microRNA expression profile analysis in human endothelial cells exposed to estradiol. Role of miR-30b-5p in the regulation of endothelin-1 expression

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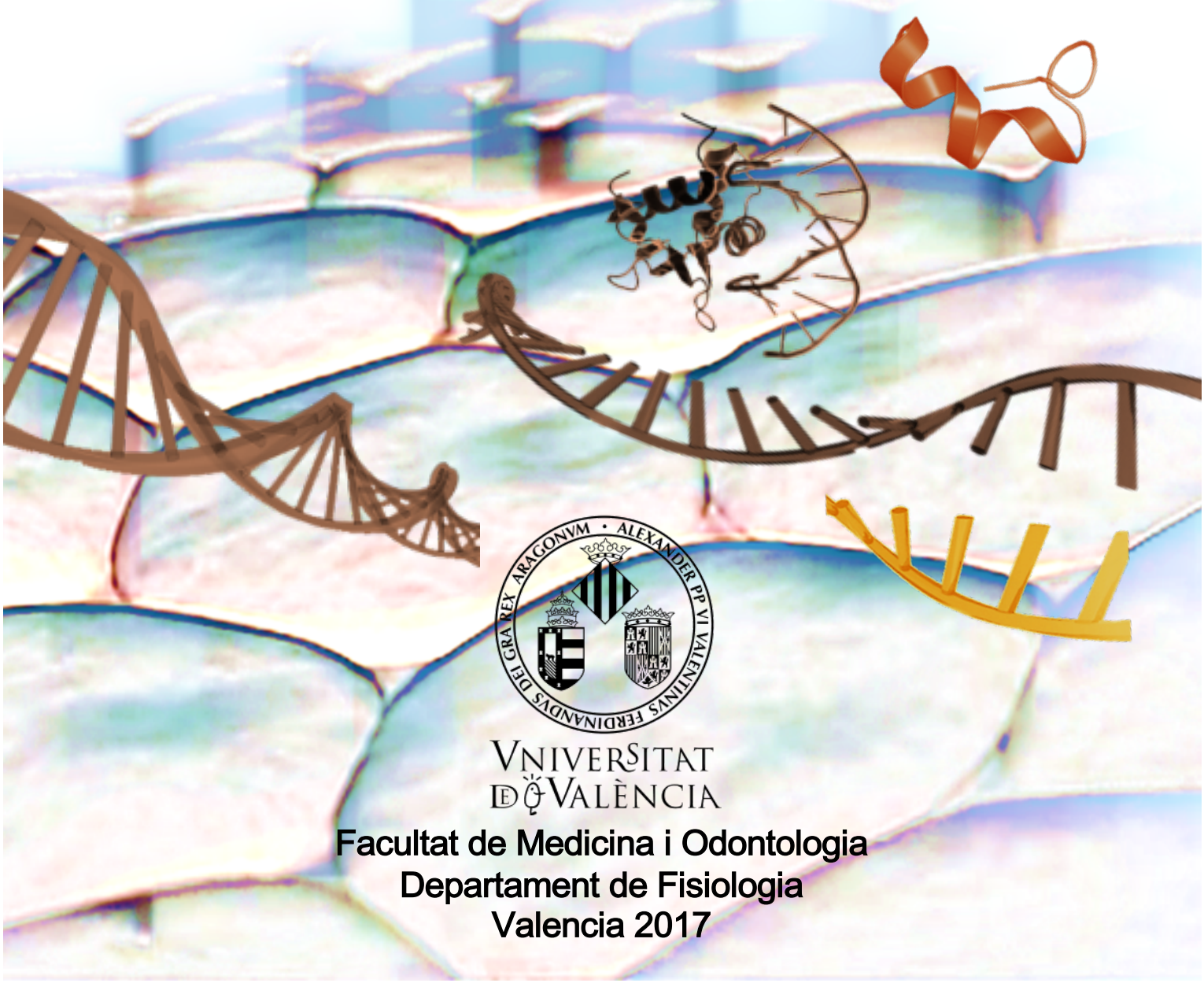

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Tesis doctoral

Análisis del perfil de expresión de microRNA en células endoteliales humanas expuestas a estradiol. Papel del miR-30b-5p en la regulación de la expresión de endotelina-1.

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CERTIFICAN:

Que D. Daniel Bernardo Pérez Cremades, Licenciado en Biología, ha realizado bajo su dirección el trabajo titulado **"Análisis del perfil de expresión de microRNA en células endoteliales humanas expuestas a estradiol. Papel del miR-30b-5p en la regulación de la expresión de endotelina-1"** para alcanzar el Grado de Doctor por la Universitat de València.

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ABBREVIATION LIST

AGO: argonaute

ANOVA: A one-way analysis of variance

AP-1: activator protein 1

AT_1R: Angiotensin receptor 1

BSA: bovine serum albumin

c-Myc: avian myelocytomatosis viral oncogene homolog

CAM: Cell adhesion molecules

cAMP: cyclic adenosine monophosphate

CDC25A: cell division cycle 25 homolog A

cGMP: cyclic guanosine monophosphate

COX: cyclooxygenases

CVD: cardiovascular diseases

CXCR4: C-X-C chemokine receptor type 4

DAPI: 4 ',6-diamino-2-fenilindol

DGCR8: DiGeorge syndrome critical region 8

DLG5: discs, large homolog 5

DLL4: Delta-like 4

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

E-selectin: endothelial selectin

ECE: endothelin-converting enzyme

EDRF: endothelium-derived relaxing factor

EDTA: ethylenediaminetetraacetic acid

EGFL7: epidermal growth factor-like domain multiple 7

EGR1: early growth response

ELK4: ETS-like transcription factor 4

eNOS: endothelial NOS

ER: estrogen receptors

ERE: estrogen response element

ERK: extracellular signal-regulated kinases

ET: endothelin

ET_A: ET type A receptor

ET_B: ET type B receptor

ETS: E twenty-six

FGF: fibroblast growth factor

FIH: factor inhibiting HIF-1

G1: (±)-1-[(3a*R**,4*S**,9b*S**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3*H*-cyclopenta[*c*]quinolin-8-yl]-ethanone

G15: (3a*S**,4*R**,9b*R**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3*H*-cyclopenta[*c*]quinoline

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GPER: G protein-coupled ER

GPR: G protein-coupled receptor

HIF: hypoxia inducible factor

HUVEC: human umbilical vein endothelial cells

ICAM: Intracellular CAM

IL: interleukin

iNOS: inducible NOS

IPA: Ingenuity Pathways Analysis

IRAK1: IL-1 receptor associated kinase

Jak: Janus activated kinases

KO: knockout

lncRNA: Long non-coding RNA

LPS: lipopolysaccharide

MAPK: mitogen-activated protein kinase

MDM4: mouse double minute 4

miRNA: microRNA

moR: miRNA offset RNA

MPP: methyl-piperidino-pyrazole

MYSM1: Myb-like, SWIRM and MPN domains 1

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

nNOS: neuronal NOS

NO: nitric oxide

NOS: enzyme NO synthase

nt: nucleotide

oxLDL: oxidized low-density lipoproteins

PACT: protein kinase R–activating protein

PAI-1: plasminogen activator inhibitor-1

PBS: phosphate-buffered saline

PCA: Principal Component Analysis

PGI_2: prostacyclin

PGIS: PGI_2 synthase

piRNA: Piwi-interacting RNA

PLA2: phospholipase A2

PLC: phospholipase C

PPAR: peroxisome proliferator-activated receptor

PPP3CA: protein phosphatase 3, catalytic subunit, alpha isozyme

PPT: 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol

PTMA: Prothymosin α

qRT-PCR: Quantitative Real Time Polymerase Chain Reaction

RHO: Ras homolog family

RISC: RNA-induced silencing complex

RNA: ribonucleic acid

RNases: ribonucleases

ROBO: Roundabout

ROCK: Rho kinase

ROS: Reactive oxygen species

RUNX: runt-related transcription factor

SAP: SRF associated protein

SDS: sodium dodecyl sulphate

SEM: Standard Error of Mean

siRNA: small interfering RNA

Smad: small mothers against decapentaplegic

SNP: Single nucleotide polymorphisms

SRE: serum response element

SRF: serum response factor

Stat: Signal transducers and activators of transcription

TBS-T: Tris-buffered saline with Tween 20

TCF: ternary complex factor

TFPI: tissue factor pathway inhibitor

TGF: transforming growth factor

TIE: tyrosine kinase with immunoglobulin like and EGF like domains

TNF: tumour necrosis factor

TRAF: TNF receptor associated factor

TRBP: TAR RNA-binding protein

TSS: transcription start site

TXA: thromboxane

UTR: untranslated region

VCAM: Vascular CAM

VE-cadherin: vascular endothelial cadherin

VEGF: vascular endothelial growth factor

VSMC: vascular smooth muscle cells

XPO5: exportin 5

ZO: zona occludens

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SUMMARY/RESUMEN

Summary

Endothelium is a highly dynamic tissue with a key role in the correct regulation of vascular function. Endothelium, which is located in the inner layer of blood and lymphatic vessels and heart, modulates several functions of cardiovascular system, such as haemostasis, regulation of vascular tone, response to inflammatory processes, as well as, regulation of vascular permeability and angiogenesis.

Endothelium acts as estrogen target since it expresses both types of estrogen receptors (ER), the classic nuclear transcription factors ERα and ERβ, and the most recently described G protein-coupled ER (GPER). Whereas ERα and ERβ modulate gene expression through binding to estrogen response elements (ERE) in the promoter region of estrogen-responsive genes, rapid estrogen signalling is mediated through membrane-associated ER and GPER.

Estrogen regulation of endothelial function is mediated by several mechanisms, by way of direct and indirect modulation of gene transcription machinery. In addition, regulation through non-coding ribonucleic acid (RNA) has emerged as a key mechanism modulating gene expression profile. Among them, microRNA (miRNA) are the most predominant class and they induce translation repression via sequence-specific interactions with target genes.

Although gene expression regulation mediated by miRNA has received a lot of attention in the last years, the role of estrogens on endothelial gene regulation through miRNA-based mechanisms has been less studied. Therefore, the main objective of the present work

was to study the role of miRNA in the estradiol-mediated effects in human umbilical vein endothelial cells (HUVEC).

First, global changes in the miRNA expression profile induced by estradiol in endothelial cells was evaluated using microarrays technology, and results were analysed using bioinformatics tools in order to obtain related pathways regulated by estradiol-sensitive miRNA. Results revealed that physiological concentration of estradiol (1 nmol/l) induced changes in the miRNA expression profile of endothelial cells. Specifically, 114 miRNA were regulated by estradiol, including 70 up-regulated and 44 down-regulated miRNA. Fold changes of estradiol-regulated miRNA range from -1.76 to 2.02. Among them, the most differentially expressed miRNA (miR-30b, miR-487a, miR-4710, miR-501, miR-378h and miR-1244) were validated by qRT-PCR. Functional analysis revealed that estradiol-modulated miRNA were associated to key molecular pathways, which were important to regulate vascular physiology in health and disease.

Additionally, the role of ER in estradiol-induced miRNA was evaluated by both an *in silico* approach evaluating specific ER binding sites in the promoter region of selected miRNA and a pharmacological approach by modulation of ER with specific agonists and antagonists. Our results show that changes in estradiol-induced miRNA expression were mediated through ER. Most of selected estradiol-regulated miRNA were regulated by ERα. Specifically, estradiol-dependent changes in miR-30b-5p, miR-487a-5p and miR-378h expression were mediated by ERα. In a lesser extent, ERβ and GPER were also implicated in miRNA expression changes after

estradiol treatment in endothelial cells. Changes in miR-501-3p and miR-4710 expression were related to ERβ and GPER action, respectively. Additionally, estradiol-dependent regulation of miR-1244 could also be mediated via the different ER in HUVEC.

Furthermore, an integrated miRNA-mRNA analysis was performed using mRNA microarray data of estradiol-treated HUVEC previously obtained by our group (Sobrino et al., 2009), increasing the process-specificity of predicted miRNA-mRNA pairings. Thereby, we identified miRNA-mRNA signature induced by estradiol as well as their implication in specific regulatory networks. Indeed, functional characterization highlighted ERK/MAPK signalling among canonical pathways in which estradiol-regulated miRNA were involved. Integrin, endothelin (ET)-1 and eNOS signalling, which regulate important vascular functions, were among regulated pathways in estradiol-treated endothelial cells.

miR-30b-5p, the miRNA that exhibited the greatest up-regulation in estradiol-exposed HUVEC, was selected to further study. For this purpose, cell transfection with miRNA inhibitor and miRNA mimic was used to determine the role of miR-30b-5p regulating predicted mRNA targets. Estradiol and miR-30b-5p mimic regulated gene expression of different miR-30b-5p predicted targets, including the transcription factor ELK4. Moreover, ET-1 was among putative estradiol-regulated genes with ELK4 binding sites after an *in silico* analysis. When endothelial cells were exposed to miR-30b-5p mimic, decreased ELK4 and ET-1 expression was observed, suggesting the

importance of miR-30b-5p/ELK4 association in estradiol-dependent inhibition of ET-1 in endothelial cells.

In summary, the obtained results provide for the first time an estradiol-mediated miRNA expression profile in endothelial cells, and information about biological processes related to estradiol-regulated miRNA. Specifically, the ET-1 regulation by estradiol-dependent miR-30b-5p has been studied. Due to increasing evidences suggesting the implication of ET-1 in endothelial dysfunction, atherosclerosis and hypertension, our results could open up an unexplored field by using miR-30b-5p as a new therapeutic approach aimed to improve endothelial function.

Resumen

El endotelio es un tejido dinámico que juega un papel fundamental en el mantenimiento de la función vascular. Está localizado en la capa interna de los vasos sanguíneos, linfáticos y corazón, y resulta clave para las diversas funciones del sistema cardiovascular, tales como la hemostasia, la regulación del tono vascular, la respuesta inflamatoria, la regulación de la permeabilidad vascular y la angiogénesis.

El endotelio actúa como una diana de los estrógenos y expresa ambos tipos de receptores de estrógenos (ER): los nucleares clásicos ERα and ERβ, que actúan como factores de transcripción, y el receptor acoplado a proteína G (GPER). Mientras que la unión de estrógenos a ERα y ERβ modula la expresión génica a través de su unión a elementos de respuesta a estrógenos (ERE), las acciones rápidas inducidas por estrógenos son llevadas a cabo a través de GPER y de otros ER asociados a membrana.

Los estrógenos regulan la función endotelial a través de diferentes mecanismos, entre los que destaca la modulación de la expresión génica a nivel transcripcional. Sin embargo, la regulación por ácidos ribonucleicos (RNA) no codificantes ha surgido como un mecanismo clave en la modulación del perfil de expresión génica. Entre ellos, predominan los microRNA (miRNA) y se caracterizan por mediar la represión de la traducción génica a través de interacciones específicas con sus genes diana.

En los últimos años, los miRNA han generado un gran interés en la regulación de la expresión génica en general, aunque su papel en la respuesta a estrógenos en células endoteliales no ha sido

estudiado. Por esta razón, el objetivo principal de esta tesis doctoral ha sido estudiar el papel de los miRNA en los efectos que ejercen los estrógenos en cultivos de células endoteliales de vena umbilical humana (HUVEC).

Para ello, se determinaron los cambios globales en el perfil de expresión de miRNA inducidos por 17β-estradiol en HUVEC mediante técnicas de microarrays. Los resultados se analizaron con herramientas bioinformáticas y se obtuvieron diferentes rutas moleculares en las que los miRNA estaban regulados por estradiol. Se observó que concentraciones fisiológicas de estradiol (1 nmol/l) inducían cambios en el perfil de expresión de miRNA. Concretamente variaron significativamente 114, de los que 70 incrementaron su nivel de expresión y 44 fueron reprimidos. El ratio de cambio varió entre -1,76 y 2,02. De entre estos 114 miRNA, los que mostraron un mayor cambio de expresión (miR-30b, miR-487a, miR-4710, miR-501, miR-378h y miR-1244) fueron validados mediante qRT-PCR. Además, el análisis funcional puso de manifiesto la asociación de los miRNA modulados por estradiol con rutas moleculares clave en la regulación de la función vascular en estado fisiológico y fisiopatológico.

Por otra parte, el papel de los ER en la regulación de miRNA fue evaluado mediante dos aproximaciones: por la presencia de sitios de unión específicos para ER en las regiones promotoras de los miRNA validados y a través de una aproximación farmacológica, mediante el uso de agonistas y antagonistas específicos de los diferentes ER. Nuestros resultados mostraron que los cambios en la expresión de miRNA inducidos por estradiol estaban mediados por

los ER. Si bien ERβ y GPER estaban implicados en cambios de expresión de algunos miRNA, como miR-501-3p y miR-4710 respectivamente, otros estaban regulados por ERα, como miR-30b-5p, miR-487a-5p y miR-378h. Finalmente, se observó en la expresión del miR-1244 una la regulación inespecífica a través de diferentes receptores de estradiol.

Mediante el análisis integrado de la interacción entre miRNA y mRNA, utilizando para ello datos de microarray de mRNA previamente publicados por nuestro grupo (Sobrino et al., 2009), se incrementó la especificidad del proceso de identificación de las interacciones miRNA-mRNA. De ese modo, se identificó el perfil de asociaciones miRNA-mRNA inducidas por estradiol, así como su implicación en rutas reguladoras específicas. La caracterización funcional destacó la ruta de señalización ERK/MAPK entre las rutas canónicas en las que estaban implicados los miRNA regulados por estradiol. Además, otras rutas de señalización vascular, como las relacionadas con integrinas, endotelina (ET)-1 y eNOS, también fueron identificadas entre las cuales los miRNA regulados por estradiol en células endoteliales estarían implicados.

Los últimos estudios se centraron en el miR-30b-5p, por ser el que más se inducía por estradiol en HUVEC. Mediante estudios de transfección celular con inhibidores y miméticos del miR-30b-5p se determinó su papel en la regulación de las dianas que previamente se habían predicho. Tanto el estradiol como el mimético de miR-30b-5p regularon la expresión génica de las dianas predichas para miR-30b-5p, como el factor de transcripción ELK4. Además, mediante el

análisis bioinformático se determinó que el gen de la ET-1, cuya expresión estaba disminuida en los datos del array de expresión de mRNA, poseía sitios de unión para ELK4. Por tanto, la disminución de la expresión de ELK4 y de ET-1 observada en células endoteliales expuestas al mimético de miR-30b-5p sugería una posible función de la interacción de miR-30b-5p con ELK4 en la inhibición de ET-1 inducida por estradiol en células endoteliales.

En resumen, los resultados obtenidos ofrecen por primera vez el perfil de expresión de miRNA inducido por estradiol en células endoteliales humanas. Además, se han identificado diferentes procesos biológicos relacionados con miRNA que están regulados por estradiol a través de sus receptores. En concreto, en este trabajo se ha descrito la regulación de ET-1 por el miR-30b-5p inducido por estradiol. Por todo ello, considerando la implicación de la ET-1 en la disfunción endotelial, aterosclerosis e hipertensión, estos resultados podrían abrir un campo inexplorado en la mejora de la función endotelial mediante la utilización del miR-30b-5p como una nueva aproximación terapéutica.

1. INTRODUCTION

Endothelium is located in the inner layer of blood and lymphatic vessels and heart between blood stream and underlying vascular smooth muscle cells (VSMC). It has been estimated that 10 trillion endothelial cells disposed as monolayer constitute the adult endothelium (Galley and Webster, 2004). Nowadays, endothelium is recognized as a highly dynamic tissue that plays a key role in the correct regulation of vascular physiology (Aird, 2007a, b)

Endothelial cells are important to the regulation of vascular homeostasis and they respond to external stimuli by the release of vasoactive compounds, which modulate the function of the neighbouring cells. Endothelium-derived factors influence in many functions of circulating cells by the modulation of the coagulation and fibrinolysis, leukocyte interaction and antigen presentation. Moreover, they can control vascular wall action by their effects on smooth muscle proliferation and contraction, extracellular matrix composition and vascular permeability (Michiels, 2003).

It has been demonstrated that vascular tissues are targets for sex hormones since specific receptors are expressed in both endothelial cells and VSMC (Khalil, 2013). For this reason, effect of

sex hormones on cardiovascular system have been extensively studied and several clinical and experimental data have demonstrated beneficial effect of estrogens on cardiovascular system through direct targeting endothelial cells (Vitale et al., 2009) (Miller and Duckles, 2008).

Estrogen regulation of endothelial function has been described to be mediated by several mechanisms, such as direct and indirect gene transcription of estrogen-responsive genes (Arnal et al., 2010; Sobrino et al., 2009). In addition, another group of important gene expression regulators receiving a lot of attention in the last years are non-coding ribonucleic acid (RNA), which can regulate gene expression via sequence-specific interactions with target genes. Among them, microRNA (miRNA) is the most predominant class. However, the role of estrogens on endothelial gene regulation through miRNA-based mechanisms has been less studied.

Therefore, in order to better understand the obtained results in this thesis, it is necessary to summarize the current knowledge about the different aspects of the role of estrogens in the regulation of endothelial function via activation of estrogen receptors (ER). Moreover, miRNA biology and important aspects in the involvement of miRNA in vascular function will be addressed.

1.1 Regulation of endothelial function by estrogens.

The role of estrogens is well described for sexual development and reproduction, but estrogens are also involved in many physiological processes, including regulation of skeletal homeostasis, lipid and carbohydrate metabolism, electrolyte balance, the central nervous system, cognition, behaviour and the cardiovascular system (Burns and Korach, 2012; Vrtacnik et al., 2014).

Role of estrogens is widespread in human physiology. Therefore, estrogen action has been related to the development of numerous diseases, which include various types of the well-known hormone-dependent cancers such as breast, ovarian, endometrial and prostate, among others. But they are also implicated in the progression of osteoporosis, neurodegenerative diseases, metabolic disorders (insulin resistance, obesity), autoimmune diseases (lupus erythematosus), endometriosis or cardiovascular diseases (CVD) (Deroo and Korach, 2006).

1.1.1 Estrogen action and estrogen receptors

Estrogens induce its action through different mechanisms. In the first described mechanism, also called “classical” mechanism of estrogen action, estrogens diffuse into the cell and bind to the ER, and this estrogen-ER complex binds to specific deoxyribonucleic acid (DNA) motifs called estrogen response elements (ERE) in the promoter region of estrogen-responsive genes. Moreover, ER transcriptional activity can also be carried out without DNA binding.

In the “tethering” mechanism, ER interacts with another DNA-bound transcription factor and modulate its activity by DNA binding stabilization and/or recruitment of co-activators/co-repressors (Klinge, 2001).

Classical mechanisms are mediated by two main ER isoforms, ERα and ERβ, encoded by separate genes located in different chromosomes. The full length of human ERα protein has 66 kDa while the molecular weight of ERβ isoform is 54 kDa. These ER are classical hormone nuclear receptors and belong to the nuclear receptor superfamily with 6 structural domains. A/B-domain contains the activation function 1 (located in the N-terminal) and is hormone-independent, C-domain is the DNA-binding domain which contains a nuclear localization sequence and the E/F-domain is the ligand-binding domain and contains the activation function 2. Structure of both ER subtypes are distinct from each other. Sequence homology is approximately 96% in the DNA-binding domains, 53% between both hormone-binding domains and 30% in the amino terminal domain (Li et al., 2004). An alternatively spliced isoform of ERα (46 kDa) with a truncated N-terminal domain has also been identified in endothelial cells (Li et al., 2003). ERα and ERβ form homo- or heterodimers before ERE binding and along with the recruitment of other coregulatory proteins (co-activators or co-repressors) induce changes in the gene expression. This described ER mechanism occurs over the course of hours and can be blocked by transcription inhibitors. For this reason, “classical” mechanism of ER has also been called the “genomic” mechanism.

Besides the classic genomic actions, ER can also trigger faster responses (minutes) through receptors that have been localized at the plasma membrane. Rapid estrogen signalling through membrane-associated ER was first identified in Clara Szego's Laboratory about 40 years ago (Pietras and Szego, 1977; Szego and Davis, 1967). Both ER isoforms have been localized in the plasma membrane and in other cytoplasmic organelles such as mitochondria and endoplasmic reticulum membranes (Levin, 2009). The ER may be targeted to the membrane by adaptor proteins such as caveolin-1. It has been described that ER-caveolin interaction modulates estrogen response due to its approximation with others signal-modulating proteins in the membrane (Razandi et al., 2002). Membrane ER can increase levels of Ca^{2+} or nitric oxide (NO), and activate different kinase pathways, such as mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and the Janus activated kinases (Jak) and Signal transducers and activators of transcription (Stat) pathway (Arevalo et al., 2015; Arnal et al., 2010). Finally, the rapid stimulation of kinases induced by membrane ER may also affect gene expression through their downstream regulation (Hammes and Levin, 2007).

Several studies have provided evidence for different physiological functions between ERα and ERβ (O'Lone et al., 2007). Indeed, the two subtypes can regulate gene expression in an opposite manner (Lindberg et al., 2003; Tsutsumi et al., 2008), but they can also mediate redundant functions (Arias-Loza et al., 2007; Lahm et al., 2008). In addition, estrogen signalling is selectively

stimulated or inhibited depending on the relative balance between ERα and ERβ expression in target organs, but also can be attributed to the presence of specific co-activators and co-repressors (Murphy and Steenbergen, 2014).

In addition, it was noted that some rapid signalling responses mediated by estrogens could not be attributed to ERα and ERβ since specific antagonists to this receptors did not block the observed estrogen-dependent effect (Wehling, 1997). For this reason, an alternative membrane-bound ER was predicted. In fact, it was in the late 1990s when it was reported a rapid estrogen-mediated activation of extracellular signal-regulated kinases (ERK) dependent on an orphan, G protein-coupled receptor (GPR) with seven transmembrane domains (Filardo et al., 2000). The receptor was known as GPR30 and was first cloned in endothelial cells in 1997 (Takada et al., 1997). Since then, accumulated research evidences suggested that GPR30 was an estrogen-binding receptor, which led to its designation as G protein-coupled ER (GPER) (Revankar et al., 2005). Indeed, many estrogen effects have been attributed to this receptor in cardiovascular system such as reduction of proinflammatory cytokines expression in myocardium, inhibition of endothelial cell and VSMC proliferation, as well as, NO-dependent vasodilatory action in humans and animal models (Prossnitz and Barton, 2011).

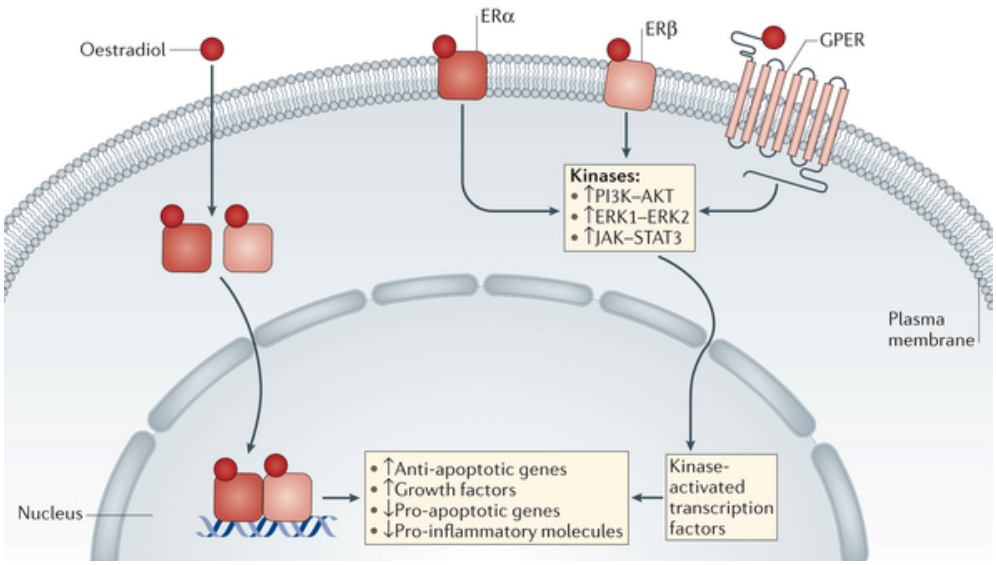


Figure 1. ER signalling. Endothelial cells express both classical ERα and ERβ, as well as, most recently described GPER. The action of estradiol is mediated through different ER mechanisms: binding to intracellular ERα and ERβ and regulation of estrogen-responsive genes at transcriptional level; binding to membrane-bound ER (ERα, ERβ and GPER) which leads to activation of second messenger pathways that produce rapid responses or gene regulation by transcription factors. Reproduced from (Arevalo et al., 2015).

1.1.2 Role of estrogens in endothelial function

Estrogens, acting through ER, have been involved in the regulation of crucial endothelial functions. Among them, maintenance of blood fluidity, vascular tone control, inflammation and vascular permeability regulation, as well as, its implication in angiogenesis processes are described in the next pages.

One of the important functions of vascular endothelium is the **maintenance of blood fluidity**. Endothelial cells have an essential role in the fluid state of the blood and promote limited clot formation when vascular damage appears after a breach in the vascular wall. Several endothelial-dependent mechanisms control coagulation in

the vascular system (Chapin and Hajjar, 2015). Among the anti-coagulant released factors are tissue factor pathway inhibitor (TFPI) that is the primary regulator of initiation of the blood coagulation process, heparin sulphate that binds anti-thrombin III, thrombomodulin and tissue-type plasminogen activator, as well as vasoactive compounds prostacyclin (PGI_2) and NO. On the other side, tissue factor, plasminogen activator inhibitor-1 (PAI-1) and von Willebrand factor (vWF) are among produced pro-coagulant molecules in endothelial cells (Bajaj et al., 2001).

The role of estrogens in haemostasis has been described with contradictory findings. Hormone therapy with estrogens has been associated to increase risk of venous thromboembolism (Daly et al., 1996). Indeed, levels of TFPI, mainly produced by endothelium, are reduced by estrogen exposition in postmenopausal women treated with estrogenic compounds (Hoibraaten et al., 2001) and in endothelial cells exposed to estradiol (Dahm et al., 2006). However, estrogen activity reduces plasma concentration of fibrinogen and anti-thrombin. Moreover, plasma estrogen levels have been associated to decreased levels of PAI-1, suggesting its role in fibrinolysis (Mendelsohn and Karas, 1999). Conversely, direct action of estrogens on endothelium has been related to increase PAI-1 expression in endothelial cells and aortic tissue from ovariectomized mice (Gopal et al., 2012).

Furthermore, endothelium has a key role in the **vascular tone** modulation. The first evidence of the involvement of endothelium in vascular tone regulation was observed by Robert Furchgott and John

Zawadzki in 1980 (Furchgott and Zawadzki, 1980). They demonstrated that removal of endothelium from isolated arteries prevents the relaxing response to acetylcholine. This phenomenon revealed the importance of endothelial – VSMC communication in vascular tone control. Indeed, endothelial cells selectively secrete several vasoactive molecules in response to different chemical and biomechanical stimuli under physiological conditions and regulate the contractile status of the underlying VSMC (Sandoo et al., 2010).

Endothelial-derived signals can be divided depending on their action on vascular tone. Among endothelium-derived vasodilator signals, NO and PGI_2 actions are the most important mechanisms. Meanwhile, endothelium-derived vasoconstrictor signals are vasoconstrictor prostanoids and endothelin (ET)-1, among others.

NO is considered the main endothelial vasodilator. NO was first termed as "endothelium-derived relaxing factor" (EDRF) and was defined as a diffusible factor that mediate relaxation (Furchgott, 1996; Palmer et al., 1987). Besides its action on vascular tone, NO also inhibits adhesion molecules expression, platelet aggregation and VSMC proliferation.

NO is produced by the enzyme NO synthase (NOS) which converts the amino acid L-arginine to NO. There are three isoforms of NOS which are classified according to the cells they were first identified: neuronal isoform (nNOS or NOS1), inducible or inflammatory NOS (iNOS or NOS2) which is expressed in cells that are exposed to inflammatory stimuli and was first observed in activated macrophages, and endothelial NOS (eNOS or NOS3) which produces

NO in endothelial cells (Li et al., 2002). NO rapidly diffuses from endothelial cells to the underlying VSMC where increases cyclic guanosine monophosphate (cGMP) levels through guanylyl cyclase activation. Then, intracellular Ca^{2+} levels are increased which lead to vascular wall relaxation (Zhao et al., 2015).

Enhanced acetylcholine-induced vasodilation in isolated arteries of estrogen-treated ovariectomized animals was one of the first evidences of the role of estrogen in vascular tone (Gisclard et al., 1987). Indeed, estradiol increases NO production in human endothelial cells (Caulin-Glaser et al., 1997). This estradiol-induced NO production is attributed to the modulation of eNOS through different mechanisms. Among them, estradiol is implicated in the increment of eNOS expression at transcriptional levels and its activation through phosphorylation (Chambliss and Shaul, 2002). Moreover, modulation of eNOS endogenous antagonists and cellular location are also regulated by estrogens (Monsalve et al., 2007; Xu et al., 2001). In this regard, ER have been involved in estradiol-dependent NO production (Guo et al., 2005). Indeed, a subpopulation of ERα protein is detected in caveolae where eNOS is located. ERα – eNOS interaction allows estradiol-dependent NO production in caveolae but not in non-caveolae membranes (Chambliss et al., 2000). In addition, studies with ER-knockout (KO) mice have demonstrated the role of ERα in estradiol-mediated vasodilation through increased NO production (Rubanyi et al., 1997).

Other important regulators of vascular tone are **prostanoids**. They are composed by prostaglandins and thromboxane (TXA) which

are synthesized from arachidonic acid in a multistep pathway that is initiated by a Ca^{2+}-mediated activation of cellular phospholipase A2 (cPLA2). Arachidonic acid is released from the cell membrane and processed by cyclooxygenases (COX), the main enzymes in the prostaglandin production. There are two main COX isoforms: COX-1 is considered the constitutive isoform in endothelium, while COX-2 is induced under pro-inflammatory conditions. However, both COX share characteristics of constitutive and inducible enzymes in endothelial cells (Ricciotti and FitzGerald, 2011). COX products cyclo-endoperoxides are rapidly converted into active compounds by means of the specific synthases. Prostanoids secreted by endothelium diffuse to VSMC and mediate their action by its binding to specific membrane receptors (Jabbour et al., 2006). PGI_2, the main vasodilatory prostaglandin released by endothelial cells, which was the first endothelial vasorelaxant molecule discovered by Moncada et al. in 1976 (Moncada et al., 1976), binds to specific receptors and activates adenylyl cyclase resulting in a cyclic adenosine monophosphate (cAMP) levels increment and vasodilation (Fetalvero et al., 2007).

PGI_2 receptor is also located in platelets and its activation leads to inhibition of platelet aggregation. In contrast to PGI_2, TXA_2 action is related to platelet aggregation and vasoconstriction through its specific receptor. In VSMC, TXA_2 receptor activation is involved in increasing intracellular Ca^{2+} levels that produce vasoconstriction (Alfranca et al., 2006).

Estrogens have also been involved in vascular prostanoid production. Indeed, studies on postmenopausal women support the role of prostanoids in acute estrogen-induced vasodilation (Calkin et al., 2002). In this way, the role of estradiol decreasing vascular tone has been described to be mediated by shifting COX-dependent vasoconstriction to vasodilation (Ospina et al., 2003). The effect of estrogen seems to be mediated mainly through an increase of COX-1 and PGI_2 synthase (PGIS) expression and activation without affecting TXA_2 production. However, contradictory results have been reported which could be related to differential effect of estrogens depending on vascular bed and conditions such as reproductive cycle phase and inflammation status (Novella and Hermenegildo, 2011).

The effects of estrogens on prostanoid pathway is related to both genomic and non-genomic effects. On the one hand, estrogens induce PGI_2 production in endothelial cells after 15 min of incubation. This increment, not related to changes in COX protein expression (Sherman et al., 2002), could be mediated by NO (Davidge et al., 1995). In addition, COX inhibition enhances the vasodilator effect of estrogen in mesenteric arteries (Tep-areenan et al., 2003), suggesting an endogenous release of vasocontractile prostanoids. On the other hand, longer estrogen exposition (hours) increases PGI_2 synthesis in endothelial cells (Jun et al., 1998; Sobrino et al., 2010). Effect that has been attributed to COX-1 expression up-regulation (Ospina et al., 2002; Sobrino et al., 2010). Meanwhile, COX-2 expression remained unaltered in endothelial cells or decreased in mesenteric arteries from ovariectomized aged rats exposed to estradiol (Armstrong et al.,

2002; Sobrino et al., 2010). Contradictory results have been reported concerning vasoconstrictor prostanoids. Indeed, estrogen exposition has been related to increase TXA_2 release in rat aorta (Li and Stallone, 2005), whereas no changes in TXA_2 levels were found in estradiol-treated endothelial cells (Sobrino et al., 2010).

Finally, **ET**, considered one of the most potent vasoconstrictors known (Levin, 1995), was identified by Yanagisawa and colleagues in 1988 (Yanagisawa et al., 1988). ET family is composed by three isoforms, ET-1, ET-2 and ET-3, being ET-1 the most abundant and widely expressed. ET-1 is predominantly synthesized by endothelial cells. However, *in vitro* VSMC and cells in the kidney, heart, lung, pancreas, spleen and brain also synthesize ET-1 (Remuzzi et al., 2002). Human *edn1* gene encodes the 212 aminoacid prepro-ET-1. A leader sequence targets prepro-ET-1 to the endoplasmic reticulum where it enters in the secretory pathway. Then, a two-steps cleavage process is mediated by proteases. First, prepro-ET-1 is cleaved into a 38 aminoacid pro-ET-1. Finally, endothelin-converting enzyme (ECE) cleaves pro-ET-1 (also called Big ET-1) in order to obtain the active ET-1 that is 21 aminoacids in length with two intrachain disulphide bonds (Stow et al., 2011).

Regulation at transcriptional level has been described to be the main mechanism modulating ET-1 bioavailability. Several studies have revealed specific binding elements upstream from the *edn1* gene that are involved in its expression through diverse transcription factors such as, hypoxia inducible factor (HIF)-1, small mothers against decapentaplegic (Smad) and activator protein 1 (AP-1),

among others (Stow et al., 2011). These transcription factors are in turn activated by upstream pathways such as, transforming growth factor (TGF) -β1, MAPK or PI3K/Akt pathways. Reactive oxygen species (ROS) signalling has also been implicated to the up-regulation of ET-1 release through activation of different transcription factors. Indeed, the well-known redox-sensitive nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) has also been involved in *edn1* gene induction. In addition, steroid hormone-mediated up-regulation of ET-1 expression has been reported to be mediated by both mineralocorticoid and glucocorticoid receptors. Finally, miRNA-mediated regulation of ET-1 expression has also been reported.

Two different secretory pathways have been proposed for the ET-1 release. First, secretory vesicles that are constitutive released through a cAMP-independent mechanism. Second, Weibel-Palade bodies, specialized regulatory granules, in which ET-1 is stored and released after specific stimuli (Russell et al., 1998; Yu and Davenport, 1995). ET-1 release can be activated in response to different stimuli, such as hypoxia, angiotensin II, thrombin, platelet products, inflammatory cytokines, norepinephrine, vasopressin, glucose, oxidized low-density lipoproteins (oxLDL) and biomechanical signals (Mombouli and Vanhoutte, 1999; Remuzzi et al., 2002).

ET-1 actions are mediated by binding to cell membrane receptors. There are two types of ET receptor: ET type A receptor (ET_A) and ET type B receptor (ET_B). ET_A is located in VSMC and mediates vasoconstriction, whereas ET_B is found in both endothelial cell and VSMC membranes where it produces vasodilatory and/or

vasocontractile action. When ET receptors are activated on VSMC membrane, intracellular Ca^{2+} levels increase by the activation of phospholipase C (PLC) and second messenger pathways which leads to short-term vasoconstriction (Tostes et al., 2007). On the other hand, vasodilatory action of endothelial ET_B is produced by inducing the release of NO and PGI_2 that negatively regulate the constrictor effect of ET-1 on VSMC (de Nucci et al., 1988; Verhaar et al., 1998). Therefore, the overall response of ET-1 on muscle tone regulation drives from the balance between vasoconstrictor action of ET-1 on VSMC through ET_A and ET_B receptors and vasodilatory molecules released after endothelial ET_B receptor binding (Tostes et al., 2007). Moreover, the expression and distribution of the different type receptors can modulate ET-1 action. Indeed, ET_A:ET_B receptor ratio depends on vascular bed, being higher in arteries compared with veins (Kedzierski and Yanagisawa, 2001).

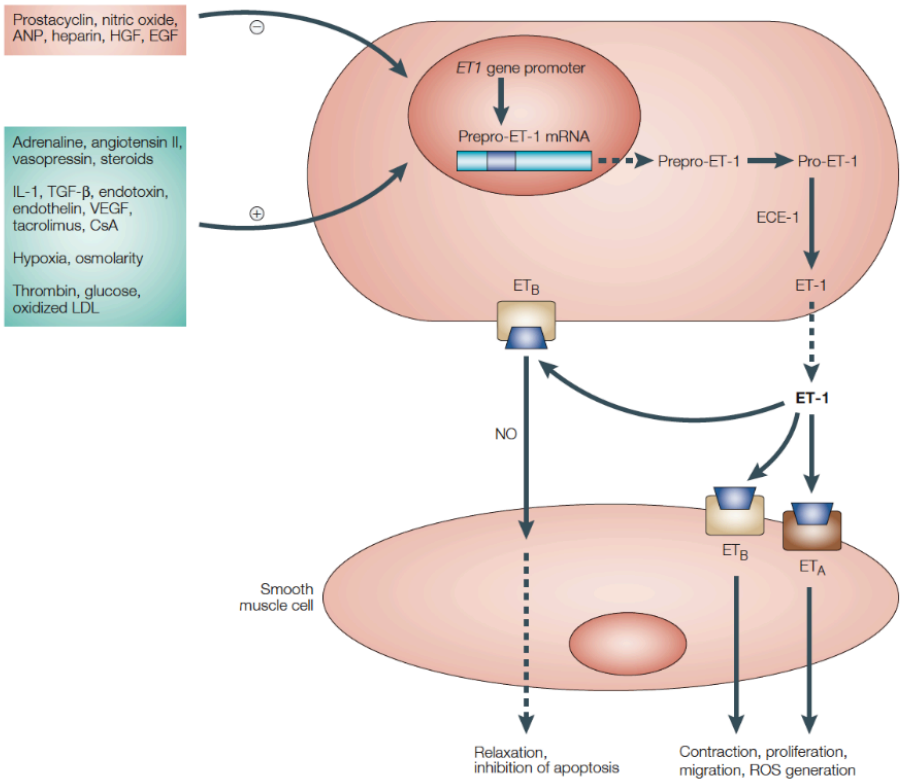


Figure 2. ET-1 pathway regulation, synthesis and action mechanisms. ET-1 production is regulated by different stimulators and inhibitors factors that modify ET-1 gene transcription. First ET-1 gene transcript is prepro-ET-1, which is cleaved in order to obtain pro-ET-1 (also known as Big ET-1). Finally, ECE-1 convert pro-ET-1 into the mature active peptide ET-1. ET-1 is secreted from endothelial cells and binds to cell membrane receptors. ET_A is located in VSMC and mediates vasoconstriction, whereas ET_B is found in both endothelial cell and VSMC membranes where it produces vasodilatory and vasocontractile action, respectively. ET receptors induce different down-stream pathways which lead to contraction, proliferation, migration and ROS generation in VSMC. Modified image from (Remuzzi et al., 2002).

Plasma ET-1 levels are higher in men than in age-matched women (Miyauchi et al., 1992), suggesting a regulatory action of estrogens on its production and release. Considering ET-1 production, estrogen is known to decrease its release in endothelial cells (Akishita et al., 1998; Pearson et al., 2008). Moreover, estrogens

attenuate ET-1-dependent vasoconstriction in blood vessels (Sudhir et al., 1997) and reduce circulating ET-1 levels in postmenopausal women receiving hormone replacement therapy (Christodoulakos et al., 2002). Additionally, estrogens can modulate ET-1 production by indirect mechanisms. In this regard, it has been reported that inhibition of NOS activity partly attenuates the estradiol-mediated decrease in ET-1 gene expression in endothelial cells (Bilsel et al., 2000), suggesting a role of NO in the inhibition of ET-1 actions.

ET-1 is not only a potent vasoconstrictor, but also is involved in VSMC proliferation and inflammation by increasing cytokine release, macrophages activation and neutrophil interaction to endothelium (Kowalczyk et al., 2015).

Regarding **inflammation**, endothelium plays a critical role in the initiation and progression of the process. Resting endothelial cells do not bind with leukocytes because interactive molecules (selectins and chemokines) are stored in Weibel-Palade bodies, in which ET-1 is also found as previously mentioned. Cell adhesion molecules (CAM) and cytokines expression is also supressed by constant NO production in endothelial cells (De Caterina et al., 1995).

After an inflammatory stimulus, endothelial cells acquire new capacities that allow the rapid recruitment and infiltration of neutrophils into the tissues. This process is known as “endothelial cell activation” and may be divided into type I activation, independent of new gene expression, and type II activation, which is dependent of transcriptional process. In both mechanisms exist an increase in local

blood flow; a localized leakage of plasma-protein-rich fluid and recruitment of circulating leukocytes (Pober and Sessa, 2007).

The first critical event in an inflammation process is the increase in endothelium regulated **vascular permeability**. Endothelial monolayer barrier is constituted by cell-cell junctions (tight, adherents and gap junctions) which permits selective transport of molecules and cells between blood and tissues. Changes in surface cadherins or occludins distribution are associated with regulation of vascular permeability. In addition, focal adhesion bond stabilization and activation of matrix metalloproteases are also key events in this process (Park-Windhol and D'Amore, 2016).

Ras homolog family (RHO) activation and elevated cytosolic Ca^{2+} levels are the main factors that regulate vascular permeability. Both mechanisms activate myosin light chain which leads to the contraction of actin filaments, opening the gaps between adjacent endothelial cells (Stevens et al., 2000).

In the case of a more persistent inflammatory signal such as tumour necrosis factor (TNF) α and interleukin (IL) -1 derived from activated leukocytes, type II endothelial activation is initiated. Both cytokines bind to their specific receptors, induce kinase signalling cascades and activate transcription factors NF-κB and AP-1. Finally, transcription factors translocate to nucleus where they produce new gene expression (Pober and Sessa, 2007). PGI_2 synthesis is produced by induction of COX2 up-regulation at sites of inflammation. In this regard, COX2-selective inhibitors are used as anti-inflammatory drugs inhibiting PGI_2-dependent blood flow increment (Mitchell et al.,

1995). Moreover, synthesis and display of chemokines and CAM on the cell membrane surface lead to a more effective neutrophil recruitment (Muller, 2003).

Estrogens has an anti-inflammatory effect that has been described in *in vitro* assays as well as in many vascular injury models (Bakir et al., 2000; Miller et al., 2004; Straub, 2007; Xing et al., 2004). In this regard, estradiol reduces induced CAM expression in endothelial cells exposed to IL-1 and lipopolysaccharide (LPS) treatment (Caulin-Glaser et al., 1996; Simoncini et al., 2000) and significantly decreases cytokine-induced adhesion of monocytes to human aortic endothelial cells (Mikkola and St Clair, 2002). Moreover, estradiol modulates inflammatory mediator expression, neutrophil chemotaxis (Miller et al., 2004) and leukocyte infiltration (Xing et al., 2004) after balloon injury of rat carotid artery. eNOS and COX pathway activation by estrogen have been implicated in the inhibition of leukocyte – endothelial cell interaction (Alvarez et al., 2002). Estrogen treatment also attenuates neointima formation after vascular damage (Mori et al., 2000). Furthermore, it has been observed that estrogen-induced vasoprotection in vascular injury is mediated through an ER-dependent mechanisms (Bakir et al., 2000).

Inflammation can evolve to a chronic process if inflammatory response fails to resolve the triggering stimulus. In that case, endothelial antigen presentation and angiogenesis processes have been associated with chronic inflammation. On the one hand, it has been suggested that endothelial cells might present antigens to circulating memory T cells (Choi et al., 2004). On the other hand,

generation of new vessels at the site of inflammation is required for survival of inflammatory cells (Costa et al., 2007).

Angiogenesis is referred to the formation of new blood vessels from pre-existing ones. Although during adulthood endothelium remains quiescent, it is essential during embryonic development and in tissue repair processes, endometrial growth during menstrual cycle and tumour growth, among others. New vessels formation is necessary due to the increased oxygen and nutrient demand of the tissue (Lamalice et al., 2007).

Endothelial cells play a key role during angiogenesis which is dependent on proliferation, migration and differentiation of these cells (Potente et al., 2011). Migration is regulated by chemotactic stimuli and growth factors. Degradation of the extracellular matrix through metalloproteases released by endothelial cells is also necessary to enable cell progression (van Hinsbergh and Koolwijk, 2008). Concerning angiogenic factors, they are cytokines such as vascular endothelial growth factor (VEGF) -A, which binds to VEGF receptor (R) -1 and VEGFR-2, fibroblast growth factor (FGF)-2 which activates FGFR-1, and angiopoietins which signal through tyrosine kinase with immunoglobulin like and EGF like domains (TIE) -2. All receptors are tyrosine kinase receptors that dimerize and initiate a phosphorylation cascade that in turn regulates angiogenesis through different signalling pathways (Khurana et al., 2005).

Estrogen promotes angiogenic activity in endothelium (Losordo and Isner, 2001). The mechanism includes up-regulation of VEGF release and receptor expression (Gargett et al., 2002).

Moreover, estrogens are involved in VEGF release through NO production, since it has been described that NO activity is necessary to allow VEGF-mediated angiogenesis (Papapetropoulos et al., 1997). Estrogens also increase integrins expression (Cid et al., 1999) which are important mediating cell attachment and migration. In addition, it has been described an enhanced endothelial recovery after arterial injury mediated by estradiol (Krasinski et al., 1997). Indeed, estrogens promote re-endothelialization by endothelial progenitor cells mobilization after vascular injury (Masuda et al., 2007).

Estradiol induces migration and proliferation in endothelial cells via RhoA/Rho kinase (ROCK) pathway activation (Oviedo et al., 2011) which is involved in the regulation of the actin cytoskeleton as well as in cell-cycle progression (Coleman et al., 2004). In contrast, estrogens inhibits the mitogenic action of growth factors on VSMC (Suzuki et al., 1996). Indeed, estrogen have been implicated in attenuation of neointimal proliferation after injury of rat carotid artery (Chen et al., 1996).

1.2 miRNA and vascular system

As described before, estrogen signalling regulates several physiological processes by directly and indirectly target gene transcription. Another group of important gene expression regulators widely studied in recent years consists on epigenetic mechanisms. Molecular basis of epigenetics includes three pathways: DNA methylation; histone density, variants and post-translational modifications; and RNA-based mechanisms (Yan and Marsden, 2015). Together, they are characterized by their ability to influence gene expression without changing the DNA sequence and have been established as fundamental determinants of endothelial gene expression in health and diseases (Matouk and Marsden, 2008). Among them, RNA-based mechanisms, which regulate gene expression via sequence-specific interactions, is the most recently described epigenetic mechanisms.

1.2.1 miRNA biosynthesis

Regulatory non-coding RNA can be classified depending on RNA length. Long non-coding RNA (lncRNA) is a heterogenic class of RNA that includes: intergenic lncRNA, antisense transcripts, and enhancer RNA. All of them are described as non-protein-coding transcripts larger than 200 nucleotide (nt) in order to differentiate them from small noncoding RNA (Mercer et al., 2009). On the other hand, small non-coding RNA include: miRNA, small interfering RNA (siRNA) and Piwi-interacting RNA (piRNA) which are defined as small (20-30 nt) and associated with argonaute (AGO) family proteins

(Kaikkonen et al., 2011). Moreover, it has recently described a new class of non-coding RNA derived from sequences located adjacent to miRNA and called miRNA offset RNA (moR). Although moR were first considered to be a by-product of miRNA biogenesis, recent studies have provided evidence that are biologically active and alter gene expression (Shi et al., 2009; Zhao et al., 2016).

Among them, miRNA is about 20-22 nt long approximately RNA and constitute the dominating class in most tissues. miRNA derive from nuclear transcripts with characteristic stem–loop structures (pri-miRNA). The first step in miRNA biosynthesis is the cleavage of pri-miRNA, which is mediated by a processing complex constituted by the RNase III Drosha and DiGeorge syndrome critical region 8 (DGCR8) (also known as Microprocessor complex). Nuclear processing involves cropping the stem-loop to release a small hairpin-shaped RNA (pre-miRNA). Then, pre-miRNA is transported into the cytoplasm through exportin 5 where maturation can be completed. Second processing step is mediated by RNase III Dicer that cleaves pre-miRNA into 22 nt miRNA duplexes. Usually, one strand from the cleavage products remains as a mature miRNA due to a selective process that depends on thermodynamic stability. Finally, RNA generated is loaded into an AGO protein to form the effector RNA-induced silencing complex (RISC) along with other component such as TAR RNA-binding protein (TRBP) or protein kinase R–activating protein (PACT). miRNA function as a guide by base pairing with its target mRNA and AGO proteins recruit factors that induce translational repression. miRNA-binding site are usually located at

the 3'- untranslated region (UTR) of the target mRNA (Kim et al., 2009).

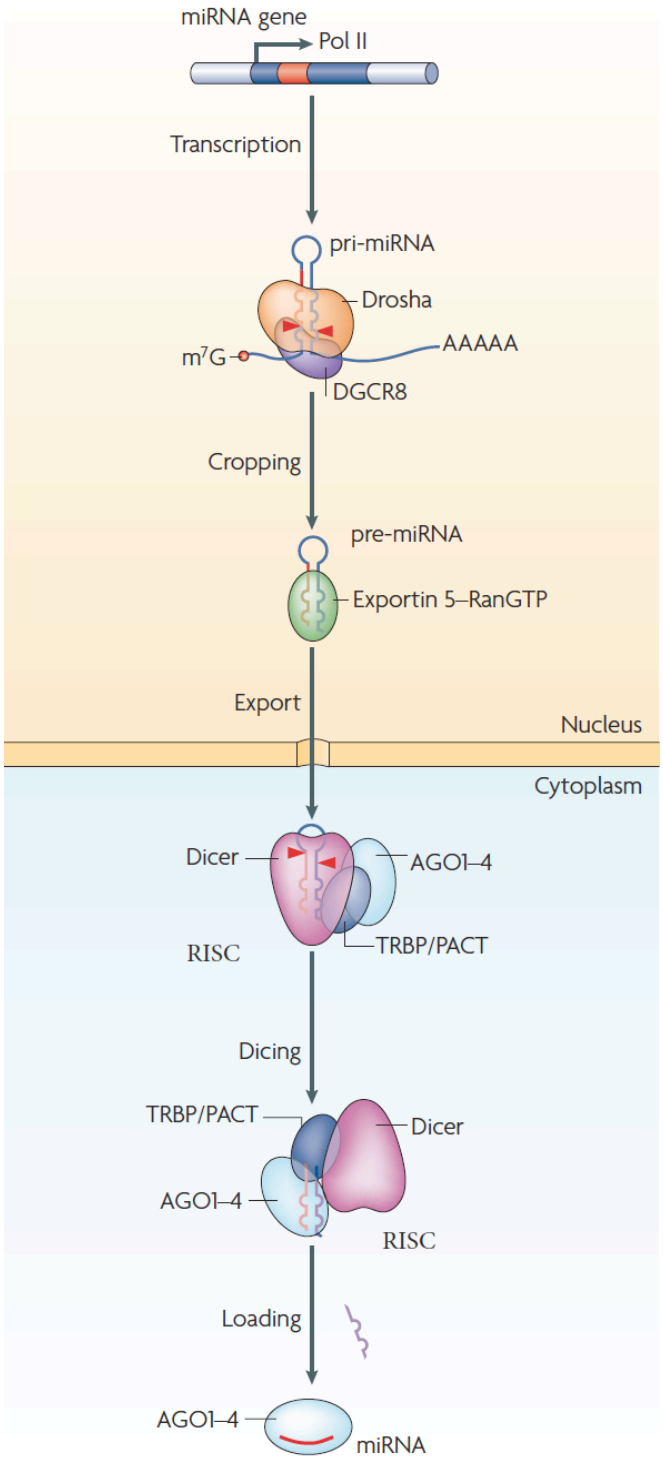


Figure 3. microRNA biosynthesis pathway. microRNA (miRNA) are transcribed by RNA polymerase II (Pol II) activity in order to generate the primary transcripts (pri-miRNA). Then, miRNA production is a two-step process that involved a nuclear cropping process and a cytosolic dicing process. First, cleavage of pri-miRNA is mediated by a processing complex constituted by the RNase III Drosha and DiGeorge syndrome critical region 8 (DGCR8) (also known as Microprocessor complex). As a result, a hairpin-shaped pre-miRNAs is generated. Pre-miRNA is recognized by the nuclear exportin 5 and exported to the cytoplasm. In the cytoplasm, RNase III Dicer cleaves pre-miRNA into 22 nucleotide miRNA duplexes. One strand from the cleavage products remains as a mature miRNA on the Argonaute (AGO) 1-4 proteins, whereas the other strand is degraded. Dicer, TAR RNA-binding protein (TRBP) or protein kinase R–activating protein (PACT), and AGO 1–4 proteins mediate the assembly of the RISC (RNA-induced silencing complex). Finally, miRNA function as a guide by base pairing with its target mRNA and AGO proteins recruit factors that induce translational repression. Modified image from (Kim et al., 2009).

miRNA sequences can be located in intronic regions of non-coding or coding transcripts, but it can be also encoded by exonic regions. Some miRNA are located in a close proximity region, constituting a polycistronic transcription unit which are usually transcribed at the same time (Ha and Kim, 2014). Precise locations of miRNA promoters have not been established. Regarding intronic miRNA, they can share regulatory regions of their host gene. However, it has been noted that miRNA have diverse transcription start sites and distinct to host gene promoter (Monteys et al., 2010).

Alterations in the nucleotide sequence of the miRNA or their target genes can modulate miRNA action. Single nucleotide polymorphisms (SNP) within the miRNA-binding sites in the 3'UTR region of target genes have been described in several cases (Saunders et al., 2007). A mutation in the 3'UTR sequence can create or destroy a binding site for a specific miRNA resulting in an altered

expression of the targeted mRNA that could be cause of a disease (Haas et al., 2012). First association between a SNP in miRNA target site (also known as miR-SNP) and a pathology was predicted by bioinformatics analysis in 2007 (Yu et al., 2007). Although miR-SNP are less frequent than in other portions of the genome, it has been found miR-SNP associated to CVD. For instance, angiotensin receptor 1 (AT_1R) is a target of miR-155, but A1166C polymorphism in the 3'UTR of AT_1R affects miR-155 binding site and consequently the ability to decrease AT_1R expression by this miRNA. Then, a SNP in AT_1R sequence leads to an enhanced expression, increased blood pressure and hypertension (Ceolotto et al., 2011).

On the other hand, SNPs in a miRNA-coding sequence might affect the expression of a great amount of different genes. An example is the polymorphism found in pre-miRNA sequence of miR-146a which impairs mature miR-146a synthesis. As a consequence, miR-146a target expression is altered and has been involved in coronary artery disease and isquemic stroke (Qu et al., 2016; Ramkaran et al., 2014).

1.2.2 miRNA regulation of endothelial function

The importance of miRNA in vascular biology was first observed by Yang et al. in 2005 (Yang et al., 2005) who observed impaired vascular formation in Dicer KO mice. In endothelial cells, Dicer knockdown showed impaired proliferation and cord formation as well as altered expression of proteins implicated in vascular tone regulation and angiogenesis, such as VEGFR2, IL-8 and eNOS

(Kuehbacher et al., 2007; Suarez et al., 2007), suggesting an important role of miRNA production in endothelial function. In Table 1 are presented the main endothelial functions regulated by miRNA and their main mRNA targets. Regarding the function of specific miRNA in endothelial biology, **miR-126** was the first described miRNA. miR-126 is highly expressed in vascularized tissues and considered an endothelial-specific miRNA that is implicated in vascular integrity and angiogenesis (Fish et al., 2008; Wang et al., 2008). miR-126 gene is located within an intron of the epidermal growth factor-like domain multiple 7 (EGFL7) gene which is mostly expressed in endothelium and involved in vascular angiogenesis. miR-126 induces angiogenic processes by targeting negative regulators of VEGF pathway. In fact, it has been reported that miR-126 increases Angiopoietin-1 signalling and therefore is involved in vessel stabilization and maturation (Sessa et al., 2012). Others miRNA involved in **angiogenesis** regulation are miR-17-92 cluster, miR-218, miR-503, miR-221, miR-16 and miR-424.

miR-17/92 cluster is a polycistronic miRNA which encodes 6 individual miRNA: mir-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a, involved in cell proliferation, cell cycle arrest and apoptosis (He et al., 2005). For example, it has been observed that members of the miRNA cluster are induced after VEGF treatment suggesting a role of VEGF-mediated angiogenesis (Chamorro-Jorganes et al., 2016). However, miR-19a has also been involved in the inhibition of cell cycle progression by directly targeting Cyclin-D1 (Qin et al., 2010), and miR-92a overexpression induced impaired endothelial sprout

formation by reducing integrin-α5 expression in endothelial cells (Bonauer et al., 2009). Moreover, it is worth mentioning that miR-17/92 cluster is regulated by oncogenic transcription factor avian myelocytomatosis viral oncogene homolog (c-Myc) at transcriptional level and also, E2F transcription factor 1, its downstream effector, is in turn repressed by some of the miRNA transcribed (He et al., 2005).

Others miRNA have also been described as anti-angiogenics through repression in cell migration and proliferation. It has been observed that decreased miR-218 expression but miR221/222 overexpression correlates with decreased endothelial cell migration (Poliseno et al., 2006; Small et al., 2010). In fact, miR221/222 cluster targets with transcription factor E twenty-six (ETS)-1 which regulates pro-angiogenic pathways (Zhu et al., 2011). Moreover, miR-503 has been described to reduce endothelial proliferation, migration and cord formation by direct targeting cell cycle-related molecules, such as cell division cycle 25 homolog A (CDC25A) and cyclin-E1 (Caporali et al., 2011).

Specifically, miR-16 and miR-424 decrease endothelial angiogenic functions via targeting VEGFR2 and FGFR1 (Chamorro-Jorganes et al., 2011). Interestingly, these miRNA are transcriptionally induced by pro-angiogenic factors such as VEGF and FGF. Therefore, as described above with miR-17/92, it seems that miRNA induction provides a tight regulation of the angiogenesis by a negative feedback loop mechanism targeting on specific molecules, such as receptors or intermediate effectors, involved in the process.

Table 1. miRNA related to endothelial function. Specific miRNA associated with different endothelial functions and their target genes are shown.

Endothelial function	miRNA	Target	References
Angiogenesis	miR-126	SPRED1	(Fish et al., 2008)
	miR-92a	Integrin-α5	(Bonauer et al., 2009)
	miR-19a	Cyclin D1	(Qin et al., 2010)
	miR-221/222	ETS-1	(Zhu et al., 2011)
	miR-503	CDC25A Cyclin E1	(Caporali et al., 2011)
	miR-16	VEGFR1	(Chamorro-Jorganes et al., 2011)
	miR-424	FGFR1	
Inflammation	miR-126	VCAM1	(Harris et al., 2008)
	miR-31	E-Selectin	(Suarez et al., 2010)
	miR-17-3p	ICAM1	
	miR-146a/b	TRAF IRAK	(Cheng et al., 2013)
	miR-155	CARHSP1	(Li et al., 2016c)
	miR-181b	Importin-α3	(Sun et al., 2012b)
Vascular permeability	miR-125b	VE-Cadherin	(Muramatsu et al., 2013)
	miR-27		(Young et al., 2013)
	miR-208	ROBO1	(Small et al., 2010)
	miR-18a	RUNX1	(Miao et al., 2015)
	miR-98	FIH-1	(Hu et al., 2015)
	miR-184	β-Catenin	(Zong et al., 2016)
Haemostasis	miR-421	PAI-1	(Marchand et al., 2012)
	miR-30c		
	miR-19b	TF	(Teruel-Montoya et al., 2015)
	miR-20a		
	miR-106b		
Vascular tone	miR-155	eNOS	(Sun et al., 2012a)
		AT_1R	(Ceolotto et al., 2011)
	miR-21	PPARα	(Zhou et al., 2011)
	miR-125a/b	ET-1	(Li et al., 2010)
	miR-199		(Yeligar et al., 2009)
	miR-1		(Feng et al., 2014)

Moreover, miR-30 family has been related in angiogenesis regulation through targeting Delta-like 4 (DLL4) (Bridge et al., 2012). In addition, endothelial miR-30 family members act in an anti-inflammatory manner by supressing angiopoietin 2 expression (Demolli et al., 2015).

First evidence of miRNA control in vascular **inflammation** was published by Lowenstein and co-workers (Harris et al., 2008) who described the role of miR-126 regulating vascular (V)CAM-1 expression in endothelial cells exposed to TNFα. Other miRNA induced by pro-inflammatory molecules such as TNFα, IL-1 or LPS are miR-17, miR-31, miR-146a, miR-155, miR-181b and miR-221/222 among others.

As seen above in the case of VCAM-1, TNFα-mediated induction of endothelial (E)-selectin and intercellular (I)CAM-1 are post-transcriptionally regulated by TNFα-induced miRNA, miR-31 and miR-17-3p respectively, providing a negative feedback control of inflammation (Suarez et al., 2010).

Other cytokine-modulated miRNA is miR-146a/b. TNFα and IL-1b induce miR-146 expression in endothelial cells which regulates endothelial activation by targeting TNF receptor associated factor (TRAF) 6 and IL-1 receptor associated kinase (IRAK1) (Cheng et al., 2013), up-stream regulators of inflammation-related NF-κB pathway. Curiously, increased miR146a/b levels are maintained in the late stages of inflammation induction even when adhesion molecules have already been down-regulated, suggesting a role of these miRNA in the resolution of vascular inflammation. Other miRNA, such as

miR-10a, miR-92a, miR-663, have been involved in adhesion of immune cells to endothelium (Fang and Davies, 2012; Fang et al., 2010; Ni et al., 2011).

Considering NF-κB activity, miR-155 and miR-181b have been described as negative regulators. miR-155 expression is transcriptionally regulated by NF-κB after cytokine stimuli and has been described as an anti-inflammatory factor in chronic inflammation (Li et al., 2016c). Moreover, miR-155 overexpression decreases endothelial activation after angiotensin II exposition (Zhu et al., 2011). On the other hand, it has also been described that miR-181b regulates the expression of importin-α3, a protein required for nuclear translocation of NF-κB (Sun et al., 2012b), a critical step in NF-κB-dependent response.

As mentioned above, loss of **barrier function** is one of the first events in inflammation process. Different works have demonstrated the role of miRNA in barrier function regulation (Lopez-Ramirez et al., 2016; Zhuang et al., 2016). Specifically, miR-125b and miR-27 have been involved in repression of vascular endothelial (VE)-cadherin (Muramatsu et al., 2013; Young et al., 2013). miR-208 has been found to target Roundabout (ROBO) signalling components involved in endothelial permeability (Small et al., 2010). Moreover, deletion of miR-150 caused irreversible increment of vascular permeability in mouse and cell models (Rajput et al., 2016). Regarding tight junctions, miR-18a and miR-98 increased permeability by negatively regulation of zona occludens (ZO)-1 via runt-related transcription factor (RUNX) 1 (Miao et al., 2015) and

factor inhibiting HIF-1 (FIH)-1 (Hu et al., 2015), respectively. Finally, miR-184-mediated down-regulation of β-catenin resulted in changed adherens junction structure in endothelial cells (Zong et al., 2016).

As mentioned before, endothelium is involved in the control of **haemostasis** by production of different coagulation factors, such as PAI-1. miR-421 and miR-30c can regulate PAI-1 production in endothelial cells by inhibition of PAI-1 mRNA translation (Marchand et al., 2012). Moreover, tissue factor, another procoagulant factor released by endothelial cells, has been regulated by miR-19b, miR-20a and miR-106b (Teruel-Montoya et al., 2015).

The first evidence regarding the role of miRNA regulating molecules involved in **vascular tone** was observed in Dicer knockdown endothelial cells in which eNOS expression was increased (Suarez et al., 2007). In fact, it has been observed that miR-155 regulates endothelium-dependent vasorelaxation by direct targeting eNOS mRNA (Sun et al., 2012a). Moreover, miR-221 and miR-222 mimic transfection increase eNOS expression in endothelial cells (Suarez et al., 2007) although this effect might be indirect since no predicted binding site has been found in eNOS sequence. Finally, it has been observed that increased miR-21 expression observed after laminar shear stress increase eNOS expression by regulating Akt pathway (Weber et al., 2010). However, oscillatory shear stress also increased miR-21 expression but promote endothelial inflammation by targeting peroxisome proliferator-activated receptor (PPAR) α (Zhou et al., 2011). These contradictory results might be explained by the difference in cell context and the magnitude of the miRNA levels.

On the other side, ET-1 expression has also been regulated by miRNA mechanisms. In this regard, translational repression of ET-1 mRNA by miR-155 and miR-199 has been described in microvascular endothelial cells exposed to ethanol (Yeligar et al., 2009). In addition, it has been reported an inversed correlation between prepro-ET-1 and miR-125a/b levels (Li et al., 2010). Moreover, overexpression of miR-1 reduced ET-1 induction in hyperglycemic conditions (Feng et al., 2014).

In addition to their role in modulation of cell functions, miRNA have been found in extracellular space and have been postulated to be mediators of **cell-to-cell communication** (Xu et al., 2013). miRNA are released by cells as miRNA-protein complexes, lipoprotein bound miRNA or associated with vesicular particles which protect themselves against degradation mediated by circulating ribonucleases (RNases) (Kosaka et al., 2010).

Extracellular vesicles transferred into recipient cells are able to modulate their function. For example, endothelial progenitor cell derived vesicles promote an angiogenic program by increasing endothelial cell survival, proliferation and organization of tubular-like structures (Deregibus et al., 2007). Interestingly, it has been hypothesized that miRNA packaging into protein complexes or vesicles is a selective process in which some miRNA are delivered to the extracellular space while other are maintained within the cell (Hergenreider et al., 2012). However, underlying mechanism of sorting and secretion of miRNA has not yet been understood.

miRNA transferred by extracellular vesicles can mediate repression of mRNA translation in the recipient cells, influencing in their physiological behaviour. This intercellular communication can be between the same or different type cells. In this regard, it has been observed that miR-214-containing exosomes suppress senescence and stimulate angiogenesis through an autocrine signal in target cells (van Balkom et al., 2013). Moreover, miR-126-enriched extracellular vesicles derived from serum-starved apoptotic endothelial cells have been related to reduced atherosclerotic lesion in mice model through changes in the expression of C-X-C chemokine receptor type 4 (CXCR4) of neighbouring cells which promote incorporation of progenitor cells (Zernecke et al., 2009).

Vesicle-mediated communication between endothelium and subjacent VSMC has been also established. miR-126 released by endothelial cells controls cell proliferation, cell cycle protein expression and apoptosis in VSMC (Zhou et al., 2013). In addition, delivery of miR-145 and miR-143 from endothelial cells to VSMC regulate its function by repression of dedifferentiation-associated genes (Hergenreider et al., 2012). Moreover, endothelial cell-derived miR-143 uptake by VSMC have been observed in pulmonary arterial hypertension where it plays a key role in paracrine signalling during remodelling pulmonary vasculature (Deng et al., 2015).

Extracellular vesicles are increased in chronic inflammatory states (Buzas et al., 2014). Vesicle-mediated communication has been described between endothelium and inflammatory cells. For example, a relationship between secreted monocytic miR-150 and

increased endothelial cell migration has also been observed (Zhang et al., 2010). In addition, extracellular vesicles released by endothelial cells can supress monocyte activation through the transfer of anti-inflammatory miRNA, such as miR-10a, miR-126 and miR-181b (Njock et al., 2015).

miRNA detected in the blood stream are known as "circulating miRNA" and changes in the profile of circulating miRNA have been identified in different pathological conditions. For this reason, circulating miRNA detection has been proposed as non-invasive biomarkers in CVD (Fichtlscherer et al., 2011).

1.2.3 miRNA and vascular estrogen action

Sex-differences in miRNA expression between male and female have been described in different physiological and pathological conditions (Guo et al., 2017; Sharma and Eghbali, 2014), suggesting a role of sex hormones in miRNA regulation. Nevertheless, the relationship between sex-dependent miRNA expression and CVD has been less explored (Sharma and Eghbali, 2014).

Regulation of miRNA expression by estrogens have been observed in different cell types and tissues (Klinge, 2015). In addition, the role of estrogens in the circulating miRNA profile has been described in ovariectomized rats and postmenopausal women using hormone replacement therapy (Chen et al., 2016; Kangas et al., 2017). Table 2 summarizes miRNA signatures that have been

associated with estrogen action on different target organs as well as estrogen-dependent effect on circulating miRNA.

Table 2. miRNA associated with estrogen action. miRNA signature associated with estrogen actions on specific target organs.

Target	miRNA	References
Uterus	miR-155 miR-429 miR-451 miR-181b miR-204	(Nothnick and Healy, 2010)
Endometrium	mir-29b miR-29c miR-30b miR-30d miR-31 miR-193a-3p miR-203 miR-204 miR-200c mir-210 miR-582-5p miR-345	(Kuokkanen et al., 2010)
	miR-30b miR-30d miR-494 miR-923	(Altmae et al., 2013)
Breast cancer	Let-7f, miR-98 and miR-21 miR-30c miR-30b miR-424 miR-27a miR-27b miR-143 miR-9	(Bhat-Nakshatri et al., 2009)
	miR-101 miR-125b-5p miR-128a mir-181b miR-205-5p mir-210 miR-221/222 mir-301 miR-519a miR-1280 Let-7 family miR-10a miR-15a/16 miR-126 miR-200b/c miR-342-3p miR-375 miR-424-3p mir-351 miR-574-3p miR-873	(Muluhngwi and Klinge, 2015)
	miR- 190b miR-101-1 miR-193b miR-342-5p miR-376c miR-451 miR-143 miR-30c miR-30e miR-26a miR-26b miR-654-3p miR-203 miR-146a miR-494 miR-338-5p miR-891a miR-1244	(Cizeron-Clairac et al., 2015)
	let-7c miR-99a miR-125b miR-148a miR-99a miR-17-92a miR-221/222 miR-497 miR-195, miR-590-3p miR-590-5p miR-30e	(Bailey et al., 2015)
Bone	miR-127 miR-133a miR-133a miR-133b miR-136 miR-206 miR-378 miR-3780 miR-204 miR-30b	(An et al., 2014)
Skeletal muscle	miRs-182 miR-223 and miR-142-3p	(Olivieri et al., 2014)
Inflammation markers	miR-21 miR-146a	(Kangas et al., 2014)
Circulating miRNA	miR-30a-5p miR-30e-5p miR-425-5p miR-142-3p miR-191a-3p miR-215 miR-29b-3p miR-30b-5p miR-26a-5p miR-345-5p miR-361-5p miR-185-5p miR-103-3p	(Chen et al., 2016)
	miR-106-5p miR-148a-3p miR-27-3p miR-126-5p miR-28-3p miR-30a-5p	(Kangas et al., 2017)

Most of described studies were performed in reproductive organs targeted by estrogens. In this regard, it has been described a hormonal regulation of miRNA expression since miRNA profiling of human endometrium reveals cyclic changes in miRNA expression through the different physiological phases (Altmae et al., 2013; Kuokkanen et al., 2010). In addition, estrogen action has also been related to regulation of specific miRNA expression in the mouse uterus (Nothnick and Healy, 2010).

The role of ER in miRNA regulation has also been extensively studied in breast cancer. Moreover, different profiles of estrogen-regulated miRNA in breast cancer cells have been described (Bhat-Nakshatri et al., 2009; Muluhngwi and Klinge, 2015). Indeed, miRNA profiling between ER+ or ER- breast cancer cells revealed significantly different expressed miRNA (Bailey et al., 2015; Cizeron-Clairac et al., 2015), suggesting a crucial role of ER in miRNA expression pattern.

miRNA have also been identified as important modulators of ER-dependent gene expression since are targeted by them. Indeed, it has been described that some estrogen-induced miRNA target and regulate ERα expression, acting as a negative feedback loop (Castellano et al., 2009). Other miRNA have also been implicated in ERα targeting, such as miR-18a, miR-22, miR-206 and miR-221/222 (Klinge, 2012). However, miR-92 and miR-424 were the only miRNA that have been identified to target ERβ (Al-Nakhle et al., 2010) and GPER (Zhang et al., 2015a), respectively.

As described above, estrogens have been used as principal constituents of hormone replacement therapy formulation. In this

regard, changes in circulating miRNA have been observed in women treated with hormone replacement therapy (Kangas et al., 2017). Indeed, beneficial effects of estrogens on osteoporosis, sarcopenia or increased inflammation markers have been attributed to miRNA in postmenopausal women using hormone replacement therapy.

A positive estrogen impact through miRNA changes has been observed in skeletal muscle of postmenopausal women using hormone replacement therapy (Olivieri et al., 2014). Moreover, changes in serum inflammatory markers and inflammatory-related miRNA has been observed in postmenopausal women using hormone therapy (Kangas et al., 2014). Finally, although estrogens levels and osteoporosis relationship have been established for decades (Richelson et al., 1984), it has been recently described changes in miRNA expression in mice bone tissue in an ovariectomy-induced osteoporosis model and postmenopausal woman (An et al., 2014).

At the cardiovascular level, some studies describe that estrogens exert its vascular protection in part via miRNA activity. The role of estrogen-induced miRNA in heart tissue, VSMC, and to a lesser extent in endothelial cells has been described. Estrogen-dependent miRNA actions in cardiovascular system are summarized in Table 3.

Table 3. miRNA associated with specific cardiovascular estrogen actions. Estrogen-dependent effect on cardiovascular tissue and the specific estrogen-related miRNA that is associated with a specific action are shown.

Estrogen action	miRNA	References
Cardiac gap junctions regulation	miR-23a	(Wang et al., 2015b)
Sex differences in heart	miR-1 miR-106b miR-720 miR-29b miR-144 miR-205 miR-222	(Evangelista et al., 2013)
Sex differences in cardiac fibrosis	miR-21 miR-24 miR-27a/b miR-106a/b	(Queirós et al., 2013)
VSMC and endothelial cell communication	miR-143 miR-145	(Deng et al., 2015)
Inhibition of VSMC proliferation	miR-203	(Zhao et al., 2013)
Endothelial cell proliferation	miR-126-3p	(Li et al., 2016a)

A protective role of estradiol-dependent regulation of miR-23a, whose regulatory regions contains ERα binding sites, has been observed in estrogen deficiency-induced damage of cardiac gap junctions in rats (Wang et al., 2015b). On the other hand, sex differences in miRNA expression have been observed in heart. In this regard, sex-dimorphic expression of miR-222 has been implicated in the modulation of cardiac expression of eNOS (Evangelista et al., 2013). In addition, the role of ERβ on miRNA expression has also been described. Sex-specific miRNA expression via ERβ has been related to sex differences in a mice model of pressure overload-induced cardiac fibrosis (Queirós et al., 2013).

In vascular tissue, VSMC proliferation was inhibited through an ER-dependent up-regulation of miR-203 (Zhao et al., 2013). Moreover, estradiol exposition also induces miR-143 and miR-145 expression in pulmonary artery VSMC via ER stimulation of specific ER binding sites located in their promoter region (Deng et al., 2015). Indeed, estradiol-treated VSMC secretes exosomes enriched with miR-143 and miR-145 regulates VSMC-endothelium crosstalk in pulmonary arterial hypertension. On the other hand, estradiol has been implicated in the increment of miR-126-3p expression in endothelial cells which is related to an increased on cell migration, proliferation and tube formation while a decreased on monocyte adhesion (Li et al., 2016a).

2. HYPOTHESIS AND OBJECTIVES

Endothelium is a highly dynamic tissue and plays a crucial role in the regulation of vascular homeostasis. Moreover, it has been demonstrated that endothelium is targeted by estrogens since specific ER are expressed in endothelial cells. Indeed, estrogen regulation of endothelial function has been described to be mediated by several mechanisms, such as direct and indirect gene transcription of estrogen-responsive genes through ER implication, as it has been previously reported by our group. In addition, another group of important gene expression regulators are non-coding RNA which can modulate cell expression profile by interfering mRNA translation. Among them, miRNA is the most predominant class. However, although gene expression regulation mediated by miRNA has received a lot of attention in the last years, the role of estrogens on endothelial gene regulation through miRNA-based mechanisms has been less studied.

For this reason, the main objective of the present work was to study the role of miRNA in some of the estradiol-mediated effects in endothelial cells.

Furthermore, different specific objectives have been determined:

1. To evaluate global changes in the miRNA profile induced by estradiol. For this propose, we will screen differentially expressed miRNA in cultured human endothelial cells exposed to physiological concentrations of estradiol using microarrays technology. Moreover, data of selected miRNA will be validated by qRT-PCR.

2. To determine targets of differentially expressed miRNA induced by estradiol and the related pathways regulated by estradiol-sensitive miRNA using transcriptome analysis.

3. To evaluate the implication of ER in estradiol-induced miRNA expression. As classical ER are transcription factors, binding sites for ERα and ERβ in the regulatory region of estradiol-regulated miRNA precursors will be evaluated by *in silico* analysis. In addition, modulation of ER with specific antagonist and agonists against ERα, ERβ and GPER will be used in order to determine the role of ER in the expression of miRNA regulated by estradiol.

4. To perform integrated miRNA-mRNA analysis using mRNA microarray data of estradiol-treated HUVEC previously obtained by our group. We will identify miRNA-mRNA signature induced by estradiol as well as their implication in specific regulatory networks.

5. Finally, highlighted miRNA and/or pathways involved in vascular function will be selected in order to further study, thereby increasing our knowledge of estradiol-dependent effects in endothelial cell function.

3. MATERIALS AND METHODS

3.1 Human umbilical vein endothelial cell culture.

Primary human umbilical vein endothelial cell (HUVEC) cultures were obtained from human umbilical cords from healthy women at Department of Obstetrics and Gynecology, "Hospital Clínico Universitario" of Valencia. After birth, umbilical cords were kept in phosphate-buffered saline (PBS) (Sigma-Aldrich, Tres Cantos, Madrid, Spain) at 4°C until further processing. Umbilical vein was cannulated using lockable one-way stopcock (VYGON, Ecouen, France) and washed with PBS in order to remove the remaining blood. Then, endothelial cells were detached using 0.1 mg/ml collagenase (Life Technologies, Carlsbad, CA, USA) incubation for 15 min at 37°C. Isolated HUVEC were cultured in Medium 199 (Sigma-Aldrich) supplemented with 20% fetal bovine serum (GIBCO, Life Technologies), endothelial cell growth supplement (Sigma-Aldrich), heparin sodium salt (Sigma-Aldrich) and antibiotics (GIBCO, Life Technologies). Cells were cultured at 37°C and 5% CO_2, and cultured media was changed every 3-4 days.

The investigation using HUVEC conforms to the principles outlined in the Declaration of Helsinki, was approved by the Ethical Committee of Clinical Research of the INCLIVA - "Hospital Clínico

Universitario" of Valencia and written informed consent was obtained from all umbilical cord donors.

Cells were identified as endothelial by their characteristic cobblestone morphology and the presence of vWF by immunofluorescence using a specific antibody (ab6994, Abcam, Cambridge, UK) and DyLight 488 conjugated anti-rabbit secondary antibody (ab96899, Abcam). Nuclear staining was achieved by using 4 ',6-diamino-2-fenilindol (DAPI) (BioLegend, San Diego, CA, USA). Pictures were obtained using an inverted fluorescence microscope (Eclipse Ti-S, Nikon Co, Japan) and NIS-Elements 3.2 software (Nikon Co, Japan). Cells used in this doctoral thesis were more than 95% vWF positive (Figure 4).

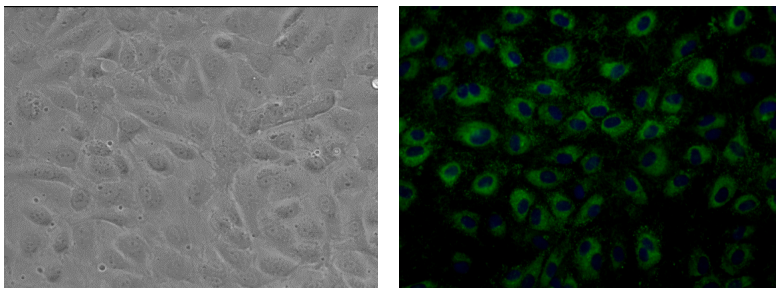


Figure 4. Representative image of HUVEC culture phenotypic characterization. Bright field picture shows cobblestone morphology cells forming a monolayer (left). Fluorescence microscopy picture for endothelial marker von Willebrand factor (vWF) expression and DAPI nuclei staining (right).

HUVEC from passages 3 to 4 were seeded onto gelatin-coated 6-well plates. When they reached about 80% confluence culture medium was exchanged for a phenol red-free Medium 199

(Sigma-Aldrich) supplemented with 20% charcoal/dextran-treated fetal bovine serum (GIBCO) ("hormone free medium") to avoid any steroid activity and maintained for 24 hours.

3.2 Exposure of HUVEC to treatments.

Cells were exposed for 24 hours to 1 nmol/l of 17β-estradiol (Sigma-Aldrich). Time and hormone concentration selected were chosen taking into account previous studies performed by our group (Sobrino et al., 2009) and are considered physiological concentrations.

In some experiments, cells were also exposed to ER antagonists 1 hour before estradiol treatment in order to study the role of hormone receptors. The unspecific antagonist of ERα and ERβ, ICI 182780 (1 µmol/l; Biogen, Madrid, Spain), the specific ERα antagonist methyl-piperidino-pyrazole (MPP) (1 µmol/l; Tocris Bioscience, Ellisville, MI, USA) and the specific GPER antagonist (3a*S**,4*R**,9b*R**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3*H*-cyclopenta[*c*]quinoline (G15) (1 µmol/l; Tocris Bioscience) were used. ER agonists were also used in order to identify estradiol specific actions. HUVEC were exposed to 1 nmol/l ER agonists for 24 hours. The selective ERα agonist 4,4',4''-(4-Propyl-[1H]- pyrazole-1,3,5-triyl)trisphenol (PPT), the selective ERβ agonist 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN) and selective GPER agonist (±)-1-[(3a*R**,4*S**,9b*S**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3*H*-cyclopenta[*c*]quinolin-8-yl]- ethanone (G1), all

obtained from Tocris Bioscience, were used in some experiments. Control cells were exposed to the same vehicles of cells treated with estradiol (0.1 % ethanol) or antagonist/agonist (0.1 % DMSO).

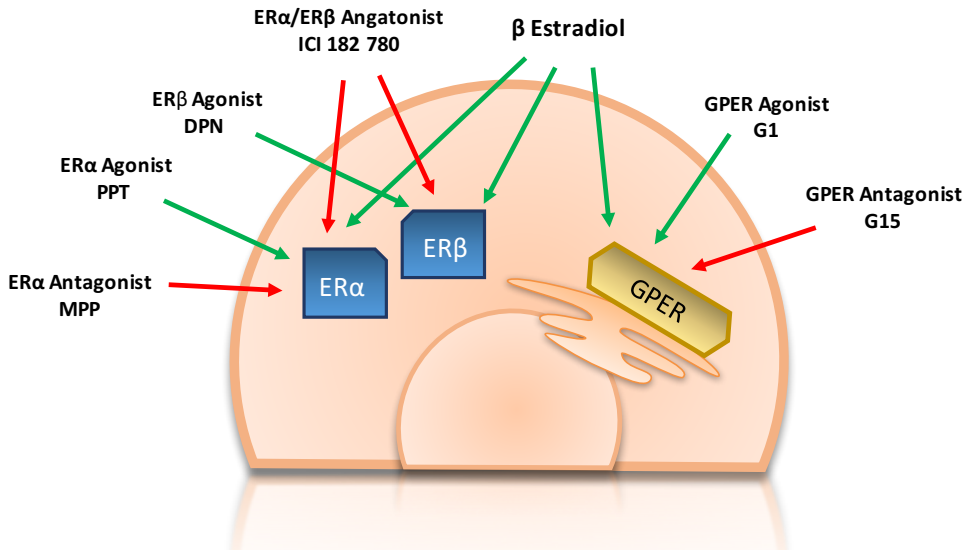


Figure 5: Schematic representation of treatments used in order to study the role of estrogen receptor. Estradiol and specific agonists of ERα, ERβ and GPER (PPT, DPN and G1) are depicted with green arrows. The unspecific ERα/ERβ antagonist ICI 182 780 and specific ERα and GPER antagonists (MPP and G15) are depicted with red arrows. Modified from (Meyer et al., 2011).

3.3 Microarray experiments.

3.3.1 Sample preparation and procedure.

Twelve different HUVEC cultures were used to perform microarray experiment. After treatments, three control (0.1 % ethanol) and three estradiol-treated cells (1 nmol/l) from three different cultures were pooled to RNA isolation, achieving four

biological replicates of both control and estradiol-treated cells. Total RNA including small non-coding RNA was isolated from cells using the QIAzol reagent and miRNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (see qRT-PCR method below for a more detailed description of the procedure).

The concentration and purity of the RNA obtained was measured as the OD260/280 ratio using a GeneQuant Pro spectrophotometer (General Electric (GE), Healthcare Life Sciences, Uppsala, Sweden). RNA integrity was determined by capillary electrophoresis using an RNA 6000 Nano Lab-on-a-Chip kit and a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA).

Small non-coding RNA expression profiling was performed using a GeneChip miRNA 4.0 Array (Affymetrix, Santa Clara, CA, USA). The array contains 30424 mature miRNA sequences from the miRBase (v20), including encoded miRNA from 203 organisms encompassing 1996 scaRNA and snoRNA (including CDBox RNA and H/ACA Box RNA), and 2025 probe sets unique to pre-miRNA hairpin sequences. Microarray experiments were conducted according to the manufacturer’s protocol. Briefly, 300 ng of total RNA was labelled with a FlashTag Biotin HSR RNA Labeling kit from Genisphere. The labelling reaction was hybridized onto the miRNA array in an Affymetrix Hybridization Oven 645 at 48 °C and 60 rpm for 18 hours. The arrays were stained using a Fluidics Station 450 with the fluidics script FS450_0002 (Affymetrix) and then scanned on a GeneChip Scanner 3,000 7G (Affymetrix), using the GeneChip Command Console Software supplied by Affymetrix to perform gene expression

analysis. MiRNA probe outliers were defined and further analysed according to the manufacturer's instructions (Affymetrix), and quality control, as well as data summarization and normalization, was carried out using the web-based miRNA QC Tool (www.affymetrix.com). Microarray experiment was performed in the multiple gene analysis core at Unidad Central de Investigación en Medicina-INCLIVA (Universitat de València, València, Spain).

3.3.2 Microarray result analysis.

3.3.2.1 miRNA profiling, Hierarchical Cluster and Principal Components Analysis.

miRNA array resulting data files (.cel) were used to analyse significant differences in miRNA expression profile between estradiol and control groups using Partek Genomic Suite (Partek Inc., St Louise, MO, USA). Paired Student's t-test analysis was performed and differences in individual miRNA expression ($p<0.05$) between estradiol and control groups were identified. Partek Genomic Suite was also used to identify global differences between samples by Principal Component Analysis (PCA) measurement. Distance between plotted samples is related to similarity between them. Hierarchical Cluster was determined to analyse expression profiles of different samples, in which branches represented in a dendrogram that are near each other had similar miRNA expression patterns.

3.3.2.2 Analysis of miRNA-predicted target interaction and gene regulation pathways.

miRNA significantly regulated by estradiol were analysed using Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA). This software uses computational algorithms to identify miRNA-targets as well as putative cellular networks related to predicted targets. IPA microRNA Target Filter tool enables prioritization of experimentally validated and predicted mRNA targets according to three different miRNA target prediction programs (TargetScan, miRecords and Ingenuity Knowledge Base) and the available database of experimentally supported miRNA targets TarBase.

3.3.3 Analysis of miRNA location and putative ER binding sites in the regulatory region of the miRNA precursors.

miRIAD software (https://www.bioinfo.mochsl.org.br/miriad) was used to know estradiol-induced miRNA location. miRIAD is a web search tool designed to obtain integrated information about intragenic microRNA and their host genes (Hinske et al., 2014). Moreover, *in silico* analysis of ER putative binding sites within the regulatory region of miRNA was performed by using available online bioinformatic tools. We used miRStart database (http://mirstart.mbc.nctu.edu.tw) (Chien et al., 2011) and Promoter 2.0 Prediction Server (http://www.cbs.dtu.dk/services/Promoter/) (Knudsen, 1999) to predict the transcription start site (TSS) of the specific miRNA. When using Promoter 2.0, sequences scoring above

1.0 (highly likely prediction), which contain about 95% of true promoter sequence, were chosen as the miRNA precursor TSS. Gene and up-stream sequences were obtained from the Ensembl browser (http://www.ensembl.org/index.html). ERα and ERβ can modulate gene expression by directly acting as transcription factors, whereas gene regulation of membrane-bound GPER is mediated through activation of down-stream transcription factors. Then, JASPAR 2016 server (http://jaspar.genereg.net/cgi-bin/jaspar_db.pl) was used to predict ERα and ERβ binding sites for binding motifs drawn from the JASPAR database (Mathelier et al., 2016).

3.3.4 Integrative analysis of miRNA-mRNA expression profile and canonical pathway determination.

Analysis of miRNA-mRNA interactions were determined based on database target predictions and mRNA expression data of HUVEC treated with 1 nmol/l estradiol for 24 hours. These mRNA microrarray data were previously published (Sobrino et al., 2009) and were deposited in NCBI's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo), accessible through GEO series accession number GSE16683. Differentially expressed miRNA and mRNA datasets were selected to perform integrative analysis using IPA software (Ingenuity Systems). Anti-correlated expression between miRNA and its predicted mRNA targets were determined. In addition, canonical pathway analysis of miRNA-mRNA pairings obtained were performed. The significance between the data and a specific canonical pathway is determined based on two parameters. p

value is calculated using Fischer's exact test to assess the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

3.3.5 Identification of estradiol-regulated genes with putative ETS-like transcription factor 4 (ELK4) binding sites.

In order to identify putative estradiol-responsive genes regulated through transcriptional activity of ELK4, gene list with predicted ELK4 binding sites in their regulatory region was obtained using JASPAR database via Harmonizome web server (http://amp.pharm.mssm.edu/Harmonizome). Harmonizome is a comprehensive resource of knowledge about genes and proteins of publicly available resources (Rouillard et al., 2016). 1441 target genes of the ELK4 transcription factor were predicted using known transcription factor binding site motif from the JASPAR Predicted Transcription Factor Targets database. This dataset was matched with estradiol-regulated genes that were obtained by mRNA microarray experiment. Then, targets found in both datasets were selected as putative ELK4-targeted estradiol-regulated genes.

3.4 Cell transfection

$1.5 \cdot 10^5$ cells were seeded onto gelatin-coated 6-well plates to perform transfection experiments. Transfection period and the dose of inhibitor was selected based on time and dose dependent experiments. Different transfection period (4, 6 and 12 hours) and

inhibitor concentration (60 and 80 nM) were assessed. After preliminary experiments, the rest of transfections were carried out for 4 hours in serum-free OptiMEM medium (GIBCO, Life Technologies) followed by 24 hours with complete medium.

HUVEC were transfected with 60 nmol/l miRNA inhibitor (miRIDIAN microRNA Human hsa-miR-30b-5p – Hairpin inhibitor) from Dharmacon (Thermo Fisher Scientific, Cultek SL, Madrid, Spain). Oligofectamine (Invitrogen, Life Technologies) was used as transfection reagent. Control samples were treated with equal concentration of a non-targeting negative control miRNA (miRIDIAN microRNA hairpin inhibitor negative control #1 from Dharmacon). qRT-PCR was used to assess miRNA expression after transfection procedure. miR-30b-5p levels were efficiently decreased after 24 hours and persisted 48 hours following inhibitory transfection. Therefore, ensuring low miR-30b-5p levels before treatments that it is also maintained throughout the exposure time. Moreover, specificity of miR-30b-5p inhibition was also tested by determining the expression of the well-known miR-92a which is highly expressed in endothelial cells. miR-30b-5p inhibition did not alter endogenous levels of endothelial miR-92a.

HUVEC were also transfected with 30 nmol/l miRNA mimic (miRIDIAN microRNA Human hsa-miR-30b-5p – Mimic, Dharmacon) using the same protocol described above. miR-30b-5p overexpression after mimic transfection could not be quantified due to lack of detection between primer used and mimic sequence. Because of this,

mRNA levels of miRNA targets in untreated cells were quantified to evaluate mimic transfection.

3.5 Gene expression analysis.

3.5.1 RNA isolation.

Total RNA including small non-coding RNA was isolated from cells using the QIAzol reagent and miRNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The chosen kit combines phenol/guanidine-based lysis of samples and silica-membrane-based purification of total RNA.

After desired treatments, medium was aspirate and cells were washed once with PBS. Then, 700 µl of QIAzol Lysis Reagent were added directly to the cell culture well in order to lyse the cells. The reagent also removes most of the cellular DNA and proteins and inhibits RNases. Then, lysate was passed through a 25-gauge (0.5 x 16 mm) needle attached to a sterile syringe at least 8-10 times to homogenize cells.

The homogenate is separated into 2 phases by centrifugation after addition of chloroform and aqueous phase is extracted. Ethanol is added and sample is applied to spin column where RNA binds to membrane. After addition of different buffers, contaminants are washed away and RNA is eluted in 80 µl of RNase-free water (Ambion, Life Technologies). RNA was stored at -80 °C until mRNA and miRNA expression level quantifications were performed.

The concentration and the purity of the RNA obtained were measured as the OD260/280 ratio using a spectrophotometer (NanoDrop 2000 UV-Vis Spectrophotometer, ThermoScientific, Rockford, IL, USA).

3.5.2 Reverse Transcription (RT).

The TaqMan miRNA Assay system (Applied Biosystems, Foster City, CA, USA) was used to miRNA levels determination. For miRNA RT reactions, RNA (100 ng) was reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). For each 15 µL reaction volume, 10 µL master mix that contains dNTPs, Buffer, RNase inhibitor and the MultiScribe™ Reverse Transcriptase, was combined with 0.5 µL of specific RT miRNA primers and 5 µL of total RNA.

RT was performed using the following parameter values to program the thermal cycler that allow primer annealing, followed by cDNA synthesis and a final inactivation step: 30 min at 16 °C, 30 min at 42 °C and 5 min at 85 °C.

In order to quantify mRNA expression, 300 ng of the purified RNA was reverse transcribed using random primers with the High-Capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. RT conditions comprised: 10 min at 25 °C, 120 min at 37 °C and 5 min at 95 °C. cDNA obtained was immediately amplified or stored at -20 °C before use.

3.5.3 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR).

Individual miRNA expression was determined by qRT-PCR analysis using an ABI Prism 7900 HT Fast Real-Time PCR System (Applied Biosystems). Gene-specific primer pairs and probes from Applied Biosystems (Table 4) were used together with TaqMan Universal PCR Master Mix (Applied Biosystems) and reverse-transcribed sample RNA in 20 µl reaction volumes. PCR conditions were 10 min at 95 °C for enzyme activation, followed by 40 cycles (15 s at 95 °C; 1 min at 60 °C). The expression levels of RNU48 was measured in all samples to normalize differences in RNA input, RNA quality, and reverse transcription efficiency. Each sample was analysed in duplicate, and the expression was calculated according to the $2^{-\Delta\Delta Ct}$ method.

miRNA	Assay ID
RNU48	001006
miR-126	000451
miR-146a	000468
miR-155	002287
miR-221	002096
miR-222	002097
miR-92a	000431

miRNA	Assay ID
miR-30b-5p	000602
miR-487a-5p	467005_mat
miR-4710	463602_mat
miR-501-3p	002435
miR-378h	464554_mat
miR-1244	002791

Table 4. Gene-specific assays used in individual miRNA expression determination by qRT-PCR.

iTaq TM Universal SYBR Green supermix (Bio-Rad Laboratories Inc., Madrid, Spain) was also used in order to determine mRNA expression. Primers used in SYBR Green-based qRT-PCR were all purchased from Sigma-Aldrich. Primers sequences are described in Table 2. PCR conditions were determined according with manufacturer's instructions. The expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured as endogenous control. Each sample was analysed in triplicate, and the expression was calculated according to the $2^{-\Delta\Delta Ct}$ method.

Symbol	Official full name	Primer sequences (5' - 3')
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	F: ACAGTTGCCATGTAGACC R: TTTTTGGTTGAGCACAGG
MYSM1	Myb-like, SWIRM and MPN domains 1	F: AAATAAGGTCAAATGCGGTC R: ACCTCATCTGTGATGTCTAC
PPP3CA	protein phosphatase 3, catalytic subunit, alpha isozyme	F: ACCTATGTGTGATATCCTGTG R: ACTGTAGAAGTATGAACACCC
ELK4	E twenty-six-like transcription factor 4	F: CTCTCCAGTATCCACTTCTG R: AACTGGAAAAGTGTGTTAGC
MDM4	mouse double minute 4	F: CCTAGAAGTAATGGCTCAAC R: CACTCATCCTCAGAGGTAAC
DLG5	discs, large homolog 5	F: CAAGAGTTGAGCTTTAAGAAGG R: TCACATATTTGCTGGGAATC
EDN1	endothelin 1	F: CAAGCAGGAAAAGAACTCAG R: CTGGTTTGTCTTAGGTGTTC
EGR1	early growth response 1	F: GACGAGTCTTTTCCTGAC R: TTGGTCATGCTCACTAGG

Table 5. Primer sequences used in mRNA expression determination by qRT-PCR (F = forward; R = reverse).

3.6 Protein expression analysis.

3.6.1 Cell lysis and protein quantification.

HUVEC were collected in RIPA buffer (Sigma-Aldrich) containing protease and phosphatase inhibitors (Roche Diagnostics, Madrid, Spain). Protein content was measured by a colorimetric method (Pierce BCA protein assay kit, ThermoScientific Inc.). In this method, bovine serum albumin (BSA) was used as a standard.

3.6.2 Western Blot.

Proteins extracts were denatured using specific buffer (Tris 40mmol/l, EDTA, bromophenol blue 0.01 %, sucrose 40 %, SDS 4 %, β-mercaptoethanol 10 %) and heated to 95°C for 5 minutes.

20 µg of protein were then separated by 12 % SDS-Polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Whatman, GE Healthcare Life Sciences). Then, the membrane was blocked with BSA 3 % in Tris-buffered saline with Tween 20 (TBS-T) for 1 hour and immunostaining was achieved using specific primary antibodies: anti-ELK4 (sc-13030) and anti-ET1 (sc-21625-R) both from Santa Cruz Biotech (Heidelberg, Germany). Actin antibody (A5441, Sigma-Aldrich) was used as endogenous control of the amount of protein. Antibody incubation was performed overnight at 4°C with agitation.

After three washes with TBS-T for 10 min, membranes were further incubated for 1 hour with a secondary mouse antibody against β-actin or rabbit antibody against ELK4 and ET-1, both conjugated with horseradish peroxidase-linked. Secondary antibody incubation was at room temperature with constant agitation.

Finally, the membrane was washed and Luminol chemiluminescent reagent was added onto the membrane (SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific Inc.), and then revealed by an image reader LAS-4000 (GE Healthcare Life Sciences). Protein levels were determined by densitometry of the bands and normalized to β-actin.

3.7 Statistics

Data shown in text, figures and tables are expressed as mean ± Standard Error of Mean (SEM). Student's t-test was applied for single comparisons. A one-way analysis of variance (ANOVA) was used to determine the difference between groups. When an interaction effect was found, multiple comparisons using Bonferroni's test was performed. Results were considered as significantly different using a p value ≤ 0.05. The significance had been indicated in each case as $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$. Statistical analysis and graphic representations were performed using Prism version 6.0c software (GraphPad Software Inc., San Diego, CA, USA).

4. RESULTS

4.1 Analysis of miRNA expression profile obtained by microarray.

4.1.1 Global analysis of miRNA expression data by hierarchical clusters and principal components.

HUVEC were treated with 1 nmol/l estradiol for 24 hours and RNA was isolated and miRNA determination was performed using GeneChip miRNA 4.0 Array as described in Methods section. miRNA expression profiles of HUVEC treated with or without estradiol were analysed using Partek Genomic Suite v6.4 software. Then, changes in the expression profile were analysed by PCA and Hierarchical clustering. Although samples are dispersed with each other, representation of global differences between samples using PCA showed treated and non-treated pools enclosed in two different sets, underlying patterns between their expression profiles (Figure 6).

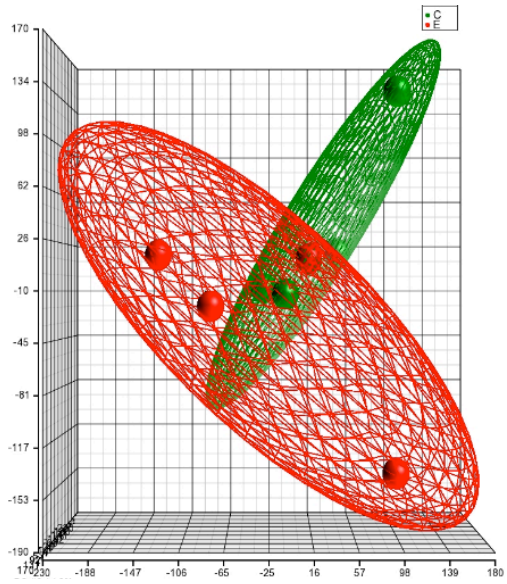


Figure 6. Principal component analysis (PCA) of estradiol-treated HUVEC. HUVEC were treated with vehicle (0.1 % ethanol) or estradiol (1 nmol/l) for 24 hours. Total RNA of 4 Control (C) sample pools (green) and 4 estradiol (E) sample pools (red) were used to perform microarray experiment. Three-dimensional plot of miRNA expression profiles obtained is shown.

Furthermore, analysis by Hierarchical clustering showed large similarities among samples of endothelial cells treated with estradiol and cells treated with vehicle. However, 298 probe sets were differentially expressed between both groups using $p < 0.05$ (Figure 7). Specifically, 114 mature miRNA, 100 stem-loop miRNA, 83 snoRNA (including 13 CDBox and 10 HAcaBOX) and 1 scaRNA were found among them. Figure 8 shows distribution of differentially expressed non-coding RNA observed in estradiol-exposed HUVEC.

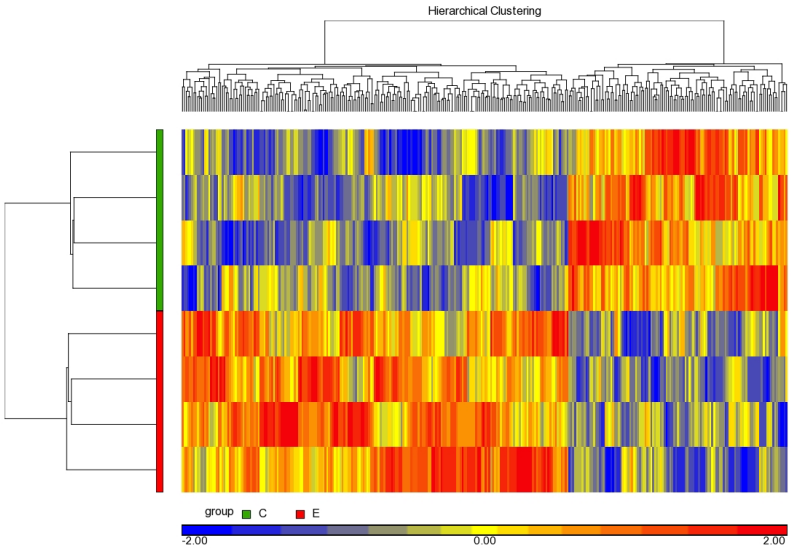


Figure 7. Hierarchical cluster of estradiol-treated HUVEC. HUVEC were treated with vehicle (0.1 % ethanol) or estradiol (1 nmol/l) for 24 hours. Total RNA of 4 Control (C) sample pools and 4 estradiol (E) sample pools were used. Differentially expressed (p value < 0.05) probe sets were used for hierarchical cluster. Each column represents an individual probe set and each row represents a pool of cells. Up-regulated (red) and down-regulated (blue) probe sets are shown.

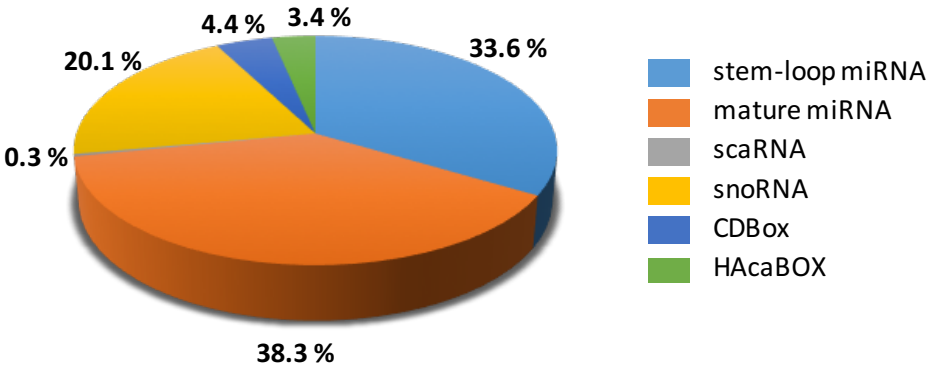


Figure 8. Distribution of differentially expressed non-coding RNA obtained using microarray of estradiol-exposed HUVEC. HUVEC were treated with vehicle (0.1 % ethanol) or estradiol (1 nmol/l) for 24 hours and non-coding RNA were determined using microarray technology. 298 probe sets were differentially expressed (p < 0.05) between both groups. Specifically, 114 mature miRNA, 100 stem-loop miRNA, 1 scaRNA and 83 snoRNA (including 13 CDBox and 10 HAcaBOX) were found among them. Percentage of differentially expressed non-coding RNA are shown.

Mature miRNA was the most abundant non-coding RNA (38 %), in which 114 miRNA were significantly regulated in HUVEC treated with estradiol compared to vehicle-treated cells (Table 6). As used microarray chips contain 2578 human mature miRNA probes, only 4.42 % of the miRNA were regulated by estradiol in endothelial cells.

Table 6. Estradiol-regulated miRNA in HUVEC. Differentially expressed ($p < 0.05$) miRNA are listed based on their p value.

miRNA name	Accession number	p value	Fold change
hsa-miR-6754-3p	MIMAT0027409	0.0013	1.57
hsa-miR-6839-3p	MIMAT0027581	0.0023	-1.14
hsa-miR-501-3p	MIMAT0004774	0.0025	1.77
hsa-miR-548al	MIMAT0019024	0.0037	-1.20
hsa-miR-219a-5p	MIMAT0000276	0.0038	1.16
hsa-miR-6721-5p	MIMAT0025852	0.0041	1.34
hsa-miR-548c-3p	MIMAT0003285	0.0047	1.13
hsa-miR-3170	MIMAT0015045	0.0055	-1.15
hsa-miR-577	MIMAT0003242	0.0057	-1.18
hsa-miR-526b-3p	MIMAT0002836	0.0059	-1.15
hsa-miR-1244	MIMAT0005896	0.0059	-1.76
hsa-miR-3975	MIMAT0019360	0.0061	1.24
hsa-miR-487a-5p	MIMAT0026559	0.0062	1.94
hsa-miR-6866-3p	MIMAT0027633	0.0073	-1.24
hsa-miR-623	MIMAT0003292	0.0077	-1.16
hsa-miR-6871-3p	MIMAT0027643	0.008	-1.14
hsa-miR-26a-5p	MIMAT0000082	0.0085	1.20

hsa-miR-6848-5p	MIMAT0027596	0.0093	1.21
hsa-miR-6750-3p	MIMAT0027401	0.0098	1.35
hsa-miR-6884-3p	MIMAT0027669	0.0108	1.47
hsa-miR-4797-3p	MIMAT0019973	0.0109	1.09
hsa-miR-520g-3p	MIMAT0002858	0.0109	1.29
hsa-miR-1224-3p	MIMAT0005459	0.0113	-1.21
hsa-miR-1973	MIMAT0009448	0.0123	-1.31
hsa-miR-4491	MIMAT0019026	0.013	1.22
hsa-miR-3120-5p	MIMAT0019198	0.0142	1.14
hsa-miR-1303	MIMAT0005891	0.0144	1.18
hsa-miR-3689f	MIMAT0019010	0.0145	1.30
hsa-miR-6782-5p	MIMAT0027464	0.0149	1.35
hsa-miR-937-3p	MIMAT0004980	0.0152	-1.13
hsa-miR-7-2-3p	MIMAT0004554	0.0163	-1.15
hsa-miR-4679	MIMAT0019763	0.0165	1.16
hsa-miR-922	MIMAT0004972	0.0165	1.18
hsa-miR-378h	MIMAT0018984	0.0169	-1.72
hsa-miR-4429	MIMAT0018944	0.0174	-1.40
hsa-miR-5008-3p	MIMAT0021040	0.0174	1.23
hsa-miR-7158-5p	MIMAT0028226	0.0196	1.44
hsa-miR-4462	MIMAT0018986	0.0197	1.42
hsa-miR-1296-5p	MIMAT0005794	0.0199	1.39
hsa-miR-4739	MIMAT0019868	0.0200	-1.15
hsa-miR-1253	MIMAT0005904	0.0204	1.20
hsa-miR-2052	MIMAT0009977	0.0214	1.10
hsa-miR-3680-3p	MIMAT0018107	0.0216	-1.24
hsa-miR-25-5p	MIMAT0004498	0.0219	1.69
hsa-miR-5684	MIMAT0022473	0.0222	-1.12
hsa-miR-4291	MIMAT0016922	0.0225	1.12
hsa-miR-4778-5p	MIMAT0019936	0.0228	-1.14

hsa-miR-4524b-5p	MIMAT0022255	0.0234	1.15
hsa-miR-3684	MIMAT0018112	0.0236	1.19
hsa-miR-34c-3p	MIMAT0004677	0.0238	1.24
hsa-miR-1263	MIMAT0005915	0.0240	-1.29
hsa-miR-4320	MIMAT0016871	0.0241	-1.33
hsa-miR-302b-5p	MIMAT0000714	0.0246	-1.21
hsa-miR-4457	MIMAT0018979	0.0249	-1.10
hsa-miR-3160-5p	MIMAT0019212	0.0255	-1.26
hsa-miR-6726-3p	MIMAT0027354	0.0262	1.38
hsa-miR-3914	MIMAT0018188	0.0265	1.30
hsa-miR-4473	MIMAT0019000	0.0266	-1.23
hsa-miR-522-3p	MIMAT0002868	0.0267	1.20
hsa-miR-1205	MIMAT0005869	0.0270	1.14
hsa-miR-5588-3p	MIMAT0022296	0.0271	-1.33
hsa-miR-124-5p	MIMAT0004591	0.0273	-1.13
hsa-miR-4264	MIMAT0016899	0.0277	1.21
hsa-miR-8089	MIMAT0031016	0.0277	1.47
hsa-miR-4460	MIMAT0018982	0.0284	1.25
hsa-miR-448	MIMAT0001532	0.0294	1.28
hsa-miR-4632-5p	MIMAT0022977	0.0296	1.34
hsa-miR-548ae	MIMAT0018954	0.0298	1.43
hsa-miR-4685-5p	MIMAT0019771	0.0298	-1.28
hsa-miR-3682-3p	MIMAT0018110	0.0312	1.27
hsa-miR-6886-5p	MIMAT0027672	0.0315	1.18
hsa-miR-130a-5p	MIMAT0004593	0.0318	1.18
hsa-miR-1287-5p	MIMAT0005878	0.0324	1.15
hsa-miR-6888-3p	MIMAT0027677	0.0326	-1.18
hsa-miR-3936	MIMAT0018351	0.0327	-1.19
hsa-miR-374a-3p	MIMAT0004688	0.0345	-1.18
hsa-miR-4298	MIMAT0016852	0.036	-1.57

hsa-miR-5007-3p	MIMAT0021036	0.0362	1.14
hsa-miR-512-3p	MIMAT0002823	0.0364	1.11
hsa-miR-5739	MIMAT0023116	0.0365	1.18
hsa-miR-3124-5p	MIMAT0014986	0.0365	1.58
hsa-miR-4710	MIMAT0019815	0.0379	1.88
hsa-miR-1294	MIMAT0005884	0.0382	1.13
hsa-miR-6734-5p	MIMAT0027369	0.0385	1.53
hsa-miR-3622b-3p	MIMAT0018006	0.0396	1.23
hsa-miR-4680-3p	MIMAT0019765	0.0399	-1.09
hsa-miR-553	MIMAT0003216	0.0405	-1.2
hsa-miR-876-5p	MIMAT0004924	0.0406	1.2
hsa-miR-6800-3p	MIMAT0027501	0.0415	1.67
hsa-miR-6747-3p	MIMAT0027395	0.0418	-1.21
hsa-miR-3125	MIMAT0014988	0.0421	-1.17
hsa-miR-6512-5p	MIMAT0025480	0.0422	1.11
hsa-miR-92a-3p	MIMAT0000092	0.0425	1.08
hsa-miR-6841-5p	MIMAT0027584	0.0431	-1.17
hsa-miR-508-3p	MIMAT0002880	0.0435	-1.09
hsa-miR-1302	MIMAT0005890	0.0439	-1.29
hsa-miR-506-5p	MIMAT0022701	0.0442	-1.33
hsa-miR-30b-5p	MIMAT0000420	0.0445	2.02
hsa-miR-885-5p	MIMAT0004947	0.0446	1.42
hsa-miR-4769-3p	MIMAT0019923	0.0446	1.41
hsa-miR-1323	MIMAT0005795	0.0446	1.24
hsa-miR-1193	MIMAT0015049	0.0457	1.17
hsa-miR-5580-3p	MIMAT0022274	0.0459	-1.23
hsa-miR-205-3p	MIMAT0009197	0.0459	1.06
hsa-miR-7849-3p	MIMAT0030424	0.046	1.11
hsa-miR-383-3p	MIMAT0026485	0.0463	-1.17
hsa-miR-126-3p	MIMAT0000445	0.0468	1.16

hsa-miR-7150	MIMAT0028211	0.047	1.32
hsa-miR-3620-3p	MIMAT0018001	0.047	1.34
hsa-miR-634	MIMAT0003304	0.0471	-1.33
hsa-miR-6808-5p	MIMAT0027516	0.0477	1.65
hsa-miR-4759	MIMAT0019905	0.0485	-1.22
hsa-miR-6729-5p	MIMAT0027359	0.0486	1.16
hsa-miR-6746-3p	MIMAT0027393	0.0493	1.48

4.1.2 Expression of key molecules in miRNA biosynthesis pathway.

In order to further evaluate the implication of estradiol in miRNA profile changes observed, we explore the expression of key molecules required for miRNA biosynthesis (Figure 3). Previous mRNA microarray data (Sobrino et al., 2009) was used to know changes in DROSHA, DGCR8, XPO5, DICER1 and AGO2 mRNA expression. As shown in Table 7, mRNA expression of DGCR8 significantly increased when cells were exposed to 1 nmol/l estradiol for 24 hours, with a fold change of 2.38. However, the other component of Microprocessor complex, DROSHA, did not change after estradiol exposition. The expression of exportin 5 (XPO5) had an increased fold change of 1.51 in estradiol-treated HUVEC compared to non-treated cells. However, it was not a significantly change. Finally, the mRNA expression of DICER1 and AGO2 were significantly down-regulated in cells exposed to estradiol, with a fold change of -1.98% (DICER1) and -1.29% (AGO2) compared to control values. DICER1 and AGO2 are two genes encoding proteins related to maturation of pre-miRNA and assembly with target mRNA,

respectively. Altogether, these results revealed an altered expression of pivotal components in the machinery of miRNA processing in estradiol-treated HUVEC.

Probe set ID	Symbol	Official Full Name	Fold change	p value
218269_at	DROSHA	drosha, ribonuclease type III	-1.117	0.586
64474_g_at	DGCR8	DiGeorge syndrome critical region gene 8	2.376	0.016
223056_s_at	XPO5	exportin 5	1.514	0.259
213229_at	DICER1	dicer 1, ribonuclease type III	-1.979	0.012
225569_at	AGO-2	Argonaute-2	-1.290	0.002

Table 7. Microarray expression data of key molecules of the miRNA biosynthesis pathway. mRNA expression data were obtained from previously published (Sobrino et al., 2009) mRNA microrarray data of HUVEC treated with 1 nmol/l estradiol for 24 hours. Probe set ID, gene symbol, official full name, p value and fold change are shown.

4.1.3 *In silico* analysis of gene regulation by estradiol-modified miRNA.

IPA microRNA Target Filter tool was used in order to know the biological networks related to observed changes in miRNA expression profile of estradiol-treated HUVEC. The software identified 112 of significantly expressed miRNA that had either predicted or validated targets (from a total of 21748 targets). In order to adopt a restrictive approach, we further filtered our miRNA targets using only experimentally observed or highly predicted target correlations, discarding the category of moderately predicted targets. After this filter, we had 112 miRNA targeting 5126 mRNA. Using the

selected miRNA-target pairing as an input, IPA analysis showed significant regulatory networks (Table 8 shows top ten networks modulated by estradiol-dependent miRNA based on their p-value). Results revealed that estradiol-dependent miRNA regulated genes are associated with biological processes that include mainly: cell death and survival, cellular assembly and organization, molecular transport, cell cycle, as well as cellular morphology and movement. Lipid and carbohydrate metabolism have also been included among the most regulated networks. Moreover, reproductive, auditory-vestibular and cardiovascular system development and function appeared as regulated by estradiol-dependent miRNA. Finally, analysis of the main networks showed that differentially expressed miRNA in HUVEC exposed to estradiol are associated to different diseases.

	Top Diseases and Functions	Score
1	Cell Death and Survival, Infectious Diseases, Organismal Development	27
2	Lipid Metabolism, Small Molecule Biochemistry, Cellular Assembly and Organization	21
3	Reproductive System Development and Function, Organ Development, Developmental Disorder	21
4	Molecular Transport, Organismal Development, Behaviour	16
5	Cell Cycle, Cellular Compromise, Cellular Function and Maintenance	16
6	Cell Morphology, Gastrointestinal Disease, Organismal Injury and Abnormalities	15
7	Cardiovascular Disease, Cancer, Dermatological Diseases and Conditions	15
8	Auditory and Vestibular System Development and Function, Carbohydrate Metabolism, Cardiovascular Disease	15
9	Cardiovascular Disease, Cardiovascular System Development and Function, Organ Morphology	13
10	Cell Cycle, Cellular Movement, Cancer	13

Table 8: Regulatory networks modulated by estradiol-dependent miRNA. miRNA significantly regulated by estradiol ($p < 0.05$) were obtained by microarray technology and analysed using Ingenuity Pathways Analysis software. IPA microRNA Target Filter tool and confidence filter were used, selecting only experimentally observed or highly predicted target correlations. Top 10 networks are listed based on their assigned score.

In order to further explore the impact of estradiol-regulated miRNA in the context of our study, predicted target genes assigned to cardiovascular pathways by IPA software were selected and functional characterization were performed. Results showed that miRNA regulated by estradiol in HUVEC were associated to different signalling pathways. Top 10 cardiovascular canonical pathways based

on their p-value are listed in Table 9. Moreover, estradiol-regulated miRNA were related to endothelin-1 signalling, nitric oxide and reactive oxygen species production, important vasoactive compounds in the regulation of endothelial homeostasis. Finally, cardiac hypertrophy signalling as well as role of NFAT in cardiac hypertrophy signalling and the role of macrophages, fibroblasts and endothelial cells in rheumatoid arthritis also appeared as the main regulated pathways.

	Canonical Pathways	-log (p value)
1	Cardiac Hypertrophy Signalling	131.0
2	Role of NFAT in Cardiac Hypertrophy	116.0
3	Thrombin Signalling	106.0
4	CXCR4 Signalling	86.0
5	Endothelin-1 Signalling	85.2
6	Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	83.9
7	Phospholipase C Signalling	83.6
8	Protein Kinase A Signalling	81.6
9	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	79.1
10	Signalling by Rho Family GTPases	75.9

Table 9: Top 10 cardiovascular related Canonical Pathways regulated by estradiol-dependent miRNA. HUVEC were treated with vehicle (0.1 % ethanol) or estradiol (1 nmol/l) for 24 hours. miRNA significantly regulated by estradiol were obtained by microarray technology and analysed using Ingenuity Pathways Analysis software. Only predicted target genes assigned to a specific cardiovascular pathways were selected and functional characterization were performed. Top 10 canonical pathways are listed based on their p-value.

4.1.4 miRNA expression profiling and microarray verification.

As describe above, differences between miRNA profiling of human endothelial cells exposed during 24 hours with or without 1 nmol/l estradiol revealed a total of 114 miRNA significantly regulated, including 70 up-regulated and 44 down-regulated miRNA. Fold changes of estradiol-regulated miRNA range from -1.76 to 2.02. Figure 3 list most differentially expressed miRNA with a fold-change greater than ± 1.5. 13 miRNA were differentially regulated by estradiol with this selected fold-change, including 10 up-regulated and 3 down-regulated miRNA (Figure 9).

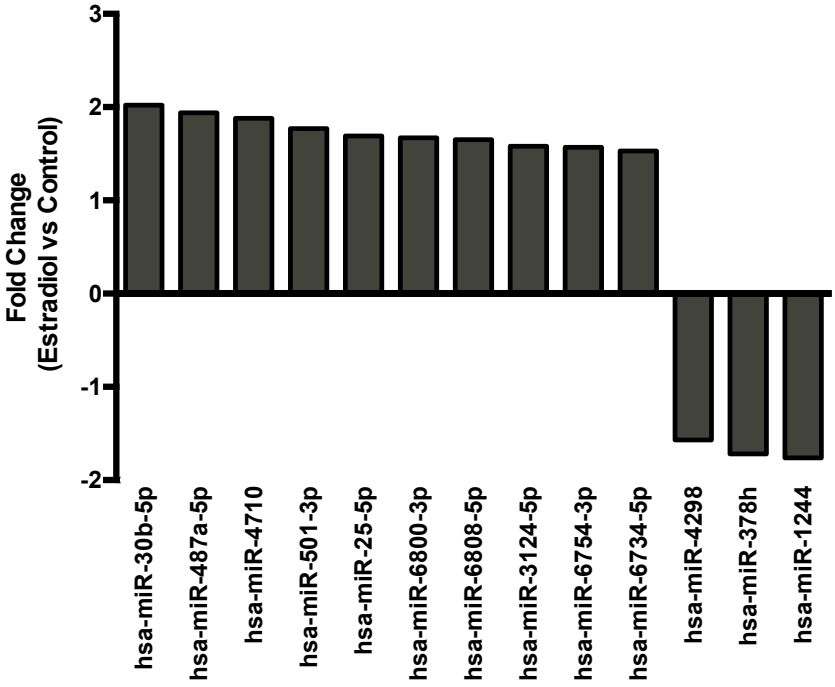


Figure 9. miRNA expression profiling in estradiol-treated HUVEC. HUVEC were exposed to estradiol (1 nmol/l) for 24 hours and total RNA was isolated. Changes in miRNA expression profile were obtained by microarray technology. miRNA with fold change greater than 1.5 were represented. Results were shown as fold change of 4 independent experiments.

In order to validate the results obtained by microarray analysis, qRT-PCR was performed using independently obtained RNA samples. The most differentially expressed miRNA obtained in microarrays (miR-30b, miR-487a, miR-4710, miR-501, miR-378h and miR-1244) as well as others miRNA based on their relevance in vascular function (miR-126, miR-146a, miR-155, miR-221 and miR-222) were chosen and analysed by qRT-PCR. Obtained results confirmed microarray data of miRNA chosen for validation, including estradiol-dependent up-regulation of 4 miRNA (miR-30b, miR-487a, miR-4710 and miR-501) (Figure 10A) and the down-regulation of 2 miRNA (miR-378h and miR-1244) (Figure 10B). Obtained results were similar to those observed in the microarray experiment, validating our miRNA expression profile data. In addition, all miRNA selected based on their importance in vascular functions remained unaltered (Figure 10C).

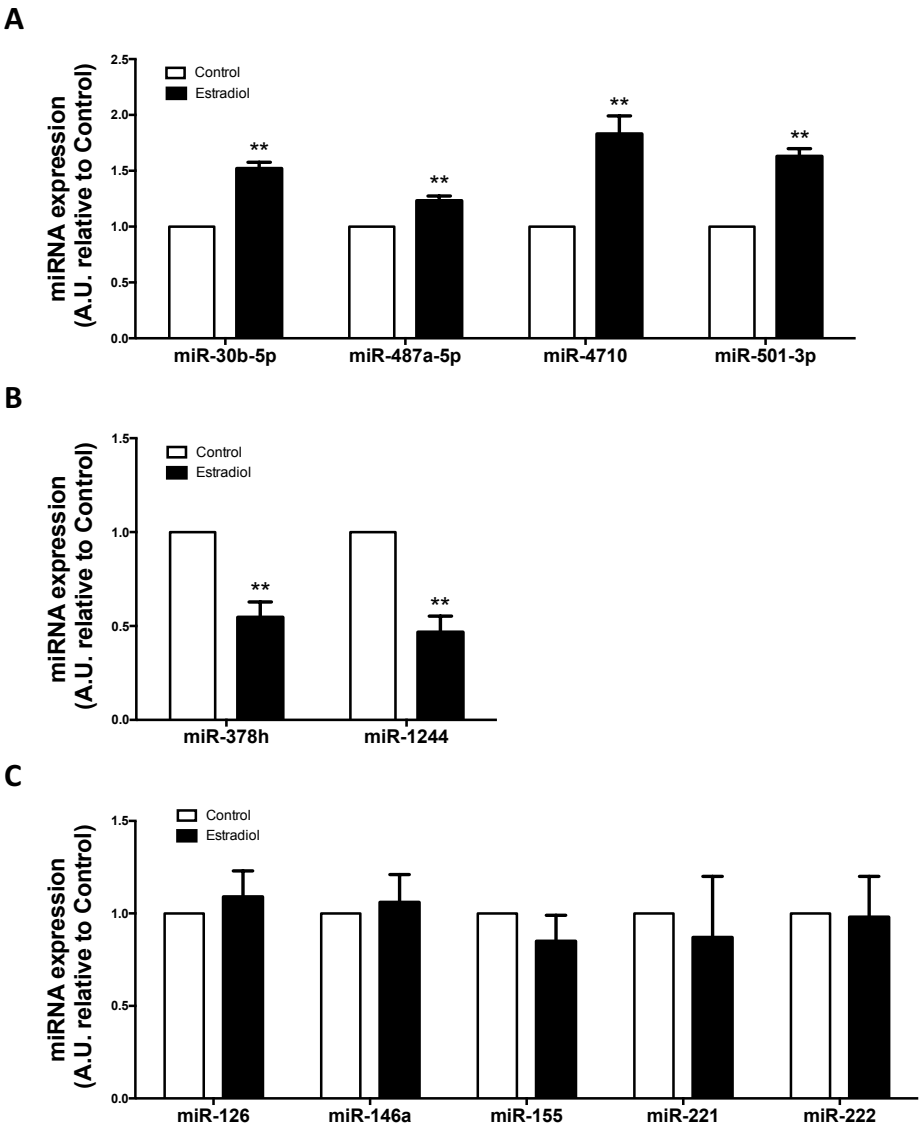


Figure 10. Validation of microarray data of estradiol-regulated miRNA in HUVEC. HUVEC were exposed to estradiol (1 nmol/l) for 24 hours and total RNA was isolated. Most differentially expressed miRNA obtained from microarray experiment and others miRNA based on their relevance in vascular function were selected and their expression was determined by qRT-PCR as described in Material and Methods. (A) Up-regulated miRNA in estradiol-treated cells (miR-30b, miR-487a, miR-4710 and miR-501). (B) Down-regulated miRNA (miR-387h and miR-1244). (C) Vascular-related miRNA (miR-126, miR-146a, miR-155, miR-221 and miR-222). Results were shown as mean ± SEM of n = 8-12 from 3 to 5 independent experiments. **p<0.01, refers to the comparison between estradiol- and vehicle-treated cells (Control).

4.1.5 Genomic location of validated estradiol-regulated miRNA.

As described before in Introduction section, miRNA sequences can be located in intronic regions of non-coding or coding transcripts, but it can be also encoded by exonic regions. We search for genomic location of the precursors of estradiol-dependent miRNA using miRIAD web service. Table 10 shows chromosomic location and information about host genes of intragenic miRNA. It is worthy to note that mature hsa-miR-1244 have 4 precursor sequences located in 3 different chromosomes.

Symbol	Mirbase accession	Location		
		Chromosome	Intragenic	Host gene
hsa-mir-30b	MI0000441	8	NO	-
hsa-mir-487a	MI0002471	14	NO	-
hsa-mir-4710	MI0017344	14	NO	-
hsa-mir-501	MI0003185	X	INTRONIC	CLCN5
hsa-mir-378h	MI0016808	5	INTRONIC	FAXDC2
hsa-mir-1244-1	MI0006379	2	EXONIC	PTMA
hsa-mir-1244-2	MI0015974	5	INTRONIC	DTWD2
hsa-mir-1244-3	MI0015975	12	NO	-
hsa-mir-1244-4	MI0031511	12	NO	-

Table 10. Location of validated miRNA regulated by estradiol in endothelial cells. The available web search tool miRIAD software was used to know genomic location of the miRNA precursors of validated estradiol-induced miRNA.

Among miRNA regulated by estradiol there are 5 miRNA precursor sequences that are located between gene sequences and 4 miRNA precursor sequences that are intragenic: hsa-miR-501, hsa-miR-378h and hsa-miR-1244-2 are intronic miRNA whereas hsa-miR-1244-1 is an exonic miRNA. Host gene of intronic miRNA are also shown in Table 5: hsa-miR-501 is located in CLCN5, hsa-miR-378h in FAXDC2, hsa-miR-1244-1 in PTMA and hsa-miR-1244-2 in DTWD2. In order to know the relationship between miRNA and host gene transcription, expression levels of these 4 host genes were obtained using previous mRNA microarray data and are shown in Table 11. The expression data of indicated host genes did not show significant differences in estradiol-exposed HUVEC, suggesting no relationship between the expression of a specific miRNA and the expression of its host gene.

Symbol	Location	microarray mRNA data		
	Host gene	Probe set ID	Fold change	p value
hsa-mir-501	CLCN5	226273_at	-1.40	0.086
hsa-mir-378h	FAXDC2	48031_r_at	-1.21	0.381
hsa-mir-1244-1	PTMA	211921_x_at	1.09	0.748
hsa-mir-1244-2	DTWD2	231277_x_at	-1.15	0.214

Table 11. Microarray expression data of estradiol-regulated miRNA host genes. mRNA expression data were obtained from previously published (Sobrino et al., 2009) mRNA microarray data of HUVEC treated with 1 nmol/l estradiol for 24 hours. In addition to miRNA and host gene symbol, Probeset ID, p-value and fold change of the specific host genes are shown.

4.2 Role of estrogen receptors in estradiol-modified miRNA.

ERα and ERβ are classic nuclear receptor that regulate gene expression by directly acting as transcription factors, whereas GPER-dependent gene regulation is mediated through activation of down-stream transcription factors. Thus, in order to study the role of different ER in the expression of estradiol-dependent miRNA, both computational and pharmacological approaches have been used. Firstly, we search for putative ER binding sites in the regulatory region of miRNA precursors using *in silico* tools. In addition, endothelial cells were exposed to different ER antagonists and agonists.

4.2.1 *In silico* analysis of putative ER binding sites in the regulatory region of estradiol-regulated miRNA precursors.

Available online bioinformatic tools were used to search for ER putative binding sites within the regulatory region of miRNA. We used miRStart database to predict the TSS of the estradiol-regulated miRNA. TSS of hsa-miR-4710, hsa-miR-378h and hsa-miR-1244-4, which were not found in miRStart database, were predicted using Promoter 2.0 Prediction Server. TSS of validated estradiol-regulated miRNA were found located in average at 8000 pb distance approximately of their precursor sequences. Ranging from 109 pb distance from the precursor sequence of hsa-miR-1244-2 up to 31696 pb distance from the hsa-miR-30b precursor (Table 12).

Regulatory region was established as 10 kb up-stream sequence from TSS of estradiol-regulated miRNA. Then, JASPAR 2016 server was used to predict ERα and ERβ binding sites for binding motifs drawn from the JASPAR database (Figure 11). ER binding sites were found located in the regulatory region of all selected estradiol-regulated miRNA. Number of ER binding sites found in promoter region is determined in Table 12. Results showed a greater number of binding sites for ERβ than for ERα in the promoter region of selected miRNA precursors. The sequence logo is a graphical representation that depicts the sizes of a nucleotide depending on their frequency, reaching a maximum of 2 bits when a nucleotide is 100 % conserved in a specific position. Therefore, the large number of ERβ binding sites found could be related to a greater variation observed in the sequence logo for the ERβ motif.

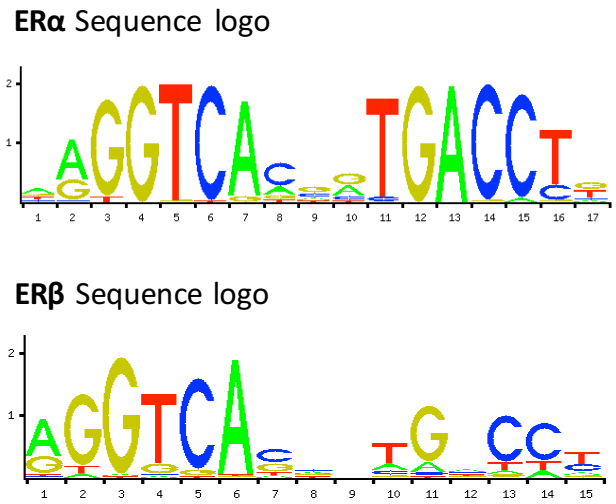


Figure 11. Sequence logo of ERα and ERβ binding motifs drawn from the JASPAR database. The sequence logo is a graphical representation of the conservation of a specific nucleotide sequence that depicts the sizes of a nucleotide depending on their frequency, reaching a maximum of 2 bits when a nucleotide is 100 % conserved in a specific position.

Symbol	TSS (bp to precursor)	Binding sites	
		ERα	ERβ
hsa-mir-30b	31696	1	16
hsa-mir-487a	14000	2	41
hsa-mir-4710	20600	4	54
hsa-mir-501	5189	1	25
hsa-mir-378h	1900	2	34
hsa-mir-1244-1	5129	2	46
hsa-mir-1244-2	109	2	30
hsa-mir-1244-3	110	3	25
hsa-mir-1244-4	1000	3	28

Table 12. Putative ER binding sites in the regulatory region of the precursors of estradiol-regulated miRNA. miRStart database and Promoter 2.0 Prediction Server were used to predict the transcription start site (TSS) of the specific miRNA precursor and the up-stream sequences were obtained from the Ensembl browser. JASPAR 2016 server was used to predict ERα and ERβ binding sites for binding motifs drawn from the JASPAR database.

4.2.2 Role of ER antagonists in estradiol-regulated miRNA expression.

In order to evaluate whether the changes in miRNA observed after exposition to 1 nmol/l estradiol are mediated through ER, HUVEC were exposed to the non-selective antagonist of ERα and ERβ ICI 182780 (1 µmol/l), the specific ERα antagonist MPP (1 µmol/l) and the specific GPER antagonist G15 (1 µmol/l) (Figure 12). miR-30b-5p, miR-378h and miR-487a-5p expression changes observed after estradiol treatment were abolished by ICI and MPP but not by G15 treatment. Results suggest a specific role of ERα in miRNA expression

changes observed in these miRNA. In addition, decreased miR-1244 expression observed after estradiol exposition was abrogated by MPP but no by ICI 182780 antagonist, suggesting also a specific role of ERα in miR-1244 regulation. Moreover, the increment in miR-501-3p expression observed when HUVEC were exposed to 1 nmol/l estradiol was reverted by using the unspecific ER antagonist, but not by MPP or G15. This finding suggests a possible role of ERβ in the regulation of miR-501-3p levels in HUVEC. Finally, neither ICI 182780 nor MPP antagonism had effect in inhibiting the miR-4710 increase observed after estradiol exposition. However, G15 treatment abolished estradiol-induced miR-4710 expression in HUVEC. Taken together, these results suggest that estradiol-dependent up-regulation of miR-4710 is probably mediated through GPER.

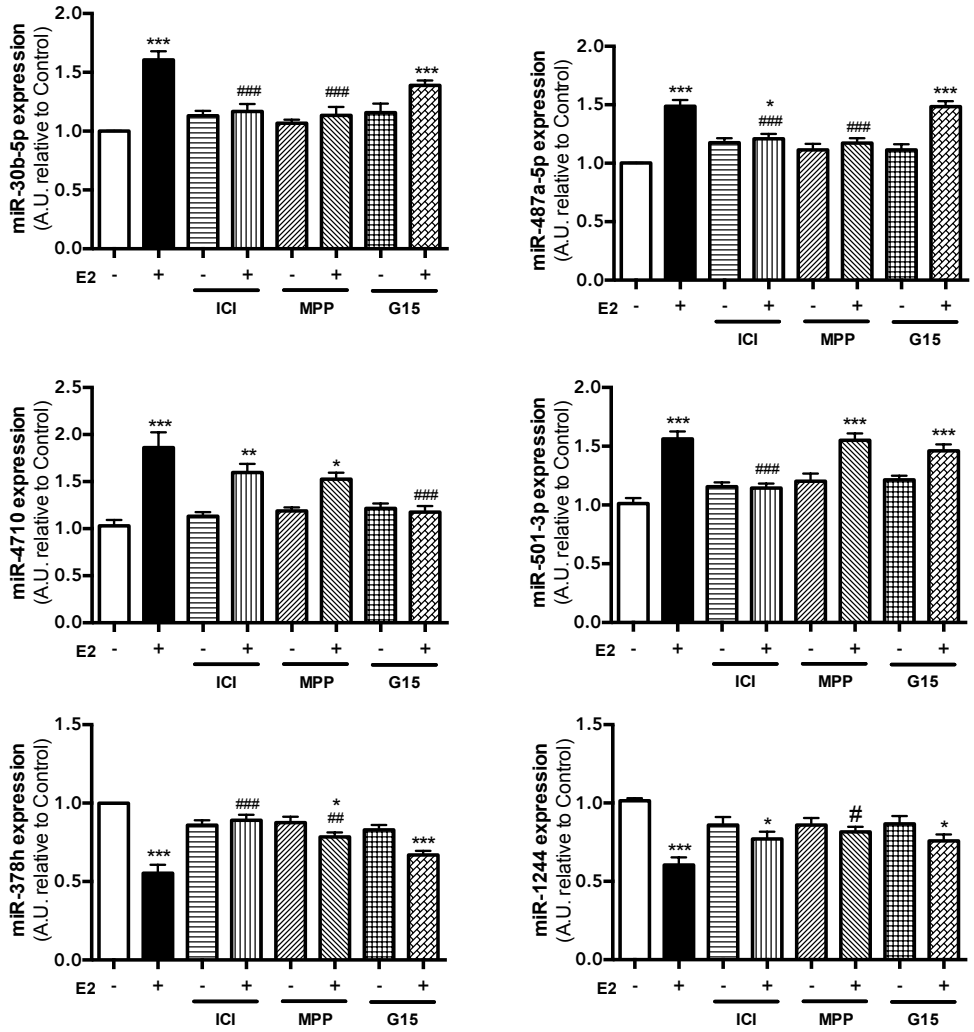


Figure 12. Role of ER antagonists in miRNA expression. HUVEC were exposed to ICI 182780 (1 µmol/l), MPP (1 µmol/l) or G15 (1 µmol/l) with or without estradiol (E2; 1 nmol/l) for 24 hours. Relative miRNA expression was measured by qRT-PCR as described in Material and methods. Data are expressed as mean ± SEM of n = 8-12 from 3 to 5 independent experiments. *p<0.05; **p<0.01; ***p<0.001 vs. Control and $^{\#}$p< 0.05; $^{\#\#}$p< 0.01 $^{\#\#\#}$p<0.001 vs. E2.

4.2.3 Role of ER agonists in estradiol-regulated miRNA expression.

The use of specific ER agonists for the different ER were used to reinforce the role of specific ER in estradiol-regulated miRNA. HUVEC were treated with the selective ERα agonist PPT (1 nmol/l), the selective ERβ agonist DPN (1 nmol/l) and the selective GPER agonist G1 (1 nmol/l) during 24 hours and the expression of validated miRNA were measured by qRT-PCR (Figure 13). Exposition to PPT, but not DPN or G1, stimulate miR-30b-5p and miR-487a-5p expression. In addition, only PPT treatment decreased miR-378h levels. These findings reproduce changes in miRNA expression observed when HUVEC were treated with estradiol and are in accordance with results obtained using specific ERα antagonist. Moreover, estradiol induction of miR-4710 was mediated through GPER, since treatment with the specific GPER agonist G1 completely mimic estradiol effect. On the contrary, miR-501-3p expression was not significantly changed after exposition to different agonists, being DPN treatment the one that results in a greater response. This observation suggests that ERβ could be related to miR-501-3p expression in HUVEC, results that are in accordance with those observed in antagonist treatments, where ICI 182780 but not MPP abolished estradiol effect in miRNA-501-3p expression. Finally, miR-1244 expression were decreased after exposition of the three different ER agonists.

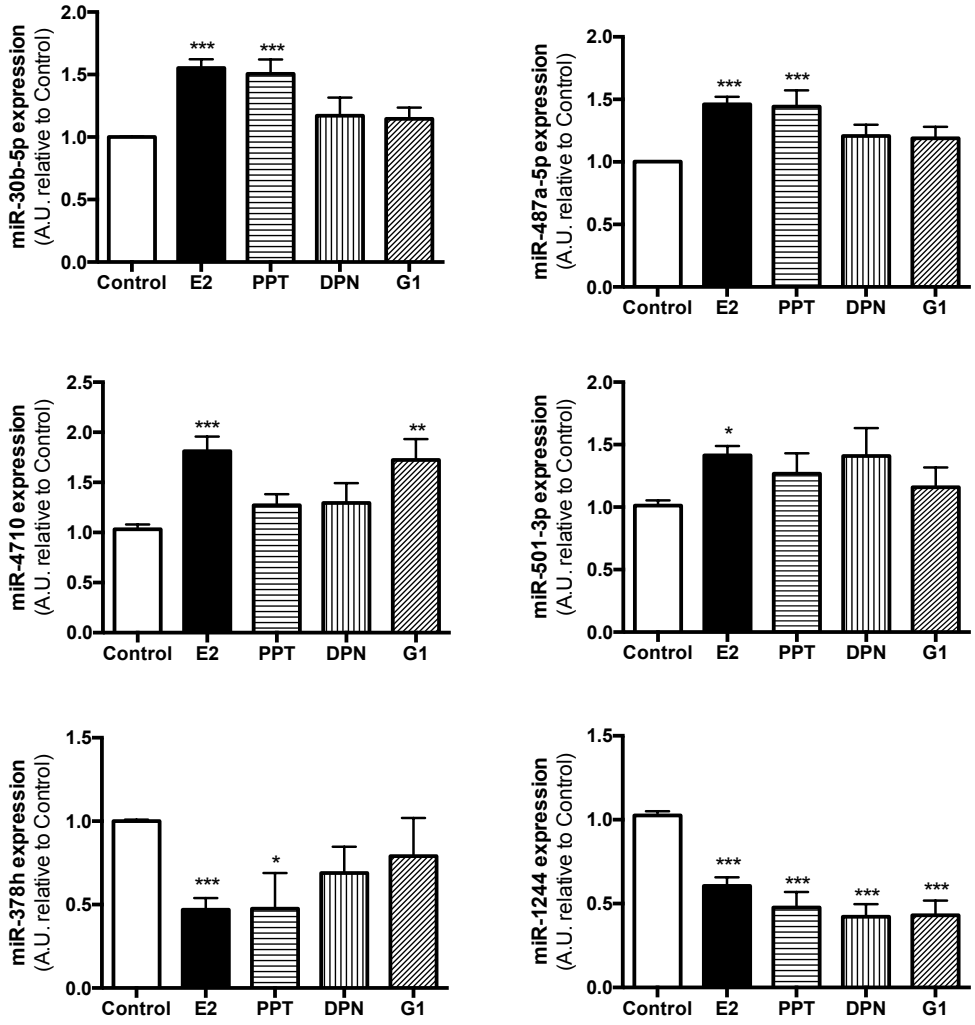


Figure 13. Role of ER agonists in miRNA expression. HUVEC were exposed to estradiol (E2; 1 nmol/l), PPT (1 nmol/l), DPN (1 nmol/l) or G1 (1 nmol/l) for 24 hours. Relative miRNA expression was measured by qRT-PCR as described in Material and methods. Data are expressed as mean ± SEM of n = 8-12 from 3 to 5 independent experiments. *p<0.05; ***p<0.01; ***p<0.001 vs. Control.

Altogether, our results demonstrated that ERα, ERβ and GPER were involved in miRNA expression in estradiol-treated HUVEC. Specifically, experiments using ER agonist and antagonists suggest that miR-30b-5p, miR-487a-5p and miR-378h expression is mediated

by ERα. Moreover, estradiol-induced changes in miR-501-3p and miR-4710 expression could be related to ERβ and GPER action, respectively. Finally, estradiol-dependent regulation of miR-1244 seems to be mediated via the different ER in HUVEC.

4.3 Integrative analysis of miRNA-mRNA expression pairings in estradiol-treated HUVEC.

Differentially expressed miRNA ($p < 0.05$) obtained in estradiol-treated HUVEC were used in order to identify either predicted or validated targets using IPA software. Moreover, adding our previous mRNA microarray data of differential mRNA expression profile in estradiol-exposed HUVEC (Sobrino et al., 2009) we combined both target predictions and experimental RNA data. Figure 14 shows a schematic of the analysis workflow implemented in integrated miRNA/mRNA expression in estradiol-treated HUVEC and obtained results.

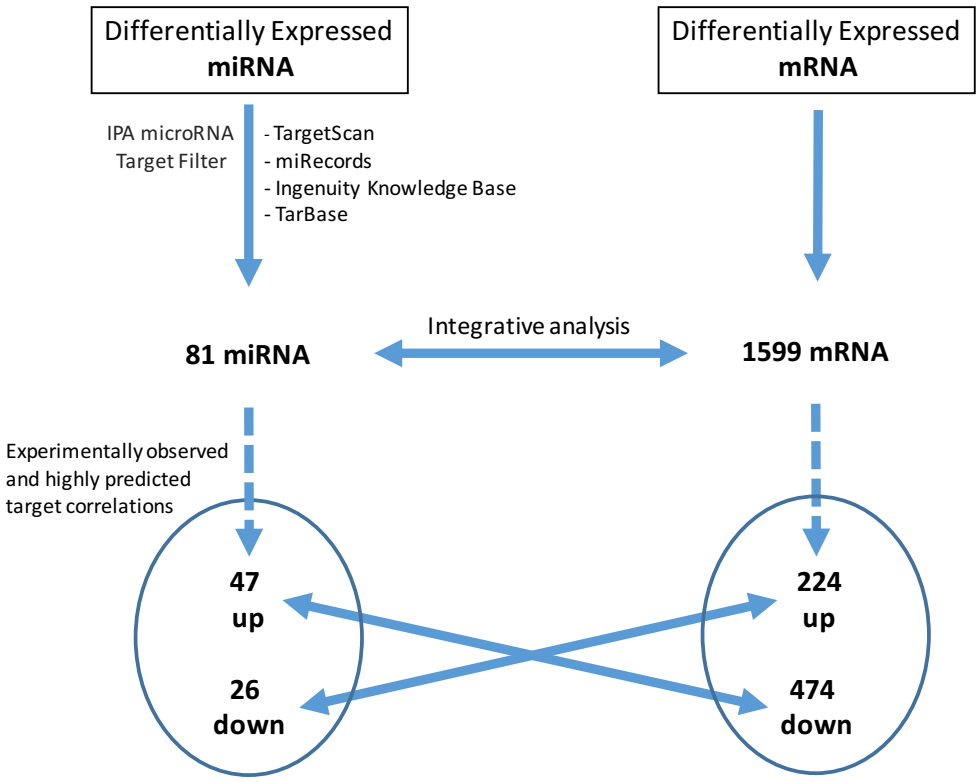


Figure 14. Workflow of integrative analysis of miRNA/mRNA expression in estradiol-treated HUVEC. Differentially expressed miRNA and mRNA from microarray data were used to obtain miRNA-mRNA interactions according to IPA microRNA Target Filter tool. Different miRNA target prediction programs (TargetScan, miRecords, Ingenuity Knowledge Base and TarBase) filtered our miRNA-mRNA pairings. Confidence filter was used by selecting only experimentally observed or highly predicted target correlations. The results show 73 differentially expressed miRNA and 698 inversely correlated mRNA targets.

Analysis of miRNA and mRNA data identified interaction of 81 miRNA with 1599 mRNA targets. As miRNA negatively regulates mRNA expression, opposite expression pairing between miRNA and mRNA levels was implemented to further analysis. IPA identified 81 miRNA with a total of 1326 mRNA. This means that 82.9% of those target genes were inversely correlated to their respective miRNA. To

adopt a restrictive approach, we further filtered our miRNA-mRNA pairings using experimentally observed or highly predicted target correlations. After this filter, we had 73 miRNA targeting 698 mRNA: 47 miRNA were up-regulated (with a total of 474 mRNA targets) and 26 were down-regulated (with a total of 224 mRNA targets).

Furthermore, canonical pathway analysis was performed using both highly predicted and experimentally observed mRNA targets using only miRNA-mRNA pair interactions inversely correlated. Among them, 57 miRNA and 243 mRNA targets were assigned to a specific metabolic or signalling pathway determined in the Ingenuity Canonical Pathway database. Results showed that estradiol-dependent miRNA-mRNA pairings were associated with different biological processes. Top 10 based on their p value are listed in Table 13. Among them, the top 5 canonical pathways are: ERK/MAPK signalling, Integrin signalling, Molecular mechanisms of cancer, HIPPO signalling and B Cell receptor signalling.

	Canonical Pathways	-log (p value)
1	ERK/MAPK Signalling	13.70
2	Integrin Signalling	12.30
3	Molecular Mechanisms of Cancer	12.20
4	HIPPO signalling	10.40
5	B Cell Receptor Signalling	10.20
6	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	9.94
7	SAPK/JNK Signalling	9.92
8	Axonal Guidance Signalling	9.80
9	HGF Signalling	9.21
10	Signalling by Rho Family GTPases	9.20

Table 13. Top 10 canonical pathway study of inversely correlated miRNA-mRNA pairs using IPA software. High predicted and experimentally observed pairings among inversely correlated miRNA-mRNA interactions were used to determine canonical pathways. p value is determined by the probability that each biological function assigned to the network is due to chance alone.

In addition, to further explore the impact of the obtained results in the context of our study, predicted estradiol-regulated miRNA-mRNA pairings assigned to cardiovascular signalling were selected and functional characterization was performed. Results showed that 30 out of 57 miRNA had targets directly involved in cardiovascular signalling pathways. Moreover, functional analysis showed 14 canonical pathways that were related to cardiovascular function (Table 14). Among them, Hypoxia signalling in cardiovascular system, Cardiac hypertrophy signalling, Thrombin signalling, HIF1α signalling and Cardiac β-adrenergic signalling were the most regulated pathways based on their p-value.

	Canonical Pathways	-log (p value)
1	Hypoxia Signalling in the Cardiovascular System	8.27
2	Cardiac Hypertrophy Signalling	7.61
3	Thrombin Signalling	4.54
4	HIF1α Signalling	4.42
5	Cardiac β-adrenergic Signalling	4.26
6	Renin-Angiotensin Signalling	4.03
7	Role of NFAT in Cardiac Hypertrophy	3.36
8	Aldosterone Signalling in Epithelial Cells	3.10
9	Factors Promoting Cardiogenesis in Vertebrates	3.06
10	P2Y Purigenic Receptor Signalling Pathway	2.32
11	Endothelin-1 Signalling	2.11
12	Angiopoietin Signalling	2.04
13	Cardiomyocyte Differentiation via BMP Receptors	1.62
14	eNOS Signalling	1.40

Table 14. Cardiovascular signalling pathways obtained of inversely correlated miRNA-mRNA pairs analysis using IPA software. High predicted and experimentally observed pairings among inversely correlated miRNA-mRNA interactions assigned to cardiovascular signalling were selected to determine canonical pathways. p value is determined by the probability that each biological function assigned to the network is due to chance alone.

Overall, our results using both the predicted targets and the experimentally obtained mRNA data increased the process-specificity of the miRNA-mRNA pairing prediction. In addition, functional analysis of these miRNA-mRNA interactions revealed specific biological processes and/or molecular pathways in which estradiol-dependent miRNA could play an important role.

4.4 Role of miR-30b-5p – ELK4 pairing in estradiol-exposed HUVEC.

4.4.1 Estradiol and miR-30b-5p regulation of predicted miR-30b-5p targets.

In order to validate results obtained from differentially expressed miRNA-mRNA associations in endothelial cells, we studied mRNA targets of miR-30b-5p, the miRNA that exhibited the greatest up-regulation in estradiol-exposed HUVEC (Figure 9). The predicted miR-30b-5p targets that were inversely correlated in HUVEC after 1 nmol/l estradiol with a fold change greater than 1.5 are listed in Table 15.

Fold change	Symbol	Official Full Name
-4.72	MYSM1	Myb-like, SWIRM and MPN domains 1
-2.47	PPP3CA	protein phosphatase 3, catalytic subunit, alpha isozyme
-2.27	ELK4	E twenty-six-like transcription factor 4
-2.16	MDM4	mouse double minute 4
-2.14	DLG5	discs, large homolog 5 (Drosophila)
-2.11	PPP1R12A	protein phosphatase 1 regulatory subunit 12A
-1.88	ENAH	enabled homolog (Drosophila)
-1.86	IL1A	interleukin 1 alpha
-1.80	ADAMTS6	ADAM metallopeptidase with thrombospondin type 1 motif 6
-1.80	PRKAR1A	protein kinase, cAMP-dependent, regulatory subunit type I alpha
-1.76	KPNA3	karyopherin alpha 3 (importin alpha 4)
-1.67	GRB10	growth factor receptor bound protein 10
-1.64	RAP2B	RAP2B, member of RAS oncogene family
-1.63	PRPF40A	pre-mRNA processing factor 40 homolog A
-1.63	CUL2	cullin 2

-1.59	NFIA	nuclear factor I/A
-1.54	SOCS6	suppressor of cytokine signalling 6
-1.51	NCOA3	nuclear receptor coactivator 3
-1.50	PDE4D	phosphodiesterase 4D

Table 15: Predicted miR-30b-5p targets that were inversely correlated in estradiol-treated HUVEC. Integrative analysis using miRNA and mRNA microarray data of HUVEC exposed to 1 nmol/l estradiol for 24 hours were used to determine putative miR-30b-5p targets. All targets with a fold change greater than 1.50 are listed.

Top 5 targets of miR-30b-5p were selected based on their fold change and qRT-PCR was performed in order to validate the effect of estradiol and miR-30b-5p on the expression of target mRNA. Specific pairing sites between miRNA and target mRNA were obtained using TargetScan 6.2 database. Figure 15 shows the predicted pairing of 3'UTR target region of selected genes and miR-30b-5p. MYSM1, ELK4 and MDM4 showed one specific pairing site for miR-30b-5p in their mRNA sequence whereas *in silico* analysis revealed two predicted pairing sites in the 3'UTR region of PPP3CA and DLG5.

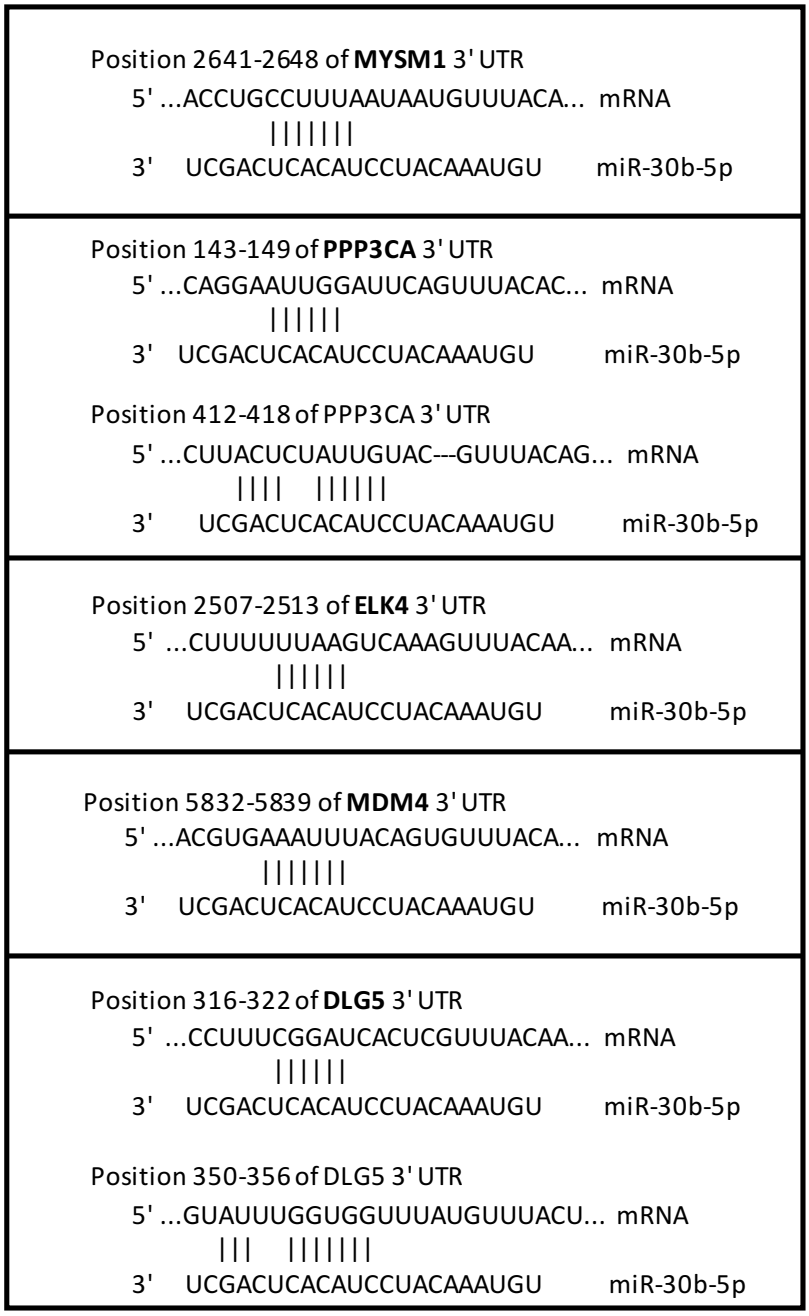


Figure 15. Predicted pairing sites in the 3'UTR region of selected targets for miR-30b-5p obtained in TargetScan 6.2 database.

To check the regulation of the different proteins by estradiol through miR-30b-5p, total RNA of HUVEC cells exposed to 1 nmol/l estradiol for 24 hours were isolated and relative expression of selected miR-30b-5p targets (MYSM1, PPP3CA, ELK4, MDM4 and DLG5) were measured by qRT-PCR. As shown in Figure 16, estradiol treatment significantly decreased mRNA expression of all of them, results that are in accordance with those observed in microarray data.

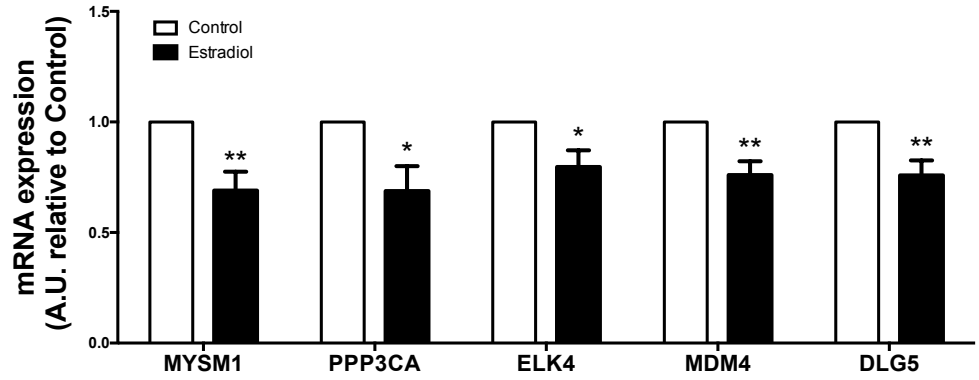


Figure 16. Validation of miR-30b-5p predicted targets regulated by estradiol in HUVEC. HUVEC were treated with ethanol (Control; 0.1 %) or estradiol (1 nmol/l) for 24 hours and total RNA was isolated. 5 predicted miR-30b-5p targets selected based on their fold change were validated by qRT-PCR as described in Material and Methods. Results are shown as mean ± SEM of n = 8 -12 from 3 to 5 independent experiments. *p<0.05, **p<0.01 vs. Control.

In addition, we examined the effect of miR-30b mimic in selected target mRNA (Figure 17). HUVEC were transfected with 30 nmol/l of miR-30b mimic (Mimic-30b) or miRNA negative control (Negative) and total RNA was isolated 48 hours after. mRNA analysis

of selected targets showed that miR-30b mimic significantly decreased expression of MYSM1, PPP3CA, ELK4 and DLG5, while MDM4 expression decreased was not significantly different (p = 0.0693). Therefore, the miR-30b-5p mimic exerts nearly the same effects than estradiol in HUVEC, supporting its involvement in estradiol-dependent regulation of the selected mRNA.

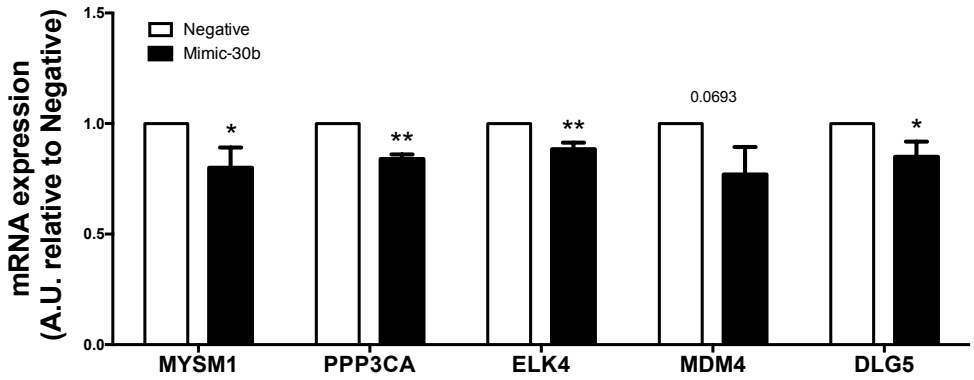


Figure 17. Role of miR-30b-5p mimic in miR-30b-5p predicted targets regulated by estradiol in HUVEC. HUVEC were transiently transfected with 30 nmol/l of miR-30b-5p mimic (Mimic-30b) or negative control miRNA (Negative) and expression of selected targets were determined by qRT-PCR as described in Material and Methods. Results were shown as mean ± SEM of n = 6 - 7 from 3 independent experiments. *p<0.05, **p<0.01 vs. Negative.

4.4.2 Estradiol-dependent down-regulation of ELK4 is mediated by miR-30b-5p in HUVEC.

ELK4 is a member of the ETS family of transcription factors. ETS members act through direct transcriptional regulation of their down-stream gene and have been involved in several biological processes (Sharrocks, 2001). Therefore, based on the interest of gene

regulation by transcription factors, we decided to focus the remaining studies on the role of estradiol-mediated regulation of miR-30b-5p and ELK4 association in endothelial cells.

To further investigate whether the estradiol-dependent decrease of ELK4 expression is mediated through miR-30b-5p up-regulation, HUVEC were transfected with a specific hsa-miR-30b-5p hairpin inhibitor. Then, different transfection period (4, 6 and 12 hours) and inhibitor concentration (60 and 80 nM) were assessed. Preliminary experiment results are shown in Figure 18. The parameters used did not show differences in the reduction of miR-30B-5p levels. Therefore, the rest of experiments were carried out using 4 hours of transfection period and 60 nM of inhibitor concentration.

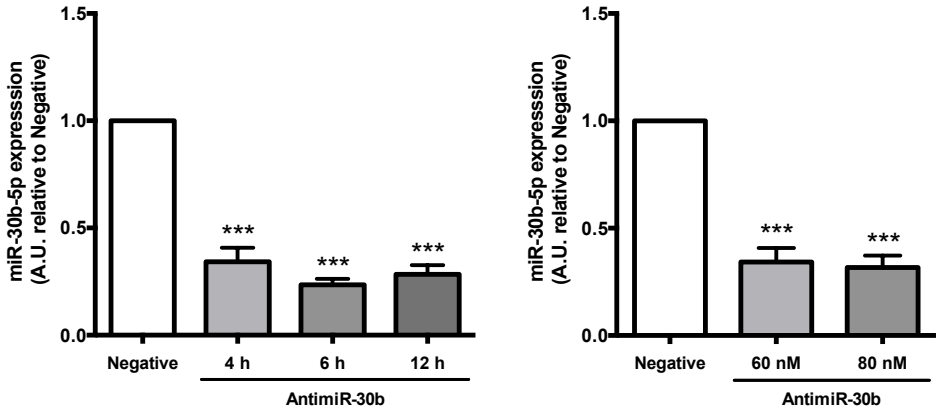


Figure 18. Preliminary results of miR-30b-5p inhibitor transfection. HUVEC were transfected with hsa-miR-30b-5p inhibitor (AntimiR-30b) or negative control miRNA (Negative) and relative expression of miR-30b-5p was determined by qRT-PCR as described in Material and Methods. Different transfection conditions were assessed by three transfection periods (4, 6 and 12 hours (h)) using miRNA inhibitor at 60 nM and two inhibitor concentrations (60 and 80 nM) during 4 hours. Results were shown as mean ± SEM of n = 2 - 4 from 2 independent experiments. ***p<0.001 vs. Negative.

Secondly, we determined the specificity of miR-30b hairpin inhibitor in decreasing miRNA expression by qRT-PCR (Figure 19). HUVEC were transfected with hsa-miR-30b-5p hairpin inhibitor and miR-30b-5p and miR-92a expression were measured. Results showed that cells transfected with miR-30b-5p inhibitor reduce significantly miR-30b-5p expression (≈ 70 %), ensuring a good down-regulation of miR-30b-5p levels. In addition, the specificity of the procedure was assessed by miR-92a expression determination. HUVEC treated with miR-30b-5p inhibitor did not affect de endogenous levels of miR-92a, confirming specific miR-30b-5p inhibition.

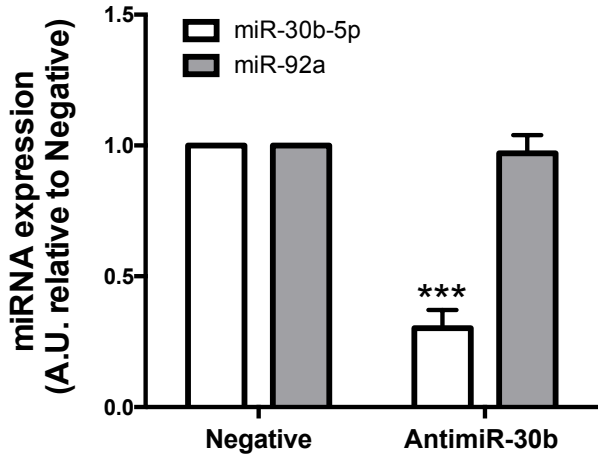


Figure 19. miR-30b hairpin inhibitor specifically decrease miR-30b-5p expression in HUVEC. Cells were transfected with hsa-miR-30b-5p inhibitor (AntimiR-30b) or negative control miRNA (Negative) and relative expression of miR-30b-5p and miR-92a were determined by qRT-PCR as described in Material and Methods. Results were shown as mean ± SEM of n = 3 - 4 from 2 independent experiments., ***p<0.001 vs. Negative.

HUVEC were transfected with hsa-miR-30b-5p inhibitor (antimiR-30b) or negative control miRNA (Negative) and were stimulated with 1 nmol/l estradiol or vehicle (0.1 % ethanol). Results showed that estradiol decreased ELK4 mRNA expression ($p < 0.01$) as in intact cells (Figure 16). However, when cells were transfected with the miR-30b-5p inhibitor (antimiR-30b), the effect of estradiol was completely abolished (Figure 20).

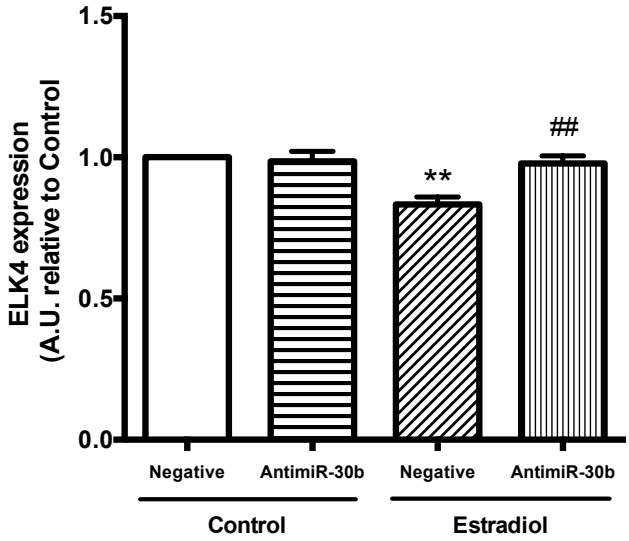


Figure 20. Estradiol-dependent decrease of ELK4 mRNA expression is mediated by miR-30b-5p. HUVEC transfected with 60 nmol/l of hsa-miR-30b-5p inhibitor (AntimiR-30b) or negative control miRNA (Negative) were treated with 1 nmol/l estradiol or 0.1 % ethanol (Control) for 24 hours and relative expression of ELK4 was determined by qRT-PCR. Results were shown as mean ± SEM of n = 8 - 9 from 5 independent experiments. **p<0.01 vs. Control and $^{\#\#}$p<0.01 vs. Negative.

In addition, the effect of estradiol on ELK4 protein level was also determined using same conditions described above. Relative levels assessed by densitometry revealed a significant decrease in

ELK4 protein expression in estradiol-treated HUVEC (25 ± 8 %, $p < 0.05$). Similar results that were obtained using non-transfected cells (NT). Moreover, estradiol-dependent ELK4 decrease was abolished when cells were previously transfected with miR-30b-5p inhibitor (Figure 21).

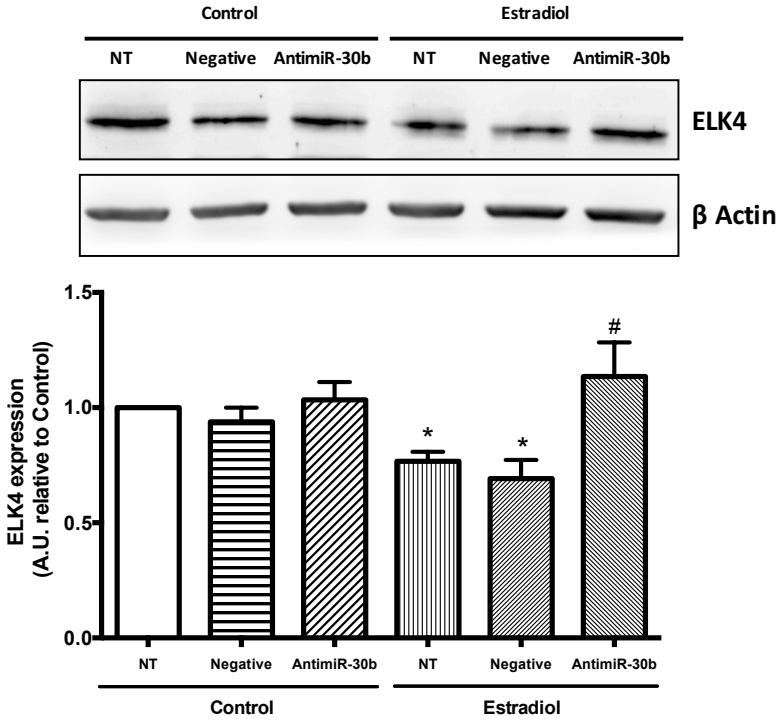


Figure 21. Estradiol-dependent decrease of ELK4 protein expression is mediated by miR-30b-5p. Non-transfected HUVEC (NT) and the transfected with 60 nmol/l of hsa-miR-30b-5p inhibitor (AntimiR-30b) or negative control miRNA (Negative) were treated with 0.1 % ethanol (Control) or 1 nmol/l estradiol for 24 hours and protein extracts (20 µg) were loaded on SDS-PAGE gels and analysed by Western blotting using anti-ELK4 antibody. β-actin was used as loading control. One representative immunoblot is shown. Relative levels assessed by densitometry are represented. Results are shown as mean ± SEM of n = 3 - 4 from 3 independent experiments. *$p<0.05$ vs. Control and $^{\#}p<0.05$ vs. Negative.

Finally, HUVEC transfected with miR-30b-5p mimic showed a decreased ELK4 protein expression ($p < 0.05$) (Figure 22). Results indicated that miR-30-5p mimic significantly decreased ELK4 expression up to 31 ± 10 %, in accordance with results obtained using mRNA determination. Overall, our results suggest that estradiol-dependent up-regulation of miR-30b-5p represses ELK4 expression in HUVEC.

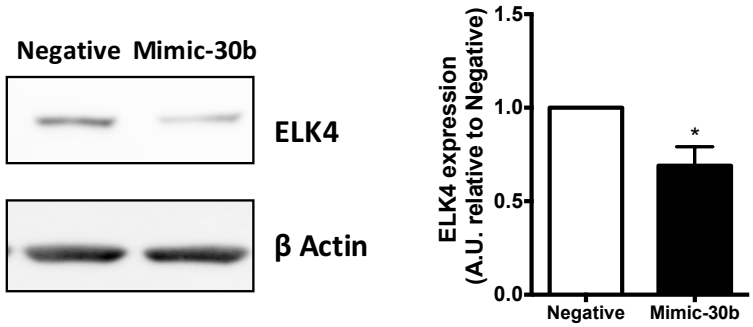


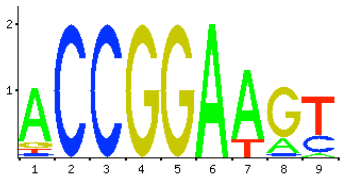
Figure 22. miR-30b-5p mimic decreases ELK4 protein expression. HUVEC were transfected with 30 nmol/l of miR-30b mimic (Mimic-30b) or negative control miRNA (Negative) and protein extracts (20 µg) were loaded on SDS-PAGE gels and analysed by Western blotting using anti-ELK4 antibody. β-actin was used as loading control. One representative immunoblotting is shown. Relative levels assessed by densitometry are represented. Results are shown as mean ± SEM of n = 4 - 6 from 3 independent experiments. *p<0.05 vs. Negative.

4.4.3 *In silico* determination of estradiol-regulated genes with ELK4 binding sites.

In order to further investigate the role of ELK4 in endothelial cells exposed to estradiol, we search for potential down-stream targets that could be regulated by the transcription factor ELK4. For this purpose, ELK4 binding sites motifs (Figure 23A) in the regulatory region of estradiol-down-regulated genes were search.

Identification of genes with putative ELK4 binding sites within the promoter region was performed using JASPAR database via Harmonizome web server as described in Methods section. We identified potential ELK4 down-stream target genes by comparing estradiol-down-regulated genes ($p < 0.05$; Fold change > 1.5) observed in mRNA microarray data and predicted target genes of ELK4 obtained using JASPAR data. Figure 23B depicts a schematic representation of the approach used and obtained results.

A



ELK4 binding motif

B

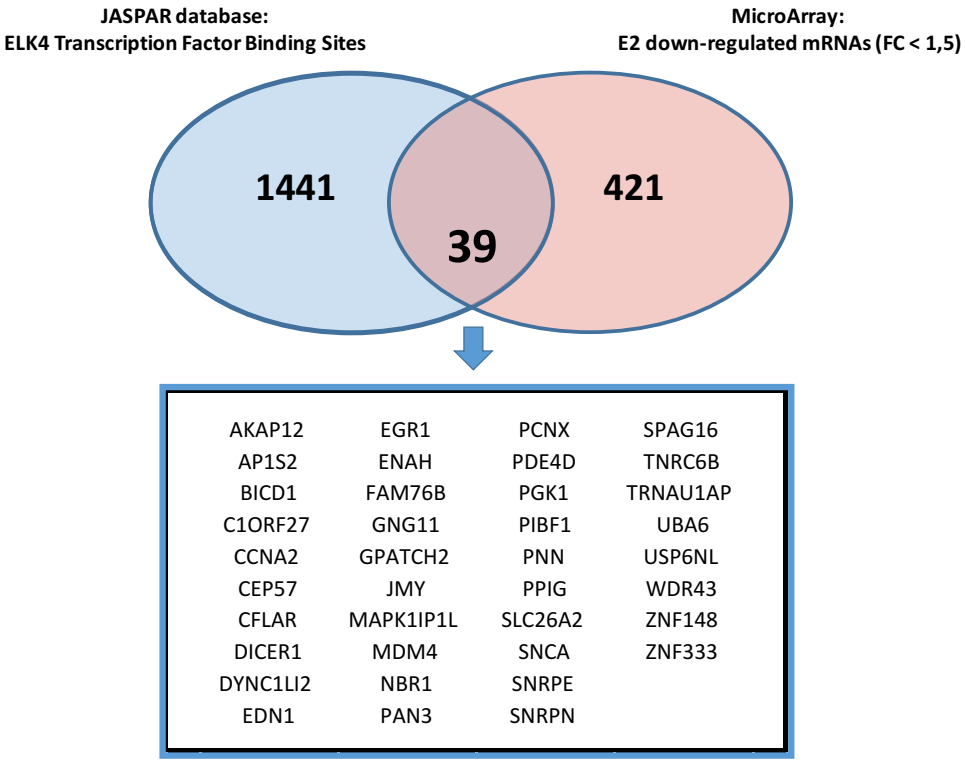


Figure 23. Determination of putative estradiol-regulated genes with ELK4 binding sites. (A) Consensus ELK4 binding motif drawn from the JASPAR database. (B) Schematic representation of approach used to obtain putative estradiol-modulated genes with ELK4 binding sites in their regulatory region. Shared genes between ELK4 binding site genes obtained from JASPAR database and estradiol down-regulated mRNA from microarray data were selected.

Results showed 39 potential ELK4 target genes regulated in estradiol-exposed HUVEC. Predicted genes based on their fold change are listed in Table 16.

p value	Fold change	Symbol	Official Full Name
0.019	-10.50	FAM76B	family with sequence similarity 76, member B
0.047	-5.78	PGK1	phosphoglycerate kinase 1
0.033	-4.78	EDN1	endothelin 1
0.006	-4.00	AP1S2	adaptor-related protein complex 1, sigma 2 subunit
0.024	-3.62	TNRC6B	trinucleotide repeat containing 6B
0.045	-3.48	SNRPN	small nuclear ribonucleoprotein polypeptide N
0.044	-3,12	PNN	pinin, desmosome associated protein
0.013	-2.90	ZNF148	zinc finger protein 148
0.005	-2.55	EGR1	early growth response 1
0.011	-2.54	ZNF333	zinc finger protein 333
0.012	-2.40	SNRPE	small nuclear ribonucleoprotein polypeptide E
0.014	-2.39	TRNAU1AP	tRNA selenocysteine 1 associated protein 1
0.032	-2.39	SLC26A2	solute carrier family 26 (anion exchanger), member 2
0.007	-2.34	AKAP12	A kinase (PRKA) anchor protein 12
0.009	-2.28	WDR43	WD repeat domain 43
0.048	-2.16	MDM4	MDM4, p53 regulator
0.027	-2.03	DYNC1LI2	dynein, cytoplasmic 1, light intermediate chain 2
0.012	-1.98	DICER1	dicer 1, ribonuclease type III
0.009	-1.96	PPIG	peptidylprolyl isomerase G (cyclophilin G)
0.000	-1.90	SNCA	synuclein, alpha (non A4 component of amyloid precursor)

0,002	-1.90	CCNA2	cyclin A2
0.034	-1.88	ENAH	enabled homolog (Drosophila)
0.049	-1.85	BICD1	bicaudal D homolog 1 (Drosophila)
0.007	-1.79	NBR1	neighbor of BRCA1 gene 1
0.001	-1.75	USP6NL	USP6 N-terminal like
0.009	-1.68	C1ORF27	chromosome 1 open reading frame 27
0.030	-1.60	PIBF1	progesterone immunomodulatory binding factor 1
0.049	-1.58	UBA6	ubiquitin-like modifier activating enzyme 6
0.021	-1.57	PAN3	PAN3 poly(A) specific ribonuclease subunit
0.006	-1.56	GPATCH2	G patch domain containing 2
0.002	-1.55	CFLAR	CASP8 and FADD-like apoptosis regulator
0.027	-1.52	GNG11	guanine nucleotide binding protein (G protein), gamma 11
0.010	-1.52	SPAG16	sperm associated antigen 16
0.027	-1.51	CEP57	centrosomal protein 57kDa
0.040	-1.50	JMY	junction mediating and regulatory protein, p53 cofactor
0,001	-1.50	PCNX	pecanex homolog (Drosophila)
0.009	-1.50	MAPK1IP1L	mitogen-activated protein kinase 1 interacting protein 1-like
0.013	-1.50	PDE4D	phosphodiesterase 4D, cAMP-specific

Table 16. Putative ELK4 target genes regulated in estradiol-exposed HUVEC. Identification of estradiol-regulated genes with putative ELK4 binding sites in their promoter region was performed using JASPAR database via Harmonizome web server. Potential ELK4 down-stream target genes were identified among estradiol-down-regulated genes ($p < 0.05$; Fold change > 1.5) obtained from mRNA microarray data. Target genes are listed based on their fold change. p value, Fold change, Gene symbol and Official full name are shown.

ET-1 was found among estradiol-regulated genes with predicted ELK4 binding motif in its regulatory region. ET-1 plays a key role in the regulation of endothelial function and it has been involved in the development of different cardiovascular diseases (Bohm and Pernow, 2007). Moreover, EGR-1, a well-known ELK4 target (Clarkson et al., 1999) was also among identified genes after *in silico* analysis. We next validated our findings by measuring mRNA expression levels of ET-1 and the previously described ELK4 target EGR-1 (Figure 24). Results showed that estradiol treatment significantly decreased mRNA expression of ET-1 (20 ± 7 %, $p < 0.05$) and EGR-1 (23 ± 8 %, $p < 0.05$) in HUVEC, validating those results obtained from microarray data.

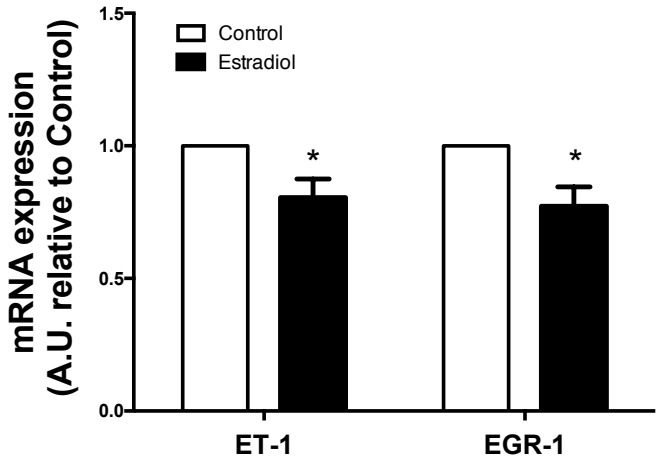


Figure 24. Validation of putative ELK4 target genes regulated in estradiol-exposed HUVEC. Cells treated with 1 nmol/l estradiol for 24 hours were collected and mRNA expression were determined by qRT-PCR as described in Material and Methods. Results are shown as mean ± SEM of n = 5 - 12 from 3 to 5 independent experiments. *p<0.05 vs. Control.

4.4.4 Role of miR-30b-5p in estradiol-dependent down-regulation of ET-1.

Given the importance of ET-1 in endothelial function, we decided to study the implication of miR-30b-5p in the decreased ET-1 levels observed in HUVEC exposed to 1 nmol/l estradiol. First, ET-1 aprotein levels were determined in estradiol-treated HUVEC. Results showed a decreased of ET-1 protein expression up to 33 ± 7 % ($p < 0.05$) when compared to control cells (Figure 25) in accordance with ET-1 mRNA expression levels.

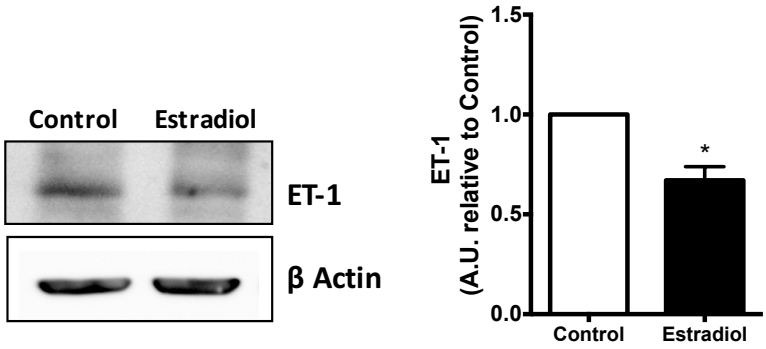


Figure 25. Estradiol decrease ET-1 protein expression in HUVEC. HUVEC were treated with 1 nmol/l estradiol or 0.1 % ethanol for 24 hours and protein extracts (20 µg) were loaded on SDS-PAGE gels and analysed by Western blotting using anti-ET-1 antibody. β-actin was used as loading control. One representative immunoblotting is shown. Relative levels assessed by densitometry are represented. Results are shown as mean ± SEM of n = 5 - 6 from 4 independent experiments. *p<0.05 vs. Control.

In addition, we used miR-30b-5p inhibitor in order to determine whether estradiol-induced miR-30b-5p is involved in ET-1 down-regulation observed in HUVEC. Decreased ET-1 mRNA expression (20 ± 4 %, P < 0.05) was abolished upon transfection of miR-30b-5p inhibitor (Figure 26).

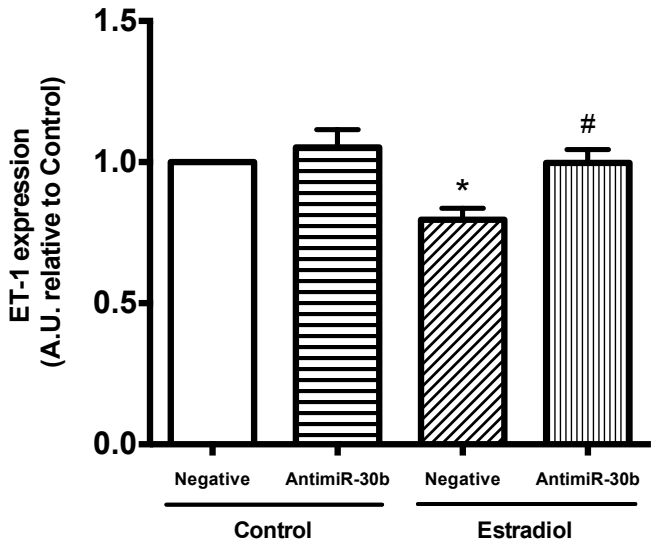


Figure 26. Estradiol-dependent decrease of ET-1 expression is mediated by miR-30b-5p. (A) HUVEC transfected with 60 nmol/l of hsa-miR-30b-5p inhibitor (miR-30b) or negative control miRNA (Negative) were treated with 1 nmol/l estradiol or 0,1 % ethanol (Control) for 24 hours and relative expression of ET-1 was determined by qRT-PCR. Results are shown as mean ± SEM of n = 5 - 7 from 4 independent experiments. *p<0.05 vs. Control and $^{\#}$p<0.05 vs. Negative.

mRNA expression results were supported by ET-1 protein expression using Western blotting, since relative levels assessed by densitometry revealed a significant decrease in ET-1 protein expression in estradiol-treated HUVEC (40 ± 6 %, $p < 0.05$) that was abolished when cells were previously transfected with miR-30b-5p inhibitor (Figure 27).

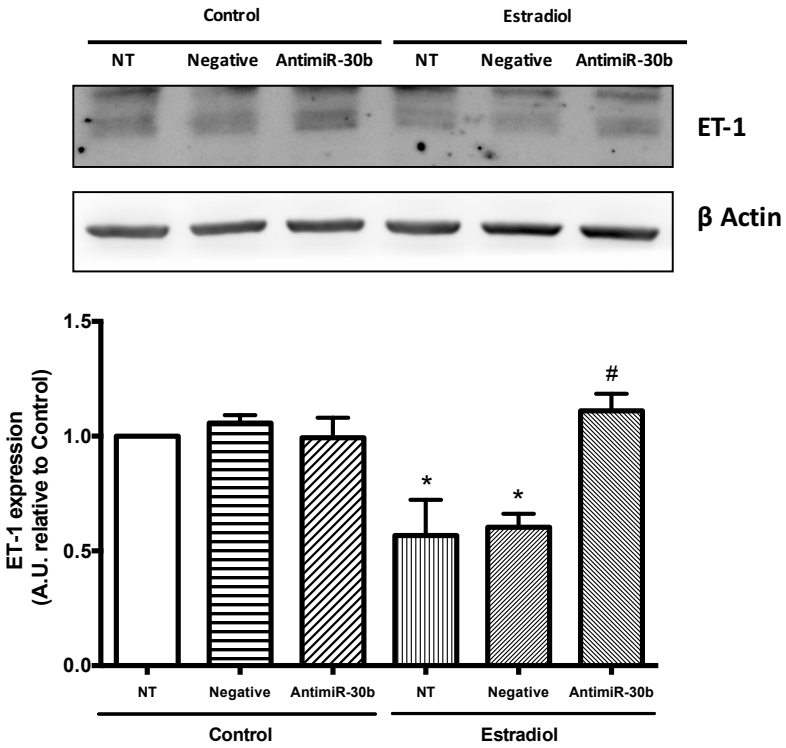


Figure 27. Estradiol-dependent decrease of ET-1 protein expression is mediated by miR-30b-5p. Non-transfected HUVEC (NT) and HUVEC transfected with 60 nmol/l of hsa-miR-30b-5p inhibitor (AntimiR-30b) or negative control miRNA (Negative) were treated with 0.1 % ethanol (Control) or 1 nmol/l estradiol for 24 hours and protein extracts (20 µg) were loaded on SDS-PAGE gels and analysed by Western blotting using anti-ET-1 antibody. β-actin was used as loading control. One representative immunoblotting is shown. Relative levels assessed by densitometry are represented. Results are shown as mean ± SEM of n = 5 - 7 from 4 independent experiments. *$p<0.05$ vs. Control and $^{\#}p<0.05$ vs. Negative.

Furthermore, we examined the effect of miR-30b mimic in ET-1 expression. mRNA analysis showed that miR-30b mimic significantly decreased mRNA expression (Figure 28) up to 23 ± 5 % ($p < 0.01$).

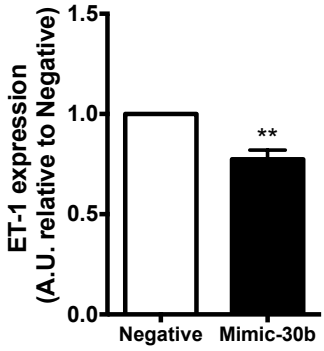


Figure 28. miR-30b-5p mimic decreases ET-1 expression. HUVEC were transfected with 30 nmol/l of miR-30b mimic (Mimic-30b) or negative control miRNA (Negative) and relative expression of ET-1 was determined by qRT-PCR. Results are shown as mean ± SEM of n = 5 - 6 from 3 independent experiments. *p<0.05 vs. Negative.

Moreover, addition of exogenous miR-30b-5p completely mirrored estradiol effect, since HUVEC transfected with miR-30b mimic also decrease ET-1 protein expression up to 27 ± 16 % (Figure 29).

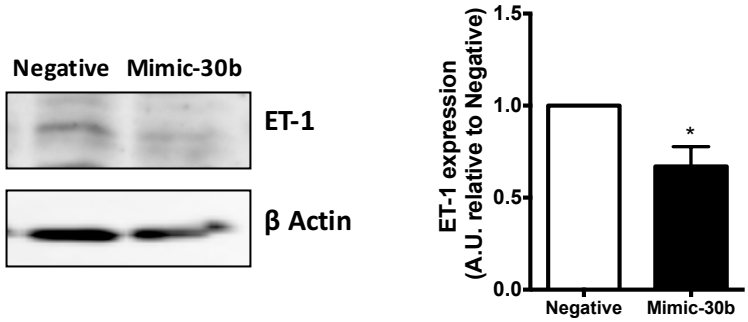


Figure 29. miR-30b-5p mimic decreases ET-1 protein expression. Protein extracts (20 μg) of HUVEC transfected with 30 nmol/l of miR-30b mimic (Mimic-30b) or negative control miRNA (Negative) were loaded on SDS-PAGE gels and analysed by Western blotting using anti-ET-1 antibody. β-actin was used as loading control. One representative immunoblotting is shown. Relative levels assessed by densitometry are represented. Results are shown as mean ± SEM of n = 5 - 6 from 3 independent experiments. *$p < 0.05$ vs. Negative.

5. DISCUSSION

In the present work, we have identified changes in miRNA expression profile in human endothelial cells exposed to a physiological concentration (1 nmol/L) of estradiol using microarray technology. Estradiol effects on complete gene (Felty et al., 2010; Sobrino et al., 2009) and protein (Felty, 2011) expression in endothelial cells have already been studied but its effect on whole miRNA expression profile has not been explored.

We first evaluated whether estradiol is involved in the expression of miRNA production machinery using mRNA microarray data. Our results revealed a deregulation of key components in **miRNA biosynthesis** in estradiol-treated HUVEC. mRNA microarray data revealed significant different expression in estradiol-treated cells in which DROSHA expression was up-regulated whereas DICER1 and AGO-2 mRNA expression were down-regulated. In this regard, differences in key miRNA processing genes have been observed between ER+ and ER- breast cancer (Cheng et al., 2009; Cizeron-Clairac et al., 2015). Specifically, DICER1, DGCR8 and DROSHA were increased whereas AGO-2 expression was reduced in ER+ versus ER- breast tumours. Indeed, it has been described that ER interact with DROSHA and modulate its activity in breast cancer cells (Paris et al., 2012). Moreover, estrogen significantly increased Exportin-5 mRNA

expression in the mouse uterus (Nothnick et al., 2010). Furthermore, unlike observed results in endothelial cells, it has been described an estradiol-dependent induction of DICER1 mRNA expression in breast cancer cells. Indeed, in the same study it is also described that DICER1 gene was the only one with an ERα binding sites in the regulatory region among miRNA processing genes (Bhat-Nakshatri et al., 2009). Additionally, it has also been described that differentially expressed miRNA in ERα- versus ERα+ breast cancer cells negatively control DICER1 expression (Cochrane et al., 2010).

On the basis of results obtained about estradiol-regulated miRNA, we have characterized functional networks in which miRNA modified by estradiol are involved. In addition, the role of ER in estradiol-mediated regulation has also been investigated. Moreover, the second part of the thesis focuses on an integrated analysis of miRNA-mRNA interactions observed in HUVEC exposed to estradiol. Finally, the role of miR-30b-5p in the regulation of the transcription factor ELK4 and its predicted down-stream gene ET-1 is studied. Therefore, results have been divided in four sections and discussed in the following pages. Finally, some aspects about relevance of obtained results in the context of endothelial dysfunction and cardiovascular pathologies are addressed.

5.1 Role of estradiol-regulated miRNA in endothelial function.

Functional analysis of predicted targets of estradiol-regulated miRNA identified different **biological processes** that include cellular assembly and organization, cellular morphology and cell movement. In that sense, the role of estradiol in endothelial cell movement has been described through actin cytoskeleton remodelling (Oviedo et al., 2011). Indeed, it has recently described that increase in cell migration and proliferation of estradiol-exposed HUVEC is mediated by miR-126-3p regulation (Li et al., 2016a).

Cell cycle, cell death and survival, as well as cancer have also been ranked in the principal estradiol-regulated networks. Estradiol has already been implicated in apoptosis via activation of miRNA-23a and p53 in liver cancer (Huang et al., 2015), and different profiles of estrogen-regulated miRNA in breast cancer cells have been described (Muluhngwi and Klinge, 2015).

Lipid and carbohydrate metabolism have also been included among the most regulated networks. In this regard, our group described the relationship between estradiol and different metabolic pathways in HUVEC through the study of mRNA microarray, such as pentose phosphate pathway, galactose metabolism, N-glycan biosynthesis and inositol phosphate metabolism (Sobrino et al., 2009). In addition, estrogens action and the implication of ER have been involved in lipid and carbohydrate metabolism in different tissues (Barros and Gustafsson, 2011). Moreover, as described in Introduction section, the effect of estrogens on lipid and carbohydrate metabolisms has been extensively described in

postmenopausal women using estrogen replacement (Salpeter et al., 2006). Since miRNA play a role in metabolic disorders (Fernandez-Hernando et al., 2013), it is feasible that modification of miRNA by estradiol could play a role in lipid metabolism regulation. In this regard, it has recently described a sex-specific regulation between ERα and miR-22 through a reciprocal feedback loop. This ERα-miR-22 interaction has been involved in the regulation of muscle lipid metabolism in male mice (Schweisgut et al., 2017).

Development and functions of reproductive system is also, and expected, among most significantly regulated by estradiol. In this regard, estrogen action has been related to regulation of specific miRNA expression in the mouse uterus (Nothnick and Healy, 2010). Moreover, changes in miRNA expression has also been described along different phases of human endometrium (Altmae et al., 2013), suggesting hormonal regulation of miRNA involved in endometrial cycle.

To focus in the context of our study and to explore the role of estradiol-regulated miRNA in cardiovascular system, predicted target genes assigned to cardiovascular pathways were selected and functional characterization performed. It is well known that sex differences exist in cardiac and vascular function, suggesting the involvement of sex hormones (Vitale et al., 2009). Moreover, estrogens also play an important role in cardiac function through the regulation of miRNA, since sex-dimorphic miRNA expression has been reported in heart tissue (Evangelista et al., 2013). Among the cardiovascular related canonical pathways, highlighted cardiac

hypertrophy signal is the number one ranked canonical pathway. Small and Olson reviewed specific signature patterns of miRNA associated to different cardiovascular disorders, including cardiac hypertrophy (Small and Olson, 2011). In addition, it has been reported a sex-specific expression of miRNA mediated through ERβ in hypertrophied heart (Queirós et al., 2013).

Furthermore, CXCR4 and Rho GTPases signalling are among the main networks regulated by differentially expressed miRNA in HUVEC exposed to estradiol. In this regard, miR-126, a recently described estradiol-dependent miRNA (Li et al., 2016a), is involved in vascular protection through CXCR4 expression modulation (Zernecke et al., 2009). In addition, estradiol exposition increase Rho-A expression and activity in endothelial cells (Oviedo et al., 2011).

Nitric oxide, reactive oxygen species and endothelin-1 signalling, three of the most important vasoactive mediators produced by endothelium, appear as regulated by estradiol-dependent miRNA. These results not only reinforce the recognized role of estradiol in the stimulation of nitric oxide, the protection against reactive oxygen species and the reduction of ET-1 (Alvarez et al., 2002), but also suggest new regulatory mechanisms. In that sense, sex differences in eNOS expression have been attributed to higher miR-222 expression observed in male (Evangelista et al., 2013). In addition, estrogens stimulate ERα-dependent inhibition of miR-22 expression, leading to an increase antioxidant activity in myocardium (Wang et al., 2015a). However, the role of miRNA in estradiol-dependent in ET-1 regulation has not been described yet.

The most up-regulated miRNA after estradiol exposition was **miR-30b-5p**. miR-30b has been previously described as an estrogen-sensitive miRNA. Estradiol treatment increases mir30b levels in breast cancer cells (Bhat-Nakshatri et al., 2009). Moreover, miRNA profiling of human endometrium reveals cyclic changes in miR-30b expression through the different physiological phases (Kuokkanen et al., 2010). In addition, increased miR-30b levels were also observed in brains of female mice compared to males (Mellios et al., 2012).

miR-30 family have been related to osteoblast differentiation (Wu et al., 2012), apoptosis (Roca-Alonso et al., 2015) and cancer (Yu et al., 2010). Furthermore, miR-30b function has been described limiting cardiac fibrosis progression by myocardial matrix remodelling regulation (Duisters et al., 2009) and attenuating myocardial ischemia-reperfusion injury in a rat model and hypoxia/reoxygenation-induced injury in cardiomyocytes (Li et al., 2015b; Song et al., 2015). In endothelial cells, miR-30 has been implicated in anti-inflammatory effects of shear stress by decreasing angiopoietin 2–induced VCAM1 expression (Demolli et al., 2015). miR-30b-over-expressing HUVEC showed an increased angiogenesis through down-regulation of DLL4 mRNA target (Bridge et al., 2012). Moreover, miR-30b has been involved in apoptosis inhibition of human coronary artery endothelial cells by the regulation of caspase 3 expression (Li et al., 2015a). Therefore, miR-30b could be involved in the inhibition of caspase 3 induced by estradiol in mesenteric microvasculature (Childs et al., 2010) and in cardiac apoptosis in ovariectomized rats (Liou et al., 2010).

miR-487a, second in the top estradiol-induced miRNA, has been recently associated to the TGF-β1 pathway (Ma et al., 2016; Wang et al., 2014), a key cytokine in vascular function (Goumans et al., 2009). miR-487a expression significantly increased after TGF-β1 treatment in breast cancer cells where it was associated to an increased migration and invasion (Ma et al., 2016). Moreover, bioinformatic analysis using serum samples of atypical coronary artery disease patients also speculated about the association between miR-487a and TGF-β1 since TAB3, a down-stream element of TGF-β1-Smad pathway, is a miR-487a target (Wang et al., 2014). This association is related to our previously published work (Sobrino et al., 2009) using mRNA microarrays analysis of estradiol-treated endothelial cells in which TGF-β1 was significantly up-regulated. Indeed, network analysis highlighted Cardiovascular System Development and Function, Cellular Growth and Proliferation and Cell Morphology as the number one ranked network, in which, TGF-β1 played a central role. Moreover, hormone replacement therapy using estradiol in postmenopausal women increased plasma levels of TGF-β1 (Djurovic et al., 2000) suggesting a possible mechanism for the vasculoprotective effect of estradiol. In this regard, TGF-β1 levels has been also involved in the anti-atherogenic effect of estradiol in apoE-/- hypercholesteraemic mice (Gourdy et al., 2007).

On the other hand, our results showed **miR-1244** as the most decreased miRNA in endothelial cells exposed to estradiol. miR-1244 was found among down-regulated miRNA in ER+ compared to ER- breast tumours. Similar results were observed in ER+ breast cancer

cells compared to ER- breast cancer cells (Cizeron-Clairac et al., 2015) suggesting the role of estrogen receptor decreasing miRNA-1244. miR-1244 has been recently related to cancer progression since overexpression using mimics can reduce the growth (Zhang et al., 2015b) and migration rate (Li et al., 2016b) in lung cancer cells, which could be related to the demonstrated role of estradiol in increasing migration and proliferation of endothelial cells (Oviedo et al., 2011).

miRNA sequences can be located in intronic regions of non-coding or coding transcripts, but it can be also encoded by exonic regions. Precursor genomic location of selected estradiol-dependent miRNA showed that hsa-miR-501-3p, hsa-miR-378h and two of the precursors of hsa-miR-1244 coding sequences have an intragenic location.

As described in Introduction section, the first step in miRNA biosynthesis is the cleavage of pri-miRNA by DROSHA-DGCR8 Microprocessor complex. Processing of a miRNA located in an intronic region occurs co-transcriptionally with the transcription of the host gene. Microprocessor complex can associate with spliceosome machinery and produce both pre-miRNA and the spliced mRNA (Kim and Kim, 2007). In the case of exonic miRNA, miRNA processing disrupts the exon of unspliced transcript and affect final protein formation. However, spliced transcripts can also be processed either to form a pre-miRNA or to be exported into the cytoplasm in order to further protein synthesis (Slezak-Prochazka et al., 2013).

The expression of **host genes** showed no significant differences in mRNA data obtained by microarray of HUVEC exposed to 1 nmol/l estradiol for 24 hours. However, some comments about data are described in the following paragraphs.

CLCN5, the miR-501-3p host gene, is one of the members of the CLCN family of voltage-dependent chloride channel genes. CLCN proteins are plasma membrane Cl-/H+ antiporter that have been related to cell volume and intracellular pH regulation, but also are involved in immune reactions, cell migration and proliferation. Their role in regulation of apoptosis and transmembrane superoxide flux have been described in endothelial cells (Hawkins et al., 2007; Liu et al., 2013). Although microarray data expression of CLCN5 was not differentially significant (p value = 0.08), expression values in estradiol-treated cells are decreased (Fold change = -1.41). Interestingly, CLCN5 has also been predicted as miR-501-3p target according to TargetScan 6.2. Therefore, it could represent a negative feedback loop between miRNA and its host gene expression which has not yet been explored. However, miR-501 expression has already been correlated with its host gene in muscle cells (Mizbani et al., 2016).

As mentioned before, miRNA genes can be located together in a close proximity region and constitute a polycistronic transcription unit. miRNA clusters can contain from two to seven genes separated by short nucleotide sequence which are usually transcribed at the same time. In particular, miR-501 is located within the miR-532/188 cluster which encodes 7 individual miRNA (miR-532, miR-188, miR-

500a, miR-362, miR-501, miR-500b, miR-660). However, we analyse the expression of the other miRNA in our miRNA microarray, and we observed that none of the other clustered miRNA were significantly expressed by estradiol exposition. Possibly, post-transcriptional mechanisms might dictate the preferential expression of miR-501-3p compared with the other intronic miRNA.

Moreover, miR-1244 host gene Prothymosin α (**PTMA**), which is related to cell survival and proliferation functions, has previously been described as estrogen-responsive gene, since estradiol exposition of breast cancer cells increased PTMA expression via ER interaction with two consensus estrogen response elements (Martini and Katzenellenbogen, 2001). However, decreased PTMA expression has also been demonstrated in ER+ tumours compared to ER- ones (Cizeron-Clairac et al., 2015). It is important to note that PTMA is related to increase of ER transcriptional activity by inhibition of REA (repressor of estrogen receptor activity) (Ueda et al., 2012).

5.2 Role of ER in miRNA expression.

Estradiol genomic effects are mainly mediated through the classical nuclear **receptors** ERα and ERβ, which function as ligand-activated transcription factors. Furthermore, estradiol can bind to the transmembrane receptor GPER, that has been implicated in rapid non-genomic signalling but, also in transcriptional regulation through activation of other down-stream transcription factors (Prossnitz and Barton, 2011). HUVEC express both classical ERα and ERβ as well as

the transmembrane GPER (Addis et al., 2014; Sobrino et al., 2009). The role of estrogen receptors in miRNA regulation has been previously described in breast cancer (Bhat-Nakshatri et al., 2009; Castellano et al., 2009; Cizeron-Clairac et al., 2015). ER has been reported to regulate the expression of miRNA in response to its ligand estradiol (Bhat-Nakshatri et al., 2009).

In silico analysis of target gene-transcription factor associations predicted using known transcription factor binding site motifs provided information about ERE sites in the regulatory region of the estradiol-induced miRNA. ERE are 15 bp palindromic sequence with high affinity to ER which are conserved across species. Different studies have provided data about distribution of ER binding sites in the regulatory regions of estrogen-responsive genes. Genome-wide mapping of ERE have shown that most of receptor binding sites were located close to TSS (1 kb), but they also appeared up to 10 kb up-stream from TSS (Bourdeau et al., 2004). However, Carroll *et al.* found that only 4% of ER binding sites mapped were located to 1 kb promoter-proximal regions. Indeed, functional ERα binding sites have been described much further (up to 200 kb) from the estrogen-regulated genes (Carroll et al., 2006). Meanwhile, it has been described that, in general, ERβ-bound regions are located more closely to TSS (Liu et al., 2008).

Using specific ER agonists and antagonists, we have demonstrated the implication of ER in the selected estradiol-induced miRNA. Our results showed that estradiol-dependent regulation of all the selected miRNA is mediated via ER. Among miRNA analysed, most

of them were regulated by ERα. However, ERβ and GPER were also found to be involved in estradiol-regulated miRNA expression. Although physiological responses require a balance between the activity of all ER, several studies have reported differences in gene expression regulation mediated by either ERα or ERβ (Arnal et al., 2010; O'Lone et al., 2007). Indeed, it has been showed that ERα and ERβ can regulate genes in opposite directions (Lindberg et al., 2003). For instance, differential regulation of iNOS expression by ER has been reported, since estradiol treatment of VSMC increased iNOS expression through an ERβ-dependent mechanism whereas it was negatively regulated by ERα (Tsutsumi et al., 2008). However, estrogen action in endothelial cells is assumed to be mainly mediated by ERα. In this regard, physiological concentration of estradiol has been related in increased NO production (Chambliss and Shaul, 2002), PGI_2 release through COX-1/PGIS pathway (Sobrino et al., 2010), up-regulation of angiotensin 1-7 production (Mompeón et al., 2016) and endothelial cell migration (Sanchez et al., 2011), among others. Moreover, studies using ERα and ERβ KO mice revealed that beneficial effects of estrogens on vascular system are mediated by ERα (Arnal et al., 2010; Pare et al., 2002).

Taking together, we demonstrated that all the ER were involved in miRNA expression in estradiol-treated HUVEC and the specific ER implication is dependent of each miRNA. According to that, miRNA profiling between ER+ or ER- breast cancer cells revealed significant differences in expressed miRNA (Bailey et al., 2015; Cizeron-Clairac et al., 2015). In fact, Bailey et al. demonstrated that

ER binding sites located near miRNA are lost in differentially decreased miRNA in ER- breast cancer cell line (Bailey et al., 2015). At cardiovascular level, VSMC proliferation was inhibited through ER-dependent miRNA regulation (Zhao et al., 2013). Moreover, a protective role of estradiol-dependent regulation of miR-23a, whose regulatory regions contains ERα binding sites, has been observed in estrogen deficiency induced damages of cardiac gap junctions in rats (Wang et al., 2015b). On the other hand, the role of ERβ on miRNA expression has also been described. For instance, sex-specific miRNA expression via ERβ has been suggested in pressure overload-induced cardiac fibrosis (Queirós et al., 2013).

5.3 Integrated analysis of miRNA-mRNA pairings in estradiol-exposed HUVEC.

Joint analysis of miRNA and mRNA microarray data obtained using the same culture conditions (HUVEC exposed to 1 nmol/l estradiol for 24 hours in hormone-free medium) increased the process-specificity of predicted miRNA-mRNA interactions. Based on both target predictions and mRNA microarray data, functional characterization of miRNA-mRNA pairing results revealed more specific pathways in which estradiol-regulated miRNA could be involved.

As expected, some canonical pathways obtained using IPA software were also found in results obtained when using estradiol-dependent miRNA profile alone. For example, cellular movement appeared as one of the most important networks regulated by

miRNA-regulated estradiol. In this regard, Signalling by Rho family GTPases and Actin cytoskeleton signalling have been involved in the regulation of endothelial cell migration mediated by estradiol, since estradiol-treated HUVEC showed activated RhoA activity which in turn increased actin cytoskeleton formation and cell migration (Oviedo et al., 2011). In addition, it has been described that formation of focal adhesion complexes, constituted by integrins and other membrane-anchoring molecules, are necessary to actin remodelling and endothelial migration induced by estradiol (Sanchez et al., 2011). Moreover, pathways such as, Glucocorticoid receptor signalling, Insulin receptor signalling or IGF-1 signalling, were listed in canonical pathways of integrated analysis. These results are in accordance with those obtained by miRNA profile analysis, since energy metabolism networks were highlighted as regulated by estradiol-dependent miRNA. In this regard, sex differences in energy balance suggest the role of estradiol. Indeed, estradiol has been involve in increased insulin-stimulated glucose uptake in skeletal muscle and glucose uptake in the heart (Murphy and Steenbergen, 2014). However, although the link between estradiol-regulated miRNA and glucose metabolism has been less studied in endothelial cells, it has been reported that miR-222-mediated inhibition of ERα is involved in glucose uptake in adipocytes (Shi et al., 2014). Finally, other pathways such as Molecular mechanisms of Cancer, Production of NO and ROS in macrophages and Role of macrophages, fibroblasts and endothelial cells in rheumatoid arthritis were also found in functional miRNA microarray analysis.

ERK/MAPK signalling was the number one ranked pathway after we performed integrated analysis. Indeed, rapid membrane-associated ER actions have been described to be mediated by different kinase cascade pathways acting as a second messenger, such as PI3K/AKT, cAMP, PKA, PKC and also ERK/MAPK (Hayashi and Yamaguchi, 2008). ERK/MAPK pathway is constituted by serine/threonine protein kinases that respond to extracellular stimuli, such as mitogens, growth factors and stress signals. ERK/MAPK cascades modulate different cell functions, such as cell cycle, proliferation, cell survival and differentiation and also apoptosis. Regarding cardiovascular system, ERK/MAPK pathway are involved in cell growth, migration, apoptosis, contraction and inflammation (Muslin, 2008).

Relationship between estrogen action and MAPK pathway was first described in mid 90s, when it was demonstrated that ER activity was modulated by a specific MAPK-dependent phosphorylation (Kato et al., 1995). Indeed, a crosstalk between both pathways has been reported, since rapid estradiol activation of MAPK signalling was first described in breast cancer cells (Migliaccio et al., 1996), but also in endothelial cells where estradiol rapidly activates eNOS through a MAPK-mediated mechanism (Chen et al., 1999). However, all mentioned studies reported a transient induction (minutes) of MAPK activity when cells are exposed to estradiol. In fact, ERK/MAPK activity duration has been described to be crucial in the final outcome of estrogen actions. In this regard, it has been reported differences between transient versus sustained activation of

ERK/MAPK pathway by estradiol. For instance, estradiol induce pro-survival ERK activation in osteoblasts, whereas increase apoptosis by sustained ERK phosphorylation in osteoclasts (Chen et al., 2005).

Different role of ER on regulating specific ERK/MAPK pathway has been identified using selective ER agonists (Shen and Shi, 2016). Activation of MAPK pathway in vascular wall has been described to be specific of ER and cell type. Indeed, it has been observed that estradiol induction of MAPK pathway in endothelial cells was mediated by ERα, whereas MAPK activity was reported to be reduced via ERβ in VSMC (Geraldes et al., 2003). Moreover, opposite actions have been reported in ERK activation depending on the ER stimulated, since down-regulation of ERα in endothelial cells reversed estradiol-dependent inhibition of ERK phosphorylation to stimulatory effects. This finding was abrogated by using G15 before estradiol treatment, suggesting a role for GPER in the activation of ERK pathway (Ding et al., 2015).

Finally, functional characterization of miRNA-mRNA pairings assigned to cardiovascular signalling revealed 14 canonical pathways. Among them, there are key pathways in the maintenance of vascular tone such as, eNOS and ET-1 signalling. Indeed, the protective action of estrogens in cardiovascular system has been attributed in part to its effect on these signalling pathways, as we mentioned in Introduction section. In addition, the role of estrogen in endothelial renin-angiotensin signalling has also been described, since estradiol, acting through ERα, increased angiotensin-(1-7) production by increasing

angiotensin converting enzymes expression and activity in endothelial cells (Mompeón et al., 2016).

5.4 Role of estradiol-induced miR-30b-5p in ELK4/ET-1 pathway down-regulation.

miR-30b-5p was the most up-regulated miRNA in HUVEC exposed to estradiol. There are five miR-30 in human genome (miR-30a, miR-30b, miR-30c, miR-30d and miR-30e). They are encoded by 6 genes which are located on 3 different human chromosomes and all of them are enclosed in a miRNA family with same seed sequence motif (-GUAAACA-). Specifically, miR-30b and miR-30d are located closed to each other in the same cluster. Therefore, as mentioned by Bridge *et al*., the levels of redundancy of miR-30 suggest a critical functional role for this miRNA family (Bridge et al., 2012).

Integrated analysis revealed inversely correlated miR-30b-5p-mRNA target associations. Among them, most differentially expressed based on their fold change were validated by qRT-PCR using estradiol treatment and miR-30b mimic. Although ELK4 was selected to further studies, comments of some validated miR-30b targets are mentioned in the following lines.

MDM4, along with its homolog MDM2, are known to be negative regulators of p53 activity. In addition, MDM4 has been involved in gene expression regulation through modulation of different transcription factors (Biderman et al., 2012). Role of MDM4 in endothelial cell has been little studied. It has been reported that endothelial cell-specific MDM4 KO mice showed decreased eNOS

phosphorylation in the aorta (Yokoyama et al., 2014). Moreover, loss of MDM4 in cardiomyocytes has been implicated in dilated cardiomyopathy in mice. Indeed, sex differences were observed in the same study, since this occurs earlier in male than in female mice, suggesting hormonal regulation (Xiong et al., 2007). In this regard, it has been described a crosstalk between ERα and MDM4 in breast cancer. In fact, Swetzig *et al.* have recently reported a negative feedback loop between both molecules, in which ERα induces MDM4 expression, and in turn, MDM4 downregulates ERα expression (Swetzig et al., 2016). In addition, ERα has also been described as regulator of p53 transcriptional activity and appears to be gene-selective by recruiting transcription corepressors or coactivators to the p53-binding sites in the promoter region of p53-target genes (Berger et al., 2013; Liu et al., 2006). Nevertheless, the specific role of reduced MDM4 expression observed in endothelial cells exposed to estradiol has to be elucidated.

On the other hand, another miR-30b target predicted with our integrated analysis was **PPP3CA**, one of the members of catalytic subunit of calcineurin. According to our findings, it has been described that miR-30 family members regulate Ca^{2+}/Calcineurin signaling by direct targeting calcineurin members in podocytes and cardiomyocytes (Wu et al., 2015). Activated PPP3CA has been associated to nucleus translocation of NFAT members. As described before, the role of NFAT in cardiac hypertrophy was among the most significant pathways regulated by estradiol-dependent miRNA. In addition, estrogen action on PPP3CA expression has already been

reported, since estradiol exposition reduced PPP3CA expression in cardiomyocytes (Donaldson et al., 2009). Indeed, in the same study, Donaldson et al. described the role of estrogens in the attenuation of PPP3CA/NFAT pathway in a mice model of cardiac hypertrophy.

Finally, **ELK4** is a member of the ETS family of transcription factors. All ETS family members are identified through a highly conserved DNA binding domain, the ETS domain. ETS family members are phosphoproteins involved in many biological processes through direct regulation of the transcription of down-stream genes. In that sense, ETS proteins have been involved in apoptosis, differentiation and survival, haematopoiesis, cell migration, proliferation and angiogenesis, wound healing, and inflammation (Sharrocks, 2001).

Additionally, ETS domain has also been involved in protein-protein interaction. Indeed, a MAPK targeting motif has been found in the ETS domain. MAPK proteins interact with ETS family members and modulate its activity by direct phosphorylation. Moreover, it has been described an association between different MAPK proteins and each ETS members. Specifically, it has been reported that ELK4 is better phosphorylated by ERK and p38 kinases than by JNK (Selvaraj et al., 2015).

Furthermore, ELK4, first known as serum response factor (SRF) associated protein (SAP)-1, is member of ternary complex factor (TCF) subfamily. In addition to ELK4, there are two other TCF: ELK1 and ELK3/SAP-2/Net. ELK1 was first described in nuclear extracts where it formed a protein complex with two molecules of SRF on the serum response element (SRE) located in the regulatory

region of FOS gene (Shaw et al., 1989). On the other hand, ELK3, unlike ELK1 and ELK4, has been described to be a strong repressor of gene transcription (Buchwalter et al., 2004). TCF-SRF complexes regulate transcription of immediate early genes, such as *c-myc, c-fos, c-jun*, by binding to the SRE sequence in their promoter region. However, TCF binding on gene regulatory regions has also been reported to be independent of SRF interaction (Buchwalter et al., 2004).

Consistent with those described above, **EGR-1**, another well-studied member of immediate early genes (O'Donnell et al., 2012), was found among estradiol-dependent down-regulated genes with predicted ELK4 binding site. Indeed, reduced EGR-1 mRNA and protein expression has been reported in ELK4-deficient cells (Costello et al., 2004), result that supports our findings. However, regarding estradiol action, rapid estradiol-induced proliferation has been involved with immediate early genes up-regulation, such as FOS, JUN and EGR-1 (Chen et al., 2004; Gopal et al., 2012). Nevertheless, Boverhof *et al.* described a temporal-dependent gene expression changes in response to estradiol (Boverhof et al., 2004). Specifically, some immediate early gene members were rapidly induced by estradiol, with a peak at 2-4 hours, and gradually decreased using longer period time of estradiol exposition. Results that are in accordance with those mentioned before about estradiol-mediated induction of MAPK pathway. On the other hand, increased EGR-1 activity has been described in response to different pathophysiological stimuli through which it induces the expression of

down-stream genes involved in inflammatory processes such as cytokines (TNFα) and adhesion molecules (ICAM-1) (Fu et al., 2003; Khachigian and Collins, 1997). In addition, estradiol has been related to inhibit pro-inflammatory action of immediate early genes such as JUN (Srivastava et al., 1999). Moreover, a reciprocal activity repression has been reported between ERα and EGR-1 (Vlaeminck-Guillem et al., 2003).

Although little is known about **cellular functions regulated by ELK4 activity**, some studies have provided information by using genome-wide screening techniques. In that sense, microarray analysis of ELK4 knockdown cells revealed implication of ELK4 target genes in cellular growth, proliferation and cell morphology among the most regulated functions (Wozniak et al., 2012). Moreover, Gene Ontology analysis of genes regulated by ELK4 transcriptional activity identified by using CHIP-seq technology showed response to stress and external infection among ELK4-regulated functions in macrophages (Xie, 2014). In addition, ELK4 has been related to immune suppression since ELK4-deficient mice showed impaired thymocyte positive selection (Costello et al., 2004).

To the best of our knowledge, there is no information about regulation of ELK4 by estrogens. However, ELK4 up-regulation has been reported to be induced by testosterone (Makkonen et al., 2008), suggesting a role of sex hormones in the regulation of ELK4 activity.

Another putative ELK4 down-stream gene regulated in estradiol-treated HUVEC is **ET-1**. As described before, ET-1 is a potent

vasoconstrictor released by endothelium and a key component in vascular tone regulation. Relationship between estradiol and ET-1 activity has been described. In endothelial cells, release of ET-1 in basal conditions has been observed to be reduced when they are exposed to estradiol. In addition, ET-1 induction observed in endothelial cells exposed to different ET-1 inducers agents, such as TNFα, thrombin, AngII and cyclic strain, was also reverted by previous estradiol exposition (Dubey et al., 2001; Juan et al., 2004).

Although the role of estradiol in ELK4-dependent regulation of ET-1 has not been described, ELK4 up-stream activator MAPK pathway components has been reported. In this regard, ET-1 production increased after cyclic strain treatment of endothelial cells has been described to be mediated through ERK activation (Cheng et al., 2001). In addition, estradiol treatment of strain-exposed cells abrogated induced ET-1 expression interfering with ERK pathway (Juan et al., 2004). Furthermore, these results were mediated by ER since estradiol-mediated effects were abrogated when cells were exposed before to the unspecific antagonist ICI 182780 (Dubey et al., 2001; Juan et al., 2004).

5.5 Relevance of obtained results in endothelial dysfunction and cardiovascular diseases.

Our results provide a new insight into the mechanism by which estradiol could regulate ET-1 expression. The balance between different vasoactive molecules released by endothelium ensures a correct vascular homeostasis. However, deregulation of endothelial balance leads to endothelial dysfunction and cardiovascular pathology. In this regard, altered ET-1 expression has been related to endothelial dysfunction.

Endothelial dysfunction has been described as loss of NO-mediated vasodilation in response to acetylcholine or simply as the decreased synthesis, release or activity of endothelium-derived NO (Liao, 2013). It has been described that ET-1 action impairs NO production in endothelial cells through down-regulation of eNOS expression (Ramzy et al., 2006). Moreover, ET-1 can decrease NO bioavailability by enhancing superoxide production through regulation of NADPH-oxidase and NOS uncoupling (Loomis et al., 2005).

In addition to its vasomotor effects, ET-1 has been associated to pro-inflammatory processes in vascular tissue (Bohm and Pernow, 2007). Increased inflammatory cytokines release has been mediated by ET-1 through activation of NF-kB transcription factor (Kowalczyk et al., 2015). Moreover, ET-1 increased expression of adhesion molecules in cell membrane of endothelial cells and, in turn, an enhanced adhesion of leukocytes to endothelium has been described

in response to ET-1 exposition (Sanz et al., 1999), suggesting the role of ET-1 in endothelial activation.

It is worth to mention the relationship between both endothelial dysfunction and activation processes. As described before, endothelial activation consists in cellular changes, such as release of chemokines and cytokines and increase expression of adhesion molecules in the cell surface, which enables to perform a host defence response. Then, endothelial activation acts as an endogenous anti-inflammatory system. However, prolonged exposure to injured stimuli can result in the exhaustion of the anti-inflammatory system and endothelium becomes dysfunctional (Deanfield et al., 2007).

The concept of endothelial dysfunction, in addition to the loss of endothelial-dependent vasodilation, describes the failure of endothelial cells to perform their normal homeostatic functions (Pober et al., 2009) and is characterized by an altered control of coagulation processes, inadequate perfusion, vascular leak and inflammation, all of them associated with poor prognosis of vascular diseases. Endothelial dysfunction is the first step in the chain of events that leads to atherosclerosis, hypertension and coronary disease. Accordingly, endothelial dysfunction has been postulated as a hallmark, and indeed a predictor of cardiovascular diseases (Vanhoutte et al., 2017).

Increased plasma levels of ET-1 was described in patients with hypertension early in the 90's (Saito et al., 1990). Since then, increased ET-1-mediated action has been implicated in the

development of different cardiovascular diseases, such as atherosclerosis, pulmonary hypertension, stroke, abdominal aortic aneurysms and coronary artery disease (Davenport et al., 2016; Miyauchi and Masaki, 1999). Indeed, ET-1 administration inhibited endothelium-dependent vasodilation in healthy patients, and ET receptor blockade significantly reduced loss of endothelium-dependent vasodilation in patients with atherosclerosis (Bohm et al., 2002). Moreover, inhibition of ET effect by using ET receptor antagonists improve endothelial function in coronary arteries of patients with coronary artery disease (Halcox et al., 2007) and decreased blood pressure in hypertensive patients (Goddard et al., 2004). However, clinical use of ET antagonists is limited and has been approved only for primary pulmonary hypertension (Maguire and Davenport, 2015).

The role of miR-30b family members in cardiovascular diseases has been described with contradictory results. Expression of miR-30 family members is decreased by balloon angioplasty injury in rat carotid arteries, and overexpression of miR-30b was related to inhibit SMC proliferation and neointima formation through directly targeting CaMKIIδ (Liu et al., 2016). In the case of myocardial infarction, down-regulated miR-30a has been related to myocardial hypertrophy (Pan et al., 2013) whereas up-regulation of miR-30c/miR-30d expression has pro-hypertrophic potential (Jentzsch et al., 2012), and miR-30b overexpression aggravated myocardial infarction injury (Shen et al., 2015). Meanwhile, miR-30b was found among down-regulated miRNA in angiotensin II-induced hypertensive

rat heart (Eskildsen et al., 2013). In addition, it has recently described lower expression of circulating miR-30 in hypertensive compared with healthy individuals (Huang et al., 2016). Indeed, miR-30 levels inversely correlated with blood pressure parameters and carotid intima-media thickness in the same study. These findings suggest a different modulation of specific members of miR-30 family in cardiovascular diseases.

Cardiovascular sex differences have been extensively reported (Mendelsohn and Karas, 2005), suggesting an important influence of sex hormones in cardiovascular system. Regarding ET-1 expression, estradiol decreases whereas testosterone increases ET-1 expression in endothelial cells. In addition, ET-1-mediated vasoconstriction was attenuated by estradiol, whereas testosterone enhanced the effect (Dubey et al., 2002; Tostes et al., 2007). Sex differences in MAPK pathway have also reported since female showed less activation of p38 MAPK signalling pathway in a cardiac hypertrophy model, that was associated to more protection compared to males (Mahmoodzadeh et al., 2012). In addition, differential action of sex hormones has also been reported, since specific MAPK-dependent responses have been reported in HUVEC treated with estradiol and testosterone. Short-term exposition to estradiol induced cell proliferation through ERK2 activation, whereas testosterone increased apoptosis via p38 and JNK activation (Powazniak et al., 2009).

Considering beneficial role of estrogens, **hormonal replacement therapies** have been used in postmenopausal women

with controversial findings (Rossouw et al., 2007; Simon et al., 2001). Actual consensus in the vascular protective effects of estrogens depend on the initiation of estrogen therapy after menopause (Lobo, 2017). The phenomenon was referred to as the "timing hypothesis", and it postulates that the beneficial effects of estrogen preventing cardiovascular disease may occur only when estrogen supplementation is initiated before detrimental effects of aging on cardiovascular system are established (Clarkson et al., 2013). In this regard, it has been reported that age moderates vasodilatory (Sherwood et al., 2007) and anti-inflammatory (Novella et al., 2012) effects of estrogens on vascular tissue in postmenopausal women.

As described before, hormone replacement therapy effects have also been attributed to be mediated by miRNA activity in skeletal and muscle tissues (An et al., 2014; Olivieri et al., 2014). Moreover, specific circulating exosome miRNA profile has been associated to serum levels of estradiol in postmenopausal women using hormone replacement therapy (Kangas et al., 2017). Altogether, results suggest a role of estradiol in the regulation of tissue miRNA expression and also in the release of miRNA-containing exosomes to the circulation during hormone replacement therapy.

miR-30b-5p expression was down-regulated in serum samples from ovariectomized rats (Chen et al., 2016), suggesting a role of sex hormones in miR-30b-5p expression regulation. These findings are in accordance with our obtained results using estradiol-exposed endothelial cells. In addition, serum miR-30b-5p expression was also found to be down-regulated in postmenopausal

osteoporotic woman (Chen et al., 2016), a disease related with decreased levels of estrogens (Richelson et al., 1984).

Regarding ET-1, different data have demonstrated that enhanced vasoconstrictor tone observed with aging is related to an increase in ET-1 system activity (Stauffer et al., 2008). In addition, it has been reported that hormone replacement therapy in postmenopausal women significantly decreased plasma ET-1 levels (Saitta et al., 2001), result that may contribute to explain the vasoprotective role of hormonal supplementation. Furthermore, despite some discrepancies in delivery system or hormone composition, most of studies indicate neutral of beneficial effect on blood pressure in normotensive as well as in hypertensive postmenopausal women using hormone replacement therapy (Cannoletta and Cagnacci, 2014). Concerning the role of miR-30 family members, it has been described lower miR-30c levels in hypertensive compared to normotensive white woman in a recent work about racial differences in miRNA expression in hypertensive woman (Dluzen et al., 2016).

In summary, obtained results provide for the first time an estradiol-mediated miRNA expression profile in endothelial cells. In addition, identification of biological processes related to estradiol-regulated miRNA and the role of ER in miRNA expression has also been described. Our results may contribute to future investigations focused on characterizing in detail the role of specific miRNA in vascular actions mediated by estradiol and provide insight into the

mechanisms by which sex hormones levels can contribute to sex-differences in cardiovascular diseases.

Considering that cardiovascular diseases are a worldwide health problem and are the leading cause of mortality in developed countries (WHO, 2016) (particularly, 29.4 % of the mortality are due to cardiovascular diseases in Spain, according to the latest available data (INE, 2017)), the results obtained in the present work lead to deeper understanding endothelial functions which are important in the development of cardiovascular pathologies.

Specifically, the regulation ET-1 by estradiol-dependent miR-30b-5p has also been described in the present study. Therefore, due to an increasing evidence suggesting the implication of ET-1 activity in endothelial dysfunction, atherosclerosis and hypertension, our results could open up an unexplored field by using miR-30b-5p as a new therapeutic approach aimed to improve endothelial function.

6. CONCLUSIONS

1. Physiological concentration of estradiol induces changes in the miRNA expression profile in human endothelial cells. 114 miRNA were regulated by estradiol, including 70 up-regulated and 44 down-regulated miRNA. Functional analysis reveals that estradiol-modulated miRNA are associated to key molecular pathways, which are important to regulate vascular physiology in health and disease.

2. Changes in miRNA expression induced by estradiol are mediated through estrogen receptors. Most of selected estradiol-regulated miRNA are regulated by ERα (miR-30b-5p, miR-487a-5p and miR-378h). ERβ and GPER control miR-501-3p and miR-4710 expressions, respectively. miR-1244 regulation could be mediated by different ER.

3. Integrated analysis using miRNA and mRNA microarray data increases the process-specificity of predicted miRNA-mRNA pairings. Functional characterization highlights ERK/MAPK signalling among canonical pathways in which estradiol-regulated miRNA are involved.

4. Estradiol regulates gene expression of different miR-30b-5p predicted targets, including the transcription factor ELK4. ET-1 was found among putative estradiol-regulated genes with ELK4 binding sites. Decreased ELK4 and ET-1 expression mediated by miR-30b-5p suggests a role of miR-30b-5p/ELK4 association in estradiol-dependent inhibition of ET-1 in endothelial cells

1. Concentraciones fisiológicas de estradiol inducen cambios en el perfil de expresión de miRNA en células endoteliales humanas. 114 miRNA fueron regulados por estradiol, de los que 70 aumentaban y 44 disminuían su expresión. El análisis funcional revela que los miRNA modulados por estradiol están asociados a rutas moleculares fundamentales en la regulación de la función vascular tanto a nivel fisiológico como fisiopatológico.

2. Los cambios inducidos por estradiol en la expresión de miRNA están mediados por receptores de estrógenos. La mayoría de los miRNA regulados por estradiol que fueron seleccionados están regulados por el REα (miR-30b-5p, miR-487a-5p y miR-378h). El REβ y GPER controlan la expresión de miR-501-3p y miR-4710, respectivamente. La regulación del miR-1244 podría estar mediada por diferentes receptores de estrógenos.

3. El análisis integrado de los datos obtenidos mediante microarrays de expresión de miRNA y de mRNA incrementa la especificidad del proceso de predicción de las parejas miRNA-mRNA. La caracterización funcional de estas interacciones destaca la ruta de señalización ERK/MAPK entre las rutas canónicas en las que el estradiol, a través de la regulación de miRNA, estaría implicado.

4. El estradiol regula la expresión de diferentes dianas pronosticadas del miR-30b-5p, entre las que se encuentra el factor de transcripción ELK4. La ET-1 aparece entre los posibles genes regulados por estradiol que tenían sitios de unión predichos para ELK4. La

disminución en la expresión de ELK4 y ET-1 mediada por miR-30b-5p sugiere la participación de este miRNA y de su diana ELK4 en la inhibición de expresión de ET-1 causada por estradiol en células endoteliales.

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