairing to the 5' region of the miRNA, the 'seed' region, searching for 6-8mer matches.[97, 98] For this, it is necessary to use existent miRNA biology tools like target prediction algorithms to find possible miRNA:mRNA interactions.

Numerous target prediction algorithms have been developed, many of them exploiting different approaches. The available algorithms might use or not the conservation comparison, influencing the outcome list of targets. Among the algorithms that use conservation criteria is TargetScan. TargetScan narrows the search to sites with full complementarity in the 'seed' region and then they are extended to 21-23 nucleotide long fragments representing true interaction. The parameters contributing to the final score are the 'seed' match, the 3' complementarity, local AU content and position contribution. The conservation of the 'seed' regions among orthologous 3'UTRs within binding regions has a major importance in outcome score.[98, 99]

1.4. miRNAs in the diseased heart

The discovery of the fundamental role of miRNAs in gene regulation led to a continuous and deep interest about these small RNAs. It did not take long to realize that these molecules are also dysregulated under stress conditions. In the heart, several studies have shown the important role of miRNAs in cardiovascular development and disease. Highly specific patterns of miRNA expression correlate with different cardiovascular disorders, such as cardiac hypertrophy, heart failure[100-103], post-myocardial infarction remodelling[104-106], and vascular remodelling[107].

Furthermore, gain- and loss-of-function miRNA studies revealed pathogenic and protective roles of miRNAs *in vivo* in the heart, directly associating specific miRNAs to specific pathologies such as arrhythmias (miR-1[108], miR-133[109], miR-208a[110]), fibrosis (miR-21[111], miR-29[105]), pressure overload-induced remodelling (miR-208[110, 112], miR-133[113]) and cardiometabolic disease (miR-33[114] miR-122[115]).

A particular family of miRNAs referred to as MyomiRs and comprising miR-208a, miR-208b and miR-499, is one of the best characterised examples of stress dependent gene regulation in the heart. These miRNAs are encoded by myosin heavy chain (*MHC*) genes, namely *α-MHC* (miR-208a), *β-MHC* (miR-208b) and *Myh7* (miR-499), and constitutes a complex regulatory circuit that controls myosin gene expression and cardiac stress responsiveness during adaptation to pathological signalling.[110, 112, 116]

MiR-199a was also characterized as a master regulator of a hypoxia-triggered pathway. This miRNA was acutely down-regulated in cardiomyocytes in hypoxia, leading to an up-regulation of hypoxia-inducible factor-1α[117] that was inverted when miR-199a was replenishing. The knockdown of miR-199a in normal conditions reproduced hypoxia conditions. MiR-199a was considered a master regulator of this pathway and a possible target to preconditioning cells against hypoxia damage.[118]

The correlation of miRNAs' action with their targets and cardiovascular phenotypes helps to the understanding of new pathways and diseases mechanisms. The manipulation of these disease-related miRNAs through the usage of miRNAs inhibitors and mimics leads to a world of possibilities in what concerns future therapies.

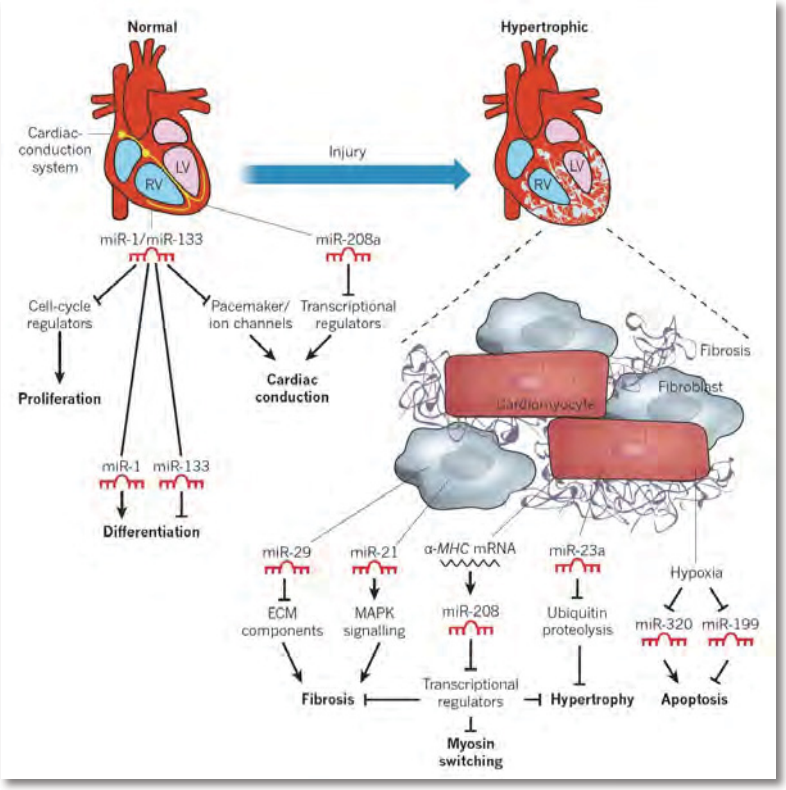


Figure 4. Roles of miRNAs in vascular disease (figure from ref. [119])

1.4.1. miRNAs and cardiac hypertrophy

MiRNAs are differentially regulated during hypertrophy and heart failure, in both rodents and humans. Given the knowledge that foetal gene expression reprogramming in the heart is an established mechanism contributing to the cardiac hypertrophy development[120, 121], an analogous change in miRNAs' expression would be expectable. Several studies have supported their role in this pathogenesis, as well as hypertrophy effects on miRNA expression.

1.4.2. Hypertrophy effect on miRNA expression

Expression profile studies of miRNAs are important to reveal novel miRNA based pathways underlying diseases. In animal models of cardiac hypertrophy, whole arrays of miRNAs have indicated that some miRNAs are typically up-, down-regulated or remain unchanged during hypertrophy, comparing to their levels in the normal heart (Table 1).

The first report of an evidence for a dysregulation of miRNA expression in cardiac remodelling in hypertrophy was in an array study based on two mouse models of pathological hypertrophy: the TAC model, as an *in vivo* model of hypertrophy induced by left ventricular pressure-overload, and a calcineurin transgenic (CnA) mouse model, a calcium-dependent model of maladaptive response. In this study, constitutive CnA signalling and TAC

resulted in the up- and down-regulation of common sets of miRNAs, suggesting that these miRNAs represent a genetic signature of the cardiac response.[100]

Another array measuring miRNA expression progression from day 1 to day 14 after TAC, revealed more than 50 miRNAs with progressive expression changes during hypertrophy development (Table 1). MiR-1 was the earliest miRNA down-regulated during hypertrophy and the study also describes several relevant targets for this miRNA (Figure 2).[122]

A comparison between 2 and 4 weeks TAC and phenylephrine (PE) treated neonatal cardiomyocytes compared miRNA expression patterns in *in vitro* and *in vivo* hypertrophy. The expression of miR-21, miR-23b and miR-125b was increased in both models, whereas miR-25 and miR-29a, highly up-regulated *in vivo*, appeared unchanged in the *in vitro* model due to their low expression in cardiomyocytes (Table 1). Additionally, the authors suggested that miR-21 was a possible negative regulator of cardiac hypertrophy.[123]

During the same year of the two previously described studies, another comparison of miRNA expression between 7, 14 and 21 days of TAC and neonatal cardiomyocytes came out. The most aberrantly expressed miRNA *in vivo*, was miR-21, but the study also identified and confirmed by northern-blot miR-27a, miR-27b, miR-146, miR-214, miR-341 and miR-424 as up-regulated in hypertrophy, while miR-29a, miR-29b, miR-29c, miR-30e, miR-126-5p, miR-133a, miR-133b, miR-149, miR-150, miR-185, miR-451 and miR-486 were significantly down-regulated after TAC (Table 1). MiR-21 was up-regulated *in vitro* by both AngII and PE, and its inhibition was able to decrease the *in vitro* hypertrophy, confirming the role of this miRNA in this pathology.[124] A summary of reported regulated miRNAs in TAC in mice is shown in Table 1.

Some of the published data are, however, contradictory. For example, miR-21 was highly up-regulated in mice heart after 1-2 weeks TAC, decreasing again to a normal level after 3-4 weeks[123], while other studies under similar conditions reported an up-regulation of miR-21 that was maintained over time.[100, 122]

In TAC, miR-1 was identified already after 1 day of TAC as one of the most down-regulated miRNAs, reaching a minimum at 1 week post-TAC and returning to near normal levels by day 14. In this analysis the expression of miR-133a/b was unchanged[122]. On the other hand, in two independent reports[100, 124] only miR-133a/b was found to be down-regulated, but not miR-1.

A possible source of differences in the expression profiles of miRNAs described above are differences in mice strain, age, time after TAC and sex, as pointed out in the footnote of Table 1.

Table 1. Reported regulation of miRNAs in mouse hypertrophy models (RNA microarrays).

miRNA	Regulation	Validation
miR-1	↓[122,113]	NB[113]
miR-10a	↓[122]	
miR-10b	↑[100] ↓[122]	
miR-103	↑[122]	
miR-106a	↑[123]	
miR-107	↑[122]	
miR-125b	↑[122,100,123]	NB[123]
miR-126-5p	↓[124]	
miR-126	↑[100]	
miR-127	↑[122]	
miR-133a	no change[122] ↓[100,124, 125] ↓ (133)[113]	NB (133)[113]
miR-139	↓[122]	
miR-140	↑[123]	
miR-140*	↑[122]	
miR-142-3p	↑[123]	
miR-146	↑[124]	
miR-149	↓[122,124]	
miR-15b	↑[122]	
miR-150	↓[122,100,124,123]	NB[100,124]; qRT-PCR[124]
miR-151	↓[122]	
miR-153	↑[123]	
miR-154	↑[100]	
miR-155	↓[122]	
miR-17-5p	↑[123]	
miR-18b	↑[123]	
miR-181b	↓[100]	NB[100]
miR-184	↑[123]	
miR-185	↓[122,124]	NB[124]; qRT-PCR[124]
miR-19a	↑[100]	
miR-19b	↑[123]	
miR-194	↓[122]	
miR-195	↑[122,100]	NB[100]
miR-199a-5p	↑[122,100, 125]	NB[100]
miR-199a-3p	↑[122,100, 125]	
miR-20b	↑[123]	
miR-200a	↑[123]	

miRNA	Regulation	Validation
miR-208	↑[123]	
miR-21	↑[122,100,124,125] (↑[123])	NB[124,123] qRT-PCR[124]
miR-210	↑[100,123]	
miR-211	↑[123]	
miR-214	↑[122,100,124, 125]	NB[100,124]
miR-217	↑[100]	
miR-218	↓[122]; ↑[100]	
miR-221	↑[122,123]	
miR-222	↑[122,123]	
miR-23a	↑[122,100,123]	NB[100,123]
miR-23b	↑[122,100]	NB[100]
miR-24	↑[122,100]	NB[100]
miR-25	↑[100,123]	NB[123]
miR-26a/b	↓[122]	
miR-27a/b	↑[122,100,124]	NB[124]
miR-29a	↑[123] ↓[122,124]	NB[124,123]
miR-29b	↓[124]	qRT-PCR[124]
miR-29c	↓[122,100,124,123]	NB[124]
miR-30a-3p	↓[122]	
miR-30a-5p	↓[122]	
miR-30b	↓[122,123, 125]	
miR-30c	↓[122,123, 125]	
miR-30d	↓[122]	
miR-30e*	↓[122]	
miR-30e	↓[122,100,124,125]	NB[124]
miR-31	↑[122]	
miR-330	↑[100]	
miR-341	↑[124]	NB[124]
miR-351	↑[122,100]	
miR-378	↓[122]	
miR-424	↑[124]	NB[124]
miR-451	↓[124]	NB[124]
miR-486	↓[124]	NB[124]
miR-93	↓[100]	
let-7b/c	↑[122]	
let-7d*	↓[122]	

NB: Northern blot. [122]:C57BL/6 mice; age and gender unknown, complete heart. [113]:10-12 weeks old C57BL/6 female mice. [100]: mice strain, age and gender unknown, cardiac tissue. [123]: 6-8 weeks old C57BL/6 male mice, heart. [124]:12 weeks old C576BJ mice; gender unknown, heart. [125]: 12 weeks old female, mice strain unknown, heart, 7 days TAC. qRT-PCR – quantitative real time Polymerase Chain Reaction

1.4.3. miRNAs with a direct action in hypertrophy

Numerous studies were conducted and several of the miRNAs found to be dysregulated during cardiac hypertrophy were characterized as pro-/anti-hypertrophic, with or without specific targets identified (Table 2), and others have just been specifically implicated in differentiation, apoptosis and fibrosis.

Initially, several miRNAs were shown to be capable of inducing hypertrophic growth *in vitro*. Over-expression of miR-23a, miR-23b, miR-24, miR-195 and miR-214, all up-regulated during cardiac hypertrophy, induced a response compared to PE in cardiomyocytes. Furthermore, a cardiac specific miR-195 over-expression *in vivo* induced cardiac growth with disorganisation and an aberrant size of cardiomyocytes, which progressed to heart failure.[100]

MiR-1 was one of the first miRNAs with identified targets directly involved in hypertrophy, being classified as anti-hypertrophic. Some of the most important targets described include Ras GTPase–activating protein (RasGAP), insulin growth factor-1 (IGF-1), calmodulin and myocyte enhancer factor-2A (Mef2A).[122, 126, 127]

Together with miR-1, miR-133a is also a muscle enriched miRNA that shares the same primary transcript and was equally early classified as anti-hypertrophic. The first interesting targets to be identified were RhoA, a GDP-GTP exchange protein regulating cardiac hypertrophy; Cdc42, a signal transduction kinase implicated in hypertrophy; and Nelf-A/WHSC2, a nuclear factor involved in cardiogenesis.[113] However, later on NFATc4 and calcineurin were described as well as miR-133a targets.[128, 129]

The first miRNA knock-out model was a miR-208a$^{-/-}$ mouse that showed reduced hypertrophy in response to pressure overload.[112] The same miRNA was later found to be sufficient to induce cardiac remodelling and modulate the expression of hypertrophy-associated genes.[110] Table 2 summarizes the miRNAs described as promoters or inhibitors of hypertrophy.

Table 2. MiRNAs studied in cardiac hypertrophy. Table adapted from ref. [86, 130, 131]

miRNA	Validated Target(s)	References	Action
miR-1	RasGAP; CdK9; fibronectin; Rheb	[122]	anti-hypertrophic
	Mef2a; Gata4; Calmodulin	[127]	anti-hypertrophic
	IGF1; IGF1R	[126]	anti-hypertrophic
	twinfillin-1	[132]	anti-hypertrophic
	---	[133]	anti-hypertrophic
miR-133a	RhoA; Cdc42; Nelf-A/WHSC2	[113]	anti-hypertrophic
	NFATc4	[128]	anti-hypertrophic
	Calcineurin	[129]	anti-hypertrophic
miR-142	p300; actinin; gp130	[134]	anti-hypertrophic
miR-145	GATA6	[135]	anti-hypertrophic
miR-19	atrogin-1; MuRF-1,	[136]	pro-hypertrophic
miR-195	---	[100]	pro-hypertrophic
miR-199a	HIF1-α	[137]	pro-hypertrophic
miR-199b	Dyrk1a	[138]	pro-hypertrophic
miR-208a	Thrap1	[112]	pro-hypertrophic
	Thrap1 and myostatin	[110]	pro-hypertrophic
miR-21	Sprouty1	[111]	pro-hypertrophic
	Sprouty2	[139]	pro-hypertrophic
miR-212/132	FoxO3	[117]	pro-hypertrophic
miR-214	---	[100]	pro-hypertrophic
miR-22	PTEN	[140]	pro-hypertrophic
	Purb	[141]	pro-hypertrophic
	PGC-1α, PPARα and SIRT1	[142]	pro-hypertrophic
	Sirt1; Hdac4	[143]	pro-hypertrophic
miR-221	p27	[144]	pro-hypertrophic
miR-23a	---	[100]	pro-hypertrophic
	MuRF1	[145]	pro-hypertrophic
	FoxO3	[146]	pro-hypertrophic
	LPA1	[147]	pro-hypertrophic
miR-23b	---	[100]	pro-hypertrophic
miR-24 …?	---	[100]	pro-hypertrophic
	JP2	[148, 149]	pro-hypertrophic
miR-26b	Gata4	[150]	anti-hypertrophic
miR-27b	PPAR-γ	[151]	pro-hypertrophic
miR-30c	CTGF	[152]	anti-hypertrophic
miR-34	VEGF; Vinculin; Pofut1; Notch1; Sema4b	[153]	pro-hypertrophic
miR-350	MAPK11/14; MAPK8/9	[154]	pro-hypertrophic
miR-378	Grb2	[155]	anti-hypertrophic
	Grb2; Igf1r; Ksr1; Mapk1	[156]	anti-hypertrophic
miR-499	---	[157]	pro-hypertrophic
	---	[158]	pro-hypertrophic
miR-9	NFATc3; myocardin	[159]	anti-hypertrophic
miR-98/let-7	Cyclin D2	[160]	anti-hypertrophic

1.4.4. miRNAs in cardiac fibrosis regulation

One of the most highly consistent up-regulated miRNA in hypertrophy is miR-21. However, miR-21 was shown to be low expressed in cardiomyocytes, high expressed in cardiac fibroblasts. Through the targeting of Sprouty homolog 1 (Spry1), a negative regulator of the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signalling pathway, miR-21 was described as a strong fibrosis regulator.[111] Furthermore, miR-21 also represses PTEN, a negative regulator of PI3K/Akt cascade.[104]

Connective tissue growth factor (CTGF), one of the main factors mediating fibrosis, was validated as a target of miR-133b and miR-30, both consistently down-regulated in several models of hypertrophy (Table 1). This inversely correlates with the up-regulation of the protein.[152] MiR-133a on its turn was described as targeting Col1A1 and is down-regulated during AngII-induced fibrosis, along with miR-29b.[161]

Other miRNAs rather interfere with ECM genes. The miR-29 family, for example, is characterized as being down-regulated after myocardial infarction, caused by an up-regulation of TGFβ. Down-regulation and over-expression of this miRNA family led to an induction and a reduction, respectively, of collagen expression. The authors concluded that miR-29 is a regulator of cardiac fibrosis and represents a potential therapeutic target for tissue fibrosis in general.[105]

Table 3 shows a summary of these and other miRNAs directly connected to fibrosis development, as well as their described targets.

Table 3. miRNAs directly involved in cardiac fibrosis.

miRNA	Validated Target(s)	Reference	Action
miR-122	TGFß	[162]	anti-fibrotic
miR-133a	Col1A1	[161]	anti-fibrotic
miR-133b	CTGF	[152]	anti-fibrotic
miR-21	Spry1	[111]	pro-fibrotic
	MMP2; PTEN	[104]	pro-fibrotic
miR-24	Furin	[163]	anti-fibrotic
miR-26a	Col1; CTGF	[164]	
miR-29	Col1A1; Col1A2; Col3A1; FBN1; ELN1	[105]	anti-fibrotic
miR-30c	CTGF	[152]	anti-fibrotic

A recent report on a miRNA expression profile of human biopsies of severe and non-severe fibrosis patients showed that miR-122 and miR-18b were down-regulated in severe compared to non-severe fibrosis patients. Additionally, the report shows in human fibroblasts the influence of miR-122 on fibrosis, targeting TGF-β1 and preventing its up-regulation.[162] MiRNAs are currently considered as important fibrosis regulators, not only on the heart but also in other tissues. Many of these fibrosis-related miRNAs are being considered for therapeutic purposes.

1.5. miRNAs regulation by E2

A number of transcription factors have been identified as stimulating or inhibiting the transcription of specific miRNAs, either in normal or disease conditions. Here we focus on the regulation by E2, studied and described in several systems. Similar to protein coding genes, E2 stimulation also modulates the transcription of several miRNAs. One of the first studies revealing E2 effect on miRNA expression, and the first *in vivo*, was performed using zebrafish and revealed a novel pathway for oestrogen regulation. The authors found an association between E2 and the expression of 25 miRNAs after 12h of treatment, some of them even showing tissue specificity.[165]

However, due to the hormone role in breast cancer, most of what is currently known about oestrogen effect on miRNA expression comes from studies performed in the scope of breast cancer. Other studies in rodents identified E2 effects on miRNA expression in the mouse uterus[166], a traditional oestrogen target tissue. Table 4 reflects some lack of consistency of oestrogen-regulated changes in miRNA expression, even within MCF-7 cells, the best studied system. These differences can eventually be attributed to different times and treatment conditions, E2 concentration, the hormone content of the serum, differences in MCF-7 cells between laboratories, the housekeeping gene and the method for miRNA quantification.

Table 4. Reported E2 effect on miRNA expression.

Reference	Cell type	Species	Sex	Induced by E2		Repressed by E2	
[167]	MCF-7	Human	♂			miR-206	
[165]	---	zebrafish	♂	miR-196b let-7h let-7d		miR-130c miR-130a miR-101a	
[168]	MCF-7	Human	♀	let-7a let-7b let-7c let-7d let-7e let-7f let-7g let-71 miR-103 miR-107 miR-17-5p	miR-200a miR-200b miR-200c miR-203 miR-21 miR-23a miR-30b miR-30c miR-424 miR-98	miR-143 miR-27a miR-27b miR-302b miR-506 miR-524 miR-9	
[169]	MCF-7	Human	♀			let-7a let-7c let-7f let-g miR-181a miR-181b miR-181d miR-193a miR-193b miR-200a miR-200c miR-203	miR-21 miR-23a miR-23b miR-24 miR-26a miR-26b miR-27a miR-27b miR-499 miR-520d* miR-98
[170]	MCF-7	Human	♀			miR-16 miR-143	

Reference	Cell type	Species	Sex	Induced by E2		Repressed by E2
						miR-203
[171]	MCF-7	Human	♀	let-7c let-7d let-7g let-7i miR-106b miR-151 miR-15a miR-15b miR-16 miR-182 miR-183 miR-195 miR-200a miR-200b miR-203	miR-20a miR-23a miR-23b miR-25 miR-26a miR-26b miR-27a miR-27b miR-30b miR-365 miR-489 miR-7 miR-92 miR-98	let-7a let-7f miR-149 miR-200c miR-21 miR-320 miR-328 miR-342 miR-423
[172]	hMSMC	Human		miR-26a		miR-21
	hLSMC	Human				miR-26a
[173]	hESC/ HGSC	Human		miR-26a		miR-20a miR-21
[174]	hESC	Human		miR-17-5p miR-542-3p		miR-23a/b
	hGEC	Human		miR-17-5p miR-23b		miR-542-3p miR-23a
[175]	MCF-7	Human	♀			miR-21

MCF-7: breast cancer cell line. hMSMC: human myometrial smooth muscle cells. hLSMC: human lung smooth muscle cells. hESC: human embryonic stem cells. hGSC: high-grade serous carcinoma cells. hGEC: human glomerular epithelial cells.

Because oestrogen does not only regulate specific miRNA expression, but may also have a global effect on their biogenesis, some of its key components were analysed concerning a possible regulation by this hormone. From these miRNA biogenesis involved components, Dicer showed to be up-regulated by E2.[168]

Due to the fact that MCF-7 cells lack of endogenous ERβ, studies often focus on ERα, and less is known about ERβ regulation of miRNAs expression. However, E2-induced ERβ binding sites were identified in MCF-7 cells engineered to express comparable levels of both receptors. In this report, miR-206 was shown to be up-regulated by ERβ selective ligand DPN.[167] Another study reported 73 miRNAs differentially expressed in $ER\beta^{+}/ER\beta^{-}$ MCF-7 cells, being 44 increased and 29 decreased.[176]

To our knowledge, no one reported previously any study on miRNA expression regulation by ERβ in a non-cancer model with the endogenous receptor. The heart, as an ERβ-expressing tissue, is thus an interesting model to study miRNA regulation by the receptor and specifically its possible role on sex-differences in cardiac hypertrophy.

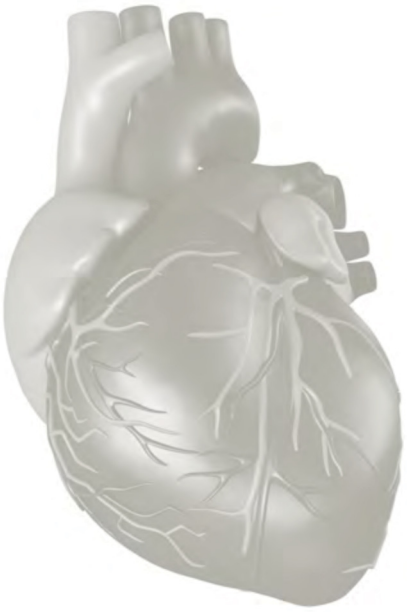
1.6. Aims of the work

The present study aims to reveal sex differences in miRNA expression in the normal and hypertrophic heart, as well as a possible role of E2 and its receptors.

To achieve these aims, we designed the project dividing it in 2 different parts, using *in vivo* samples originated for a previous project and *in vitro* experiments for the different compound treatments.

To unveil possible sex differences in miRNA expression *in vivo*, we quantified the expression of miRNAs in male and female cardiac tissue of WT Sham and TAC operated animals and compared the results with identical samples but from ERβ$^{-/-}$ animals, addressing the consequences of the receptor's deletion on miRNA expression in normal and hypertrophic hearts.

In order to analyse the effects of E2 on miRNA expression in the heart, we quantified their expression in both cardiomyocytes and cardiac fibroblasts after a treatment with the hormone and to clarify which of the receptors played a major role in each cell type, we also treated cells with ERα or ERβ specific agonists for the same measurements.



2. Material

2.1. Biological material

2.1.1. Animals

2.1.1.1. Mouse strains

The mice samples used in this project were generated for previous projects. The strain used was C57BL/6 and the animals were 12-14 weeks old at the time of the surgery.

- wildtype (WT)

Table 5. Wild type mice used.

	WT			
Sex	**Females**		**Males**	
Surgery	**Sham**	**TAC**	**Sham**	**TAC**
n=	7	10	10	10

- ERβ knock-out

Table 6. ERβ knock-out mice used.

	$ERβ^{-/-}$			
Sex	**Females**		**Males**	
Surgery	**Sham**	**TAC**	**Sham**	**TAC**
n=	8	8	8	8

- ERα knock-out

Table 7. ERα knock-out mice used.

	Sham			
Sex	**Females**		**Males**	
Genotype	**WT**	**$ERα^{-/-}$**	**WT**	**$ERα^{-/-}$**
n=	5	5	5	5

2.1.1.2. Rat strain

Adult Wistar rats were used for the isolation of cardiac fibroblasts.

2.1.2. Cardiomyocyte cell line

The cell line used was a human cardyomyocyte cell line, named AC16.[177] The cells were originated from female human ventricular tissue. These cells have retained the nuclear and the mitochondrial DNA of the primary cells, expressing myogenic markers and a fully respiratory chain. They can be frozen and thawed repeatedly, being useful in the study of developmental regulation of cardiomyocytes in normal and pathological states.

2.2. Olignucleotides for quantitative real time PCR

All the oligonucleotides were designed by us and synthesized by Thermo Scientific (Germany).

2.2.1. miRNA quantification

Table 8. Oligonucleotide sequences used for miRNA quantification.

miRNA	Oligonucluotide sequence
mmu-let-7b	TGAGGTAGTAGGTTGTGTGGT
mmu-let-7c	TGAGGTAGTAGGTTGTATGGTT
mmu-let-7d	AGAGGTAGTAGGTTGCATAGTT
mmu-let-7e	TGAGGTAGGAGGTTGTATAGTT
mmu-let-7g	TGAGGTAGTAGTTTGTACAGTT
mmu-let-7i	TGAGGTAGTAGTTTGTGCTGTT
mmu-miR-100	ACCCGTAGATCCGAACTT
mmu-miR-103	GCAGCATTGTACAGGGC
mmu-miR-106a	AAGTGCTAACAGTGCAGGTAG
mmu-miR-106b	TAAAGTGCTGACAGTGCAGAT
mmu-miR-107	GCAGCATTGTACAGGGC
mmu-miR-130a	AGTGCAATGTTAAAAGGGC
mmu-miR-133a	CCCCTTCAACCAGCTG
mmu-miR-133b	GTCCCCTTCAACCAGCTA
mmu-miR-143	TGAGATGAAGCACTGTAGCTC
mmu-miR-145	GTCCAGTTTTCCCAGGAAT
mmu-miR-149	CTGGCTCCGTGTCTTCA
mmu-miR-152	AGTGCATGACAGAACTTGG
mmu-miR-154	TAGGTTATCCGTGTTGCCT
mmu-miR-15a	AGCAGCACATAATGGTTTG
mmu-miR-15b	TAGCAGCACATCATGGTTTAC
mmu-miR-16	TAGCAGCACGTAAATATTGG
mmu-miR-181a	ACATTCAACGCTGTCGG
mmu-miR-185	AGAGAAAGGCAGTTCCTGA
mmu-miR-193	CCCACAAAGTCCCGC
mmu-miR-195	GCAGCACAGAAATATTGGC
mmu-miR-199a-3p	ACAGTAGTCTGCACATTGGTTA

mmu-miR-199a-5p	CCCAGTGTTCAGACTACCTG
mmu-miR-199b-5p	CCCAGTGTTTAGACTACCTGTT
mmu-miR-19b	TGTGCAAATCCATGCAA
mmu-miR-203	ATGTTTAGGACCACTAG
mmu-miR-208a	TAAGACGAGCAAAAAGCTTG
mmu-miR-20a	TAAAGTGCTTATAGTGCAGGTAG
mmu-miR-21	TAGCTTATCAGACTGATGTTGA
mmu-miR-212	AACAGTCTCCAGTCACGG
mmu-miR-22	AAGCTGCCAGTTGAAGAA
mmu-miR-221	AGCTACATTGTCTGCTGGG
mmu-miR-222	AGCTACATCTGGCTACTGG
mmu-miR-23a	TCACATTGCCAGGGATTT
mmu-miR-23b	ATCACATTGCCAGGGATTAC
mmu-miR-24	GGCTCAGTTCAGCAGG
mmu-miR-26a	TTCAAGTAATCCAGGATAGGCT
mmu-miR-27a	CACAGTGGCTAAGTTCCG
mmu-miR-27b	TCACAGTGGCTAAGTTCTGC
mmu-miR-29a	TAGCACCATCTGAAATCGG
mmu-miR-29b	TAGCACCATTTGAAATCAGTG
mmu-miR-29c	GCACCATTTGAAATCGGTTA
mmu-miR-290-5p	CAAACTATGGGGGCACTT
mmu-miR-301a	CAGTGCAATAGTATTGTCAAAG
mmu-miR-30a	GTAAACATCCTCGACTGGAAG
mmu-miR-30b	TGTAAACATCCTACACTCAGCT
mmu-miR-30c	TGTAAACATCCTACACTCTCAGC
mmu-miR-30d	ACATCCCCGACTGGAAG
mmu-miR-30e	TGTAAACATCCTTGACTGGAAG
mmu-miR-34a	GCAGTGTCTTAGCTGGTTGT
mmu-miR-378	ACTGGACTTGGAGTCAGAAG
mmu-miR-486	TACTGAGCTGCCCCGA
mmu-miR-497	CAGCAGCACACTGTGGTTT
mmu-miR-499	TTAAGACTTGCAGTGATGTTT
mmu-miR-99a	ACCCGTAGATCCGATCTTG
Universal Primer	
Universal Primer	Qiagen

Table 9. Endogenous reference genes oligonucleotides used for quantification

Endogenous reference genes (miScript PCR controls)	
HS_RNU1A-1	Qiagen
HS_RNU6B-2	Qiagen

2.2.2. mRNA quantification

Table 10. Oligonucleotide sequences used for mRNA quantification.

Gene	Oligonucleotide sequence
mm-Col1	FW: TGTAAACACCCCAGCGAAGAA
	RV: CTGAGTTGCCATTTCCTTGGA
mm-Col3	FW: CTCACCCTTCTTCATCCCACTCTTA
	RV: ACATGGTTCTGGCTTCCAGACAT
mm-Hprt	FW: GCTTTCCCTGGTTAAGCAGTACA
	RV: ACACTTCGAGAGGTCCTTTTCAC

2.3. Consumables and chemicals

2.3.1. Buffers and media

DMEM medium	Sigma-Aldrich, St. Louis, USA
DMEM medium without Phenol red	Sigma-Aldrich, St. Louis, USA
DMEM/F12	Gibco® Life technologies™, USA
Dulbecco's PBS (1x), ohne Ca & Mg	PAA

2.3.2. Chemicals

Angiotensin II	Calbiochem, USA
Dextrin	Sigma-Aldrich, St. Louis, USA
Chloroform	Carl Roth, Karlsruhe, Germany
DEPC-treated H_2O	Carl Roth, Karlsruhe, Germany
Estradiol, water soluble	Sigma-Aldrich, St. Louis, USA
Ethanol (pure)	Merck, Darmstadt, Germany
Ethanol (denatured)	Herbeta Arzneimittel Detlef Karlowski
FCS	Biochrom AG, Germany
FCS-CS	Life Technologies, Carlsbad, USA
Isopropanol	Carl Roth, Karlsruhe, Germany
Norepinephrin	Sigma-Aldrich, St. Louis, USA
Penicillin/Streptomycin	PAA
PSG (L-Glutamine with Penicillin/Strep)	PAA
Power Sybr Green PCR Master Mix	Life Technologies, Carlsbad, USA
RNA-Bee, RNA Isolation Reagent	Tel-Test
SYBR® Green PCR Master Mix	Qiagen, Hilden, Germany
Trypsin/EDTA	Sigma-Aldrich, St. Louis, USA
Water for molecular biology	Millipore
DNAse I	Roche, Mannheim, Deutschland

NaCl	Sigma-Aldrich, St. Louis, USA
Na_2HPO_4	Sigma-Aldrich, St. Louis, USA
HEPES	Sigma-Aldrich, St. Louis, USA
Glucose	Sigma-Aldrich, St. Louis, USA
BSA	PPA, Brussels, Belgium
Collagenase type I	Worthington , Lakewood, USA
Dispase	Gibco, Darmstadt, Germany
$CaCl_2$	Sigma-Aldrich, St. Louis, USA

2.3.3. Kits

Agilent RNA 6000 Nano Ladder	Agilent Technologies, Santa Clara, USA
RNA 6000 Nano Kit	Agilent Technologies, Santa Clara, USA
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems, USA
Fast SYBR® Green Master Mix	Life Technologies, Carlsbad, USA
miScript I RT Kit	Qiagen, Hilden, Deutschland
miScript SYBR Green PCR Kit	Qiagen, Hilden, Deutschland
Power SYBR® Green PCR Master Mix	Life Technologies, Carlsbad, USA

2.4. Devices

7300 Real-Time PCR System	Life Technologies, Carlsbad, USA
Agilent 2100 Bioanalyzer	Agilent Technologies, Santa Clara, USA
Analytical Balance ALT 220-5DAM	Kern & Sohn GmbH, Balingen, Germany
Eppendorf Mastercycler® gradient	Eppendorf, Hamburg, Deutschland
Eppendorf Centrifuge 5417 R	Eppendorf, Hamburg, Germany
HeraCell Incubator	Heraeus, Germany
Hettich Microcentrifuge 22R	Tuttlingen, Germany
NanoDrop ND-1000	Thermo Fisher Scientific, Waltham, USA
StepOnePlus™ System	Life Technologies, Carlsbad, USA
Holten Laminair	Holten Lamina Air, Denmark
Waterbath	Memmert, Germany

2.5. Software and databases

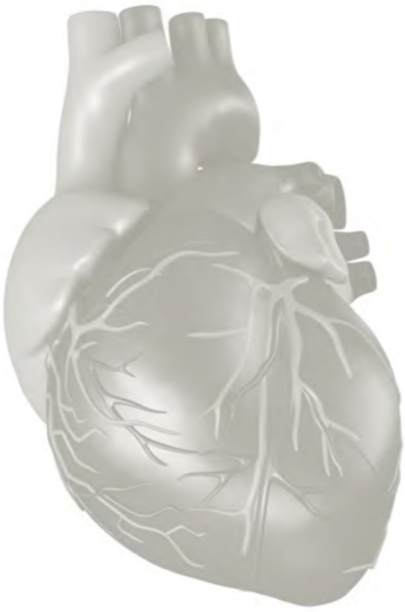
GraphPad Prism 6	La Jolla, USA

ABI Prism Taqman	Applied Biosystems
EndNote	Thomson Reuters

http://www.ncbi.nlm.nih.gov/pubmed, National Institutes of Health, USA
http://www.targetscan.org, Whitehead Institute for Biomedical Research, MIT, USA
http://cpdb.molgen.mpg.de, Max-Planck Institute for Molecular Genetics, Germany

2.6. Other

Adhesive Seal Applicator	Life Technologies, Carlsbad, USA
Cell culture plates and flasks	Biochrom, Berlin, Germany
Combitips Plus	Eppendorf, Hamburg, Germany
Eppendorf Pipette tips	Eppendorf, Hamburg, Germany
Eppendorf reaction tubes	Eppendorf, Hamburg, Germany
Falcon tubes	BD Biosciences, NJ, USA
MicroAmp Optical 96-Well Reaction Plate	Life Technologies, Carlsbad, USA
MicroAmp Fast Optical 96-well Reaction Plate	Life Technologies, Carlsbad, USA
Optical Adhesive Covers	Life Technologies, Carlsbad, USA
Serological pipettes	Carl Roth, Karlsruhe, Germany



3. Methods

3.1. Computational analysis of mRNA microarrays results

The starting point for this project was the gene expression profiling (microarrays) already published.[38] The samples used for the array, were cardiac tissue (LV) from C57BL/6N mice, males and females, 9 weeks after TAC operation, using Sham operated animals as a control group. The genes were selected by 30% up- or down-regulation when comparing TAC to Sham operated mice.

The results were analysed by the online software TargetScan Mouse database (http://www.targetscan.org/mmu_61). This software predicts possible biological targets for miRNAs, detecting the presence of 7- or 8-mer sites in the gene‘s 3’ UTRs that match the seed region of each miRNA.

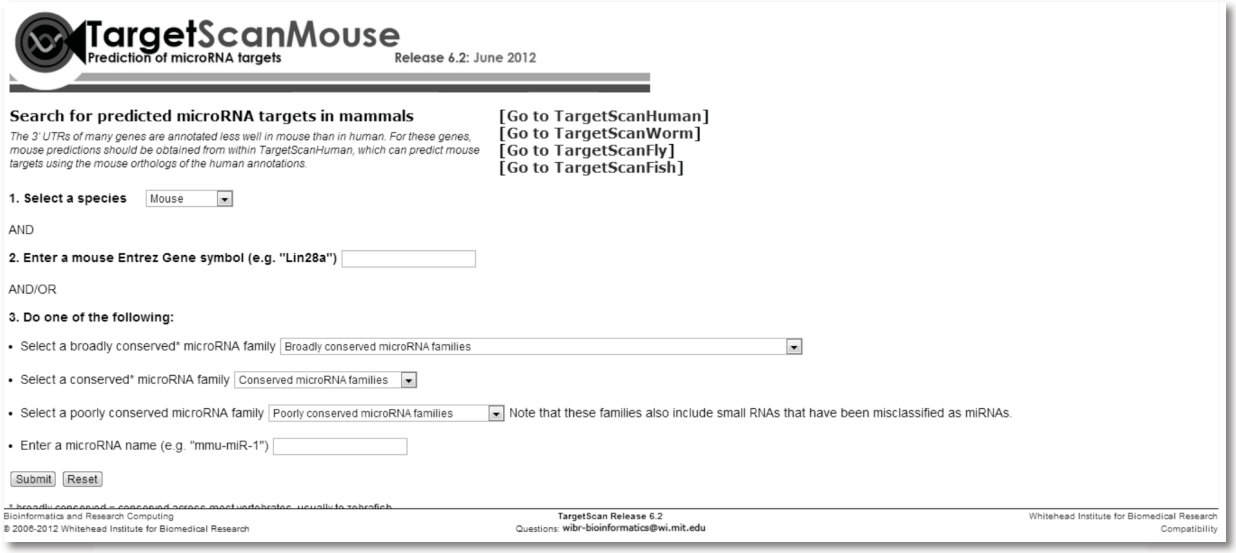


Figure 5. TargetScan start screen. This online software allows the prediction of possible biological targets for miRNAs, through the detection of binding sites.

The search was then limited to “conserved sites” and “miRNAs families broadly conserved among vertebrates”.

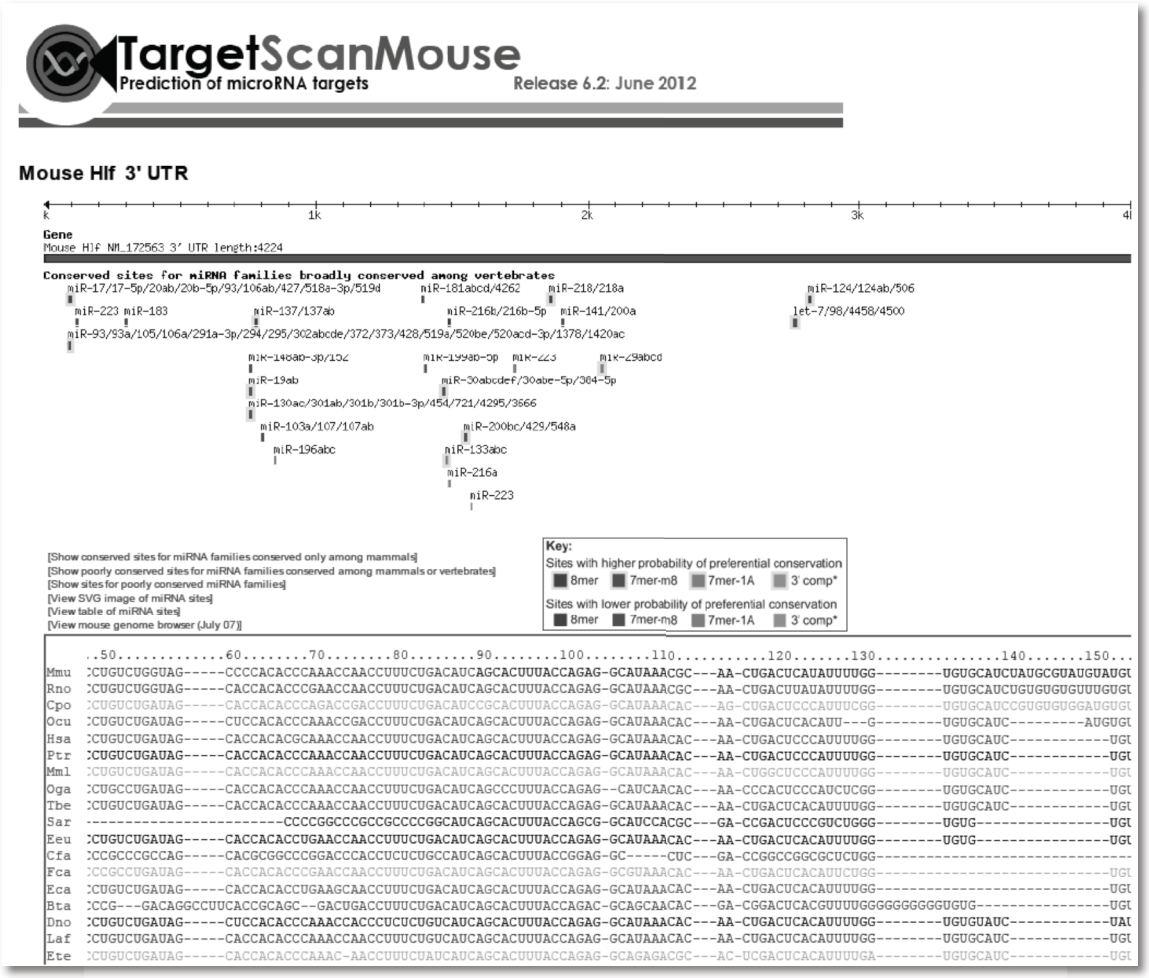


Figure 6. TargetScan results screen.

The final list of miRNAs selected for qRT-PCR was obtained by selection of the miRNAs that are expressed in the heart, according to a miRNA microarray previously done in our group.

3.2. Over-representation/enrichment analysis of genes

The over-representation/enrichment pathway analysis of the genes putatively targeted by miRNAs with significant sex*surgery interaction effect was performed with the ConsensusPathDB-mouse (http://cpdb.molgen.mpg.de/ MCPDB).[178-181]

This database integrates different types of interactions from numerous resources into a global network. In this network, physical entities (genes, proteins, metabolites, etc.) from different sources are matched depending on their accession number and interactions are matched depending on their participants to reduce data redundancy. ConsensusPathDB allows searching, visualising and retrieving the integrated interaction data, and might complement other tools that use these data for interaction- and pathway-centric analysis of genes, proteins and metabolites.[178-181]

In order to perform this analysis, the group miRNAs with significant sex*surgery interaction effect was analysed with TargetScan to find the genes that could be putatively targeted by them. This obtained list was then shortened, excluding the genes that were only targeted by one miRNA.

The final gene group was analysed by ConsensusPathDB-mouse through the over-representation analysis and the pathways were selected according to their involvement in cardiac hypertrophy.

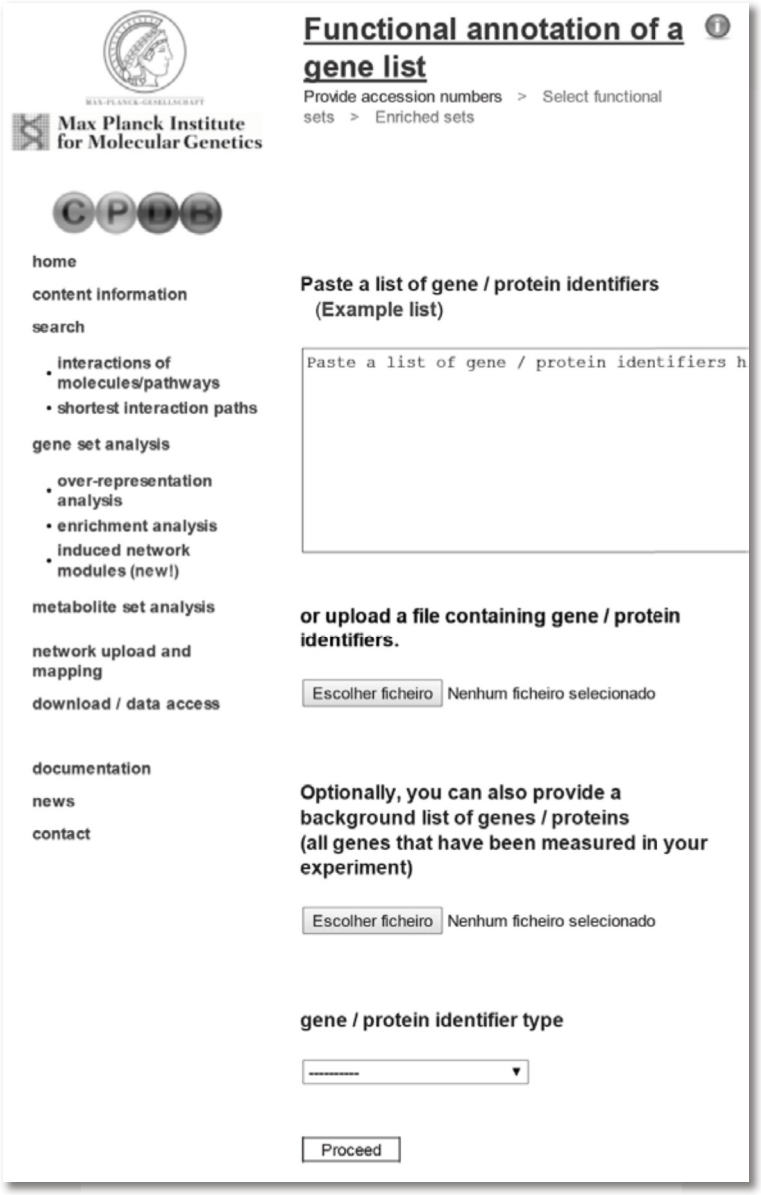


Figure 7. ConsensusPathDB: over-representation analysis start screen.

3.3. Rat cardiac fibroblasts isolation

3.3.1. Buffers and solutions

3.3.1.1. Collagenase/Dispase buffer

Table 11. Collagenase/Dispase buffer components I.

Compound	Quantity
DNAse	20mg (>2000U/mg)
NaCl	16g
KCl	0.74g
Na_2HPO_4	0,2g
HEPES	12g
Glucose	4g
Water	up to 2L

- set to pH 7,3
- sterile filtered
- stored at -20ºC in 500mL aliquots

- added before starting:

Table 12. Collagenase/Dispase buffer components II.

Compound	Quantity
BSA	5g/500mL
Collagenase type I (187U/mg)	2mg/mL
Dispase (6U/mg)	2mg/mL

3.3.1.2. Solutions

CaCl2 solution (sterile filter) 0.1M

3.3.2. Preparation

Before starting, we prepared per each heart:

- 1x 50mL Falcon tube with 25mL Collagenase/Dispase buffer (room temperature) + 120µL 2.5% Trypsin/EDTA + 188µL 0.1M $CaCl_2$
- 1x 50mL Falcon tube with 25mL PBS (on ice)
- 1x 15mL Falcon tube with 10mL PBS (on ice)
- 1x 50mL Falcon tube with 15mL FCS
- 1x 50mL Falcon tube with 10mL DMEM-Medium (without supplements)

3.3.3. Medium

Table 13. Rat cardiac fibroblasts medium components.

Compound	Quantity (per 100mL)
DMEM (high Glucose)	88mL
FCS (10%)	10mL
PSG (1%)	1mL
HEPES (1%)	1mL

3.3.4. Procedure

The rats were euthanized by throat cut after narcotisation with isofluoran. The heart was immediately removed and the LV was transferred to a 25mL Falcon tube with cold DMEM medium (10mL). Next, the erythrocytes were washed in a new Falcon tube containing 25mL of PBS (cold) and placed in a Petri dish with 10mL of new PBS (cold) to dissect in 1-2mm^2 fragments. The tissue fragments and the PBS were transferred with a pipette into a 15mL Falcon, left to sediment, washed repeatedly with PBS until the erythrocytes were completely eliminated and placed on ice.

After a new wash with PBS, 5mL of Collagenase/Dispase buffer were added and the Falcon tube was placed in the water bath (37ºC; agitation) for 15min. The tubes were vigorously shaken, and left to sediment. The supernatant (containing the fibroblasts) was removed for a new 50mL tube containing FCS (15mL) and the procedure was repeated until the fragments were totally digested.

After all the tissue was digested, the tubes were centrifuged (500xg at 4ºC) for 5min. The medium was removed and the pellet washed with 10mL of PBS. After a new centrifugation (500xg at 5min), the supernatant was removed and the pellet re-suspended in 10-20mL of full medium and kept on ice.

In order to obtain homogeneous samples, the biological samples were pooled (male and female cells kept separate), plated in 10cm cell culture dishes (10mL/dish) and incubated (37ºC; 5% CO_2). After 1h, the medium was removed, the cells were washed with PBS (2x 10mL/dish) and new full medium was added (passage 0). During the first two days, the medium was changed daily and after every second day.

When full confluence was achieved, the medium was removed and the plate washed with PBS (10mL/dish). The cells were then detached using 2mL Trypsin/EDTA (37ºC; 5%CO_2; ≈5min) and the reaction was stopped with full medium (6mL). The cell suspension was transferred to 50mL Falcon tubes and centrifuged (500xg at 10ºC). The supernatant was removed and new full medium was added (20mL per number of initial dishes). The cell suspension was then distributed (10mL/dish) for a 1:2 passage (passage 1) and incubated (37ºC; 5% CO_2). The different treatments were performed at passage 1.

3.4. Cell culture

3.4.1. Cardiomyocyte cell line

The AC16 cells were cultured in DMEM/F12 medium, supplemented with 12.5% FCS and 1% Penicillin/Streptomycin, in T75 bottles. They were incubated under at 37ºC and 5% CO_2.

The cells were detached using 3mL of Trypsin, during 3-5 min at 37ºC, 5%CO_2 and the reaction was stopped using 9mL of supplemented medium. They were then seeded in 24-well plates in supplemented medium and incubated O.N. at 37ºC, 5%CO_2.

24h before the start of each stimulation, the medium was changed for a starvation medium, which consisted of DMEM/F12 medium without phenol red, supplemented with 2.5% FCS and 1% Penicillin/Streptomycin.

The stimulations were performed adding 10^{-8}M of E2 or 10^{-7}M of ERα or ERβ agonist to a DMEM/F12 medium without phenol red, supplemented with 2.5% FCS and 1% Penicillin/Streptomycin, during 3, 6, 18, 24 and 48h. The medium was changed every day.

The cells were collected using 500µL of RNAzol per well, for total RNA isolation.

3.4.2. Primary rat cardiac fibroblasts

Rat primary cardiac fibroblasts were cultured in DMEM medium with phenol red, supplemented with 10% FCS, 1% PSG and 1% HEPES.

Prior to the cell treatment (with 10^{-8}M E2, 10^{-7}M ERα agonist, 10^{-7}M ERβ agonist, 10^{-7}M AngII or 10^{-8}M E2 + 10^{-7}M AngII) the cells were washed with PBS (10mL/dish) and starved for a period of 24h in phenol red-free medium containing 2.5% FCS-CS, 1% PSG and 1% HEPES (37ºC; 5% CO_2).

The cells were treated for an additional time of 24h in every case. In the case of the co-treatment with E2 and AngII, the cells were pre-incubated with E2 for 1h before AngII was added. The samples were collected using 500µL of RNAzol per well, for total RNA isolation.

3.5. Methods with RNA, cDNA and miRNA

3.5.1. Total RNA isolation

For 1000µL of cell lysate in RNAzol, 500µL of chloroform were added, homogenised through 2min of a strong vortexing and left for 5 min on ice. The lysates were then centrifuged 20800xg for 10 min at 4ºC. The aqueous phases were separated into new tubes. After the addition of an equal volume of 100% isopropanol, the samples were left O.N. at -20ºC for precipitation.

The day after, the samples were centrifuged 20800xg for 30 min at 4ºC. Next, the samples were washed with 750µL of ethanol and centrifuged 6800xg for 5 min at 4ºC. After the repetition of the washing step, the RNA was air-dried and resuspended in 20-50µL of DEPC-treated H_2O.

3.5.2. Qualitative and quantitative measurement of RNA

The quantitative measurement of the obtained RNA was performed using a NanoDrop ND-1000 device and the respective program (ND-1000 V3.3.0).

When a qualitative RNA measurement was required, we used a Agilent 2100 Bioanalyzer and the corresponding RNA 6000 Nano Kit.

3.5.3. Reverse transcription of mRNAs and miRNAs into cDNA

The reverse transcription (RT) of the RNA was performed using miScript I RT Kit and following the manufacturer's protocol. When RT reactions are performed using this kit, all RNA species are converted into cDNA. Mature miRNAs are polyadenylated by poly(A) polymerase and reverse transcribed into cDNA using oligo-dT primers. The oligo-dT primers have a 3' degenerate anchor and a universal tag sequence on the 5' end allowing amplification of mature miRNA in the qRT-PCR step. This allowed the miRNA quantification to be done using the sequence of the miRNA of interest as a 5' primer and the commercially available miScript Universal Primer as 3' primer. All other RNA species (including precursor miRNA, other noncoding RNA, and mRNA) are also converted into cDNA using oligo-dT and random primers. Polyadenylation and RT are performed in parallel in the same tube.

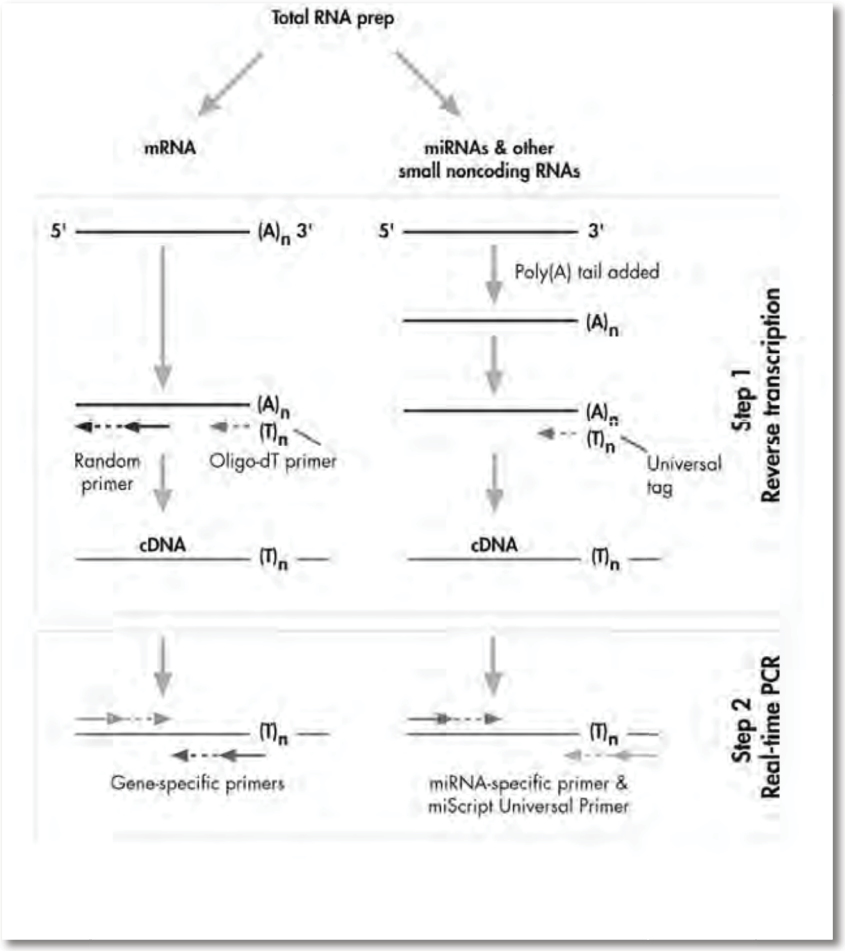


Figure 8. Reverse Transcription of mRNAs and miRNAs in cDNA with miScript I RT Kit (Qiagen).

3.5.4. RT reaction mix

Table 14. Reverse transcription reaction mix components.

Component	Vol/Reaction (µL)
miScript RT Buffer, 5x	2
miScript Reverse Transcriptase Mix	0,5
RNase-free water	(variable)
Template RNA	500ng
Total	10

3.5.5. RT Reaction protocol

Table 15. Reverse transcription reaction protocol.

Step	Temperature (ºC)	Duration (min)
1	37	60min
2	95	5min
3	4	∞

3.5.6. Quantitative real time PCR

3.5.6.1. Quantitative real time reaction mix

The quantitative real time PCR (qRT-PCR) reaction was performed using the following mix, in a total volume of 20 µL.

Table 16. Quantitative real time reaction mix components.

Component	Volume (µL)
PowerSYBR Green / FastSYBR Green	10
3' Primer (5 pmol/µL)	0,8
5' Primer (5 pmol/µL)	0,8
H2O	4
cDNA (125ug/uL)	4
Total	20 µL

3.5.6.2. Quantitative real time PCR protocol

3.5.6.2.1. Endogenous small RNAs control

Table 17. Small RNAs control quantitative real time PCR protocol.

Function	Temperature (ºC)	Duration (min)	Number of cycles
Denaturing	95	10	1
Denaturing	95	00:15	40
Annealing	55	1	
Elongation	70	1	
Dissociation curve	95	00:15	1
	60	1	
	95	00:15	

3.5.6.2.2. General miRNA quantification

Table 18. General miRNA quantification real time protocol

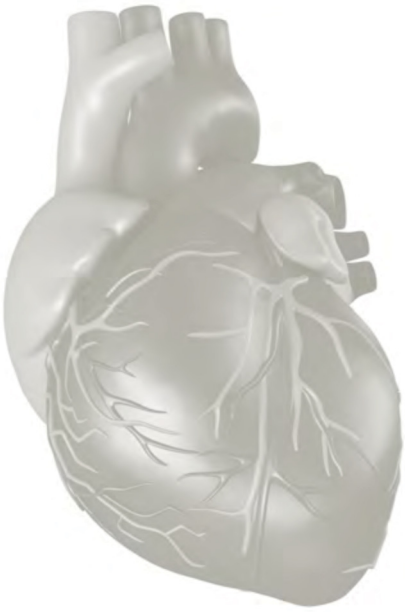
Function	Temperature (ºC)	Duration (min)	Number of cycles
Denaturing	95	10	1
Denaturing	95	00:15	40
Annealing	55	1	
Dissociation curve	95	00:15	1
	60	1	
	95	00:15	

3.6. Statistical analysis

The statistical analysis was performed using the software GraphPad Prism, version 6.03 (GraphPad Software Inc.). The statistical tests were chosen according to the groups of samples in each experiment.

Table 19. Statistical analysis performed in the results of each experiment.

Experiment	Statistical Test	Post-Hoc test
WT mice, Sham/TAC, Male/Female	two-way ANOVA	Bonferroni
ERβ$^{-/-}$ mice, Sham/TAC, Male/Female	two-way ANOVA	Bonferroni
Female mice; WT/ERβ$^{-/-}$	unpaired t-test	-
Sham mice; WT/ERβ$^{-/-}$; Male/Female	two-way ANOVA	Bonferroni
Female cardiomyocytes (AC16 cells)	unpaired t-test	-
Primary cardiac fibroblasts; E2 treatment	unpaired t-test	-
Primary cardiac fibroblasts; ERα and ERβ treatments	one-way ANOVA	Bonferroni
Sham mice; WT/ERα$^{-/-}$; Male/Female	two-way ANOVA	Bonferroni
Primary cardiac fibroblasts; AngII and AngII+E2 treatments	one-way ANOVA	Bonferroni



4. Results

4.1. Computational analysis of mRNA microarrays results

4.1.1. Genes show sex differences in their expression 9 weeks after TAC

Our group described previously sex-differences in gene expression in mice, 9 weeks after TAC surgery[60]. In this study, WT males exhibited a stronger induction of matrix related and cell proliferation genes as well as a repression of mitochondrial genes than female animals. ERβ$^{-/-}$ male mice revealed a stronger expression of pro-apoptotic genes with a higher expression in males. After TAC male WT animals developed higher fibrosis than their female siblings, but in the absence of ERβ the sex differences in cardiac fibrosis were abolished. WT TAC females also exhibited, 9 weeks after TAC, a better maintenance of cardiac energy metabolism, having mitochondrial function encoding genes less suppressed than males.

In order to identify the miRNAs with a possible sex-specific regulation in TAC, the genes that were sex-specifically regulated, up-/down-regulated in one of the sexes or regulated in an opposite way, were identified to analyse them concerning the existence of binding sites for miRNAs. Table 20 shows the list of sex-differently regulated genes utilized in the next step (TargetScan).

Table 20. Genes dysregulated in opposite ways in males and females.

Analysed genes							
Abca6	Cables1	D18Ertd289e	Fsip1	Lman1l	Olfr690	Rrbp1	Thoc1
Abcc3	Cacna1a	Dbil5	Fst	LOC230010	P2ry10	Rttn	Timp4
Abl1	Cacng6	Dbp	Gabrp	LOC667085	Padi4	Runx1	Tmem100
Ace2	Calcb	Dcamkl1	Gcnt2	LOC77413	Panx1	S100a8	Tmem108
Actn1	Ccdc16	Ddx3y	Glt25d2	Loxl2	Pax5	S100a9	Tmod3
Adamts6	Ccdc83	Depdc5	Gm129	Lrba	Pcdhb16	Scel	Tnfrsf9:
Adat1	Ccl28	Dirc2	Gm410	Mamdc1	Pdk4	Sele	Tnrc6c
Adh1	Ccnd3	Dlg2	Gnb3	Marcksl1	Per2	Senp8	Trim24
Agbl3	Ccnjl	Dnajc2	Gng8	Mark1	Phc2	Serpina1c	Trpm3
AI646023	Cdc2l5	Dnase1l2	Gp1ba	Mfap4	Phospho1	Serpina3n	Tsc22d3
Akr1c20	Cdca5	Dnm1	Grem1	Micalcl	Pik3r1	Serpine1	Ttr
Alg14	Cdon	Dock2	H2-K1	Mon2	Pkib	Sfrs12	Txnip
Angptl4	Cebpb	Dpp6	Hdh	Mpa2l	Plcxd3	Skp2	Tyr
Apitd1	Centd1	Dppa3	Hipk2	Mrc2	Pou2af1	Slc13a3	Uap1
Apoc3	Ces3	Edn3	Hlf	Ms4a1	Pou2f2	Slc26a3	Ubtf
Aqp4	Ces5	Egfr	Hmmr	Ms4a6d	Ppfia1	Slc2a10	Ucp3
Arsg	Chl1	Eif2s3y	Hsd17b11	Mt1	Prepl	Slc35f1	Ugcgl2
Atg9b	Clasp2	Enpp2	Hspa1a	Mtap1b	Prokr1	Slc39a6	Uty
Atp2c1	Clcn2	Evc2	Hspa1b	Mup1 /// Mup2	Psmd7	Slc7a9	Vav2
B3gat3	Cldn22	Eya4	Igfbp5	Myl7	Ptgs2	Snrpn	Vps33a
BB045044	Cntn4	Ezh1	Igk-V28	Nbr1	Punc	Snx15	Wdr20a
BC030477	Cpsf6	Fbxo17	Ints8	Ncor1	Pygo1	Sort1	Wnt5b
BC059841	Crispld1	Fetub	Iqwd1	Nek11	Rad50	Spbc25	Xist
Becn1	Cugbp1	Fgf14	Isg20l1	Nkd2	Rasgef1a	Stfa2l1	Xlkd1
Brms1	Cugbp2	Fgf7	Itga9	Nox4	Rbx1	Stmn3	Zbtb16
Brunol4	Cxcl7	Fkbp1b	Jarid1d	Npas2	Rhod	Sunc1	Zc3h13
Bst1	Cxxc6	Flrt2	Kcnh3	Npnt	Robo1	Syp	Zfp192
Bub3	Cyp20a1	Fmr1nb	Klf6	Nr4a1	Rorb	Sytl3	Zkscan1
C1qtnf3	Cyp2c38	Frem1	Klhdc8a	Nr4a3	Rps10	Tcte1	Zmynd19
C79407	D16Bwg1494e	Fscn1	Lima1	Olfm1	Rptn	Tef	

4.1.2. miRNAs predicted by TargetScan binding site analysis

The TargetScan analysis (gene target → miRNA) of the dysregulated genes in hypertrophy (previously published data; Table 20) allowed identifying miRNAs that could contribute to it, therefore being also possibly dysregulated in a sex-specific manner. The genes that did not have any predicted binding site and the genes that only showed putative binding sites for miRNAs that are not expressed in the heart have been withdrew. The analysis of the remaining 80 genes identified 157 different miRNAs with putative binding sites in their 3'UTR sequence. From these, 97 miRNAs were discarded because they were not detected in the heart. The remaining 60 miRNAs were selected for quantification using qRT-PCR, (Table 21).

Table 21. MiRNAs selected for further quantification

mmu-let-7b	mmu-miR-15b	mmu-miR-24
mmu-let-7c	mmu-miR-16	mmu-miR-26a
mmu-let-7d	mmu-miR-181a	mmu-miR-27a
mmu-let-7e	mmu-miR-185	mmu-miR-27b
mmu-let-7g	mmu-miR-193	mmu-miR-29a
mmu-let-7i	mmu-miR-195	mmu-miR-29b
mmu-miR-100	mmu-miR-199a-3p	mmu-miR-29c
mmu-miR-103	mmu-miR-199a-5p	mmu-miR-290-5p
mmu-miR-106a	mmu-miR-199b-5p	mmu-miR-301a
mmu-miR-106b	mmu-miR-19b	mmu-miR-30a
mmu-miR-107	mmu-miR-203	mmu-miR-30b
mmu-miR-130a	mmu-miR-208a	mmu-miR-30c
mmu-miR-133a	mmu-miR-20a	mmu-miR-30d
mmu-miR-133b	mmu-miR-21	mmu-miR-30e
mmu-miR-143	mmu-miR-212	mmu-miR-34a
mmu-miR-145	mmu-miR-22	mmu-miR-378
mmu-miR-149	mmu-miR-221	mmu-miR-486
mmu-miR-152	mmu-miR-222	mmu-miR-497
mmu-miR-154	mmu-miR-23a	mmu-miR-499
mmu-miR-15a	mmu-miR-23b	mmu-miR-99a

4.2. Altered miRNA expression in a hypertrophy mouse model

The first experimental approach of this project was made as a screening of the selected miRNAs in a mouse hypertrophy model. The samples consisted of 4 groups: WT males/females, Sham/TAC operated. The statistical analysis performed was two-way ANOVA, followed by Bonferroni post-hoc test.

The results are shown first as two-way ANOVA analysis results (Table 22). This analysis determines how a response is affected by two different factors, the sex and the surgery in this case, independently, as well as the sex*surgery interaction effect. The latter is a test whether the average surgery effect is the same for each sex or not.

Table 22. Two-way Anova analysis of miRNA quantification.

miRNA	two-way ANOVA		
	Surgery	Sex	Sex*Surgery Interaction
let-7b	***	0,068	ns
let-7c	ns	ns	ns
let-7d	0,068	ns	ns
let-7e	****	***	**
let-7g	*	**	ns
let-7i	***	ns	ns
miR-100	ns	ns	ns
miR-103	*	0,053	ns
miR-106a	*	*	ns
miR-106b	*	*	0,098
miR-107	ns	ns	ns
miR-130a	***	**	*
miR-133a	****	**	**
miR-133b	*	ns	*
miR-143	ns	*	0,052
miR-145	ns	0,084	***
miR-149	ns	ns	ns
miR-15a	**	*	ns
miR-15b	**	0,060	ns
miR-152	*	ns	ns
miR-154	*	ns	ns
miR-16	***	**	ns
miR-181a	ns	ns	ns
miR-185	ns	0,054	ns
miR-19b	*	*	0,069
miR-193b	ns	ns	ns
miR-195	***	ns	0,071
miR-199a-3p	***	ns	ns
miR-199a-5p	***	ns	ns

miRNA	two-way ANOVA		
	Surgery	Sex	Sex*Surgery Interaction
miR-199b-5p	***	ns	*
miR-20a	*	*	ns
miR-203	ns	ns	ns
miR-208a	ns	*	ns
miR-21	****	***	*
miR-212	*	ns	ns
miR-22	*	*	0,096
miR-221	***	ns	ns
miR-222	ns	ns	0,084
miR-23a	0,062	ns	**
miR-23b	*	*	*
miR-24	**	**	ns
miR-26a	**	*	0,067
miR-27a	*	**	ns
miR-27b	**	*	ns
miR-29a	***	**	*
miR-29b	ns	*	ns
miR-29c	0,090	ns	ns
miR-290-5p	****	ns	ns
miR-30a	ns	ns	ns
miR-30b	ns	ns	*
miR-30c	ns	ns	ns
miR-30d	ns	ns	ns
miR-30e	ns	**	0,052
miR-301a	*	0,078	ns
miR-34a	0,079	ns	ns
miR-378	ns	*	ns
miR-486	ns	ns	ns
miR-497	***	ns	**
miR-499	ns	*	ns
miR-99a	**	ns	ns

All the 60 miRNAs quantified by qRT-PCR in WT, male/female, Sham/TAC operated mice. The statistical analysis was performed with two-way ANOVA: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Note: The values $0.05<p<0.1$ were not discarded, as they could be important hints for further analysis.

4.2.1. Sex and LVH influence miRNA expression – summary of the two-way ANOVA analysis

4.2.1.1. Surgery effect – miRNAs dysregulated in hypertrophy

This statistical analysis of surgery effect compiles operated and non-operated animals in two different groups, without distinguishing males and females. The surgery effect was significant in 35 cases in the two-way ANOVA analysis (see Table 22), namely:

- let-7b, let-7e, let-7g, let-7i, miR-103, miR-106a, miR-106b, miR-130a, miR-133a, miR-133b, miR-15a, miR-15b, miR-152, miR-154, miR-16, miR-19b, miR-195, miR-199a-3p, miR-199a-5p, miR-199b-5p, miR-20a, miR-21, miR-212, miR-22, miR-221, miR-23b, miR-24, miR-26a, miR-27a, miR-27b, miR-29a, miR-290-5p, miR-301a, miR-497, miR-99a.

Figure 9 illustrates the surgery effect results. Males and females are represented together, according to the type of operation. The relative expression is represented as fold of the average of male and female Sham.

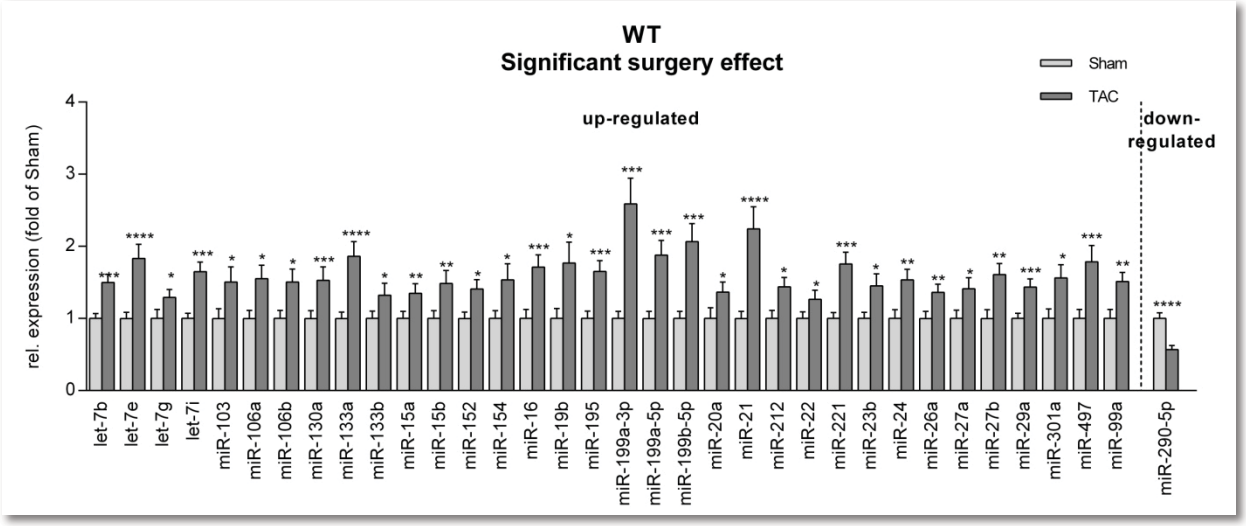


Figure 9. 35 miRNAs showed a significant surgery effect after two-way ANOVA analysis. 34 miRNAs presented an up-regulation as a TAC effect and only one was down-regulated after surgery (miR-290-5p).
* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Four miRNAs, let-7d, miR-23a, miR-29c and miR-34a, showed p-values between 0.1 and 0.05, being considered borderline values to a significant surgery effect. Figure 10. is a graphical representation of surgery effect on these miRNAs.

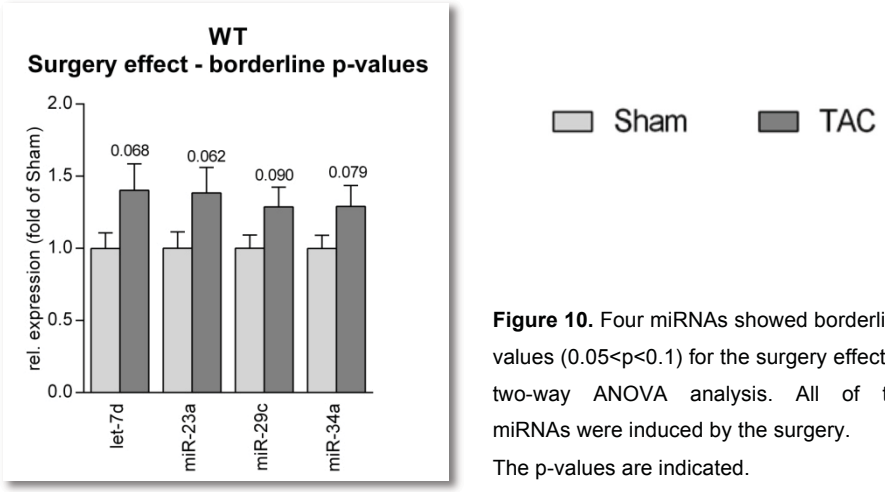


Figure 10. Four miRNAs showed borderline p-values (0.05<p<0.1) for the surgery effect after two-way ANOVA analysis. All of these miRNAs were induced by the surgery.
The p-values are indicated.

Finally, surgery didn't produce any significant effect in 21 miRNAs of the 60 analysed (Figure 11).

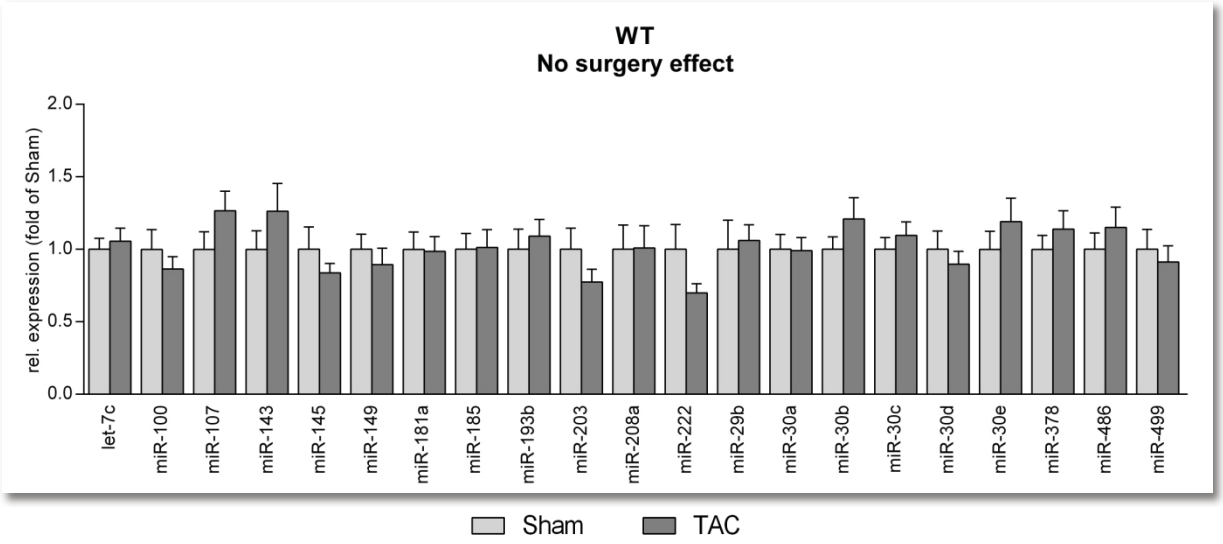


Figure 11. Twenty-one miRNAs didn't show surgery effect after two-way ANOVA analysis.

4.2.1.2. Sex effect – Sex differences in the miRNA expression in control and hypertrophic hearts

In the two-way ANOVA analysis the sex effect was significant for the expression of 24 miRNAs. In this case, the analysis compiles males and females in 2 different groups, without distinguishing between Sham and TAC surgery.

The sex effect might be due a number of different causes. In this project we focused on the E2 effect on miRNA expression. The miRNAs with a significant sex effect are:

- let-7e, let-7g miR-106a, miR-106b, miR-130a, miR-133a, miR-143, miR-15a, miR-16, miR-19b, miR-20a, miR-208a, miR-21, miR-22, miR-23b, miR-24, miR-26a, miR-27a, miR-27b, miR-29a, miR-29b, miR-30e, miR-378, miR-499

Figure 12 is a graphical representation of the sex effect on miRNA expression. Sham and TAC operated animals are represented together, according to the sex. The average expression of Sham and TAC male animals are represented as fold of the expression in female mice.

Interestingly, all 24 miRNAs with significant sex effect were higher expressed in males than in females.

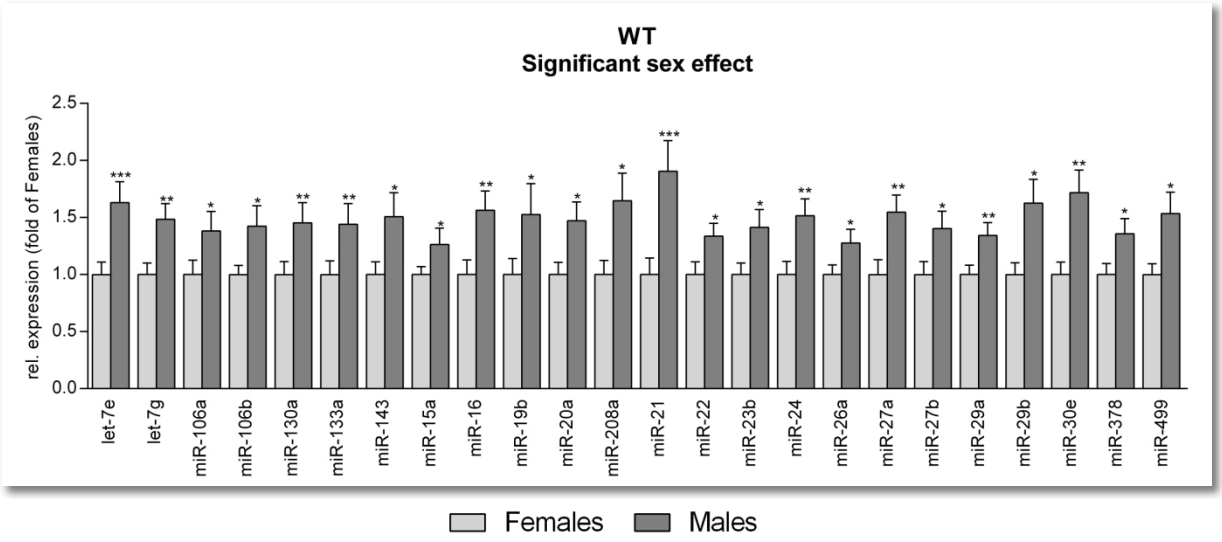


Figure 12. Twenty four miRNAs are stronger expressed in male mice. The values of Sham and TAC are represented together, according to the sex.

Two-way ANOVA; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Six miRNAs (let-7b, miR-103, mir-145, miR-15b, miR-185 and miR-301a) presented a borderline significance for the sex effect. Figure 13 shows the results for these miRNAs graphically.

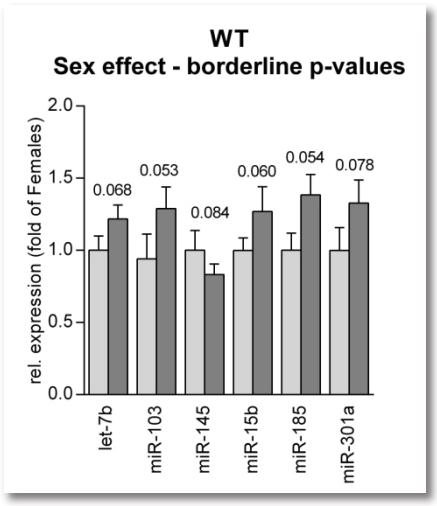


Figure 13. Six miRNAs showed a borderline p-value (0.05<p<0.1) for the sex effect after two-way ANOVA analysis. Let-7b, miR-103, miR-15b, miR-185 and miR-301a are higher expressed in males than in females, while miR-145 shows the opposite result.
The p-values are indicated.

In the case of 30 of the 60 miRNAs, sex didn't have any significant effect (Figure 14).

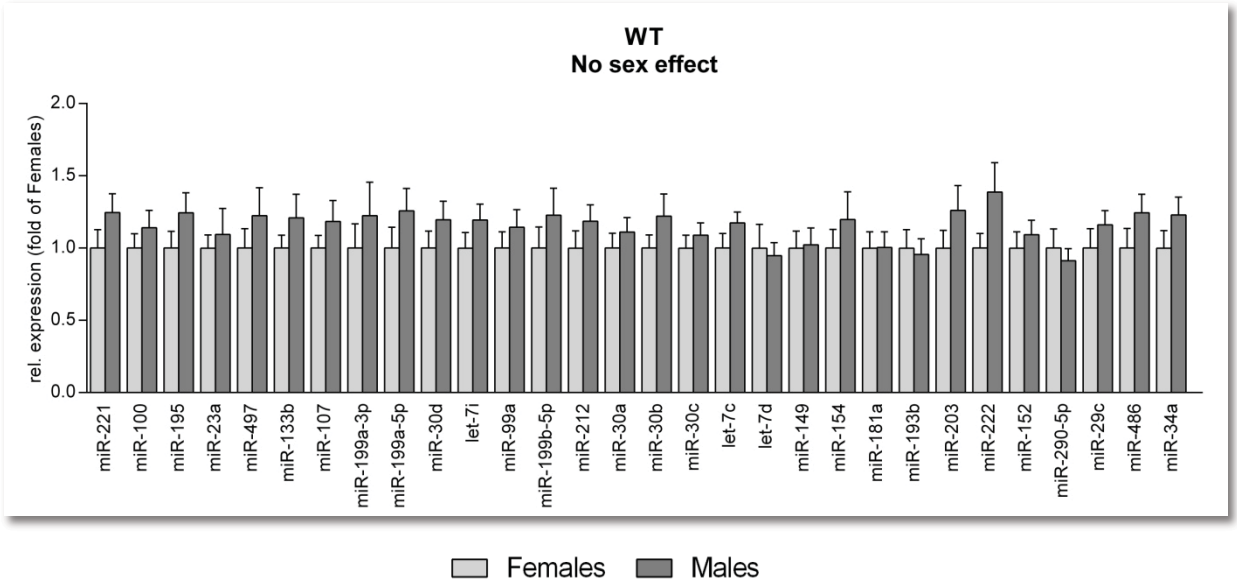


Figure 14. Thirty miRNAs didn't show a significant value for the sex effect after two-way ANOVA analysis.

4.2.1.3. Sex*Surgery interaction effect – Sex specific effect after surgery

The two-way ANOVA analysis shows also the effect of the interaction of the two factors explored in the experiment, sex and surgery. There were 12 miRNAs with a significant interaction effect, which means that the surgery had an opposite or at least a different effect in males and females, i.e. they represent a sex-specific effect of surgery in miRNA expression:

- let-7e, miR130a, miR-133a, miR-133b, miR-145, miR-199b-5p, miR-21, miR-23a miR-23b, miR29a, miR30b, miR-497

Other eight miRNAs presented borderline p-values, between 0.1 and 0.05: miR-106b, miR-143, miR-19b, miR-195, miR-22, miR-222, miR-26a and miR-30e.

The Venn diagram (Figure 15) shows a summary of the obtained data of the two-way ANOVA results in a simplified way.

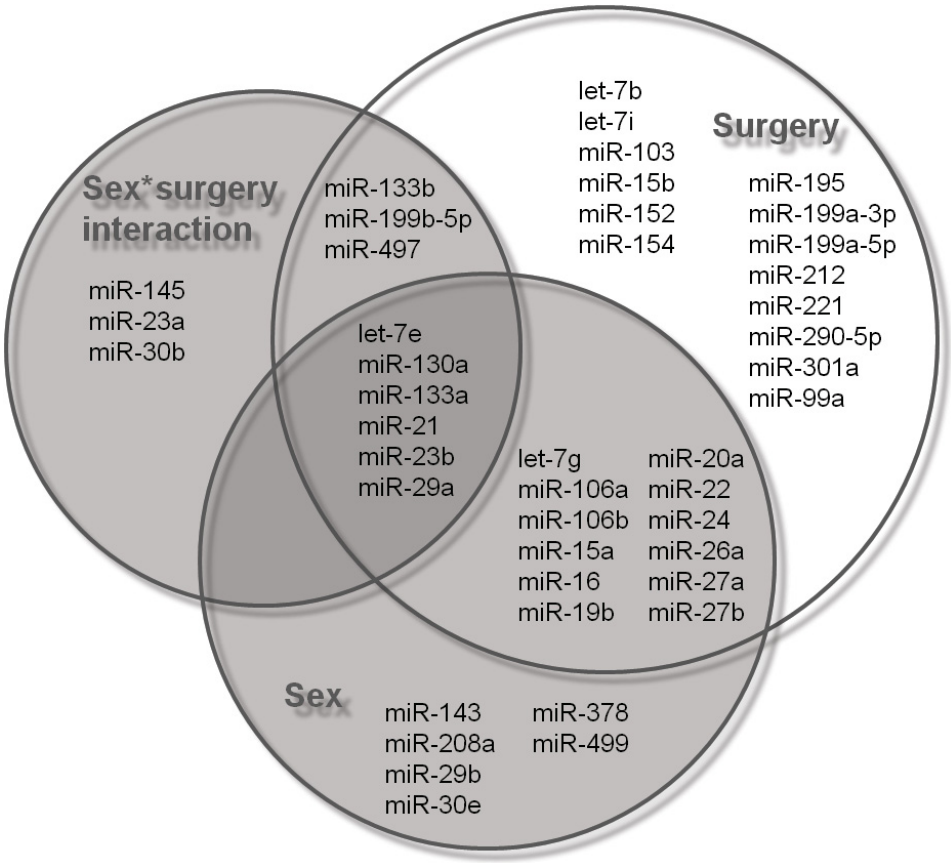


Figure 15. Summary scheme of the statistical analysis results of the miRNA quantification in WT mice. 30 miRNAs presented significant sex and/or sex*surgery interaction effect.
Two-way ANOVA analysis.

4.2.1.3.1. miRNAs with sex*surgery interaction effect – over-representation/enrichment analysis

In order to understand the possible effects of this sex-specific regulation of miRNA expression in hypertrophy, an over-representation/enrichment analysis of putatively targeted genes in known pathways using ConsensusPathDB-mouse (http://cpdb.molgen.mpg.de/MCPDB) was performed.[178-180] The TargetScan analysis of putative targets of those miRNAs with significant sex*surgery effect (let-7e, miR130a, miR-133a, miR-133b, miR-145, miR-199b-5p, miR-21, miR-23a miR-23b, miR29a, miR30b and miR-497) was compiled, counting 932 different genes. Assuming that important genes are regulated by more than one miRNA, this list was then filtered according to the number of miRNAs with the same putative target (≥2), giving a final list of 127 genes for analysis. The final gene list was inserted in the ConsensusPathDB-mouse software and the results were selected according to the relevance in cardiac hypertrophy. Table 23 shows selected hypertrophy-relevant pathways obtained by the analysis.

Table 23. Pathway enrichment analysis results after analysis using ConsensusPathDB-mouse. MAPK and PI3K-Akt signalling pathways presented the biggest set size with the lowest p-value, according to KEGG pathway database.

Pathway name (*Mus musculus* (mouse))	Set size	Candidates contained	p-value	q-value	Pathway source
MAPK signalling pathway	257	8 (3.1%)	3.64e-05	0.000692	KEGG
PI3K-Akt signalling pathway	356	9 (2.6%)	5.33e-05	0.000868	KEGG
Oxidative Damage	17	3 (17.6%)	7.89e-05	0.000999	Wikipathways
TGF-beta Receptor Signalling Pathway	150	6 (4.0%)	0.000102	0.00122	Wikipathways
Wnt Signalling Pathway	60	4 (6.7%)	0.00023	0.00238	Wikipathways
MAPK Cascade	29	3 (10.3%)	0.000406	0.0037	Wikipathways
Elastic fibre formation	31	3 (9.7%)	0.000496	0.00419	Reactome
oestrogen signalling	74	4 (5.4%)	0.000515	0.00419	Wikipathways
p38 MAPK Signalling Pathway	34	3 (8.8%)	0.000653	0.0048	Wikipathways
Mitochondrial Gene Expression	19	2 (10.5%)	0.00406	0.0162	Wikipathways
Ras signalling pathway	230	5 (2.2%)	0.00561	0.0194	KEGG
Signalling by FGFR	144	4 (2.8%)	0.00579	0.0194	Reactome
Signalling by FGFR in disease	155	4 (2.6%)	0.0075	0.0229	Reactome
Calmodulin induced events	27	2 (7.4%)	0.00812	0.0229	Reactome
CaM pathway	27	2 (7.4%)	0.00812	0.0229	Reactome
Apoptosis	83	3 (3.6%)	0.00843	0.0234	Wikipathways
Oxidative Stress	28	2 (7.1%)	0.00872	0.0239	Wikipathways
Ca-dependent events	29	2 (6.9%)	0.00933	0.0247	Reactome

Uploaded list: 127; mapped entities: 69; enriched pathway-based sets: 87

According to ConsensusPathDB-mouse, the selected miRNAs have the majority of the putative targets related to MAPK and PI3K-Akt signalling pathways. MAPKs are known to be intimately involved in cardiac remodelling. PI3K-Akt pathway, on its turn, regulates

cardiomyocyte size, survival, angiogenesis, and inflammation in both physiological and pathological cardiac hypertrophy. Among other results, oestrogen signalling is of interest and it has also been shown previously that E2 influences MAPK activity, through both phosphorylation and deactivation proteins.[182] Moreover, it has also been shown the ability of this hormone to activate Akt through a direct, non-nuclear pathway involving the regulatory subunit of PI3K, as well as its effects on MAPK signalling pathway.[183]

Furthermore, a significant number of mitochondrial genes were previously shown by our group to be sex-differently regulated in a mouse hypertrophy model[60], for what the appearance of mitochondrial gene expression in the over-representation analysis is not surprising.

4.2.2. Sex and LVH influence miRNA expression – identical expression patterns comparison

4.2.2.1. Excluded miRNAs

From the 60 miRNAs quantified, 16 miRNAs didn't show any significant effect (Table 24) and 14 miRNAs showed only significant surgery effect after two-way ANOVA analysis (Table 25). These miRNAs were not considered relevant for the questions to be answered in this study, where the effects of interest are sex and sex*surgery interaction effects, and were discarded from the subsequent analysis

Table 24. Sixteen miRNAs in WT mice without any significant effect after two-way ANOVA analysis.

miRNA	two-way ANOVA (p-value)			Bonferroni post-hoc test			
				TAC effect		Sex-differences	
	Surgery	Sex	Sex*Surgery Interaction	Females	Males	Sham	TAC
let-7c	ns	ns	ns				
let-7d	0,068	ns	ns	↑			
miR-100	ns	ns	ns				
miR-107	ns	ns	ns				
miR-149	ns	ns	ns				
miR-181a	ns	ns	ns				
miR-185	ns	0,054	ns				
miR-193b	ns	ns	ns				
miR-203	ns	ns	ns				
miR-222	ns	ns	0,084		↑	(♂>♀)	
miR-29c	0,090	ns	ns				
miR-30a	ns	ns	ns				
miR-30c	ns	ns	ns				
miR-30d	ns	ns	ns				
miR-34a	0,079	ns	ns				
miR-486	ns	ns	ns				

Two-way ANOVA; Bonferroni post-hoc test; ns – not significant. ↑↓ significant up-/down-regulation; (♂>♀) borderline p-values.

Table 25. Fourteen miRNAs in WT mice with only significant surgery effect after two-way ANOVA analysis.

miRNA	two-way ANOVA (p-value)			Bonferroni post-hoc test			
				TAC effect		Sex-differences	
	Surgery	Sex	Sex*Surgery Interaction	Females	Males	Sham	TAC
let-7b	***	0,068	ns		↑		♂>♀
let-7i	***	ns	ns		↑		♂>♀
miR-103	*	0,053	ns				
miR-15b	**	0,060	ns		↑		♂>♀
miR-152	*	ns	ns		↑		
miR-154	*	ns	ns				
miR-195	***	ns	0,071		↑		♂>♀
miR-199a-3p	***	ns	ns		↑		
miR-199a-5p	***	ns	ns		↑		(♂>♀)
miR-212	*	ns	ns		↑		
miR-221	***	ns	ns		↑		
miR-290-5p	****	ns	ns	↓	(↓)		
miR-301a	*	0,078	ns				
miR-99a	**	ns	ns		↑		

Two-way ANOVA; Bonferroni post-hoc test; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. ♂>♀ significant sex differences; ↑↓ significant up-/down-regulation; (↑↓) and (♂>♀) borderline p-values.

4.2.2.2. miRNAs with sex and/or sex*surgery interaction effect

Table 26 shows the results of Bonferroni post-hoc test performed on miRNAs that showed significant sex and/or sex*surgery interaction effect after the two-way ANOVA analysis, as well as the ratios between the comparable groups. The FT/FS and the MT/MS ratios show the dysregulation of miRNAs under TAC conditions in females and males, respectively. The FS/MS and the FT/MT ratios are used to reveal the sex differences of the miRNA expression in basal and hypertrophic conditions, respectively.

Table 26. MiRNA expression ratios in WT mice.

miRNA	Ratios (Bonferroni post-hoc test)			
	Ratio TAC/Sham *(p-value)*		Ratio Female/Male *(p-value)*	
	Females	Males	Sham	TAC
	- FT/FS -	- MT/MS -	- MS/FS -	- MT/FT -
let-7e	1,38 *ns*	2,24 ****	1,22 *ns*	1,98 ****
let-7g	1,40 *ns*	1,37 *ns*	1,58 *0,097*	1,54 *
miR-106a	1,55 *ns*	1,63 *	1,39 *ns*	1,46 *ns*
miR-106b	1,14 *ns*	1,78 **	1,10 *ns*	1,72 *
miR-130a	1,26 *ns*	1,97 ***	1,21 *ns*	1,89 ***
miR-133a	1,32 *ns*	2,47 ****	0,99 *ns*	1,85 ***
miR-133b	1,02 *ns*	1,79 **	0,92 *ns*	1,62 *
miR-143	0,92 *ns*	1,72 *	1,13 *ns*	2,10 **
miR-145	0,52 **	1,46 *ns*	0,48 **	1,36 *ns*
miR-15a	1,22 *ns*	1,63 **	1,13 *ns*	1,52 **
miR-16	1,81 *ns*	1,79 **	1,67 *ns*	1,64 **
miR-19b	1,25 *ns*	2,34 **	1,07 *ns*	2,00 *
miR-199b-5p	1,29 *ns*	3,17 ****	0,66 *ns*	1,63 *
miR-20a	1,52 *ns*	1,42 *ns*	1,62 *ns*	1,52 *0,078*
miR-208a	0,85 *ns*	1,19 *ns*	1,39 *ns*	1,93 *0,072*
miR-21	1,80 *ns*	2,53 ****	1,55 *ns*	2,18 ***
miR-22	1,06 *ns*	1,53 *	1,11 *ns*	1,60 *
miR-23a	0,81 *ns*	2,47 **	0,53 *ns*	1,64 *
miR-23b	1,07 *ns*	1,77 **	1,06 *ns*	1,75 **
miR-24	1,51 *ns*	1,72 **	1,49 *ns*	1,70 **
miR-26a	1,12 *ns*	1,63 **	1,04 *ns*	1,51 *
miR-27a	1,41 *ns*	1,50 *	1,54 *ns*	1,63 *
miR-27b	1,39 *ns*	1,84 **	1,22 *ns*	1,62 **
miR-29a	1,17 *ns*	1,73 ***	1,07 *ns*	1,59 ***
miR-29b	1,21 *ns*	1,13 *ns*	1,75 *ns*	1,62 *ns*
miR-30b	0,83 *ns*	1,66 *	0,80 *ns*	1,61 *
miR-30e	0,82 *ns*	1,53 *	1,22 *ns*	2,26 **
miR-378	0,99 *ns*	1,32 *ns*	1,18 *ns*	1,56 *
miR-497	1,14 *ns*	2,97 ****	0,68 *ns*	1,79 **
miR-499	0,94 *ns*	0,95 *ns*	1,51 *ns*	1,54 *ns*

WT mice. Eypression ratios and corresponding Bonferroni post-hoc test performed on the 32 miRNAs that showed sex and/or sex*surgery effect after two-way ANOVA analysis. FT/FS, MT/MS, FS/MS and FT/MT ratios and p-values represented.

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

As seen in Table 26, the strongest TAC effects in males (MT/MS≥2.00) were on the expression of let-7e, miR-133a, miR-19b, miR-199b-5p, miR-21, miR-23a and miR-497. Concerning the sex differences in TAC the biggest differences between males and females (MT/FT≥2.00) were found in miR-143, mR-19b, miR-21 and miR-30e.

The following tables and graphics show the results in different groups of miRNAs according to similar results between them.

4.2.2.2.1. miRNAs with sex-differences in TAC caused by an up-regulation in males and lack of TAC effect in females

Twenty-two miRNAs showed significant sex differences between TAC groups. This difference was always due to an up-regulation in males TAC, that didn't occur in females. The following tables and graphics show these miRNAs grouped according to the ANOVA result.

Group 1 (Table 27 and Figure 16.) comprises six miRNAs (let-7e, miR-130a, miR-133a, miR-21, miR-23b and miR-29a) that, besides the sex differences and the up-regulation in males after TAC, show both significant sex and sex*surgery interaction effects. All of these miRNAs show also a significant surgery effect.

Table 27. Group 1: miRNAs with significant sex and sex*surgery effect.

miRNA	two-way ANOVA (p-value)			Bonferroni post-hoc test			
				TAC effect		Sex-differences	
	Surgery	Sex	Sex*Surgery Interaction	Females	Males	Sham	TAC
let-7e	****	***	**		↑		♂>♀
miR-130a	***	**	*		↑		♂>♀
miR-133a	****	**	**		↑		♂>♀
miR-21	****	***	*		↑		♂>♀
miR-23b	*	*	*		↑		♂>♀
miR-29a	***	**	*		↑		♂>♀

Two-way ANOVA; Bonferroni post-hoc test; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.♂>♀ significant sex differences; ↑↓ significant up-/down-regulation.

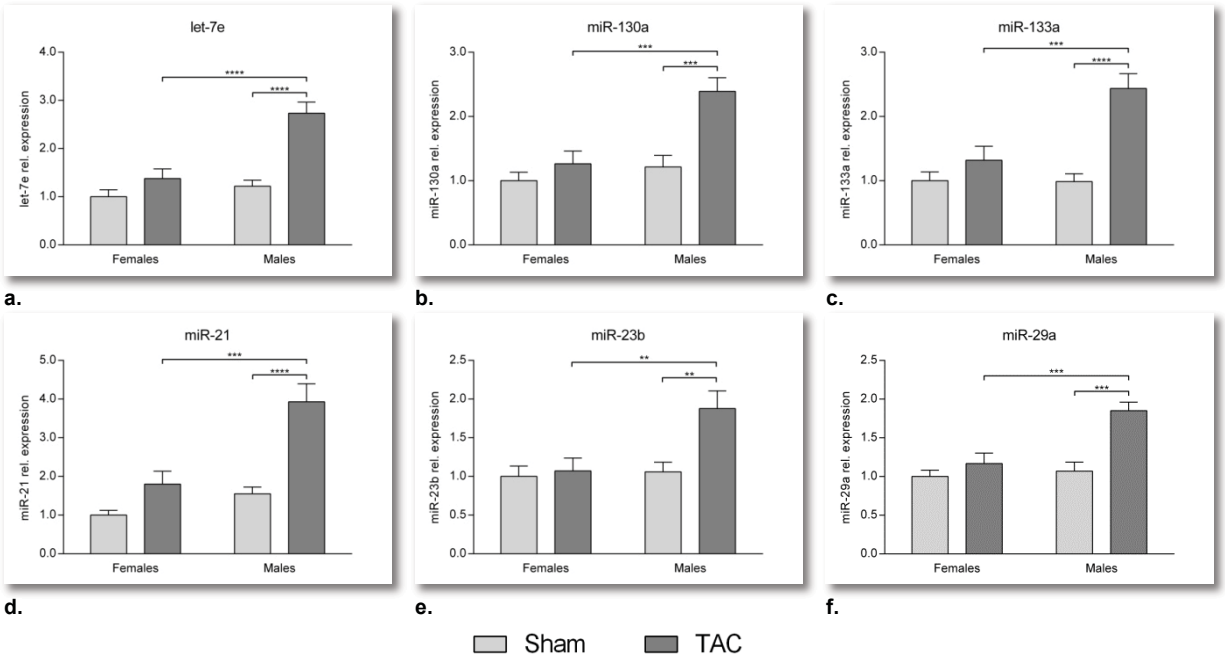


Figure 16. Graphic representation of group 1: miRNAs with significant sex and sex*surgery effect.

Bonferroni post-hoc test; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Group 2 (Table 28 and Figure 17) includes other six miRNAs (miR-106b, miR-143, miR-19b, miR-22, miR-26a and miR-30e) with a similar profile to group 1, but with a borderline p-value for sex*surgery interaction effect (0.05<p<0.1). MiR-143 and miR-30e didn't show significant surgery effect.

Table 28. Group 2: miRNAs with significant sex effect and borderline p-value for sex*surgery interaction effect.

miRNA	two-way ANOVA (p-value)			Bonferroni post-hoc test			
				TAC effect		Sex-differences	
	Surgery	Sex	Sex*Surgery Interaction	Females	Males	Sham	TAC
miR-106b	*	*	0,098		↑		♂>♀
miR-143	ns	*	0,052		↑		♂>♀
miR-19b	*	*	0,069		↑		♂>♀
miR-22	*	*	0,096		↑		♂>♀
miR-26a	**	*	0,067		↑		♂>♀
miR-30e	ns	**	0,052		↑		♂>♀

Two-way ANOVA; Bonferroni post-hoc test;* p<0.05, ** p<0.01, ns - not significant; numbers indicate borderline p-values. ♂>♀ significant sex differences; ↑↓ significant up-/down-regulation; (↑↓) and (♂>♀) borderline p-values.

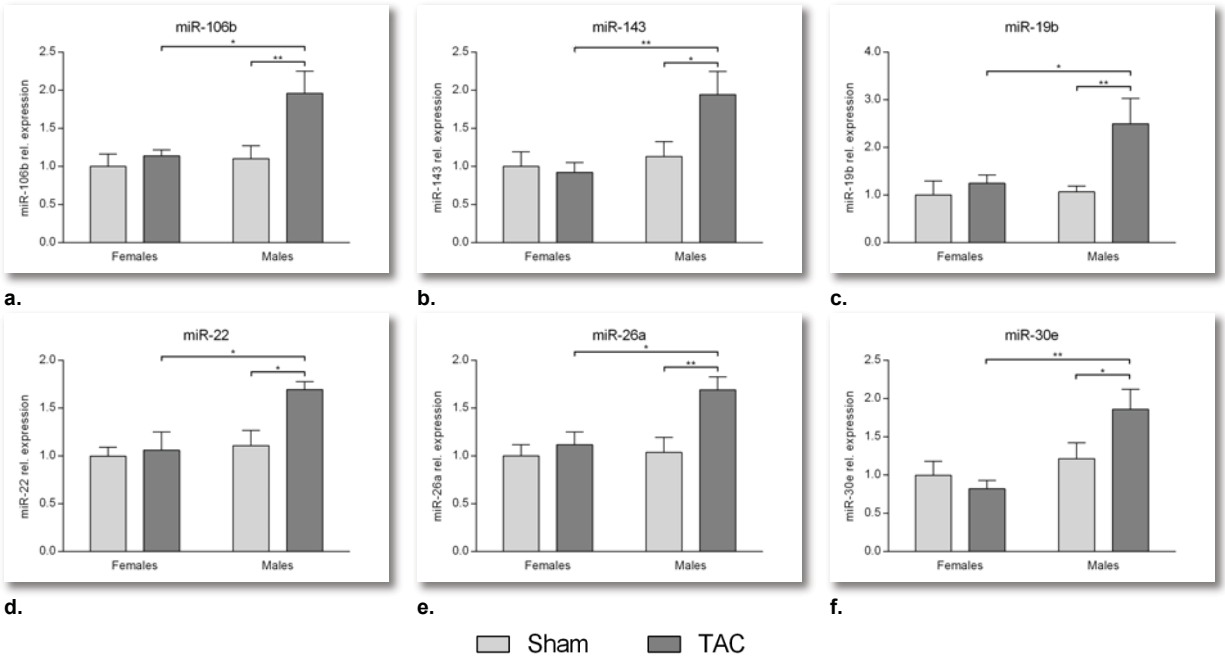


Figure 17. Graphic representation of group 2: miRNAs with significant sex effect and borderline p-value for sex*surgery interaction effect.

Bonferroni post-hoc test; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Group 3 (Table 29 and Figure 18) contains five miRNAs (miR-133b, miR-199b-5p, miR-23a, miR-30b and miR-497) with significant sex*surgery interaction effect but no sex effect. Surgery effect was not significant for miR-30b expression and for miR-23a showed a borderline p-value.

Table 29. Group 3: miRNAs with significant sex*surgery interaction effect and no sex effect.

miRNA	two-way ANOVA (p-value)			Bonferroni post-hoc test			
				TAC effect		Sex-differences	
	Surgery	Sex	Sex*Surgery Interaction	Females	Males	Sham	TAC
miR-133b	*	ns	*		↑		♂>♀
miR-199b-5p	***	ns	*		↑		♂>♀
miR-23a	0.0617	ns	**		↑		♂>♀
miR-30b	ns	ns	*		↑		♂>♀
miR-497	***	ns	**		↑		♂>♀

Two-way ANOVA; Bonferroni post-hoc test;* p<0.05, ** p<0.01, *** p<0.001, ns - not significant. ♂>♀ significant sex differences; ↑↓ significant up-/down-regulation; (↑↓) and (♂>♀) borderline p-values.

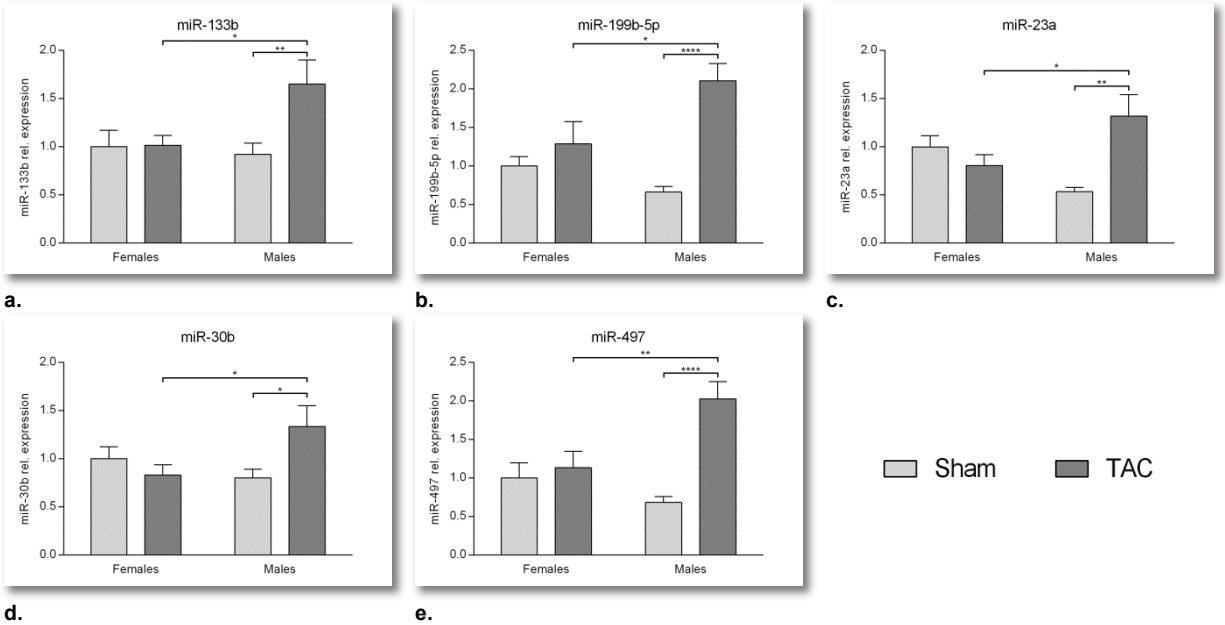


Figure 18. Graphic representation of group 3: miRNAs with significant sex*surgery interaction effect and no sex effect. Bonferroni post-hoc test; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Group 4 (Table 30 and Figure 19) includes five miRNAs (miR-15a, miR-16, miR-24, miR-27a and miR-27b) with significant sex effect but no interaction effect. All five miRNAs show significant surgery effect and a tendency for an up-regulation also in females.

Table 30. Group 4: miRNAs with significant sex effect and no sex*surgery interaction effect.

miRNA	two-way ANOVA (p-value)			Bonferroni post-hoc test			
				TAC effect		Sex-differences	
	Surgery	Sex	Sex*Surgery Interaction	Females	Males	Sham	TAC
miR-15a	**	*	ns		↑		♂>♀
miR-16	***	**	ns		↑		♂>♀
miR-24	**	**	ns		↑		♂>♀
miR-27a	*	**	ns		↑		♂>♀
miR-27b	**	*	ns		↑		♂>♀

Two-way ANOVA; Bonferroni post-hoc test; * p<0.05, ** p<0.01, *** p<0.001, ns - not significant. ♂>♀ significant sex differences; ↑↓ significant up-/down-regulation; (↑↓) and (♂>♀) borderline p-values.

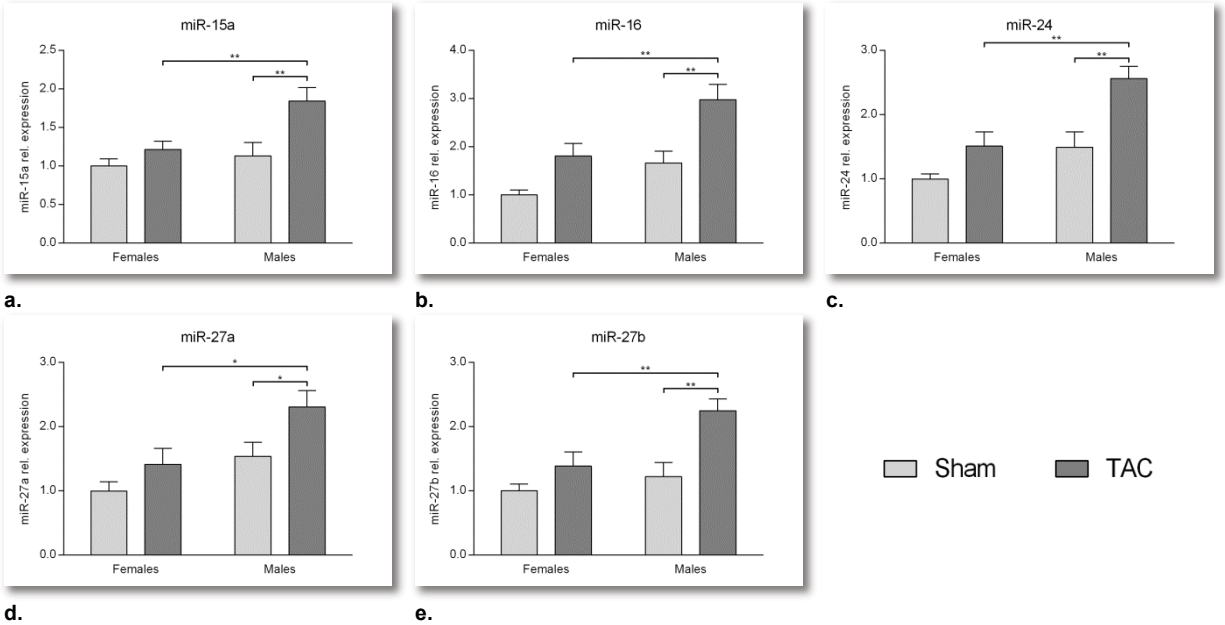


Figure 19. Graphic representation of group 4: miRNAs with significant sex effect and no sex*surgery interaction effect. Bonferroni post-hoc test; * p<0.05, ** p<0.01.

All the miRNAs of all previous groups have in common the up-regulation in males after TAC surgery, the non-existence (or non-significant) effect in females and the significant difference of their expression between both sexes after TAC surgery. The next groups will show miRNAs with different characteristics.

4.2.2.3. miRNAs with sex differences in TAC or up-regulation in males

This section encloses smaller groups, where all miRNAs have in common the significant sex effect, no sex*surgery interaction effect but they either show up-regulation in males after TAC, sex differences in TAC or no other significant effect.

Group 5 (Table 31 and Figure 20) contains one miRNA (miR-106a) with significant sex effect but no significant sex differences in TAC. However, like the miRNAs in 2.2.2.1., still presents an up-regulation in males and no effect in females after TAC.

Table 31. Group 5: miR-106a showed significant up-regulation in males but no significant sex differences after TAC.

miRNA	two-way ANOVA (p-value)			Bonferroni post-hoc test			
				TAC effect		Sex-differences	
	Surgery	Sex	Sex*Surgery Interaction	Females	Males	Sham	TAC
miR-106a	*	*	ns		↑		

Two-way ANOVA; Bonferroni post-hoc test; * p<0.05, ns - not significant. ♂>♀ significant sex differences; ↑↓ significant up-/down-regulation; (↑↓) and (♂>♀) borderline p-values.

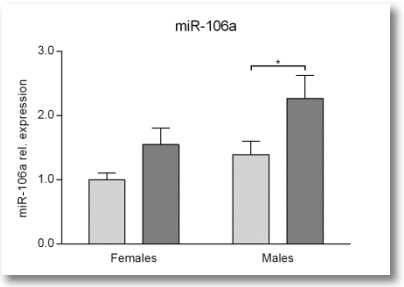


Figure 20. Graphic representation of group 5: miR-106a showed significant up-regulation in males but no sex differences after TAC. Bonferroni post-hoc test; * p<0.05

Group 6 (Table 32 and Figure 21) comprises two miRNAs (let-7g and miR-378) with significant sex differences in TAC but no significant up-regulation in males after surgery. However, let-7g presented a borderline p-value for sex differences in Sham animals.

Table 32. Group 6: miRNAs with significant sex differences after TAC but no significant up-regulations in males.

miRNA	two-way ANOVA (p-value)			Bonferroni post-hoc test			
				TAC effect		Sex-differences	
	Surgery	Sex	Sex*Surgery Interaction	Females	Males	Sham	TAC
let-7g	*	**	ns			(♂>♀)	♂>♀
miR-378	ns	*	ns				♂>♀

Two-way ANOVA; Bonferroni post-hoc test; * p<0.05, ** p<0.01, ns - not significant. >♀ significant sex differences; ↑↓ significant up-/down-regulation; (↑↓) and (♂>♀) borderline p-values.

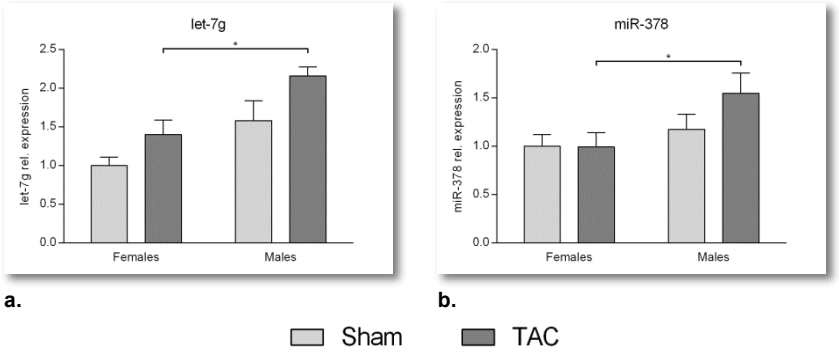


Figure 21. Graphic representation of group 6: miRNAs with significant sex differences after TAC but no significant up-regulations in males.
Bonferroni post-hoc test; * p<0.05

4.2.2.3.1. miRNAs with no significant effects after TAC

Group 7 (Table 33 and Figure 22) includes four miRNAs (miR-20a, miR-208a, miR-29b and miR-499) with no up-regulation or sex differences after TAC. However, the significant sex effect is visible in the higher expression in males compared to females, either in Sham or TAC operated mice, but this difference was not significant in the Bonferroni post-hoc test.

Table 33. Group 7: miRNAs with no significant up-regulation in males or sex differences after TAC.

miRNA	two-way ANOVA (p-value)			Bonferroni post-hoc test			
				TAC effect		Sex-differences	
	Surgery	Sex	Sex*Surgery Interaction	Females	Males	Sham	TAC
miR-20a	*	*	ns				(♂>♀)
miR-208a	ns	*	ns				(♂>♀)
miR-29b	ns	*	ns				
miR-499	ns	*	ns				

Two-way ANOVA; Bonferroni post-hoc test; * p<0.05, ** p<0.01, ns - not significant. (♂>♀) borderline p-values.

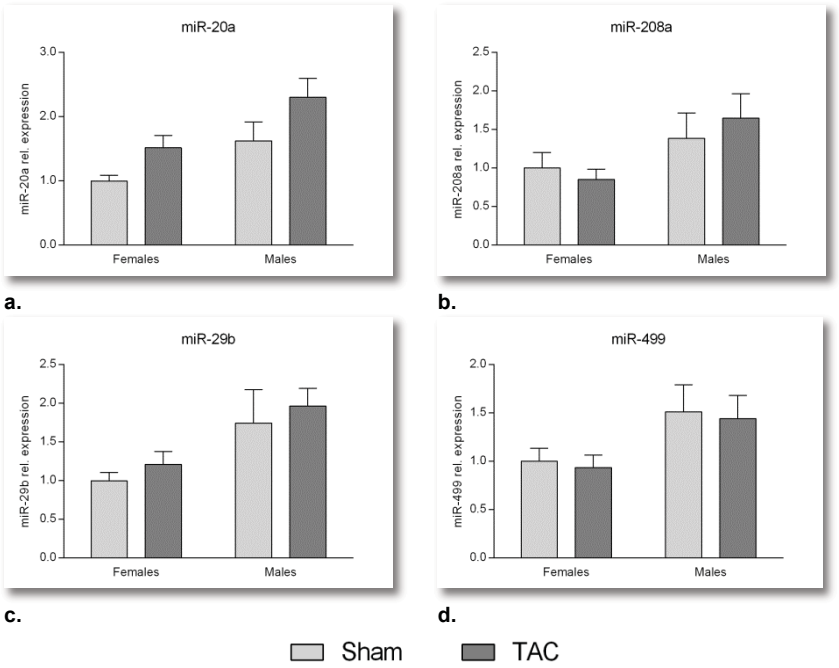


Figure 22. Graphic representation of group Group 7: miRNAs with no significant up-regulation in males or sex differences after TAC.

4.2.2.3.2. miRNAs with sex differences in Sham

Group 8 (Table 34 and Figure 23) includes the only miRNA (miR-145) with a significant down-regulation in TAC. MiR-145 presented a remarkably different expression profile, being down-regulated after TAC in female mice and showing no significant effect in males. In Sham operated animals significant sex differences were observed, being higher in females than in males. Sex*surgery interaction effect was significant and sex effect showed a borderline p-value.

Table 34. Group 8: miR-145 was the only miRNA down-regulated after TAC in female mice and with sex differences in Sham operated mice.

miRNA	two-way ANOVA (p-value)			Bonferroni post-hoc test			
				TAC effect		Sex-differences	
	Surgery	Sex	Sex*Surgery Interaction	Females	Males	Sham	TAC
miR-145	ns	0,084	***	↓		♂<♀	

Two-way ANOVA; Bonferroni post-hoc test; * p<0.05, ** p<0.01, *** p<0.001, ns - not significant. ♂>♀ significant sex differences; ↑/↓ significant up-/down-regulation; (↑/↓) and (♂>♀) borderline p-values.

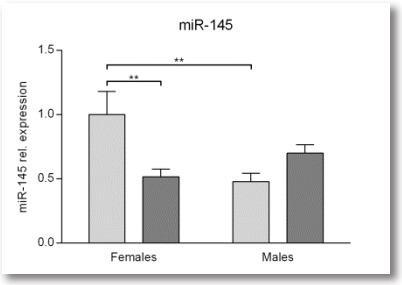


Figure 23. Graphic representation of group 8: miR-145 was the only miRNA down-regulated after TAC in female mice and with sex differences in Sham operated mice. Bonferroni post-hoc test; ** p<0.01.

4.2.2.3.3. Summary of the TAC effects and sex differences in WT mice

Table 35 is a summary of the significant TAC effects in males and females. The majority of miRNAs are up-regulated in males and only one miRNA showed a down-regulation after TAC.

Table 35. Summary of the TAC effects in WT mice by sex. Bonferroni post-hoc test results.

Females		Males	
Down-regulated	Up-regulated	Down-regulated	Up-regulated
miR-145		---	let-7b let-7i miR-106a miR-106b miR-133b miR-143 miR-15a miR-15b miR-152 miR-16 miR-19b miR-195 miR-199a-3p miR-199a-5p miR-199b-5p miR-212 miR-22 miR-221 miR-23a miR-24 miR-26a miR-27a miR-27b miR-30b miR-30e miR-497 miR-99a

Table 36 summarizes of the significant sex differences observed in Sham and TAC operated mice. The majority of the miRNAs showed sex differences after TAC and only one miRNA presented significant sex differences in Sham operated animals.

Table 36. Summary of the sex differences in Sham and TAC operated mice. Bonferroni post-hoc test results.

Sham	TAC	
miR-145	let-7e miR-106b miR-130a miR-133a miR-133b miR-143 miR-15a miR-16 miR-199b-5p miR-19b miR-21	miR-22 miR-23a miR-23b miR-24 miR-26a miR-27a miR-27b miR29a miR-30b miR-30e miR-497

4.3. ERβ is required for sex differences in miRNA expression

4.3.1. Sex and sex*surgery interaction effects disappear in the absence of ERβ

After a careful statistical analysis of the data obtained in WT mice, most of the interesting miRNAs (sex*surgery interaction and/or sex significant effect) were measured in ERβ$^{-/-}$ mice. As already mentioned above, for the aims of this project these two groups were considered the most interesting. Sex*surgery interaction effect group shows the sex-specific effect on miRNA expression after TAC and on the other hand, sex effect group is a good hint for a possible regulatory effect of sex hormones on these miRNAs. The two-way ANOVA results of the miRNAs quantification in ERβ$^{-/-}$ mice are represented in Table 37.

Table 37. Two-way ANOVA analysis of miRNA quantification in ERβ$^{-/-}$ mice.

miRNA	two-way ANOVA (p-value)		
	Surgery	Sex	Sex*Surgery Interaction
let-7e	*	ns	ns
miR-106a	ns	0,0835	ns
miR-106b	**	ns	ns
miR-130a	ns	ns	ns
miR-133a	*	ns	ns
miR-133b	ns	0,0505	ns
miR-143	*	ns	0,0883
miR-145	ns	ns	ns
miR-16	*	ns	ns
miR-199b-5p	**	ns	ns
miR-20a	**	ns	ns
miR-208a	0,056	ns	ns
miR-21	*	ns	ns
miR-23a	*	ns	ns
miR-24	ns	ns	ns
miR-27a	0,0823	ns	ns
miR-27b	**	ns	ns
miR-29a	ns	ns	ns
miR-29b	**	ns	ns
miR-30e	**	ns	ns
miR-378	**	ns	ns
miR-497	*	ns	ns

Two-way ANOVA;* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

From the quantification in ERβ$^{-/-}$ mice of 22 selected miRNAs (with sex and/or interaction effect in WT mice) it's possible to make a first and major observation, which is the disappearance of all these significant effects with the absence of ERβ. The only significant effects observed after two-way ANOVA analysis is surgery effect.

Surgery effect was found significant in the expression of 14 miRNAs, (Table **37**), however not all of these miRNAs showed results identical to the observed in WT animals. Six miRNAs (let-7e, miR-106, miR-133a, miR-16, miR-20a and miR-21) show significant surgery effect in both phenotypes, but in contrast to what happens in WT the TAC effect observed in males is a down-regulation or a tendency for it. Four miRNAs (miR-143, miR-29b, miR-30e and miR-378) didn't show this effect in WT, but in ERβ$^{-/-}$ all of them presented significant (or tendency for) a down-regulation in males.

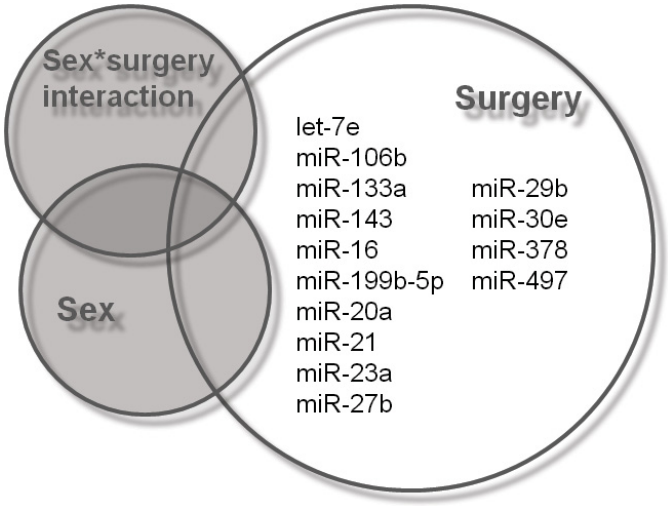


Figure 24. Scheme of the statistical analysis (two-way ANOVA) results of the measurements in ERβ$^{-/-}$ mice.
Sex and sex*surgery circles are empty due to the lack of significant results.

4.3.2. Sex differences in miRNA expression after TAC also disappear in the absence of ERβ

Table 38 shows the Bonferroni post-hoc test results after two-way ANOVA analysis. These results help to the understanding of the different expression patterns that led to the differences observed between WT and ERβ$^{-/-}$ mice.

Table 38. Bonferroni post-hoc test performed on 22 miRNAs in ERβ$^{-/-}$ mice.

miRNA	Ratios (Bonferroni post-hoc test)			
	Ratio TAC/Sham		Ratio Female/Male	
	Females	Males	Sham	TAC
	- FT/FS -	- MT/MS -	- MS/FS -	- MT/FT -
let-7e	0,73 *ns*	0,81 *ns*	0,86 *ns*	0,95 *ns*
miR-106a	0,95 *ns*	0,83 *ns*	1,28 *ns*	1,11 *ns*
miR-106b	0,63 *	0,72 *0,053*	1,08 *ns*	1,22 *ns*
miR-130a	0,77 *ns*	0,91 *ns*	0,96 *ns*	1,13 *ns*
miR-133a	0,91 *ns*	0,79 *0,0925*	0,96 *ns*	0,83 *ns*
miR-133b	1,11 *ns*	0,93 *ns*	1,27 *0,0671*	1,06 *ns*
miR-143	0,88 *ns*	0,47 *	1,30 *ns*	0,70 *ns*
miR-145	1,03 *ns*	0,84 *ns*	1,09 *ns*	0,89 *ns*
miR-16	0,70 *	0,84 *ns*	0,86 *ns*	1,04 *ns*
miR-199b-5p	2,07 *0,0593*	2,11 *	1,19 *ns*	1,22 *ns*
miR-20a	0,62 *	0,66 *	1,03 *ns*	1,10 *ns*
miR-208a	0,56 *ns*	0,75 *ns*	1,20 *ns*	1,60 *ns*
miR-21	2,27 *ns*	2,57 *ns*	0,97 *ns*	1,09 *ns*
miR-23a	1,27 *ns*	1,31 *ns*	1,00 *ns*	1,04 *ns*
miR-24	1,16 *ns*	1,16 *ns*	1,01 *ns*	1,01 *ns*
miR-27a	1,16 *ns*	1,39 *ns*	0,97 *ns*	1,16 *ns*
miR-27b	1,37 *ns*	1,50 *	0,90 *ns*	0,99 *ns*
miR-29a	0,83 *ns*	0,87 *ns*	0,86 *ns*	0,89 *ns*
miR-29b	0,75 *ns*	0,68 *	1,12 *ns*	1,01 *ns*
miR-30e	0,68 *0,0709*	0,63 *	0,93 *ns*	0,86 *ns*
miR-378	0,75 *ns*	0,62 *	0,98 *ns*	0,82 *ns*
miR-497	1,86 *	1,30 *ns*	1,33 *ns*	0,93 *ns*

FT/FS, MT/MS, FS/MS and FT/MT ratios and p-values represented. Bonferroni post-hoc test; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001; ns – not significant.

Comparing the significant results in the post-hoc test of ERβ$^{-/-}$ mice with the results in WT mice it is possible to observe many differences in the regulation of the expression of miRNA in response to TAC.

In female WT mice, miR-106b, miR-16 and miR-20a showed no significant effect after TAC, or at least a tendency for up-regulation (Table 26. Ratios FT/FS: 1.14, 1.81 and 1.52, respectively). However, these miRNAs were significantly down-regulated after TAC surgery in knock-out female mice (Ratios FT/FS: 0.63, 0.70 and 0.62, respectively).

MiR-497 was the only miRNA significantly up-regulated in ERβ deficient females after TAC (FT/FS=1.86).

In ERβ$^{-/-}$ males, miR-143, miR-20a, miR-29b, miR-30e and miR-378 were significantly down-regulated (Ratios MT/MS: 0.47, 0.66, 0.68, 0.63 and 0.62, respectively), while in WT animals they were significantly or tended to an up-regulation (Ratios MT/MS: 1.72, 1.42, 1.13, 1.53 and 1.32, respectively).

MiR-199b-5p and miR-27b were significantly up-regulated in males in both models, WT and ERβ$^{-/-}$ mice.

4.3.3. Summary of the TAC effects and sex differences in ERβ$^{-/-}$ mice

Table 39 represents a summary of all significant up-/down-regulated miRNAs after TAC in ERβ deficient females and males. The majority of miRNAs up-regulated after TAC in WT males, is not up-regulated in ERβ$^{-/-}$.

Table 39. TAC effects in ERβ$^{-/-}$ mice by sex.

Females		Males	
Down-regulated	**Up-regulated**	**Down-regulated**	**Up-regulated**
miR-106b miR-16 miR-20a	miR-497	miR-143 miR-20a miR-29b miR-30e miR-378	miR-199b-5p miR-27b

There were no significant sex differences in miRNA expression in Sham or TAC operated animals.

4.4. Direct comparison of WT and ERβ$^{-/-}$ female mice confirms the involvement of the receptor on miRNA expression

Given the observed differences between the WT and the ERβ$^{-/-}$ mice, the female Sham groups were then directly compared through qRT-PCR. Some of the miRNAs presenting surgery and sex effect were measured. All the significant sex effects were abolished in the knock-out model. The ratio ERβ$^{-/-}$/WT was calculated and the groups were compared through unpaired T-test.

Table 40. Direct comparison of WT and ERβ$^{-/-}$ female Sham mice.

miRNA	Ratio ERβ$^{-/-}$/WT	Unpaired t-test (p-value)
let-7e	2,77	***
miR-106a	0,90	*ns*
miR-106b	1,17	*0,0952*
miR-130a	1,37	*
miR-133a	1,70	***
miR-20a	1,69	***
miR-21	1,65	***
miR-24	1,70	***
miR-27a	0,95	*ns*
miR-27b	1,62	***
miR-29a	2,15	***

T-test; * p<0.05, ** p<0.01, *** p<0.001; ns – not significant.

This comparison revealed the expected differences: most of miRNAs analysed were higher expressed in the ERβ deficient females (Table 40 and Figure 25). A higher basal (Sham operated animals) expression in ERβ$^{-/-}$ females is one of the mechanisms responsible for the different miRNA expression profile from the WT mice.

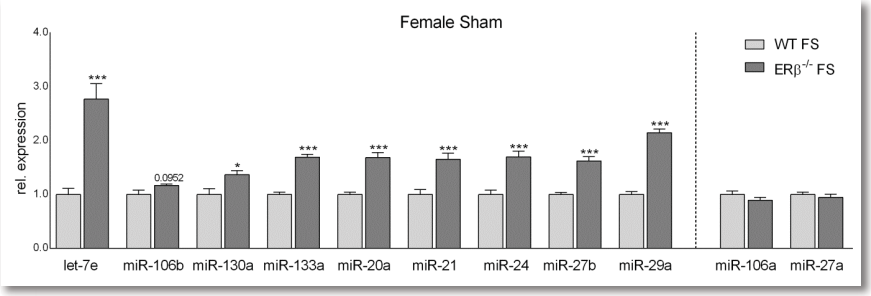


Figure 25. Direct comparison of WT and ERβ$^{-/-}$ female Sham mice revealed a higher expression in ERβ deficient mice. WT and ERβ$^{-/-}$ FS animals directly compared by qRT-PCR. T-test; * p<0.05, ** p<0.01, *** p<0.001.

4.5. ERβ plays a role in miRNA expression in Sham operated mice

After the confirmation that the expression of several miRNAs with surgery and sex effect is different in WT and ERβ$^{-/-}$ animals, all the values of the previous measurements of WT and ERβ$^{-/-}$ males and females Sham operated mice were corrected in order to be directly compared. The correction factor used was calculated in the following way:

$$\frac{mean\ Sham\ females\ (2nd\ measurement)}{mean\ Sham\ females\ (1st\ measurement)}$$, for WT and ERβ$^{-/-}$ females.

After the correction, the values were analysed by two-way ANOVA (Table 41). Table 42 shows the ratios between ERβ$^{-/-}$ and WT or females and males, as well as Bonferroni post-hoc test results.

Table 41. Two-way ANOVA analysis of corrected WT and ERβ$^{-/-}$ miRNA expression values.

miRNA	two-way ANOVA (p-value)		
	Sex	Genotype	Sex*Gnotype Interaction
let-7e	ns	****	ns
miR-	*	ns	ns
miR-	ns	ns	ns
miR-	ns	ns	ns
miR-	ns	****	ns
miR-20a	ns	0,085	ns
miR-21	ns	ns	ns
miR-24	ns	*	ns
miR-27a	ns	*	0,099
miR-27b	ns	*	ns
miR-29a	ns	****	ns

* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001; ns – not significant.

Table 42. MiRNA expression ratios.

miRNA	Ratios *(p-value)*			
	Females ERβ$^{-/-}$/WT	Males ERβ$^{-/-}$/WT	WT M/F	ERβ$^{-/-}$ M/F
let-7e	2,64 ***	1,86 **	1,22 *ns*	0,86 *ns*
miR-106a	0,89 *ns*	0,82 *ns*	1,39 *ns*	1,28 *ns*
miR-106b	1,12 *ns*	1,09 *ns*	1,10 *ns*	1,07 *ns*
miR-130a	1,29 *ns*	1,02 *ns*	1,21 *ns*	0,96 *ns*
miR-133a	1,65 **	1,60 **	0,99 *ns*	0,96 *ns*
miR-20a	1,72 *ns*	1,09 *ns*	1,62 *ns*	1,03 *ns*
miR-21	1,60 *0,070*	1,00 *ns*	1,55 *0,094*	0,97 *ns*
miR-24	1,66 *0,060*	1,13 *ns*	1,49 *ns*	1,01 *ns*
miR-27a	0,89 *ns*	0,56 *	1,54 *0,057*	0,97 *ns*
miR-27b	1,59 *	1,18 *ns*	1,22 *ns*	0,90 *ns*
miR-29a	2,13 ***	1,71 **	1,07 *ns*	0,86 *ns*

Corrected WT and ERβ$^{-/-}$ miRNA expression ratios values (ERβ$^{-/-}$/WT and Males/Females) and the corresponding Bonferroni post-hoc test; * p<0.05, ** p<0.01, *** p<0.001; ns – not significant.

4.5.1. miRNAs without genotype or sex effect

MiR-106b, miR-130a and miR-21 didn't present any significant effect of ERβ in Sham operated animals (Figure 26).

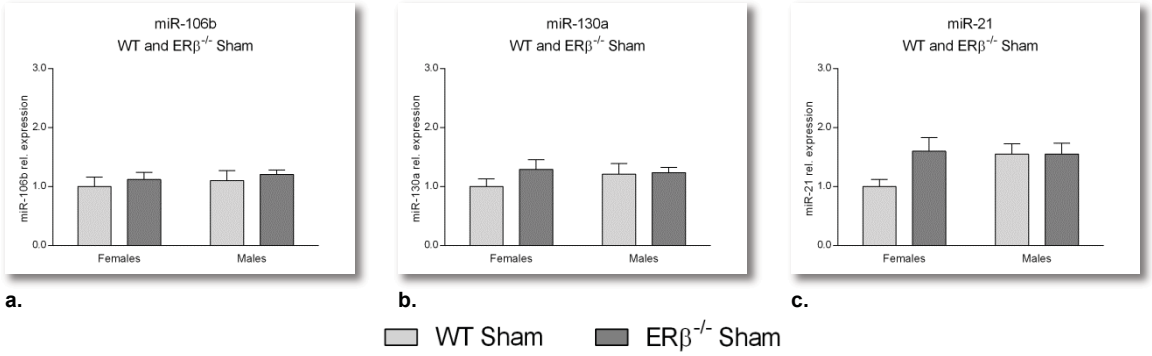


Figure 26. Graphical representation of WT and ERβ$^{-/-}$ Sham operated animals of miRNAs without sex or genotype effect.

The higher expression of miR-106a in male mice is independent of the genotype. MiR-106a was the only miRNA that showed a sex effect, being higher expressed in males than in females. This sex effect was already visible in WT Sham/TAC animals, as well as it was almost significant in ERβ$^{-/-}$, and for that reason this effect was expected.

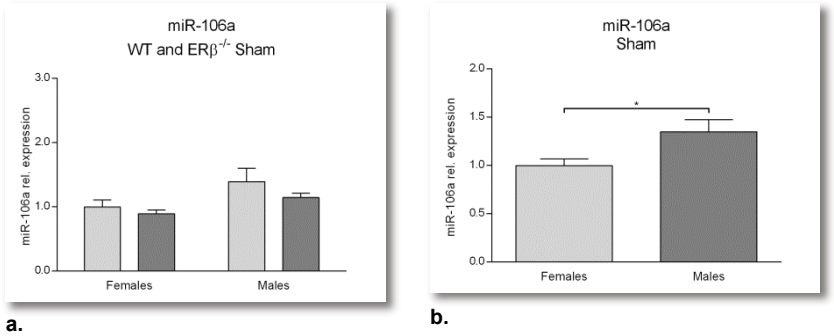


Figure 27. MiR-106a was the only miRNA with a significant effect in this analysis. a. Graphical representation of WT and ERβ$^{-/-}$ Sham operated animals of miR-106a. b. Graphical representation of the sex effect in miR-106a.
WT and ERβ$^{-/-}$ are represented together, according to the sex.
Two-way ANOVA; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

4.5.2. Genotype effect on miRNA expression

The main expected effect was the genotype effect (different expression in WT than in ERβ$^{-/-}$ independent of the sex), which shows that the receptor affects miRNA expression. From the six miRNAs (let-7e, miR-133a, miR-24, miR-27a, miR-27b and miR-29a) of the direct comparison showing a genotype effect (Figure 28.), five presented a higher expression in ERβ$^{-/-}$ animals, when compared to WT. MiR-27a was the only one with a lower expression in the ERβ deficient mice.

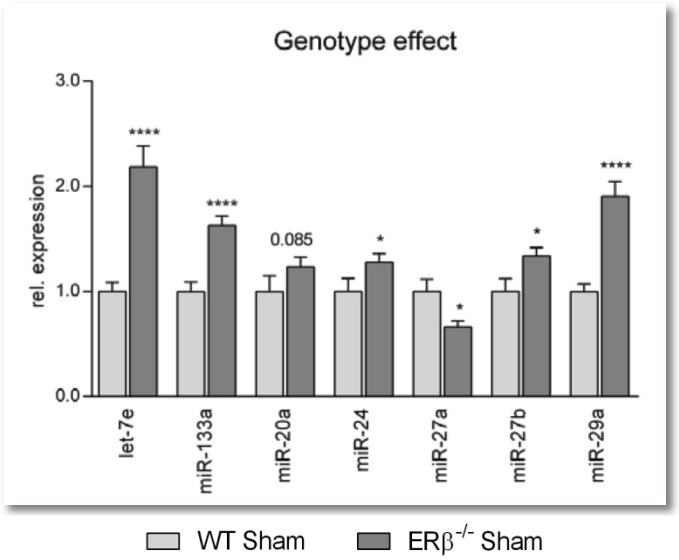
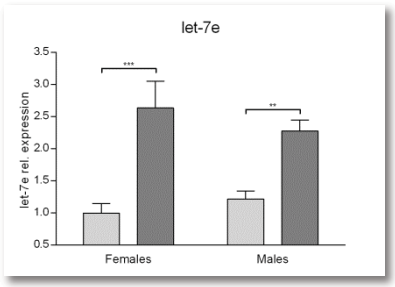
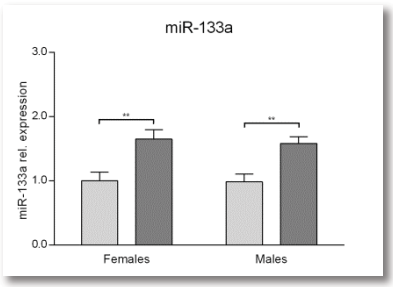


Figure 28. 6 miRNAs showed a significant genotype effect in two-way ANOVA analysis.
The values of females and males are represented together, according to the genotype.
* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

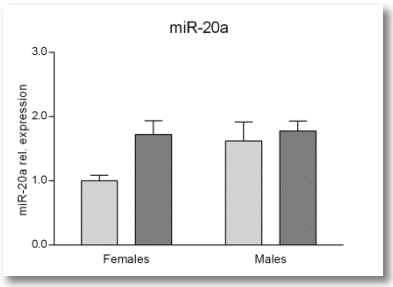
After a detailed analysis of the expression of these six miRNAs with significant genotype effect we looked for the sex effect in each genotype. A general higher expression in ERβ$^{-/-}$ females (borderline p-value in the case of miR-24, p=0.06) was observed. In males, the up-regulation with the lack of ERβ was only observed in 3 miRNAs (let-7e, miR-133a and miR-29a).



a.



b.



c.

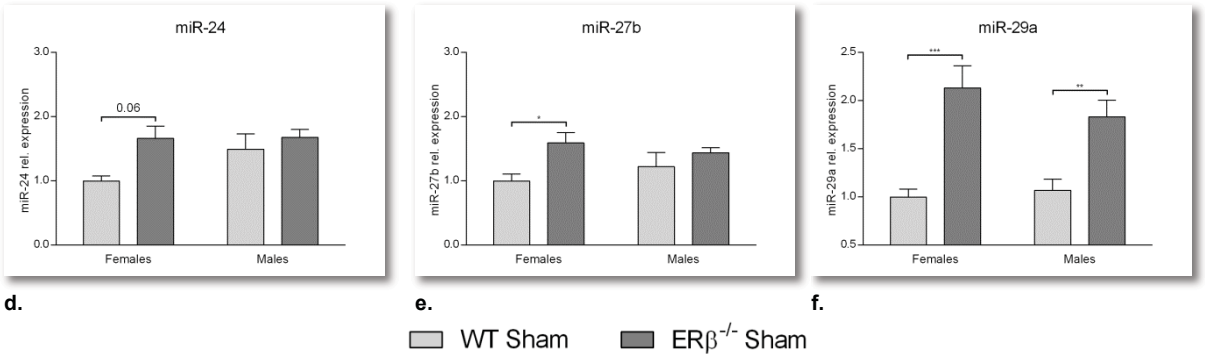


Figure 29. Most miRNAs with genotype effect after two-way ANOVA are higher expressed in female ERβ$^{-/-}$ mice.
Bonferroni post-hoc test; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Finally, miR-27a was the only miRNA with a borderline value for sex*genotype interaction effect in the two-way ANOVA analysis. This miRNA presented a significant lower expression in ERβ$^{-/-}$ than in WT males, but the same didn't happen in females. In the latter, the sex differences in miR-27a expression observed in WT animals (higher expressed in males than females), disappeared in the absence of ERβ.

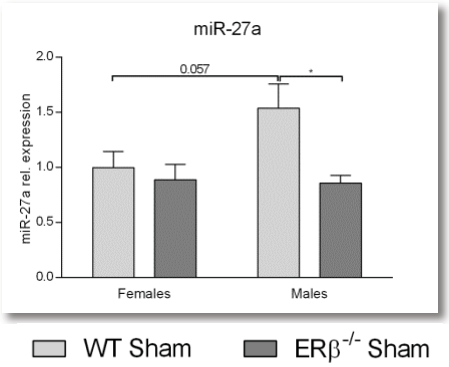


Figure 30. miR-27a was the only miRNA with a borderline value for sex*genotype interaction effect in the two-way ANOVA analysis.
Bonferroni post-hoc test; * p<0.05.

4.6. Summary of the *in vivo* results

A general overview of the *in vivo* results of the ERβ deletion effect is given in the following table.

Table 43. Overview of the *in vivo* effects of ERβ deletion.

miRNA	Sham and TAC		Sham only			
	WT and ERβ$^{-/-}$ initial screening		WT and ERβ$^{-/-}$ Sham comparison		ERβ deletion effect by sex (Bonferroni post-hoc test)	
	Sex effect		Sex effect	Genotype effect	Females	Males
	WT	ERβ$^{-/-}$				
let-7e	♂>♀	♂=♀	♂=♀	ERβ$^{-/-}$ > WT	↑	↑
miR-106a	♂>♀	(♂>♀)	♂>♀	-	-	-
miR-106b	♂>♀	♂=♀	♂=♀	-	-	-
miR-130a	♂>♀	♂=♀	♂=♀	-	-	-
miR-133a	♂>♀	♂=♀	♂=♀	ERβ$^{-/-}$ > WT	↑	↑
miR-20a	♂>♀	♂=♀	♂=♀	(ERβ$^{-/-}$ > WT)	-	-
miR-21	♂>♀	♂=♀	♂=♀	-	↑	-
miR-24	♂>♀	♂=♀	♂=♀	ERβ$^{-/-}$ > WT	↑	-
miR-27a	♂>♀	♂=♀	♂=♀	ERβ$^{-/-}$ < WT	-	↓
miR-27b	♂>♀	♂=♀	♂=♀	ERβ$^{-/-}$ > WT	↑	-
miR-29a	♂>♀	♂=♀	♂=♀	ERβ$^{-/-}$ > WT	↑	↑

Note: Effects between () refer to borderline p-values.

4.7. E2, ERβ and ERα affect miRNA expression in female cardiomyocytes

After the observed sex differences in miRNA expression in hypertrophy and the determinant influence of ERβ on them, the question whether the effect of the hormone was cell-specific and whether both receptors are involved in similar way arose. For this purpose AC16[177], a human female cardiomyocyte cell line, was used, although being aware of the inability of testing sex-differences in this model.

AC16 cells were treated with E2 or specific agonists for ERβ or ERα for 48h. Table 44 shows miRNA expression of the 11 miRNAs described in Table 41 after 48h treatment with the 3 compounds as ratios of the corresponding control group (vehicle). The statistical analysis is also depicted. Of note, all these miRNAs presented surgery and sex effect in WT mice.

Table 44. The treatment of AC16 cells with E2, ERβ and ERα specific agonists reduces miRNA expression

miRNA	E2	ERβ ag	ERα ag
	Ratio 48h treat/ vehicle	Ratio 48h treat/ vehicle	Ratio 48h treat/ vehicle
	p-value	*p-value*	*p-value*
let-7e	0,81 *	0,72 *	1,02 *ns*
miR-106a	0,65 *	0,54 **	0,56 **
miR-106b	0,72 *	0,68 **	0,75 *
miR-130a	0,85 *	0,99 *ns*	0,79 **
miR-133a	0,87 *ns*	0,75 *	1,01 *ns*
miR-20a	0,72 **	0,78 *	0,76 *
miR-21	0,80 *	0,76 *	0,66 *
miR-24	0,74 *	0,70 **	0,75 *
miR-27a	0,76 *	0,64 *	0,97 *ns*
miR-27b	0,70 *	0,66 *	0,78 *
miR-29a	0,91 *	0,78 *	0,70 ***

T-test; * p<0.05; ** p<0,01; *** p<0.001.

Each of the 11 selected miRNAs showed a significant response to at least one of the treatments, always being repressed after treatment. After E2 treatment all except miR-133a were significantly down-regulated.

All miRNAs were down-regulated after ERβ specific agonist treatment with the exception of miR-130a with an unaltered expression.

Concerning ERα specific agonist, a significant down-regulation compared to the control was observed in a group of eight miRNAs (miR-106a, miR-106b, miR-130a, miR-20a, miR-21, miR-24, miR-27b and miR-29a).

The results summary is represented in Figure 31 as a Venn diagram. MiR-106a, miR-106b, miR-20a, miR-21, miR-24, miR-27b and miR-29a were repressed after treatment with E2 and both ER specific agonists. Let-7e and miR-27a showed effects with both E2 and ERβ specific agonist but not with ERα, whereas miR-130a responded only to E2 and ERα specific agonist. Finally, the expression of miR-133a was affected only by ERβ specific agonist.

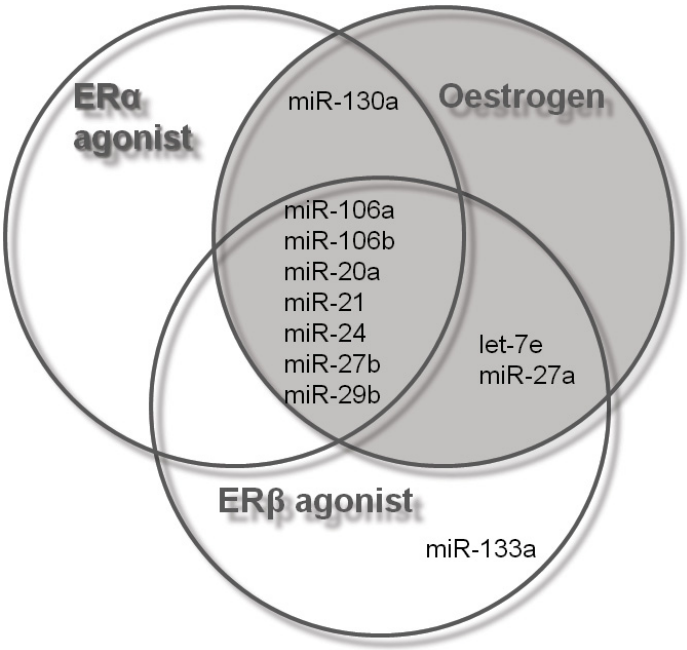


Figure 31. MiRNAs down-regulated in AC16 cells by 48h treatment with E2, ERβ or ERα specific agonists.

4.8. E2 and ERβ regulate fibrosis related miRNAs

A previous study from our group, based in the same biological samples, showed for the first time that ERβ plays different actions in each sex and plays a role in sex differences in the development of LVH and heart failure[60]. Male WT mice presented a more pronounced hypertrophy than females and these differences were specifically found in myocyte hypertrophy as well as in fibrosis content. The deletion of ERβ reduced these differences, promoting fibrosis in females.

MiR-21 was previously identified as a fibrosis regulator. This miRNA was shown to be an inducer of interstitial fibrosis, augmenting cardiac MAPK/ERK activity in fibroblasts through the inhibition of sprouty homologue 1 (SPRY1).[111]

Because nothing is known about sex-specific expression of miR-21, the role of this and other miRNAs putatively regulating the same pathway was investigated.

4.8.1. miR-21, a validated fibrosis inducer, is regulated by ERβ

Given the high importance of the role of miR-21 in fibrosis we recalled the comparison of its expression in both genotypes. In this project, miR-21 expression was clearly affected by ERβ, showing significant sex and sex*surgery interaction effects in WT animals (Table 22) that were abolished in ERβ$^{-/-}$ mice (Table 37). This effect was mainly due to an induced expression in female ER deficient mice. Both expression profiles are represented in Figure 32.

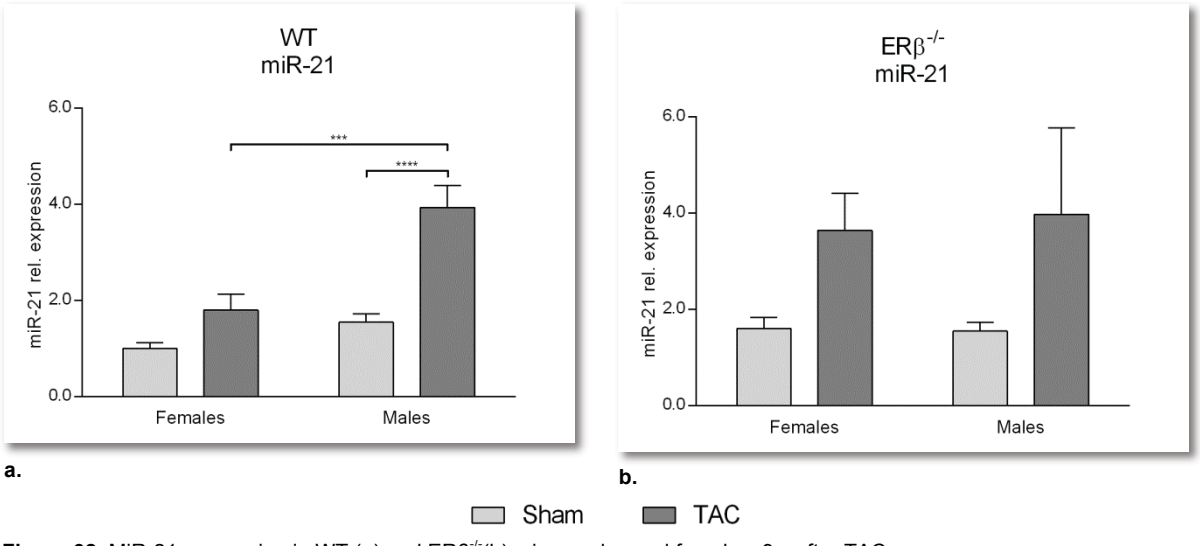


Figure 32. MiR-21 expression in WT (a) and ERβ$^{-/-}$(b) mice, males and females, 9w after TAC.

Although male WT mice showed a higher expression in Sham (FS=1.00; MS=1.55) and TAC (FT=1.80; MT=3.93), this difference was only significant under hypertrophic conditions. In contrast, in ERβ deficient animals the sex differences in hypertrophy disappeared, mainly because in hypertrophic females the expression of miR-21 was higher

than in WT and similar to the MT group (FT=3.64; MT=3.93). Moreover, in ERβ$^{-/-}$ Sham animals the expression in both sexes had also similar mean values (FS=1.60; MS=1.55) (Table 45).

Table 45. MiR-21 expression in WT and ERβ$^{-/-}$ mice..

Genotype	Females		Males	
	FS	FT	MS	MT
WT	1,00	1,80	1,55	3,93
ERβ$^{-/-}$	1,60	3,64	1,55	3,98

The expression is represented as mean values after the correction performed in 1.5. Values normalized to WT FS group.

As seen in point 4.4, miR-21 expression was higher in the ERβ$^{-/-}$ than in the WT female animals (Figure 25). Figure 33 shows the two-way ANOVA analysis which confirms that surgery effect prevails between genotypes, but not the sex effect.

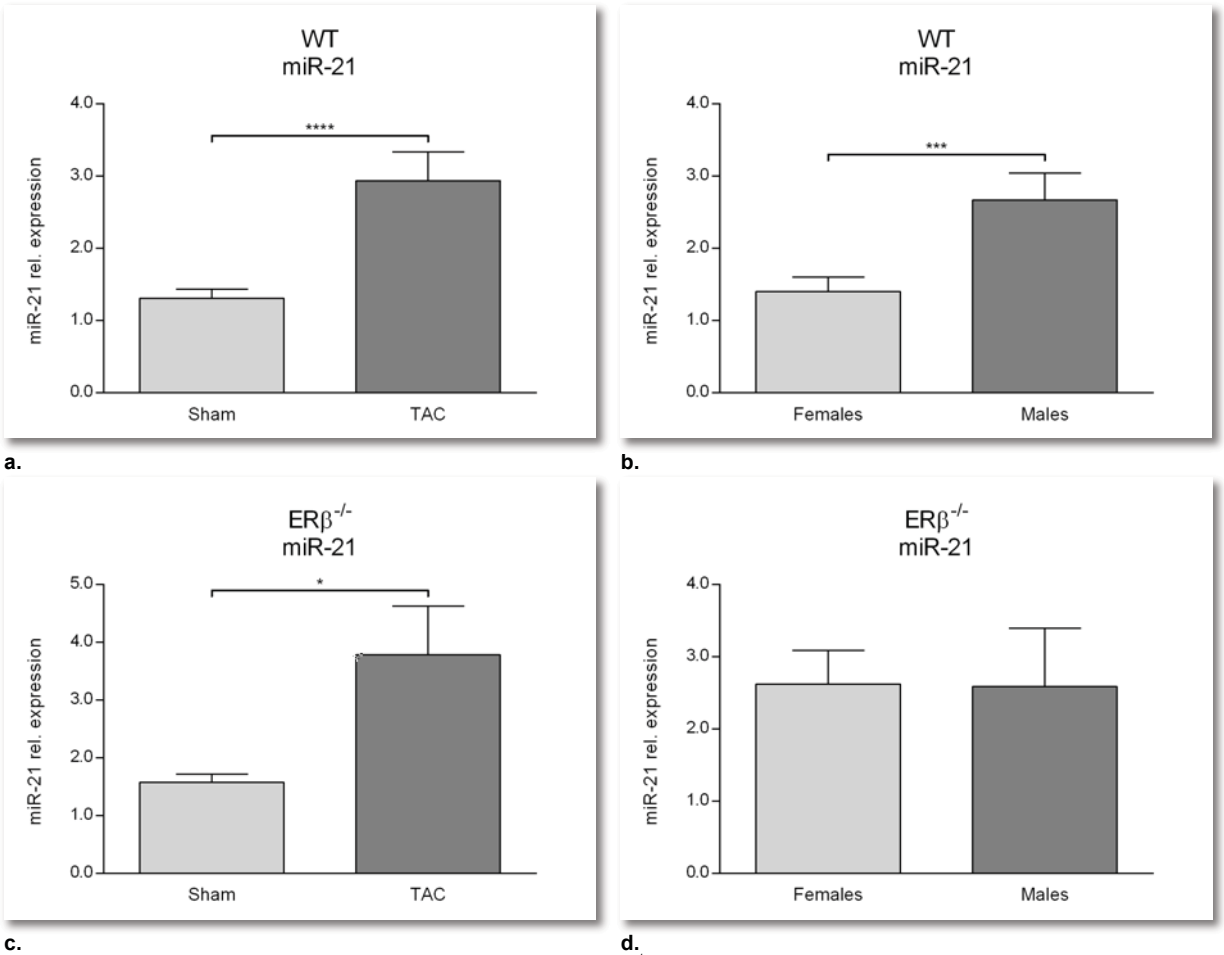


Figure 33. Sex differences in miR-21 expression disappear in ERβ$^{-/-}$ mice.

Two-way ANOVA results, after the correction (see point 4.5). Only TAC effect persists with the absence of ERβ.

These data correlate with the results concerning fibrosis described before that show in WT animals the strong development of fibrosis occurring only in males while in ERβ$^{-/-}$ animals it occurs similarly in both sexes.[60]

Knowing that MAPK/ERK pathway has other negative regulators than SPRY1 and the knowledge that miRNAs frequently act in networks, encouraged us to look for other miRNAs that could target other genes directly involved in this pathway.

4.8.2. miR-21 is not the only miRNA which targets MAPK/ERK pathway negative regulators

Analysing MAPK/ERK pathway and its negative regulators other than SPRY1, we selected other possible targets as interesting for the study, namely SPRY2, RASA1 and RASA2 (RAS p21 protein activator 1 and 2). These repressors were analysed with TargetScan in order to find which miRNAs could putatively target them (Figure 34).

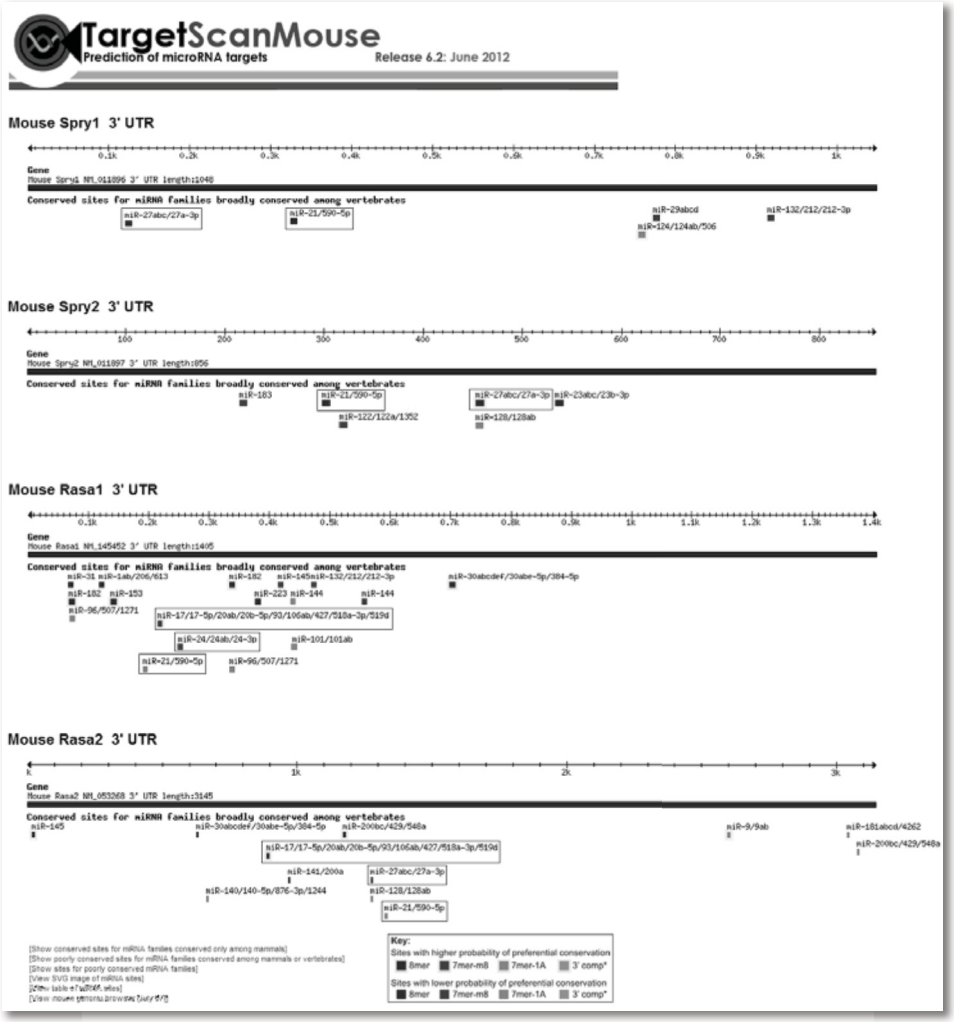


Figure 34. Partial screenshots of the TargetScan analysis of SPRY1, SPRY2, RASA1 and RASA2. miRNAs of interested highlighted.

SPRY2 was selected because, as SPRY1, this gene was already a described target for miR-21.[139] Apart of miR-21, the TargetScan analysis of the 3'UTRs of these genes, predicted the existence of binding sites for miR-27 in both sequences.

On the other hand the TargetScan analysis of RASA1 and RASA2 3'UTRs also identified putative binding sites for miR-21 and in the case of RASA2 for miR-27 as well. Surprisingly, all the three miRNAs (miR-21, miR-27a and miR-27b) have similar expression profiles in the WT TAC model previously analysed.

Other interesting miRNAs expressed in the heart, with RASA1 and RASA2 as putative targets and with similar expression profiles in the studied model include miR-106 and miR-24. RASA1 has putative conserved binding sites for miR-24 and miR-106, whereas RASA2 has putative conserved binding sites for miR-106. The summary of the miRNAs with putative binding sites in all the analysed MAPK/ERK pathway inhibitors are represented in Table 46.

Table 46. MiRNAs with binding sites in the selected MAPK/ERK pathway inhibitors, SPRY1, SPRY2, RASA1 and RASA2.

MAPK/ERK signalling pathway inhibitor	miRNAs with binding sites in the 3'UTR
RASA1	miR-21, miR-106, miR-24
RASA2	miR-21, miR-27, miR-106
SPRY1	miR-21, miR-27
SPRY2	miR-21, miR-27

4.8.3. ERβ regulates the miRNAs with putative binding sites on fibrosis repressors in mice

All miRNAs selected in 4.8.2 and represented in Table 46, have similar expression profiles in the mouse TAC models. In WT mice, the six miRNAs show significant surgery and sex effect, with an up-regulation observed only in male TAC animals and only miR-106a didn't present significant sex differences in TAC.

Table 47. Statistics analysis summary of the six miRNAs expression in WT mice.

miRNA	two-way ANOVA (p-value)			TAC effect		Sex-differences	
	Surgery	Sex	Sex*Surgery Interaction	Females	Males	Sham	TAC
miR-106a	*	*	ns		↑		
miR-106b	*	*	0,098		↑		♂>♀
miR-21	****	***	*		↑		♂>♀
miR-24	**	**	ns		↑		♂>♀
miR-27a	*	**	ns		↑		♂>♀
miR-27b	**	*	ns		↑		♂>♀

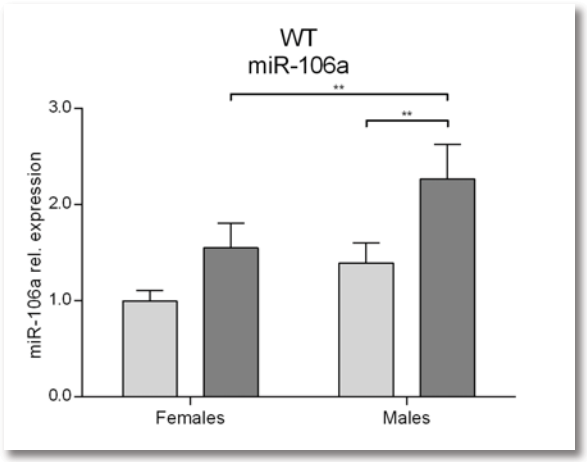
* p<0.05; ** p<0,01; *** p<0.001; ns – not significant.

Table 48. Statistics analysis summary of the six miRNAs expression in ERβ$^{-/-}$ mice.

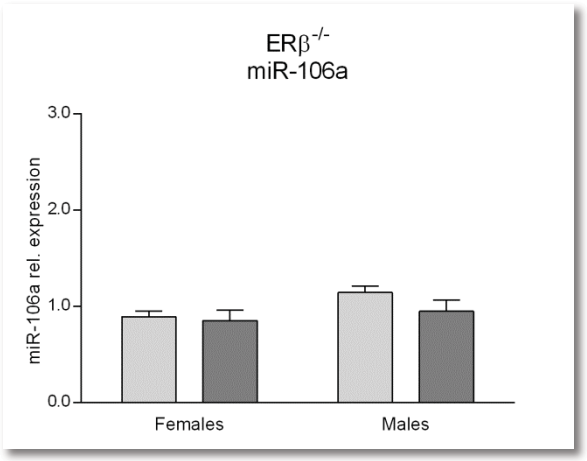
miRNA	two-way ANOVA (p-value)			TAC effect		Sex-differences	
	Surgery	Sex	Sex*Surgery Interaction	Females	Males	Sham	TAC
miR-106a	ns	0,0835	ns				
miR-106b	**	ns	ns	↓			
miR-21	*	ns	ns				
miR-24	ns	ns	ns				
miR-27a	0,0823	ns	ns				
miR-27b	**	ns	ns		↑		

* p<0.05; ** p<0,01; *** p<0.001; ns – not significant.

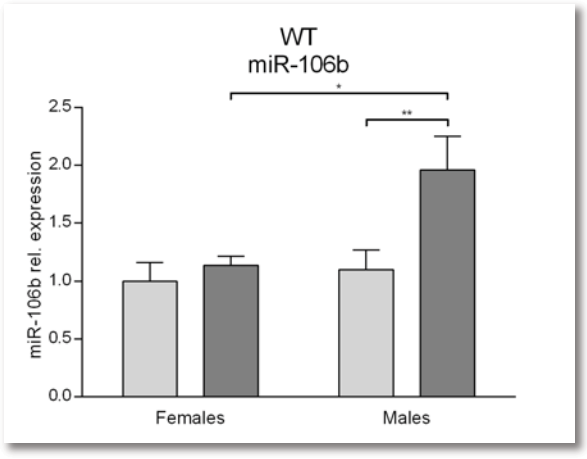
As shown in Table 47 and Table 48, all the sex and surgery effects were abolished with the absence of ERβ and only miR-27b maintained the significant up-regulation in males. The absence of ERβ also led to a significant down-regulation of miR-106b in female TAC operated animals. Figure 35 shows the individual expression profiles in both genotypes.



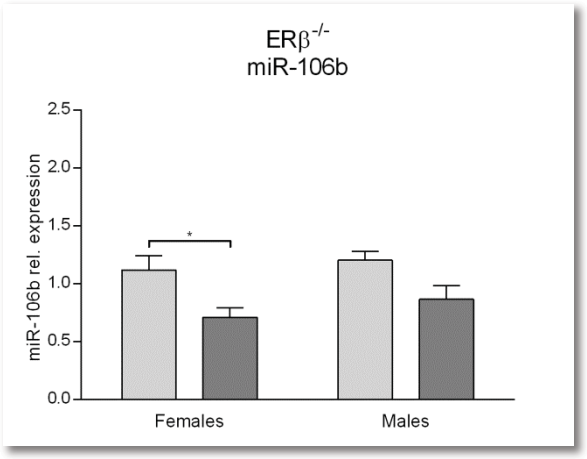
a.



b.



c.



d.

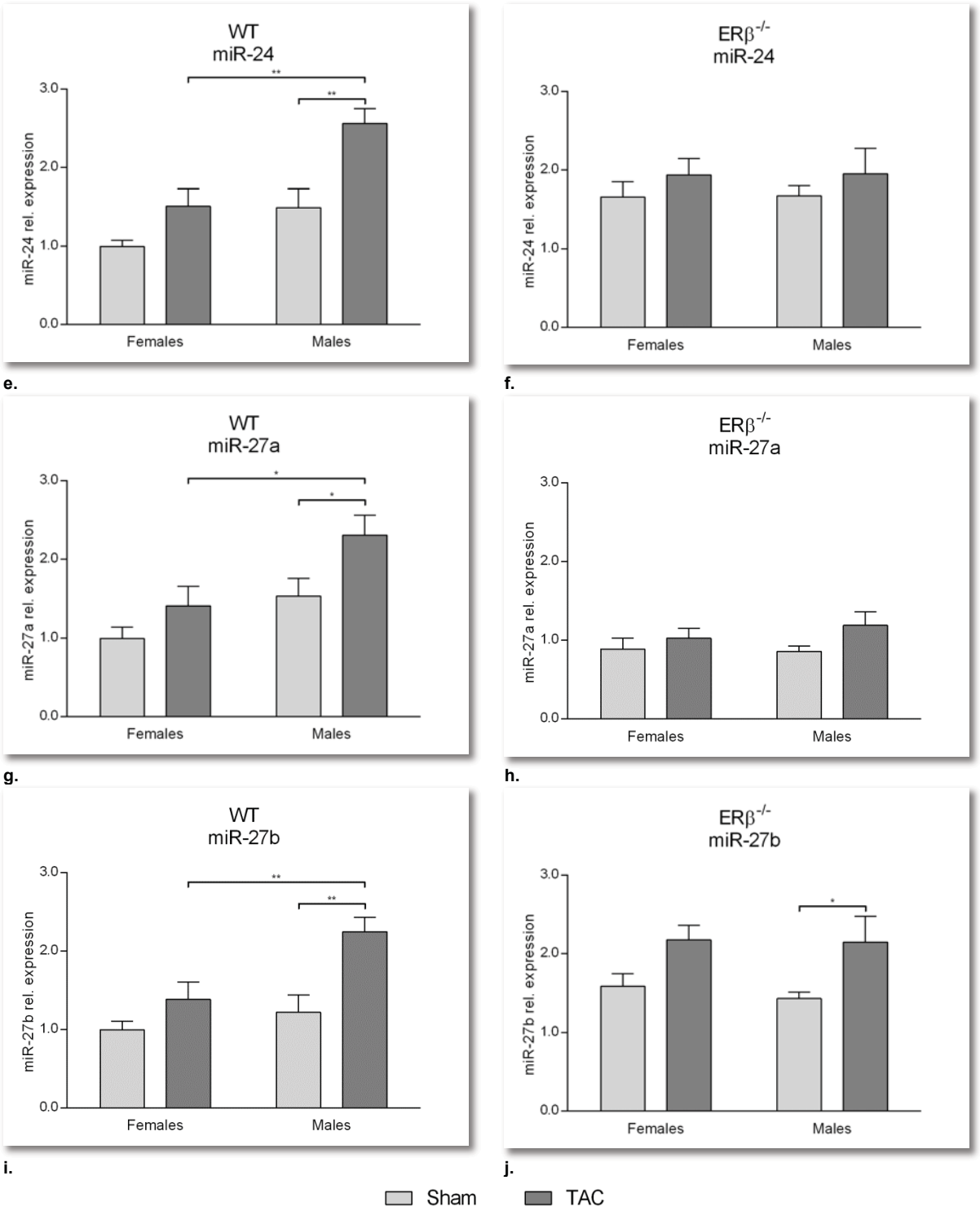
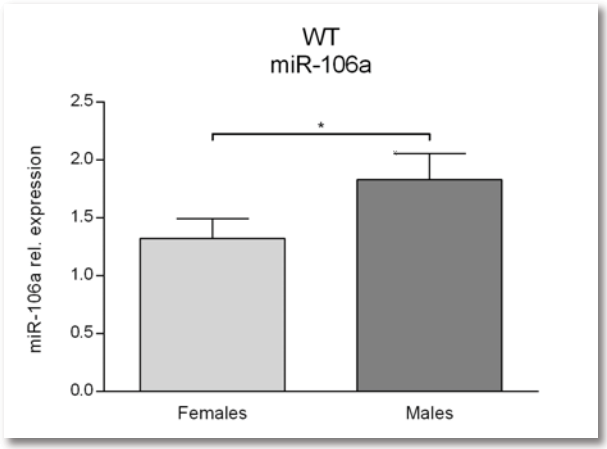
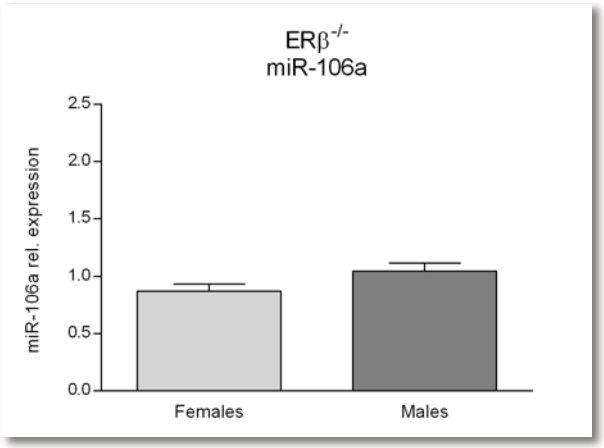


Figure 35. miRNA expression in Sham and TAC operated WT and ERβ$^{-/-}$ mice.
Two-way ANOVA; Bonferroni post-hoc test; * p<0.05, **p<0.01, ***p<0.001.

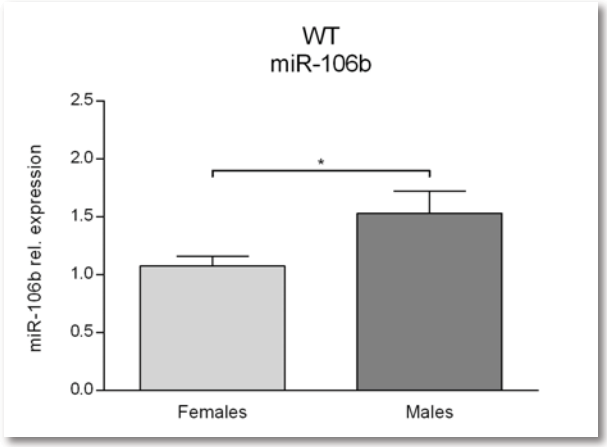
As observed in miR-21, ERβ deletion had also an effect in the expression of all five miRNAs (Figure 35), abolishing the surgery and the sex effects either through a higher basal expression in female Sham operated animals (miR-106b, miR-24 and miR-27b), as seen in point 4.4, or through a lower expression in male Sham (miR-106a and miR-27a). Once again, ERβ deletion also abolished the sex effect observed in these miRNAs expression in WT mice (Figure 36)


WT
miR-106a
miR-106a rel. expression
2.5
2.0
1.5
1.0
0.5
0.0
*
Females
Males

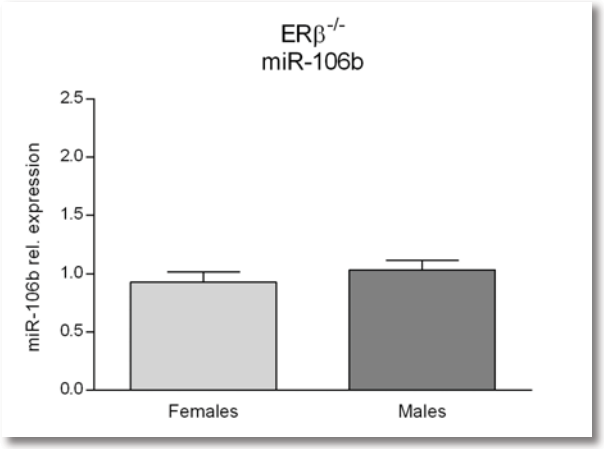
a.


ERβ-/-
miR-106a
miR-106a rel. expression
2.5
2.0
1.5
1.0
0.5
0.0
Females
Males

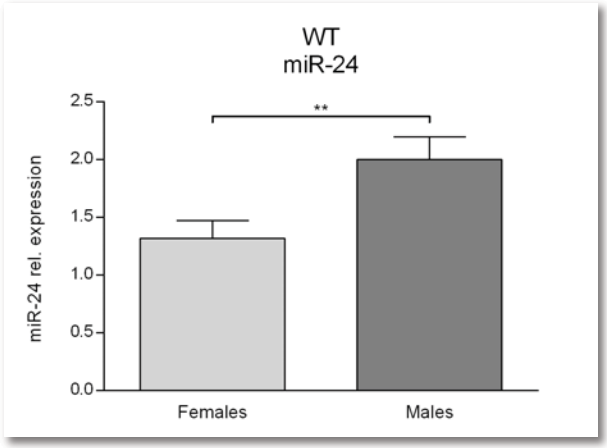
b.


WT
miR-106b
miR-106b rel. expression
2.5
2.0
1.5
1.0
0.5
0.0
*
Females
Males

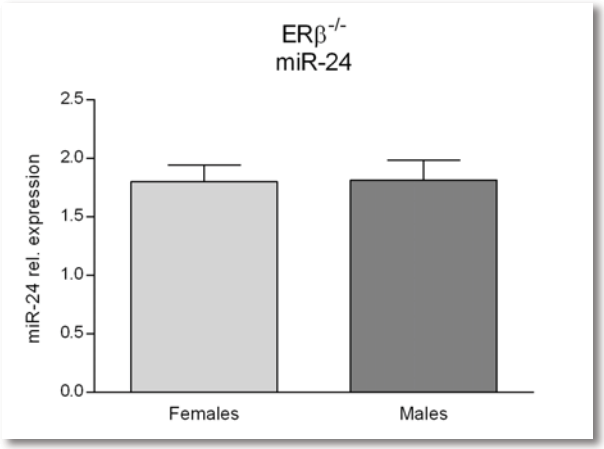
c.


ERβ-/-
miR-106b
miR-106b rel. expression
2.5
2.0
1.5
1.0
0.5
0.0
Females
Males

d.


WT
miR-24
miR-24 rel. expression
2.5
2.0
1.5
1.0
0.5
0.0
**
Females
Males

e.


ERβ-/-
miR-24
miR-24 rel. expression
2.5
2.0
1.5
1.0
0.5
0.0
Females
Males

f.

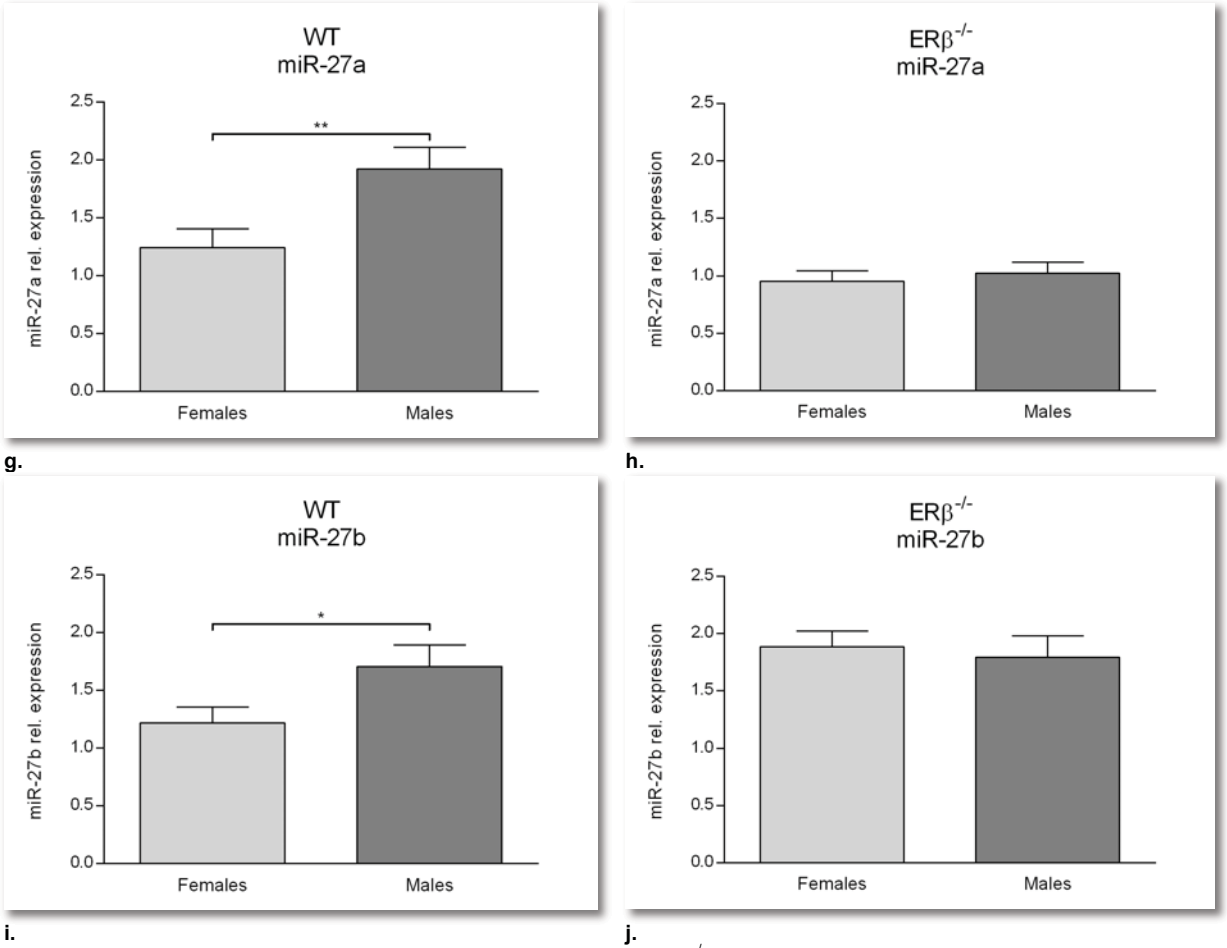


Figure 36. Genotype influence on sex effect. WT (a, c, e, g, i) and ERβ$^{-/-}$ (b, d, f, h, j) mice, Sham and TAC operated animals are represented together, according to the sex.

Two-way ANOVA; Bonferroni post-hoc test; * p<0.05, **p<0.01.

These comparisons demonstrated that the selected miRNAs with MAPK/ERK pathway repressors as possible targets are regulated by ERβ. However it was necessary to prove this hypothesis in the main cell type involved in fibrosis development, the fibroblasts (*in vitro*).

4.8.4. E2 regulates miRNA expression in cardiac fibroblasts in different ways according to the sex

Knowing that ERβ has an effect in fibrosis[60] and given the visible effect on miRNA expression in the whole heart, it was expectable that E2 would have also an effect in collagen producer cells, cardiac fibroblasts. For this, primary cardiac fibroblasts were treated with E2 for 24h to measure its effect on the expression of Col1, Col3 and the selected miRNAs.

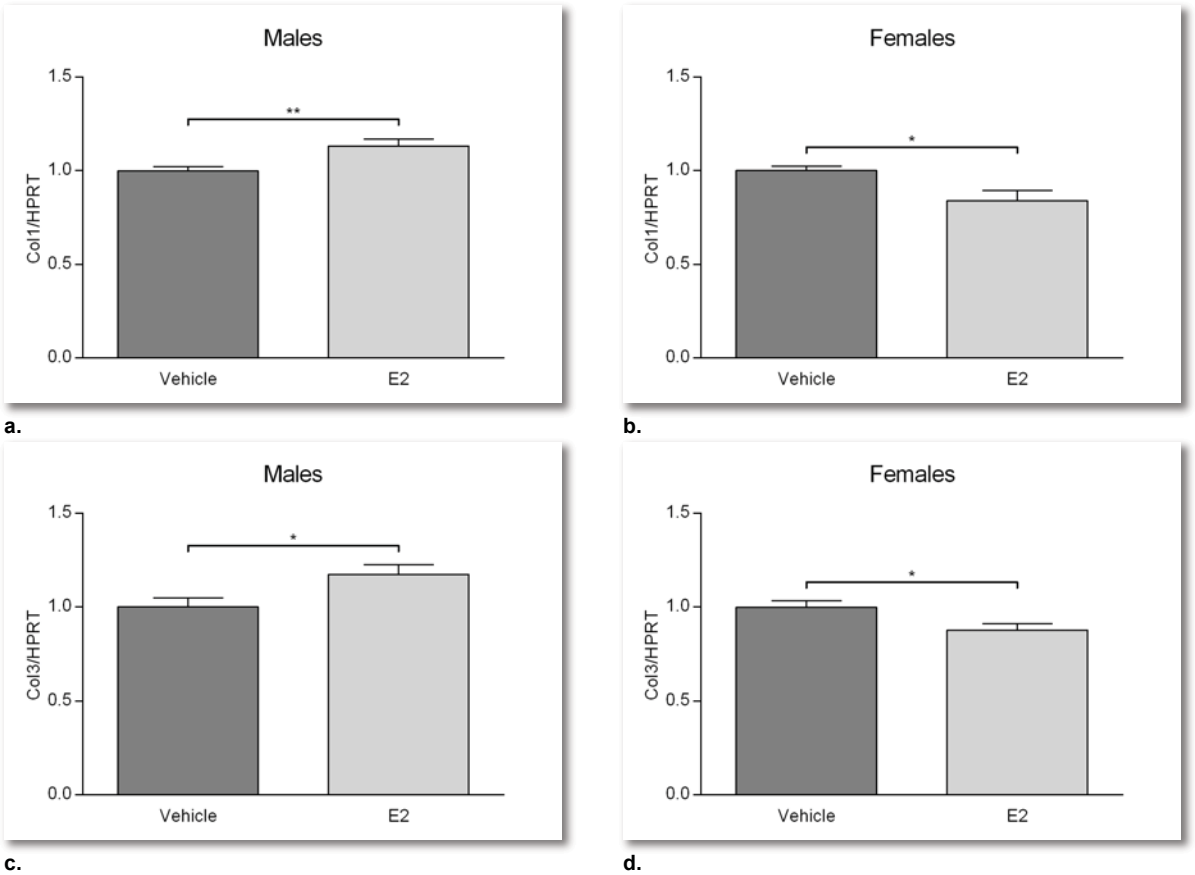


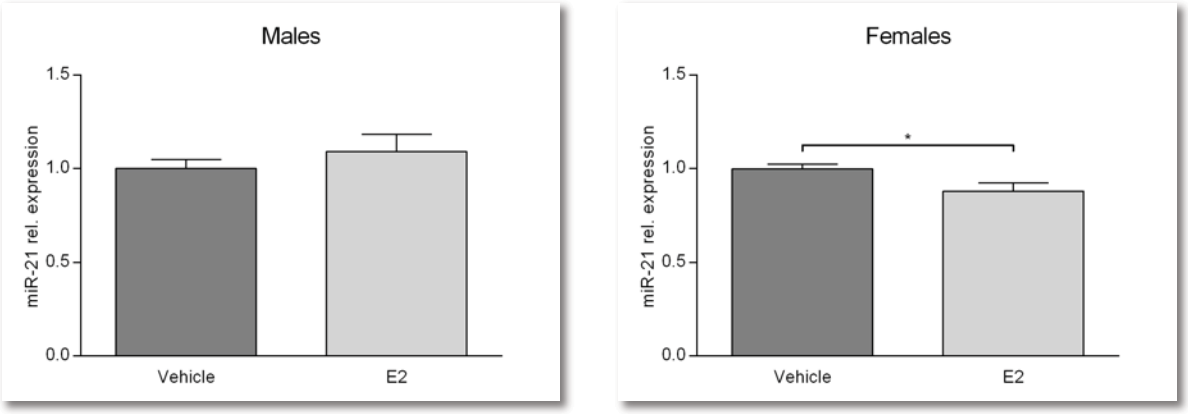
Figure 37. E2 induces Col1 and Col3 only in male primary cardiac fibroblasts. Col1 (a, b) and Col3 (c, d) expression in male and female primary cardiac fibroblasts, treated with E2.

T-test; * $p<0.05$, ** $p\leq0.01$.

As shown in Figure 37 and also by our group previously[184], 24h of E2 treatment caused an up-regulation of Col1 and Col3 mRNA in male fibroblast, whereas in female fibroblasts the expression of both collagens was down-regulated by the hormone. These results were used as positive control for the fibroblasts treatment in order to investigate the direct effects of E2 on miRNA expression in isolated fibroblasts.

In the case that these miRNAs influence fibrosis regulating the expression of the MAPK/ERK pathway, an increase in miRNA expression will reduce the amount of repressor molecules, inducing fibrosis. In contrast, a reduction of these miRNAs will facilitate the expression of the repressor, reducing fibrosis.

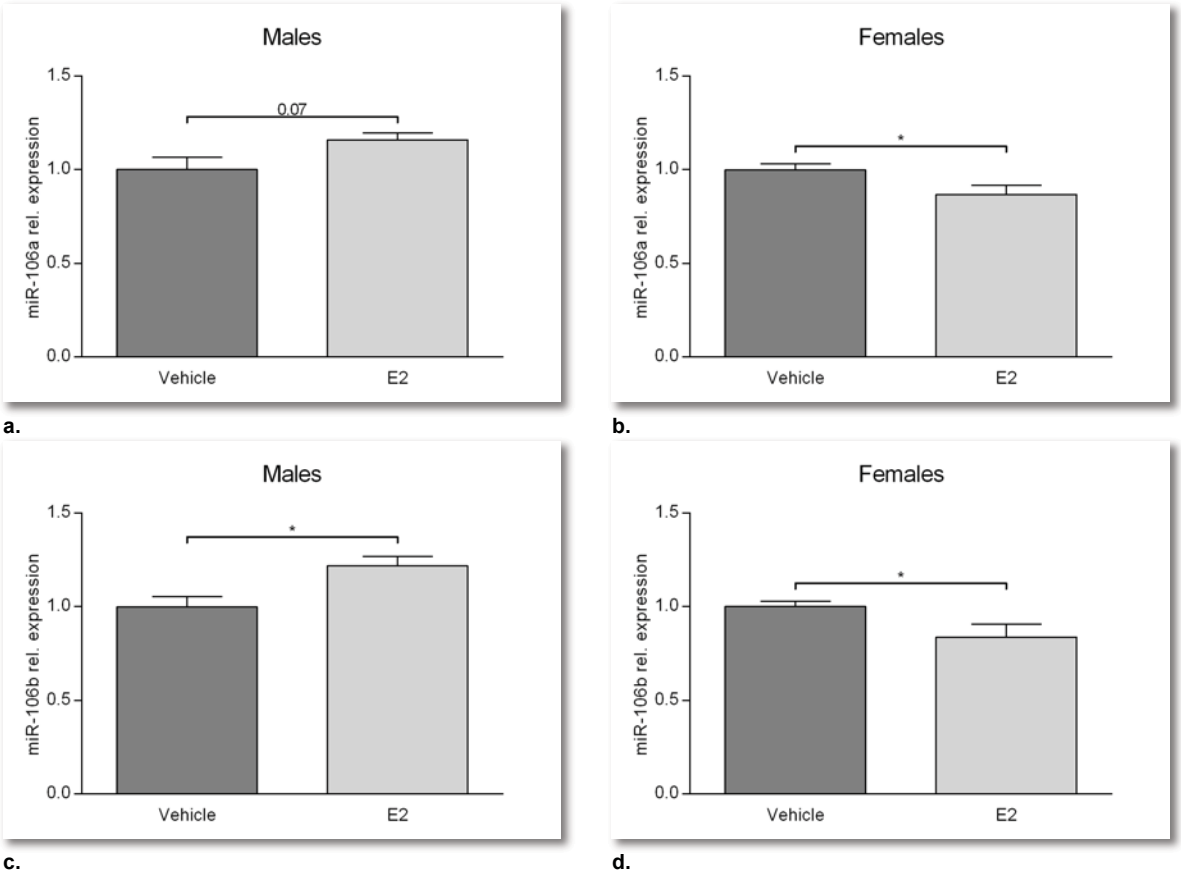
The first *in vitro* evidence of sex differences in these selected miRNAs appeared with the measurement of miR-21. E2 treatment had no effect in male fibroblasts, while in females it caused a down-regulation of this miRNA (Figure 38).



a. b.

Figure 38. MiR-21 expression in male and female primary cardiac fibroblasts, treated with E2.
T-test; * $p<0.05$.

Like miR-21, also miR-106a, miR-106b, miR-24, miR-27a and miR-27b were down-regulated by E2 treatment in female fibroblasts. However, the same treatment had a different effect in male cells, causing an up-regulation of these miRNAs (Figure 39).



a. b.

c. d.

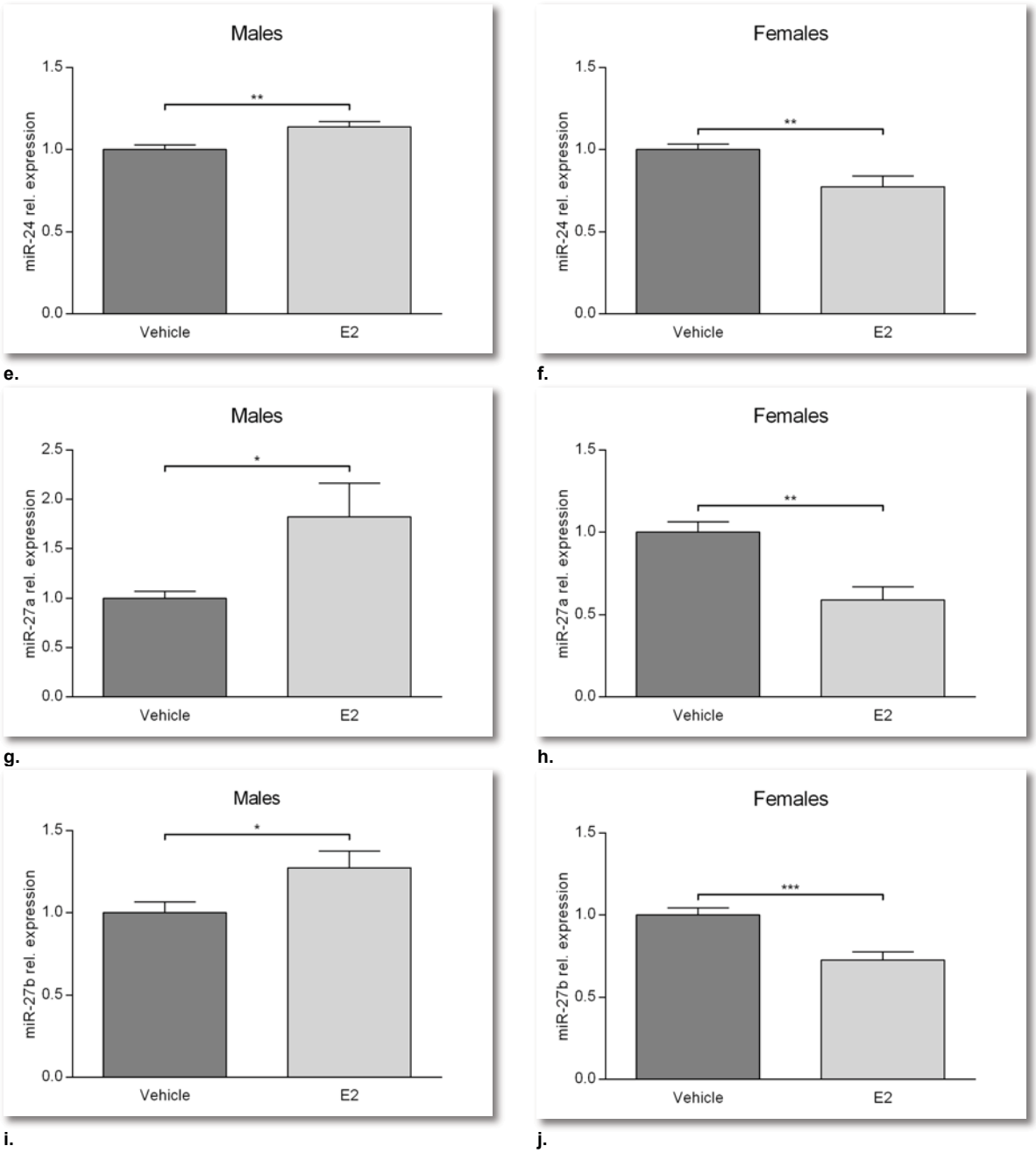
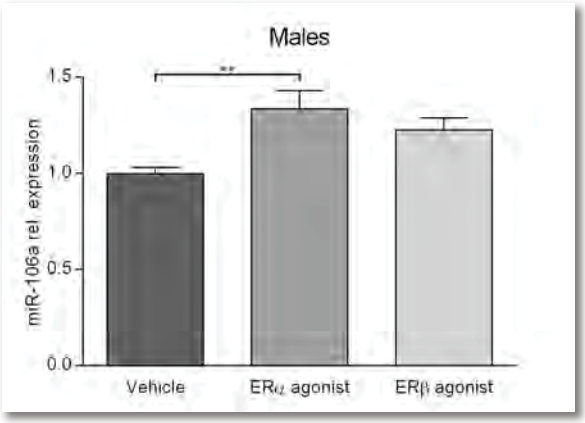


Figure 39. MicroRNAs expression is down-regulated by E2 in female and up-regulated in male primary cardiac fibroblasts MiR-106a (a, b), miR-106b (c, d), miR-24 (e, f) miR-27a (g, h) and miR-27b (i, j).

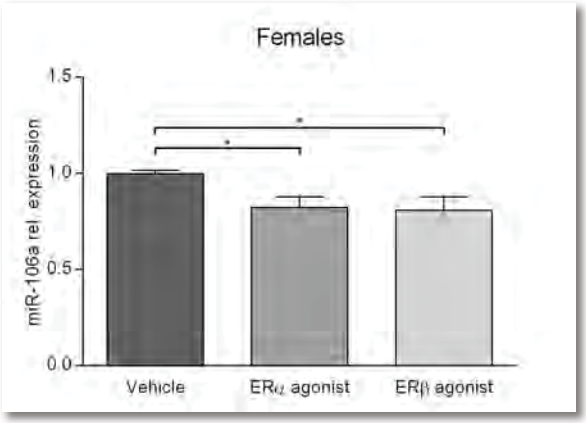
T-test; * $p<0.05$, ** $0.001<p\leq0.01$.

4.8.5. ERβ and ERα regulate miRNA expression in cardiac fibroblasts in different ways according to the sex

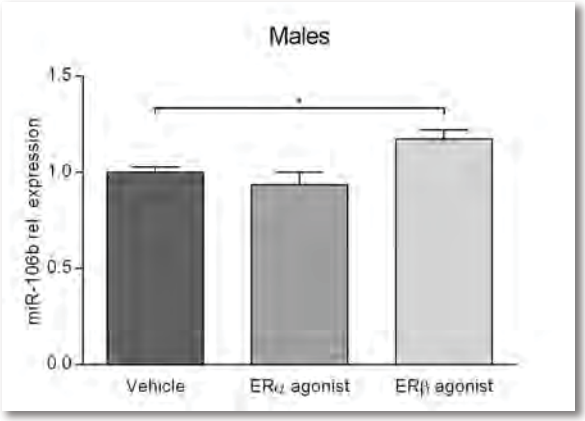
In order to unveil the role of each ER subtype in fibroblasts in each sex, primary rat cardiac fibroblasts were stimulated for 24h with ERα (PPT) or ERβ (Comp A) specific agonists.


Males
miR-106a rel. expression
1.5
1.0
0.5
0.0
**
Vehicle
ERα agonist
ERβ agonist

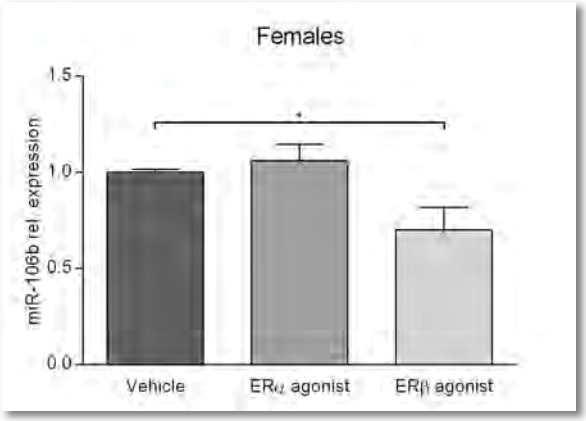
a.


Females
miR-106a rel. expression
1.5
1.0
0.5
0.0
*
*
Vehicle
ERα agonist
ERβ agonist

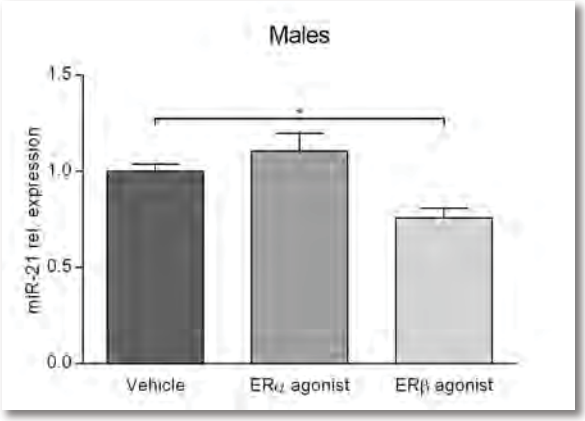
b.


Males
miR-106b rel. expression
1.5
1.0
0.5
0.0
*
Vehicle
ERα agonist
ERβ agonist

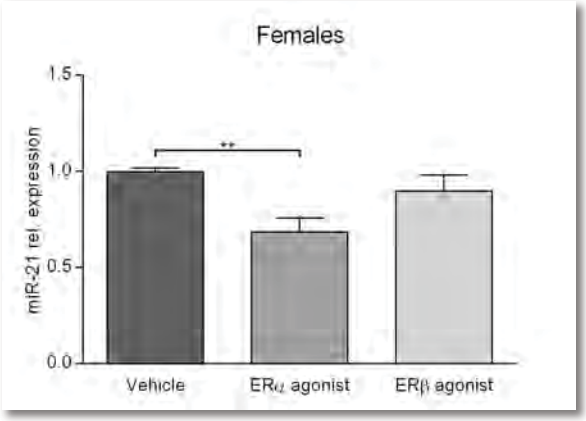
c.


Females
miR-106b rel. expression
1.5
1.0
0.5
0.0
*
Vehicle
ERα agonist
ERβ agonist

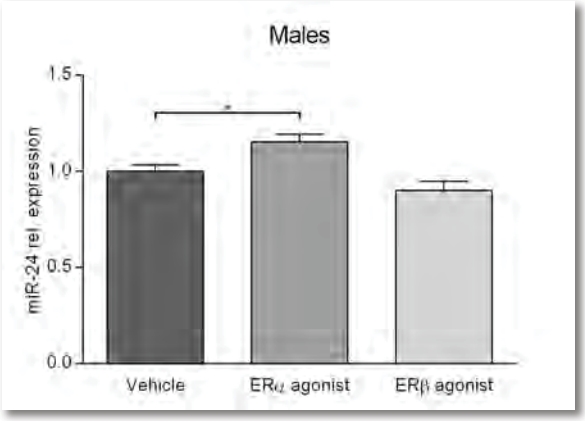
d.


Males
miR-21 rel. expression
1.5
1.0
0.5
0.0
*
Vehicle
ERα agonist
ERβ agonist

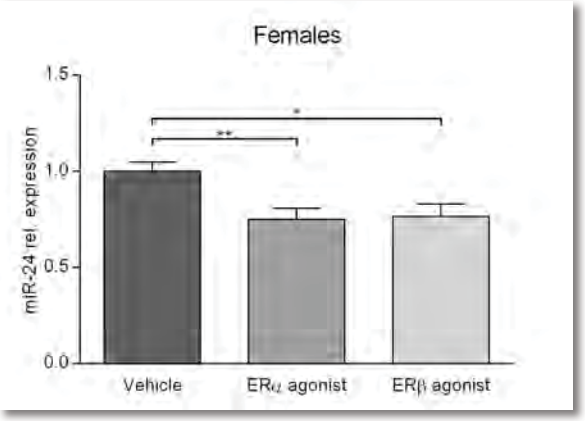
e.


Females
miR-21 rel. expression
1.5
1.0
0.5
0.0
**
Vehicle
ERα agonist
ERβ agonist

f.


Males
miR-24 rel. expression
1.5
1.0
0.5
0.0
*
Vehicle
ERα agonist
ERβ agonist

g.


Females
miR-24 rel. expression
1.5
1.0
0.5
0.0
*
**
Vehicle
ERα agonist
ERβ agonist

h.

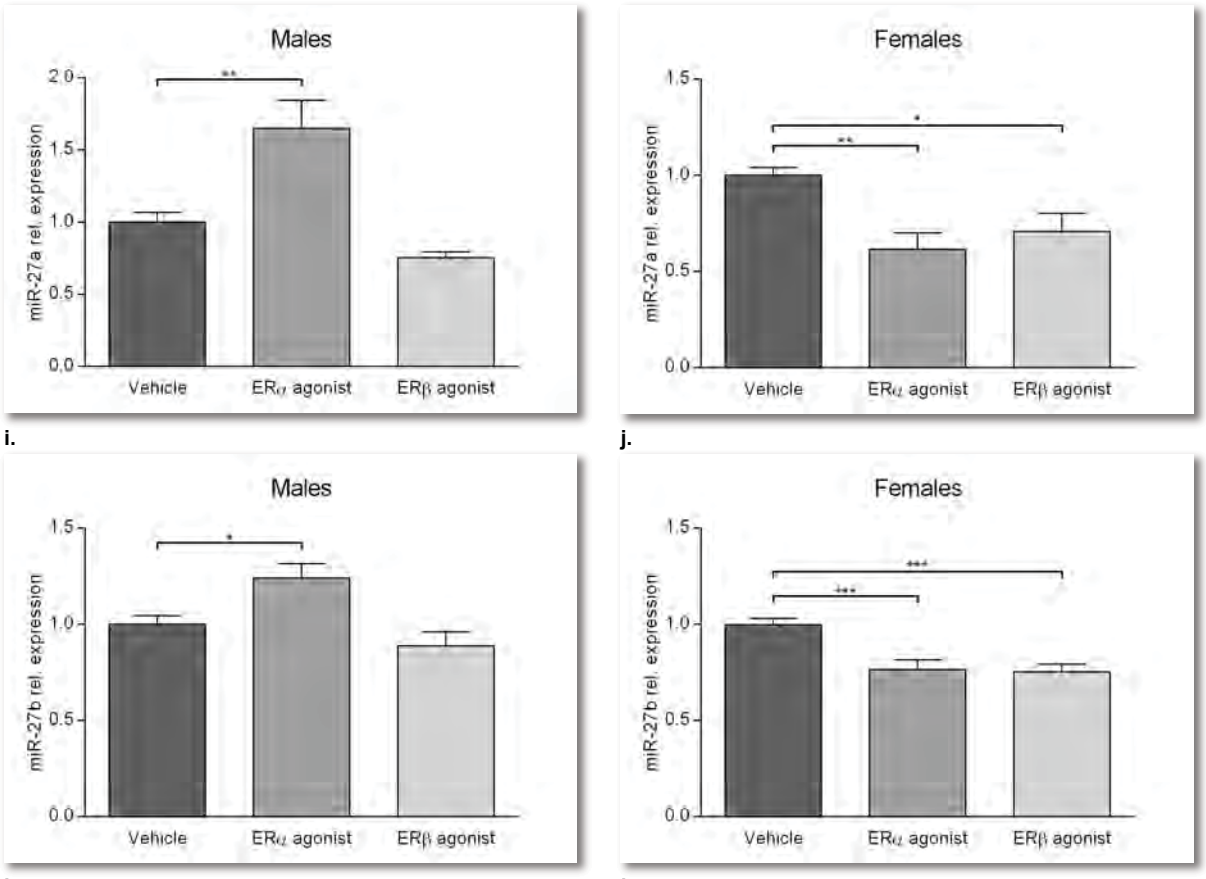


Figure 40. MiR-106a (a, b), miR-106b (c, d), miR-21 (e, f), miR-24 (g, h), miR-27a (i, j) and miR-27b (k, l) expression in male and female primary cardiac fibroblasts, treated with ERα or ERβ specific agonists.
One-way ANOVA; Bonferroni post-hoc test; * $p<0.05$, ** $0.001<p\leq0.01$, *** $p\leq0.001$.

A clear sex different response to the treatment was observed. In female cardiac fibroblasts, the treatment with ERβ-specific agonist caused a significant down-regulation of miR-106a, miR-106b, miR-24, miR-27a and miR-27b expression and ERα specific agonist caused also a significant down-regulation of miR-106a, miR-21, miR-24, miR-27a and miR-27b expression. None of the 6 miRNAs was induced by the agonist treatment in female cells.

Male cardiac fibroblasts had a remarkably different response to these compounds. The only down-regulation observed was caused by the ERβ-specific agonist on miR-21 expression. This same agonist also caused a significant up-regulation of miR-106b in comparison to the vehicle. Moreover, ERα-specific agonist significantly up-regulated the expression of four miRNAs (miR-106a, miR-24, miR-27a and miR-27b).

In general, the responses of the analysed miRNAs to the E2 and ER agonists are different between male and female fibroblasts. E2 caused down-regulations in female cells and up-regulations in male cells (except miR-21). In what concerns to ERs, both receptors, or at least one, caused down-regulation of the analysed miRNAs' expression in female cells while in males a majority of these miRNAs are up-regulated by the ERα agonist.

4.8.6. Summary of the sex-specific effect of E2 and ERβ effect on miRNA regulation in fibroblasts

Table 49 represents a summary of all significant up-/down-regulated miRNAs in cardiac fibroblasts, after treatment with E2, ERβ or ERα specific agonists. The three treatments caused down-regulations of all six miRNAs in female cells, except miR-21 and miR-106b that were not affected by ERβ and ERα specific agonists, respectively.

Table 49. E2, ERβ and ERα sex-specific effect on cardiac fibroblasts

miRNA	Cardiac fibroblasts					
	Females			Males		
	E2	ERα specific agonist	ERβ specific agonist	E2	ERα specific agonist	ERβ specific agonist
miR-21	↓	↓	-	-	-	↓
miR-24	↓	↓	↓	↑	↑	-
miR-27a	↓	↓	↓	↑	↑	-
miR-27b	↓	↓	↓	↑	↑	-
miR-106a	↓	↓	↓	↑	↑	-
miR-106b	↓	-	↓	↑	-	↑

T-test results; ↑/↓ significant up-/down-regulation.

4.9. Analysis in ERα$^{-/-}$ mice confirms the effect observed in fibroblasts

The results showed above describe very distinct effects of the ER subtypes depending on the sex. This opens the question of whether the influence of ERα on the expression of miRNAs in primary cardiac fibroblast is comparable to the LV. A set of samples of ERα$^{-/-}$ animals, males and females, Sham operated, was compared to the corresponding WT siblings.

The analysis was restricted to the same group of miRNAs selected by a possible relation to fibrosis (miR-21, miR-24, miR-27a, miR-27b, miR-106a and miR-106b) analysed previously.

Table 50. ERα deletion effect on miRNA expression in the LV. Two-way ANOVA results.

miRNA	two-way ANOVA (p-value)		
	Sex	Genotype	Sex*Genotype Interaction
miR-106a	***	ns	ns
miR-106b	*	*	ns
miR-21	ns	*	**
miR-24	**	ns	*
miR-27a	***	ns	*
miR-27b	**	*	0,0545

WT and ERα$^{-/-}$ miRNA expression values analysed by two-way ANOVA. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001; ns – not significant.

Table 51. ERα deletion effect on miRNA expression in LV. Bonferroni post-hoc test.

miRNA	Ratios *(Bonferroni post-hoc test result)*			
	Females ERα$^{-/-}$ /WT	Males ERα$^{-/-}$ /WT	WT M/F	ERα $^{-/-}$ M/F
miR-106a	0,93 *ns*	0,97 *ns*	0,75 *	0,78 *
miR-106b	0,95 *ns*	0,80 *	0,95 *ns*	0,81 *
miR-21	1,08 *ns*	0,58 ***	1,46 **	0,78 *ns*
miR-24	1,06 *ns*	0,77 *	0,96 *ns*	0,70 **
miR-27a	1,07 *ns*	0,71 *	0,91 *ns*	0,60 ***
miR-27b	0,97 *ns*	0,68 *	0,90 *ns*	0,63 **

ERα$^{-/-}$/WT and Males/Females ratios and the corresponding Bonferroni post-hoc test; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001; ns – not significant.

Interestingly, in female mice the absence of ERα does not influence the expression of any of these six miRNAs. In contrast, significant effects of this deletion were detected in male animals. MiR-106b, miR-21, miR-24, miR-27a and miR-27b were down-regulated in male ERα$^{-/-}$ animals, when compared to the WT siblings. Besides the significant sex effect, miR-106a also showed significant sex differences in both ERα$^{-/-}$ and WT animals. On the other hand, miR-106b, miR-24, miR-27a and miR-27b presented sex differences in the ERα$^{-/-}$ animals, not observed in WT (Figure 41).

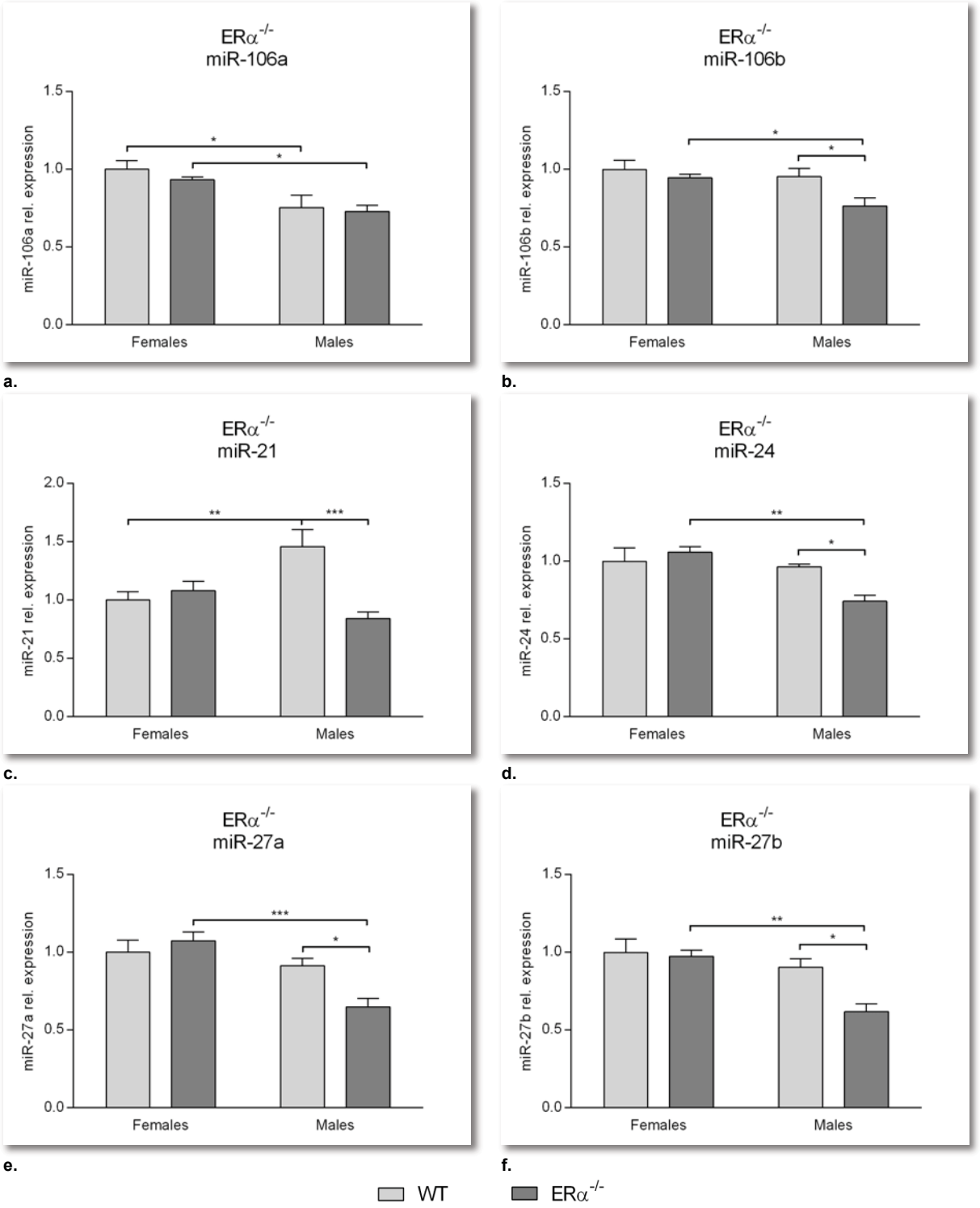


Figure 41. ERα deficiency affects only male mice . WT and ERα$^{-/-}$ mice, Sham operated animals.

Two-way ANOVA analysis; Bonferroni post-hoc test; * p<0.05, **p<0.01, ***p<0.001.

4.10. AngII regulates miRNA expression in cardiac fibroblasts in different ways according to the sex

AngII is described as an important inducer of fibroblast growth and Col1 synthesis.[185] In order to induce fibrosis *in vitro* and to understand the possible protective role of E2 in fibroblasts with a fibrotic stimulus in each sex, primary cardiac fibroblasts were treated with AngII or co-treated with AngII and E2.

The expression of Col1 and Col3 was quantified by qRT-PCR and used as a control for these treatments.

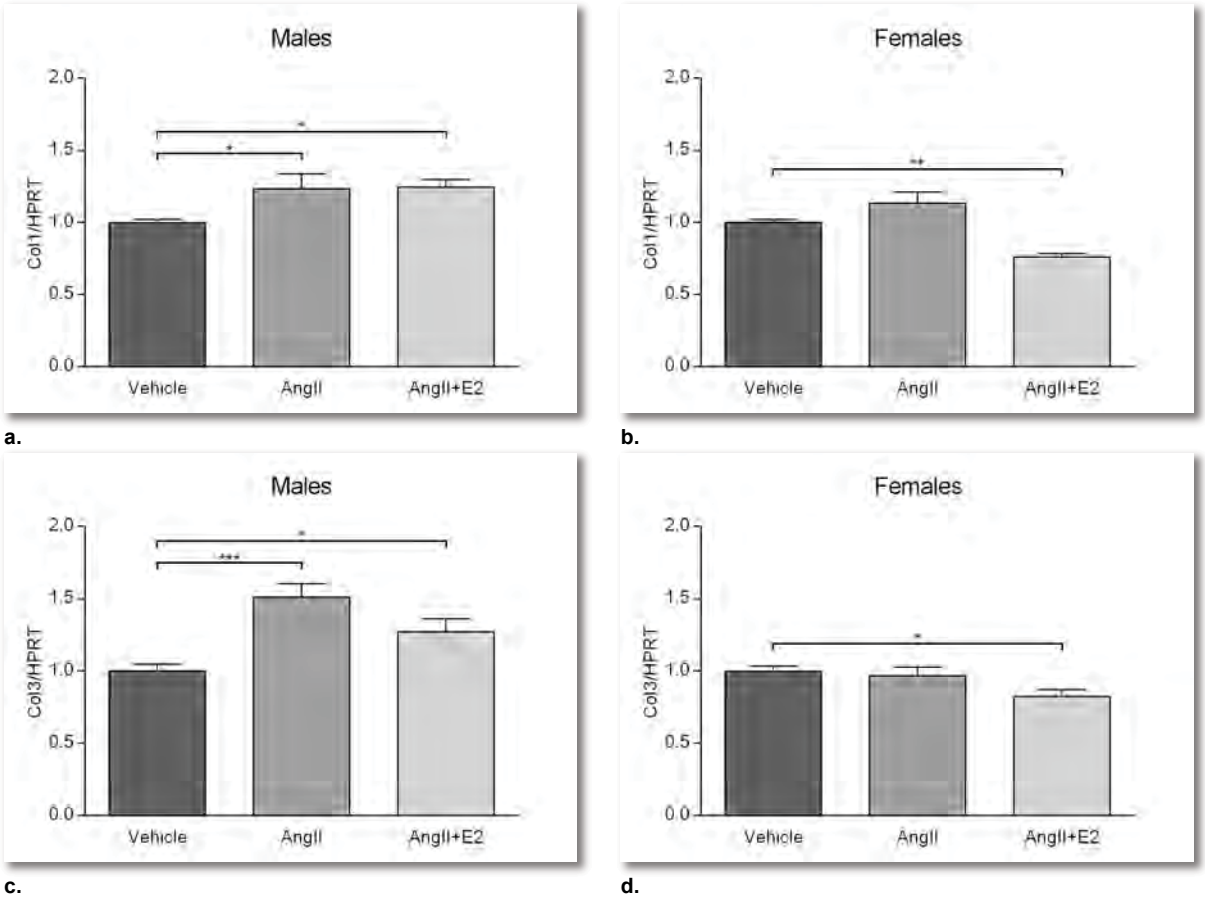
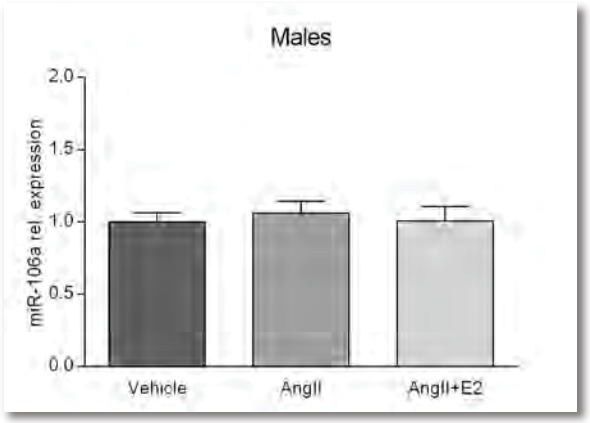


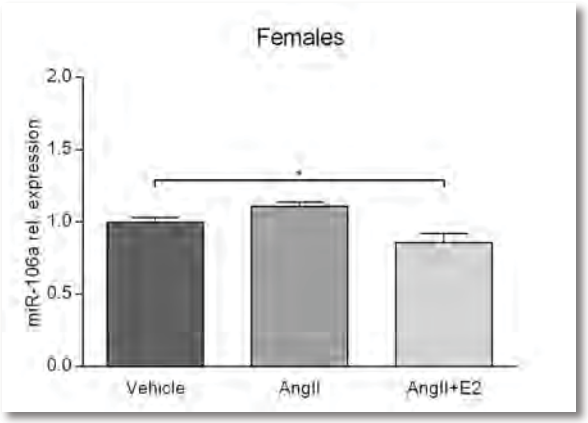
Figure 42. Col1 (a, b) and Col3 (c, d) expression in primary cardiac fibroblasts after treatment with Ang II and E2.
The statistical analysis was performed using 1-way anova, followed by Bonferroni post-hoc test. ; * p<0.05, **p<0.01, ***p<0.001.

The treatment with AngII induced Col1 and Col3 in male fibroblasts (Figure 42 a, c) but had no effect on collagen expression in female cells (Figure 42 b, d). The co-treatment with E2 did not compensate the effect of AngII in male fibroblasts, but in female fibroblasts E2+AngII treatment down-regulated both Col1 and Col3 expression.

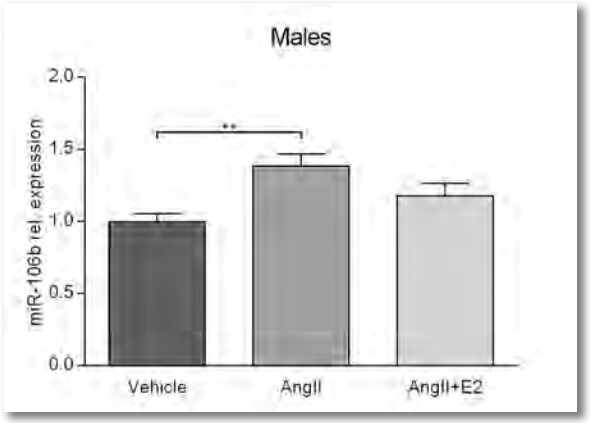
Next, miRNAs (miR-21, miR-24, miR-27a, miR-27b, miR-106a and miR-106b) expression was quantified.



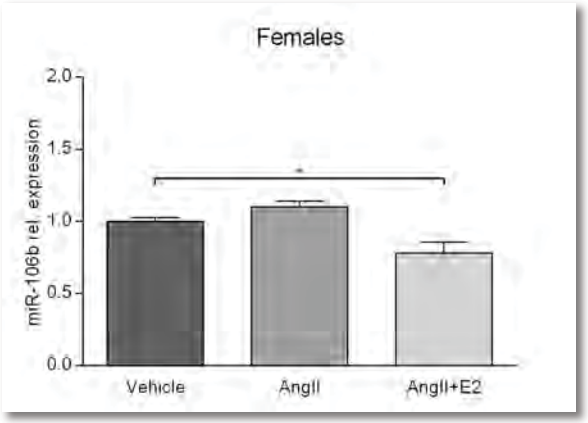
a.



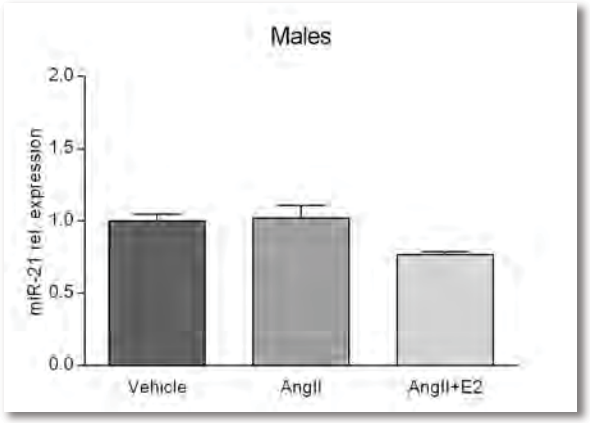
b.



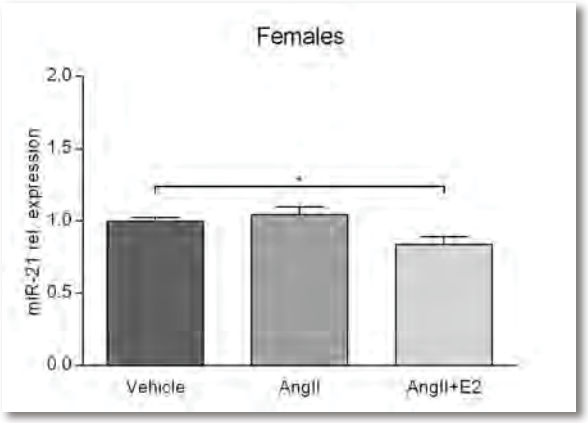
c.



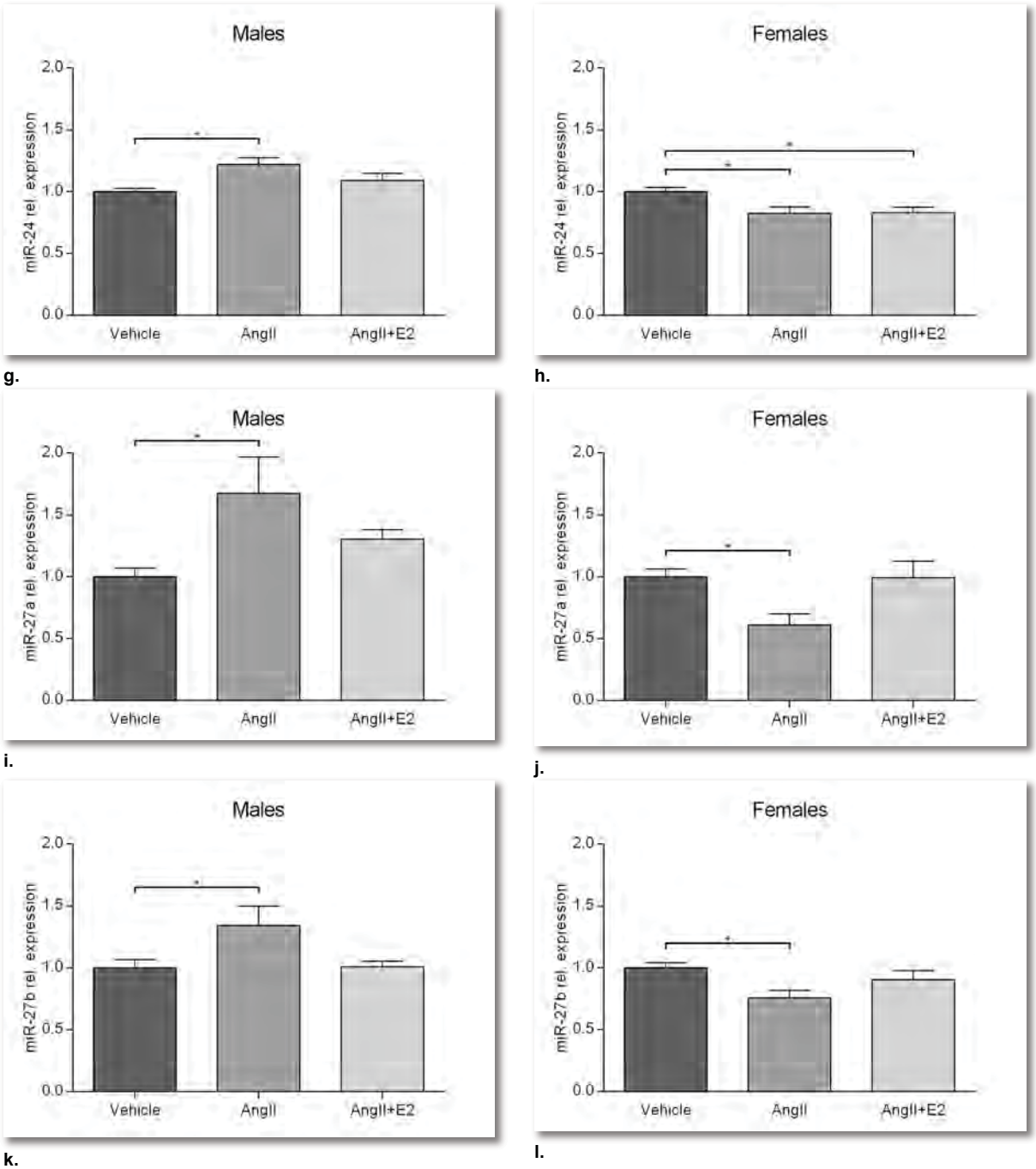
d.



e.



f.



g. h. i. j. k. l.

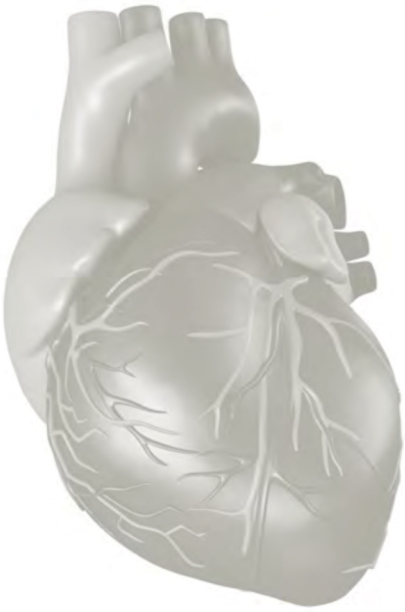
Figure 43. MiRNA expression in primary cardiac fibroblasts after treatment with AngII and E2. MiR-106a (a, b), miR-106b (c, d), miR-21 (e, f), miR-24 (g, h), miR-27a (i, j) and miR-27b (k, l).

The statistical analysis was performed using 1-way anova, followed by Bonferroni post-hoc test. ; * $p<0.05$.

The treatment with AngII affected the expression of miRNAs on cardiac fibroblast in a completely different manner depending on the sex of the cells. In male cardiac fibroblasts, AngII caused an induction of miR-106b, miR-24, miR-27a and miR-27b. The co-treatment with AngII and E2 of male fibroblasts attenuated or even abolished the effect of AngII on miR-106b, miR-24, miR-27a and miR-27b. Once again, there was no visible effect on miR-106a expression and miR-21 showed a tendency for a down-regulation in comparison to the control. MiR-106a and miR-21 didn't show any significant effect with any of the treatments. In contrast to the effect on male cells in female fibroblasts AngII didn't induce any of the six

measured miRNAs. Instead, a down-regulation was observed in three of the six miRNAs (miR-24, miR-27a and miR-27b). In female fibroblasts, the co-treatment down-regulated miR-106a, miR-106b, miR-21 and miR-24 in comparison to the vehicle and abolished the effect of AngII alone on miR-27a and miR-27b expression.

The present results show clear sex-differences in miRNA expression in the heart. Both sex and ERβ were shown to influence their response to a hypertrophic stimulus (TAC). In individual female cell types, E2 and ERα and β agonists tend to down-regulate miRNA expression in both cardiomyocytes and fibroblasts while in male fibroblasts E2 and ERα are mostly causing up-regulations of miRNA expression. However, the response a fibrotic stimulus (AngII) can be compensated by E2 to basal levels in both sexes.



5. Discussion

This study reports for the first time sex differences in miRNA expression in a mouse hypertrophy model. The sex differences were shown to be ERβ-dependent, as they were abolished in its absence. Furthermore, E2 and ERs specific agonists presented a strong effect on miRNA expression in female cardiomyocytes, as well as a sex-specific regulation of their expression in cardiac fibroblasts.

5.1. miRNAs are sex-differently expressed in cardiac hypertrophy

Our group described previously that sex-differences are present in a late stage model of cardiac hypertrophy, in morphology as well as gene expression level. After 9 weeks of TAC, in WT animals, males presented, among other differences, a more pronounced hypertrophy than females, associated with greater myocyte hypertrophy and higher fibrosis level.[60] As in the last few years the understanding of the role of miRNAs as regulators of gene expression has gained importance, the first proposed aim in the present study was to elucidate whether it exists a sex-specific regulation of miRNAs in a late stage of cardiac hypertrophy mouse model that could explain the observed sex difference. As expected the results described in this report show that an important group of miRNAs are sex-differently expressed in TAC. More than the half of the miRNAs selected for analysis was dysregulated in hypertrophy, being the majority up-regulated in males and showing no TAC effect in females.

5.1.1. Incomplete definition of experimental conditions and different methodologies lead to a difficult comparison of the results

Many studies have described miRNAs' dysregulated expression in mouse models of hypertrophy but they often omit the sex, the age and even the strain of the animals, as seen in Table 1.[100, 122-125]

We published recently that the genetic background influences oestrogen signalling and that it plays a role in the E2-dependent regulation of postnatal cardiac growth mediated by β-catenin.[186] Concerning the sex of the animals used for the various experiments, males are commonly used, despite not always reported. This factor was an important trigger for the present study. Up to our knowledge this is the first report distinguishing males and females in a late time point of hypertrophy. Other determining factors may also vary, like the methods and the duration used for the *in vivo* hypertrophy induction. Typically, TAC surgery is the most common method used, but various transgenic models and different chemical treatments might also be

used (e.g. isoproterenol, phenylephrine), thus respectively affecting different genes, pathways and consequently different miRNAs. The choice of the 9 week TAC model for this study was mainly due to our previously reported results that show sex differences in LV/TL at 6 and 9 weeks after TAC, but not on the early stage of 2 weeks.[38]

MiRNA's relevance in cardiac hypertrophy is also often studied, or at least confirmed, using *in vitro* methods. The variety of *in vitro* methods used may be even wider, going from gain-/loss of function of genes or miRNAs, to chemical treatments (aldosterone, phenylephrine, isoproterenol, lysophosphatidic acid, etc.) applied either in immortalized cell lines or in isolated cardiomyocytes/fibroblasts.

Despite the methodological differences found, the role of the miRNAs in cardiac development and disease is nowadays not questioned and has been object of numerous studies. As seen in Table 2, many miRNAs have been shown to target genes directly involved in cardiac hypertrophy and through *in vivo* or *in vitro* gain/loss of function experiments been proved to cause or to inhibit cardiac hypertrophy (pro- and anti-hypertrophic miRNAs respectively).

In the present report, 50% of the miRNAs (30/60) selected for analysis in the WT TAC model, presented significant sex and/or sex*surgery interaction effect after 2-way ANOVA analysis, which means that they are either generally higher expressed in one of the sexes than in the other or that they have a sex-dependent response to TAC, respectively.

5.1.2. miRNAs with sex-differences in TAC that are directly related to hypertrophy in other reports

Several previously published studies (Table 2) make direct associations of specific miRNAs and cardiac hypertrophy. In general, to prove a pro-hypertrophic action of a miRNA, the studies show clear hypertrophic responses (e.g. HW/BW, cardiac function and ANP and BNP expression) caused by over-expression of a specific miRNA. As for an anti-hypertrophic effect demonstration, usually the methods include a hypertrophic response to an external factor (TAC, transgenic animals, chemical induction) that is abolished or attenuated by the over-expression of a specific miRNA. Some of our results and methodology are worth to correlate or compare with previously published data.

5.1.2.1. miRNAs previously shown as pro-hypertrophic

Among the analysed miRNAs that present sex and/or sex*surgery interaction effect, up-regulation in males, no effect in females and significant sex differences in TAC are miR-19(b), miR-199b-5p, miR-21, miR-22, miR-23a, miR-23b, miR-24 and miR-27b, which are all described as being pro-hypertrophic miRNAs (Table 2).

Their described targets include genes like MuRF-1 (miR-19b[136] and miR-23a[145]), Atrogin-1 (miR-19b[136]), Dyrk1a (miR-199b[138]), Sprouty1 (miR-21[111]), Sprouty2 (miR-21[139]), PTEN, Purb, PGC-1α, PPARα, SIRT1 and Hdac4 (miR-22[140-143]), FoxO3 (miR-23a[146]), LPA1 (miR-23a[147]), JP2 (miR-24[148, 149]) or PPARγ (miR-27b[151]), and with exception of miR-199b and miR-22, all of them were previously described as up-regulated in mouse models of TAC.[100, 122-125]

The methodology used for these reports does, however, differ. In the animal experiments used for the cited reports, the mouse TAC model is the most commonly used and unless some exceptions that did not perform any *in vivo* experiments[100, 136, 140, 147], only the reports that show the direct targeting of MuRF1 by miR-23a[145] and the targeting of Sirt1 and Hdac4 by miR-22[143] use an alternative model of hypertrophy, with an isoproterenol infusion. The duration of the TAC induction also varies in these reports from 2 to 6 weeks, which means that none of them achieved the late stage of hypertrophy presently studied. Most of the above mentioned reports specify the usage of C57BL/6 male animals, from 8[138, 145, 146, 151] to 12 weeks-old[111, 139], one does not specify the strain but specifies the age of 12 weeks[141], other does not specify the sex[143] and one demonstrating the targeting of Dyrk1a by miR-199b[138] that does not refer the sex of the animals. The report of the targeting of PGC-1α, PPARα and SIRT1 by miR-22 uses FVB mice instead but also does not refer the sex of the animals.[142] The same happens in the previous reports that describe simply the up-regulation in hypertrophy (Table 1): the age of the animals varies between 6[122] and 12 weeks[124, 125], but as discussed above the strain, the gender and the age of the animals at the time of TAC is not always referred.

The *in vitro* experiments are most commonly performed in isolated neonatal cardiomyocytes, but the studies mentioned above differ from mouse[111, 146] to rat [100, 136, 138, 140, 142, 143, 145, 147, 148, 151], one report does not specify whether they used mouse or rat cells[139] and others use only or as an additional system adult cardiomyocytes from adult mice[141] or rats[142]. Despite the male sex being the most commonly used, none of these reports discriminates whether the cells were isolated from male or female animals.

The fact that all these miRNAs were up-regulated in our male mice, but not in females, together with the fact that they were all considered as pro-hypertrophic and found to be up-regulated in experiments where only male animals were used, suggests that more importance

should be given to the sex differences in cardiovascular research, specifically in cardiac hypertrophy.

MiR-208a and miR-499 are other miRNAs previously reported as pro-hypertrophic and showing sex effect in ANOVA analysis, but unlike the previously mentioned miRNAs did not present significant up-regulation in males nor the significant sex differences after TAC, as it has been previously published.[123, 157] However, The study describing the up-regulation of miR-208 in TAC does not discriminate whether it corresponds to miR-208a or miR-208b[123] and the report about miR-499 includes only human samples.[157] MiR-208b was described as up-regulated in female TAC animals, but this miRNA was not included in our analysis.[125] MiR-208a and miR-499, together with miR-208b, are of a high importance in heart diseases, especially in cardiac hypertrophy as they have in common the fact that they belong to particular family of miRNAs referred to as MyomiRs. As mentioned in the introduction, this family is encoded by MHC genes, and they act within a network to control myosin expression and skeletal myofiber phenotypes through the repression of a collection of transcriptional repressors of slow myofiber genes. Thus, myosin genes control muscle gene expression and performance through this network of intronic miRNAs.[116] MiR-208a induces cardiac remodelling and modulates the expression of hypertrophy-associated genes and its deletion in mice reduces pressure overload-induced hypertrophy[187], through a post-transcriptional repression of Thrap1, a component of the thyroid hormone nuclear receptor complex, and myostatin, a repressor of hypertrophic growth in skeletal muscle.[110, 112] Elevated levels of miR-499 in hearts of transgenic mice result in cardiomyocyte hypertrophy and stress-dependent cardiac dysfunction, through an alteration of the immediate early gene response to cardiac stress.[157] In this report, when analysing sexes separately, both miRNAs show at least a tendency for sex differences either in Sham and in TAC operated animals (miR-208a: MS/FS=1.39 and MT/FT=1.93; miR-499: MS/FS=1.51 and MT/FT= 1.54), being p-value for the sex differences of miR-208a in TAC animals a borderline p-value. It is known that gender and β1-AR-mediated signalling control cardiac β-MHC levels under physiological and pathological conditions. The levels of β-MHC, but not α-MHC, were shown to be 10-fold higher in the LV of fertile female mice compared with the age matched males and these differences disappeared after ovariectomy or in immature mice.[188, 189] As other studies have shown the beneficial effects of β-MHC in the heart[190-192], it has been suggested that the greater expression of β-MHC in the fertile female LV could have a role in the anti-hypertrophic effect of oestrogen.[189] Knowing that this regulatory network works as a positive feedback loop where miR-208a regulates the expression of two slow myosins and their intronic miRNAs, Myh7/miR-208b and Myh7b/miR-499 respectively[116], the sex effect observed in the

expression of miR-208a and miR-499 in WT animals (and eventually miR-208b that was not analysed in this study) might as well be related with the oestrogen protective role.

5.1.2.2. miRNAs previously shown as anti-hypertrophic

MiR-145, let-7 family, miR-133a and miR-378, are miRNAs previously described as having anti-hypertrophic characteristics that show also relevant sex differences in our screening.

MiR-145 was the only miRNA that presented sex differences in Sham operated mice besides a TAC effect in females (down-regulation) but not in males. This miRNA was recently described as an important cardiac hypertrophy regulator, being considered an anti-hypertrophic miRNA. Mir-145 was shown to regulate both expression and localisation of GATA6, thus protecting the heart against isoproterenol (ISO)-induced cardiomyocyte hypertrophy. However, the same study shows a dynamic pattern of miR-145 expression in ISO-treated cardiomyocytes and in the hearts of TAC mice. In the latter, the expression of miR-145 is up-regulated after 1 week of TAC, but this expression goes down to values similar to Sham 4 weeks after TAC operation.[135] The expression pattern observed in our experiments for miR-145, distinct from all the other miRNAs analysed, does not correlate with the described above and might have different interpretations. On one hand, the TAC stress might cause the up-regulation observed in males in a later time of hypertrophy, not analysed by the cited authors, as a later intent for an anti-hypertrophic response. On the other hand, we would expect also an up-regulation in female animals after TAC, which was not observed, at least after 9 week TAC. The higher expression in females in Sham animals might be associated with a possible protective role in a basal state, which is not existent anymore after 9 weeks of TAC stress. Once again, the animals used by the authors were only male, and although the age is slightly different (8- to 12-week old, while ours are 12- to 14-week old) nothing is described about the behaviour of miR-145 in female animals. Further experiments would be necessary to find an explanation for this discrepancy.

Let-7 family, as part of a bigger miRNA family (miR-98/let-7) is also associated to an anti-hypertrophic effect. Thioredoxin (Trx1), a cardiac hypertrophy suppressor with cell protective actions in the heart, up-regulates the expression of the miR-98/let-7 family which in turn inhibits AngII–induced cardiac hypertrophy. The up-regulation of miR-98/let-7 family, together with the inhibition of cyclin D2, a component of the cell cycle machinery and a validated target of this family, appears to play a role in mediating the suppression of cardiac hypertrophy by Trx1. Although miR-98 and let-7f are the top 2 miRNAs of this family up-regulated by Trx1 in transgenic mice (tg-Trx1) in the referred study, let-7e (but not let-7g) is also one of the family members responding to Trx1.[193] The results obtained in our WT model, are apparently not in

accordance to the published data. Similarly to miR-145, we would expect a significant up-regulation in females but not in males of the miRNAs of this family, to justify the anti-hypertrophic role in the TAC-induced hypertrophy. Different hypertrophic stimuli induce different pathways and in this case it might be the explanation for the discrepant results.

MiR-133a, a muscle specific miRNA[194] and one of the most studied miRNAs in the context of cardiovascular diseases, is another miRNA previously characterised as anti-hypertrophic[113, 128, 195], was shown previously to have no changes[122] or to be down-regulated[100, 124, 125] in TAC. Another report describes the same down-regulation but does not discriminate the miRNA, referring to it only as miR-133.[113] In our experiments, the results are not consistent with the previously described, being miR-133a significantly up-regulated in males and although there is the same tendency in females, the sex differences are significant after 9 weeks of TAC. The results might differ, for example, due to the hypertrophy model, including time of TAC. The reports mentioned either omit the time of TAC[113], either use between 1 and 3 weeks of TAC[122, 124, 125] or even use transgenic mouse models of hypertrophy (Cna Tg[100]). Moreover, these reports often omit the sex of the animals[100, 122, 124] or use only female animals[113, 125]. MiR-133a is considered as an anti-hypertrophic miRNA, as result of *in vitro* and *in vivo* experiments.[113] In one study, miR-133a was shown to target genes that are relevant for cardiac hypertrophy, as RhoA and Cdc42, which are both genes associated with cytoskeletal and myofibrillar rearrangements during hypertrophy, and Whsc2, whose overexpression up-regulates myocardial foetal gene expression.[113] Others described that the negative regulation of NFATc4 by miR-133a, contributes to a hypertrophy repression[128] as well as reciprocal repression between miR-133 and calcineurin regulates cardiac hypertrophy.[129] However, contradictory results have been published: one study measured miR-133a's expression in four different forms of murine hypertrophy matched for age and genetic background. MiR-133a was down-regulated in TAC and ISO-induced hypertrophy, but not in two genetic hypertrophy models, suggesting that miR-133a can be a double-edged sword, depending on the pathophysiological context.[196]

MiR-378 was previously shown to be down-regulated in hypertrophy[122, 156] in reports that either used male animals[156] or did not specify the sex of the animals.[122] Thus, our results are, once again, not consistent with these: females do not show any alteration of the expression of miR-378 and males show a tendency for an up-regulation after TAC. Highly abundant in the heart, this miRNA is considered to have an anti-hypertrophic effect, being associated with postnatal cardiac remodelling and with the regulation of cardiomyocyte survival during stress. By targeting IGF1, miR-378 was shown to be involved in a negative feedback loop between miR-378, IGF1R, and IGF1.[197] Furthermore, miR-378 was found to be both necessary and sufficient to repress cardiomyocyte hypertrophy in isolated primary cardiomyocytes and the

restoration of miR-378 levels significantly attenuated *in vivo* TAC-induced cardiac hypertrophy. MiR-378 exerted its activity by targeting four key components of the MAPK pathway: MAPK1 itself, IGFR1 (Insulin-like Growth Factor Receptor 1), GRB2 (Growth Factor Receptor-Bound protein 2), and Ksr1 (Kinase Repressor of Ras 1).[156] The expression of miR-378 is down-regulated during the development of hypertrophy and in heart failure, which prevents Ras activation and its over-expression inhibits hypertrophic growth of cardiomyocytes, by interfering with the nuclear accumulation of NFAT and induction of the fetal gene program.[155]

Other miRNAs with sex and/or sex*surgery interaction effect do not have yet described targets or functions, but the all these sex differences observed in TAC (MT/FT or interaction effect) as well as the sex differences independent from TAC (sex effect) suggest a potential role of sex, sex hormones or their receptors.

5.2. Sex differences in miRNA expression in TAC are ERβ-dependent

The second aim of this study was to figure out the putative role of ERβ in the sex differences observed in the WT mice. Interestingly, the sex differences observed in WT hypertrophic mice were totally abolished in the absence of the receptor. Besides the effect in the hypertrophic response, the sex effect observed in WT animals as well as the significant sex differences in Sham mice, were not present in ERβ$^{-/-}$ animals.

The fact that these differences disappeared in the absence of ERβ correlates with the protective role of this receptor, especially in females, as it has been suggested.[57-60]

Sex differences between men and pre-menopausal women in the normal heart exist (Figure 2) and that they are particularly relevant in what concerns to the amount of oestrogen, testosterone, and ERβ. However, the higher amount of ERβ in males[28] does not necessarily implicate that they benefit of a protective role of ERβ. We showed before that in human hypertrophic hearts ERβ mRNA amount is increased in both sexes, but as the basal amount is significantly higher in males the increment observed in females is more pronounced.[28] A following report using only female animals showed that ERβ, but not ERα, has an important protective role in the female heart.[58] Our own studies went further and using animals from both sexes we identified the hormone receptor as an important regulator of sex differences in cardiac hypertrophy. Once again, based on the fact that the protective role was observed only in females and in our *in vitro* experiments results using cardiac fibroblast isolated from male and female rats reported here, demonstrated that the observed sex different effect of ERβ is not simply due to a different availability of E2 (discussed below), but also the factor sex is also

essential. Endogenous ERβ was shown to act differently in males and females, influencing cardiac remodelling, limiting cardiac fibrosis, apoptosis and the development of cardiac hypertrophy in a sex-specific manner. The study proposes that ERβ predominantly contributes to the maintenance of energy homeostasis and limits the development of eccentric cardiac hypertrophy and fibrosis in females, while in males it restricts cardiomyocyte hypertrophy and apoptosis.[60]

The mechanisms through which ERβ plays this protective role are still not completely known but some of the studied mechanisms at the moment involve lipoprotein lipase (LPL), calcineurin and histone deacetylase proteins (HDACs).

ERβ has been suggested to be involved in a complex relationship between gender, hypertrophy and lipoprotein lipase (LPL), a protein proposed to having a role modulating cardiovascular diseases, like hypertrophy and others. This protein was found to be down-regulated at the baseline in ERβ$^{-/-}$ females, but not in males, in comparison to WT animals. On the opposite, males, and not females, showed an up-regulation of LPL in ERα$^{-/-}$ animals. Besides, LPL's down-regulation after TAC was more pronounced in WT males than in females and within females subjected to TAC ERβ$^{-/-}$ had the most substantial down-regulation.[57]

A protective effect of ERβ by inhibiting calcineurin activation has also been suggested. E2 significantly inhibited AngII-stimulated calcineurin in WT and ERα$^{-/-}$ mice, but had little effect in the absence of ERβ. This inhibition occurred through the up-regulation of the modulatory calcineurin interacting protein (MCPI1) gene. The report proposes that oestrogen, when acting through ERβ, stimulates MCIP1 gene via PI3 kinase signalling, the protein product of which binds/clamps the catalytic activity of calcineurin. This prevents NFAT translocation to the nucleus inhibiting the expression of hypertrophic genes required for cardiomyocyte to increase in size (hypertrophy). When activating both ERα and ERβ, oestrogen stimulates ANP and BNP genes, whose secreted protein products bind the guanylate cyclase A receptor and inhibit AngII-induced ERK activation.[198]

Other studies confirm the protective effect of ERβ showing for example that engaging ERβ with an agonist significantly inhibits the ability of AngII to stimulate hypertension, cardiac hypertrophy, and cardiac fibrosis in female animals[49] and even showing ERβ agonist as a potential treatment for cardiac diseases, acting as a regulator of histone deacetylase proteins (HDACs), important modulators of hypertrophy.[199]

Like the studies of E2 effects on miRNAs (Table 4), also the effects of ERs on miRNA expression are mainly performed towards cancer research, with a greater incidence in breast cancer and a high usage of MCF-7 cells. Breast cancer cells often lack endogenous ERβ, but its presence correlates with a better prognosis and a less aggressive clinical outcome of the

disease. MCF-7 cells do not express this receptor and studies often focus on ERα. However, using engineered cells expressing equal amounts of both receptors reports show that ERβ influences miRNAs' biogenesis, binding in close proximity of several miRNA genes[200] and controls synthesis, maturation and steady-state levels of a significant number of miRNAs, by interfering with ERα activity (for the miR-23b/27b/-24-1 cluster) or acting autonomously (for miR-30 gene). The authors demonstrated a profound effect on miRNome expression and activity in tumours expressing ERβ, which could help to explain their less aggressive phenotype.[176] Furthermore, knowing that ERβ, and not ERα, shows significant oestrogen-independent activities including the ability to inhibit cell cycle progression and to regulate gene transcription in the absence of the ligand, the following studies revealed a significant effect of ligand-free ERβ on breast cancer cell functions. This occurred via modulation of the cell proteome, suggesting that miRNA regulation may represent a key event in the control of the biological and clinical phenotype of hormone-responsive breast cancer by this nuclear receptor.[201] Other studies than breast cancer studies found relationship between ERβ and miRNAs in colorectal cancer[202, 203], prostate cancer[204] lung adenocarcinoma[205].

Given all the suggestions of a protective role of ERβ in the heart, the several reports indicating a miRNA regulation by this receptor in different systems and the lack of knowledge about miRNA regulation by ERβ in the heart and heart diseases, the study here described has a major importance as part of the necessary understanding of the sex differences in cardiac hypertrophy, as well as the role of oestrogen in females.

5.3. Pathway enrichment analysis of miRNAs with significant sex*surgery interaction effect

The group of 12 miRNAs with significant sex and sex*surgery effect in WT mice (two-way ANOVA) was found particularly interesting for this study. As mentioned before, these miRNAs responded to TAC in a sex-specific manner in WT mice, but they did not present the same effect in ERβ$^{-/-}$ animals. In order to try to understand better the possible roles of these sex-specifically regulated miRNAs in TAC, we performed a pathway enrichment analysis, using their putatively predicted targets by TargetScan and analysing them through ConsensusPathDB.

The analysis revealed an interesting output, showing a great number of pathways hypertrophy-related. The most prominent results point out the strong association of the putative targets with MAPK and PI3K-Akt signalling pathways and oxidative damage. Among others, the results also include oestrogen signalling or mitochondrial gene expression.

Intracellular MAPK signalling cascades likely play an important role in the pathogenesis of cardiac and vascular disease. All the three major MAPK pathways are activated in cardiac tissue of mice under TAC conditions.[206] Over-expression studies lead to the model that the simultaneous activation of ERK, JNK and p38 MAPK in heart after pressure overload contribute to the development of pathological cardiac hypertrophy. In this model, ERK activation promotes the growth of cardiomyocytes, JNK activation leads to reduced gap junction formation, p38 MAPK activation promotes cardiac fibrosis, and activation of all three pathways promotes reduced diastolic compliance.[207] MAPK-ERK1/2 pathway was shown to be regulated by miR-21 through the direct targeting of Spry1, one of the pathway's negative regulators.[111] We found sex differences in the regulation of this and other miRNAs with putative targets in other MAPK inhibitors[208] and this will be discussed ahead in this report.

Concerning mitochondrial gene expression, it is an expected result since our own data shows that gene encoding for mitochondrial function and fatty acid oxidation were less down-regulated in female hearts, compared to male.[60] Among the genes belonging to the mitochondrial fatty acid oxidation that have putative targets for several of the analysed miRNAs are:

Decr1 (2,4-dienoyl CoA reductase 1),
Hadha (hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase alpha subunit),
Hadhb (hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase beta subunit).
Ndufs4 (NADH dehydrogenase (ubiquinone) iron-sulfur protein 4) and
Auh (AU RNA binding protein/enoyl-CoA hydratase).

The questions whether these miRNAs binding sites are functional or not remain unknown and will be under investigation in the near future.

5.4. ERβ represses miRNA expression in Sham animals

The direct comparison of WT and ERβ$^{-/-}$ female Sham, as first analysis of the receptor's role, revealed a higher expression of 9 of the 11 selected miRNAs in the absence of the receptor (see 4.4, Figure 25), even though miR-106b had a borderline p-value. The exceptions were observed for miR-106a and miR-27a that did not show any difference between the expression in WT and ERβ$^{-/-}$ females. MiR-27a's expression was only affected by the deletion of ERβ in male animals and not in females.

The different behaviour of miR-106a and miR-27a under the deletion of ERβ could eventually have an explanation with a look over the effect of E2, or the ERs agonists on cardiomyocytes and cardiac fibroblasts from both sexes. In fibroblasts, both miRNAs are down-regulated by E2, ERα and ERβ specific agonists in female cells. In males, both are up-regulated by E2 and ERα and none of them suffers any change by ERβ specific agonist. However, this is also a pattern shared with miR-24 and miR-27b (Table 49). In female cardiomyocytes, we saw a down-regulation of miR-106a under the 3 different stimulations and a down-regulation of miR-27a by E2 and ERβ agonist. As the stimulations in cardiomyocytes were only performed in female cells, it remains unknown what would happen in male cells. Further experiments using cells from both sexes would be necessary to unveil whether a different behaviour of these miRNAs is also observed between male and female cardiomyocytes, thus influencing the overall expression in the heart tissue.

Few published studies correlate these two miRNAs with heart, oestrogen or ERs. In cardiomyocytes, miR-27a was previously found to strongly up-regulate β-MHC, but not α-MHC. This regulation occurs by direct targeting of thyroid hormone receptor β1 (TRβ1), which negatively regulates β-MHC transcription.[209] In breast cancer studies, miR-27a was shown to indirectly regulate E2-responsiveness in MCF-7 cells through suppression of ZBTB10, a specificity protein (Sp) repressor, thereby enhancing expression of ERα.[210] E2 treatment can also up-regulate the expression of miRNAs belonging to miR-106a≈363 (and miR-17≈92) cluster in ERα-positive breast cancer cells, and this is mediated by direct recruitment of oestrogen-inducible c-MYC to the promoter region of these miRNA cluster.[211] However, due to the fact that negligible levels of c-myc were observed in quiescent cell of non-cancer tissue[212], the influence of c-MYC on miRNA expression at cardiac tissue level is limited to proliferating cells.

5.5. Estradiol repression of miRNAs as a possible cause of the sex effect observed in WT mice

Another exciting observation in this study was that almost half (24/60) of the miRNAs analysed in WT animals presented a sex effect in the statistical analysis (and 6 more miRNAs had a borderline value for this effect). Interestingly, with the exception of miR-145, all the other miRNAs were higher expressed in male than in female animals (Table 26).

Sex hormones, particularly oestrogen, are generally expected to mediate sex differences. The remarkable number of miRNAs showing sex effect correlates with other reports that demonstrated previously a modulation of the transcription of several miRNAs by E2 stimulation.

One report suggested widespread repression of miRNAs in ERα-positive breast cancer cells, which included miR-21, miR-23b, miR-26a, miR-26b from our list of miRNAs with sex effect.[169] E2 was also shown to induce the expression of the let-7 family members and miR-21 in MCF-7 cells[168], which does not correlate with our data, but the differences are sometimes due to the array platform used, the data analysis method or even the cells used (cell lines express c-MYC and quiescent cells not[213]) and sub-clones used. The same study also shows that Dicer is associated with ERα-binding sites and found to be induced by E2, showing that oestrogen does not only regulate the expression of specific miRNAs, but might also have global effects on miRNA-regulated gene expression by altering their rate of processing.[168] A differential expression of miRNA biogenesis pathway genes was found between ERα-positive and ERα-negative breast cancer cells. ERα-positive cells showed increased DICER and TRBP expression, but also decreased Ago1 and Ago2 expression, suggesting that the lower abundance of the Ago proteins in ERα-positive tumours could limit the functional activity of the RISC complex without repression of miRNA maturation by Dicer.[214] Later, E2 was found to regulate the distribution and activity of all three RNA polymerases and virtually every class of non-coding RNA that has been described to date, also in MCF-7 cells.[215]

Interestingly, many of the miRNAs negatively regulated by E2 are predicted to target several components of the miRNA biogenesis pathway.[111] Targeting of Dicer mRNA by E2-regulated let-7, miR-29a and miR-21 has been confirmed experimentally[216-218], suggesting a potential negative feedback mechanism between miRNAs and their biogenesis machinery which may be influenced by oestrogen signalling.

While all these studies were performed in breast cancer cells, we could not find published data about oestrogen-regulated miRNAs in the heart. However, our results obtained with E2-stimulation of female cardiomyocytes are in accordance with the ones mentioned above. We show a repression of the expression of 10 miRNAs, most of which are also repressed by ERα and ERβ specific agonists. Although we could not include male cardiomyocytes within our experiments, the higher level of oestrogen in females than in males might not be the main cause for the sex effect observed. As seen on our cardiac fibroblasts experiments, the same amount of E2 can have different effects in male and female cells.

5.6. E2 and ERs regulate miRNA expression in cardiac fibroblasts in different ways according to the sex

Among our results, we found an up-regulation of miR-21 nine weeks after TAC, in a sex-dependent manner. MiR-21 presented a statistically significant higher up-regulation in males

than in females, and this sex difference was abolished in the absence of ERβ, with a higher up-regulation of this miRNA by TAC in ERβ$^{-/-}$ females in comparison to WT. Furthermore, our results show that the basal expression of miR-21 is already higher in knock-out females, and that E2 has a repressing effect over miR-21 expression in female cardiomyocytes.

MiR-21 is a miRNA previously linked to fibrosis, known to be up-regulated in fibroblasts of the failing heart augmenting mitogen-activated protein kinase (MAPK)-ERK1/2 activity. Through the inhibition of the MAPK/ERK pathway negative regulator sprouty homologue 1 (Spry1), miR-21 contributes to myocardial disease affecting the proliferation and survival of cardiac fibroblasts.[111] Some contradictory results have however been published, showing that miR-21 knock-down had no significant effect under similar pressure overload conditions.[219] This might have an explanation in the different biological half-lives of the antagomiRs used in both studies[220], in a suppression of multiple miRNAs with the same seed sequence as miR-21 or even in non-specified effects of high levels of a cholesterol modified antagomiR on the heart.[221] Finally, the sex of the animals, which is not specified, could play a significant role.

Knowing that MAPK/ERK pathway has other negative regulators than Spry1 and that miRNAs frequently act in networks, we searched for miRNAs that could putatively target Spry2, Rasa1 and Rasa2. The finding of 5 other miRNAs with putative targets within these genes, with a similar expression pattern to miR-21 in WT and also a loss of the sex differences and the sex effect in the knock-out animals, made us believe that these miRNAs could really act as a fibrosis-regulating network, mediated by oestrogen and ERβ.

Primary fibroblasts with cardiac origin isolated from male and female animals allowed the *in vitro* study of the influence of E2 and its receptors independently from the endogenous hormonal effect *in vivo*, treating cells from both sexes with the same amount of E2 or ER specific agonists.

We reported previously that the effects of E2 on collagen synthesis differ between male and female adult rat fibroblasts *in vitro*. E2 increases Col1 and Col3 expression in cardiac fibroblasts from male rats, whereas it suppresses Col1 and Col3 in cells from female animals.[184] In fact, like for Col1 and Col3 expression, we also found a sex-specific effect of E2 on miRNA expression in cardiac fibroblasts. While E2 causes in female cells a down-regulation of all the miRNAs selected (miR-106a, miR-106b, miR-21, miR-24, miR-27a, miR-27b), in males cells the general effect is an up-regulation, with the only exception observed in miR-21. Consistently, also the effect of ERα and ERβ showed a sex-specific effect on cardiac fibroblasts. While in females all the 6 miRNAs are negatively regulated by both or at least one of the ER specific agonists, in males the tendency is for an up-regulation by ERα agonist.

By demonstrating this targeting, a down-regulation of this group of miRNAs with MAPK/ERK pathway negative regulators as common targets would mean that the expression of the regulators would be higher, thus causing a higher inhibition of the pro-fibrotic MARP/ERK signalling pathway and consequently less fibrosis (in females) and vice-versa (in males). In fact, in a recent publication where we published some of these results, we also confirmed the targeting of Rasa1 by miR-21 and miR-24 and the targeting of Rasa2 by miR-21, miR-27 and miR-106. Moreover, miR-21, miR-24, miR-27a and miR-106a were able to induce ERK1/2 phosphorylation in cardiac cells.[208]

Sex differences in the miRNA processing machinery could be an explanation, as oestrogen was previously demonstrated to exert a post-transcriptional control of the maturation of miRNAs by attenuation of the processing of pri-miRNAs through oestrogen-dependent association of the ERα with the Drosha complex.[222] It is important to mention that for the referred study the authors used female mice and although the study does not include data about ERβ, our results with ERα specific agonist in female cardiac fibroblasts also correlate with them.

This present study indicates that E2 may influence the development of fibrosis in a sex-specific manner, regulating the expression of different repressors of the MAPK/ERK signalling cascade, through miRNAs. Sex is an important factor to determine whether the mediation of oestrogen occurs via ERα or ERβ, since ERβ appears to have a protective role in females and ERα to lead to an opposite effect in males. The analysis of these miRNAs in an ERα$^{-/-}$ mouse model confirmed that the absence of this receptor, only affects male heart tissue.

5.7. miRNA therapeutics applied to cardiac hypertrophy and fibrosis

Cardiac hypertrophy and fibrosis therapeutically approaches based on miRNAs have nowadays a growing interest. The first report of a miRNA knock-down *in vivo* in a mammalian system used a cholesterol-conjugated antagomiR to obtain an inhibition of miR-122, and abundant liver miRNA. Nonetheless, the same report shows that cholesterol-based chemistry was also able to knock-down miRNA expression in cardiac tissue after intravenous injection.[223]

After this important report, an antagomiR against miR-133 was implanted subcutaneously in mice, using osmotic mini-pumps for a continuous delivery. After one month of treatment, the mice presented an increase in cardiac hypertrophy, suggesting a high importance of this miRNA in preventing the pathology.[113]

In vivo inhibition of miR-199b by a specific intraperitoneally administrated antagomiR normalized reduced NFAT (nuclear factor of activated T-cells) activity and caused inhibition and

even reversal of cardiac hypertrophy and fibrosis in mouse models of heart failure. MiR-199b is a direct target of calcineurin/NFAT with an increased expression during heart failure, thus being an interesting potential target for therapies.[138]

Studies using systemic delivery of an LNA-modified antagomiR subcutaneously delivered showed that therapeutic silencing of miR-208a, a cardiac specific miRNA, prevents pathological cardiac remodelling, functional deterioration and lethality during diastolic heart disease.[187]

The *in vivo* knock-down of miR-21 with an antagomiR was initially demonstrated to blunt cardiomyocyte hypertrophy, inhibit and reverse interstitial fibrosis and attenuate cardiac dysfunction after TAC.[111] However, others demonstrated that both genetic deletion and antagomiR mediated inhibition of miR-21 failed to block cardiac remodelling during stress.[219] These differences were attributed to different chemistries and nucleotide sequences used, that led to different efficacies.[220]

The knowledge of miRNAs' role and regulation in diseases is extremely important for a better understanding of the mechanisms involved and the identification of new targets for therapeutic approaches. Advances in the miRNA delivery systems are needed to improve the specificity, efficacy and the efficiency of the targeting. However, the fact that each miRNA can target several different mRNAs and that each mRNA might be targeted by several miRNAs is yet an obstacle to these therapies.

5.8. Conclusions and implications for further research

Sex differences in cardiac hypertrophy exist and oestrogen and its receptors play a role in them. We show that ERβ plays a protective role mainly in the female heart, by regulating the expression of hypertrophy and fibrosis linked miRNAs. This effect was also observed *in vitro*, in cardiomyocytes and cardiac fibroblasts. This protective role leads to sex differences, in normal and hypertrophic hearts. In contrast, ERα affects only the expression of fibrosis linked miRNAs in the male hearts. Furthermore, E2, ERα and ERβ regulate a miRNA network in a sex specific manner

The mechanisms through which this sex-specific miRNA regulation occurs remain unknown. Concerning ERβ, some possibilities worth to investigate could be either a post-translational modification of ERβ that could influence its activity or cellular levels, or a regulation of the length of the mRNA 3'UTR sequence by utilization of alternative polyadenylation sites. These topics are interesting approaches for further investigation in the near future.

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Appendix

I. List of figures

II. List of tables

III. Selbstständigkeiterklährung:

Hiermit erkläre ich, die vorliegende Arbeit selbständig angefertig zu haben. Ich habe keine unerlaubten sowie unerwähnten Hilfen benutzt.

Berlin, den

Ana Maria Gomes Capelo Carregal Queirós

IV. Publication list

Ana Maria Queirós*, Claudia Eschen*, Daniela Fliegner, Georgios Kararigas, Elke Dworatzek, Christina Westphal, Hugo Sanchez Ruderisch, Vera Regitz-Zagrosek. *Sex- and estrogen-dependent regulation of a miRNA network in the healthy and hypertrophied heart*. Int J Cardiol, 2013. 169: p-331-8.

Ana Maria Queirós, Daniela Fliegner, Claudia Eschen, Georgios Kararigas, Hugo Sanchez Ruderisch, Vera Regitz-Zagrosek. *Oestrogen receptor β inhibits microRNAs induction in the female heart under pressure overload*. (Manuscript in progress)

Previous publications

Laura Vilarinho, **Queirós A**, Paula Leandro, Isabel Tavares de Almeida, Isabel Rivera. *Fenilcetonúria Revisitada* (Review). Arq Med, Vol 20 (5-6) pp161-72, 2006. (in Portuguese)

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Berlin, den

Ana Maria Gomes Capelo Carregal Queirós

V. *Curriculum Vitae*

VI. Acknowledgments

To the successful accomplishment of this work, many people have contributed in numerous ways and to them I would like to express my gratitude and leave my sincere thanks.

To Prof. Dr. Vera Regitz-Zagrosek, I would like to thank for the opportunity to develop a project under this extremely interesting subject and to evolve scientifically in her excellent working group in the Institute of Gender in Medicine, Center for Cardiovascular Research, Charité Universitätsmedizin Berlin. I thank also for the constructive discussions, reviews and evaluation of this work.

A special thank to Dr. Hugo Sanchez Ruderisch, for the supervision, continuous support, trust and friendship. Without his comprehensive knowledge in practical and theoretical issues, the endless discussions and precious help, this work would have never been possible.

I would like to thank to the Deutsche Forschungsgemeinschaft (DFG) for the fellowship and the entire graduate program GK-754-III financing and mediation. To Prof. Dr. Hermann-Georg Holzhütter I would like to thank for his willingness to examine my work.

A special thank to Claudia Eschen, my companion in miRNAs' studies, for the excellent teamwork, the helpful advices, dedicated friendship and for the numerous hours spent together in the lab and out of it.

I would like to acknowledge all the working group members, especially to Elke Dworatzek for teaching and helping me with the cardiac fibroblasts isolation and experiments, and to Daniela Fliegner for the animal samples and all the wonderful help, support and advices, from the very beginning of this adventure. To Britta Fielitz, Jenny Thomas and Arne Kühne for the entire lab work support and precious help with the early morning PCR runs.

To Valeria, Karima, Caterina, Veronica, Daniel, Karina, Svetlana, my CCR crew, and to all my friends in Berlin, a huge thank for their friendship, incredible days and nights and tremendous support in good and bad moments, you are all much more important for this achievement than you will ever know. A special thank to Ana S., Ana T. and Manel for making hard times so much easier.

To all my family and friends in Portugal, thank you so much for the continuous support, every single message, phone call, visit and even “welcome home” hugs during the past years were essential for my well being and made me stronger to face daily challenges and difficulties. Close or far, you were always in my heart.

Last but not least, I would like to thank to the two most important persons in my life, the ones that are and always will be there for me, no matter where I am and what my choices are. To my parents, Jaime and Lucinda, for believing, for loving, for supporting, for encouraging, for suffering, for cheering with me and above all, for making me a fighter. To you I owe everything I am and all my life achievements!

A todos muito obrigada!

Ana