REGULATION OF ANGIOGENESIS, BREAST CANCER AND INFLAMMATION BY ANGIOPOIETIN-1

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I. ABSTRACT

Angiogenesis and inflammation are hallmarks of several pathologies including breast cancer. The angiopoietins and Tie receptors have emerge as alternative targets for therapeutical intervention due to their function as essential regulators of angiogenesis, vascular homeostasis and inflammation. Angiopoietin-1 (Ang-1), the main agonist of Tie-2 receptors, promotes vessel growth, inhibits inflammation and maintains vessel stability. Although important advances have been made in understanding the functions of Ang-1 in the vasculature the intracellular signaling pathways activated by Ang-1, as well as its role in breast cancer and inflammation, remain largely unexplored.

Using human umbilical vein endothelial cells (HUVECs), we identified dual-specificity phosphatases (DUSPs) that negatively regulate mitogen-activated protein kinase (MAPK) signaling pathways activated by Ang-1. Specifically we found that Ang-1 increased the expression (mRNA and Protein), as well as the activity of DUSP1, DUSP4 and DUSP5. Knocking down these phosphatases using siRNA revealed that DUSP1 mainly inactivates p38, DUSP4 dephosphorylates ERK1/2, p38 and SAPK/JNK, and DUSP5 is ERK-specific. Furthermore DUSP1, DUSP4 and DUSP5 had distinct functions in Ang-1-dependent survival and migration of endothelial cells (ECs).

Because of the importance of angiogenesis in tumor progression, we then investigated the influence of estradiol (E2) on the expression of angiopoietins in breast cancer cell lines. We found Ang-1 mRNA and protein expressions to be lower in estrogen receptor (ERα) positive cells than in ERα negative cells. Additionally, we observed that both tumor size and Ang-1 production were reduced in ERα+ cell-derived xenografts in mouse mammary pads when

compared to those derived from ERα- cells; and this effect was inhibited when the mice were ovariectomized.

Since a large portion of the genome is under the regulation of microRNAs (miRNAs), we hypothesized that Ang-1 induces the expression of miRNAs to protect the endothelium against *E. Coli* lipopolysaccharide (LPS)-induced inflammation. We found that treating HUVECs for 24h with Ang-1 reduced LPS-induced phosphorylation of p38 and SAPK/JNK, and the activation of nuclear factor kappa b (NF-κB). Ang-1 also decreased the expression of pro-inflammatory cytokines, adhesion molecules and leukocyte adhesion *in vitro*. Our findings suggest that miR-146b-5p is induced by Ang-1 as a mechanism to control Toll-like receptor (TLR) 4 signaling and the expression of pro-inflammatory mediators by targeting the signaling proteins interleukin-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6).

We conclude that Ang-1 induces the expression of DUSP1, DUSP4 and DUSP5 to coordinate its anti-apoptotic and migratory response. Ang-1 is also an important modulator of growth and progression of ERα- breast cancers. Finally, Ang-1 treatment antagonizes pro-inflammatory pathways activated by LPS through the induction of miR-146b-5p which targets the TLR signaling proteins IRAK1 and TRAF6.

II. RESUMÉ

L'angiogenèse et l'inflammation sont caractéristiques de plusieurs pathologies, dont le cancer du sein. Les angiopoiétines et les récepteurs Tie ont émergé comme cibles d'intervention thérapeutique alternatives en raison de leur fonction de régulateurs essentiels de l'angiogenèse, de l'homéostasie vasculaire et de l'inflammation. L'angiopoiétine-1 (Ang-1), l'agoniste principal du récepteur Tie-2, favorise la croissance des vaisseaux, inhibe l'inflammation et maintient la stabilité vasculaire. Bien que des progrès importants aient été réalisés dans la compréhension des fonctions d'Ang-1 dans le système vasculaire, les voies de signalisation intracellulaires activées par Ang-1 ainsi que son rôle dans le cancer du sein et l'inflammation sont largement inexplorées.

Utilisant des cellules endothéliales de veine ombilicale (CEVOH), nous avons identifié des phosphatases à double spécificité (DSPs) qui régulent négativement les voies de signalisation des protéines kinase activée par mitogènes (MAPKs) activées par Ang-1. Plus précisément nous avons trouvé que l'Ang-1 a induit l'ARNm, l'expression des protéines et l'activité de DSP1, DSP4 et DSP5. Réduire l'expression de ces phosphatases à l'aide de ARNi a révélé que DSP1 inactive principalement p38, DSP4 déphosphoryle ERK1/2, p38 et SAPK/JNK et DSP5 est spécifique pour ERK. En outre DSP1, DSP4 et DSP5 possèdent des fonctions distinctes pour la régulation de la migration, la survie, la formation de tube capillaire et la perméabilité vasculaire dépendante d'Ang-1.

En raison de l'importance de l'angiogenèse dans la progression tumorale, nous avons ensuite étudié l'influence de l'estradiol (E2) sur l'expression des angiopoiétines dans des lignées cellulaires du cancer du sein. Nous avons trouvé que le niveau de transcrits d'ARNm ainsi que l'expression protéique d'Ang-1 étaient réduits dans cellules positives pour les récepteurs des œstrogènes (ERα) par comparison aux cellules négatives pour ERα. En outre, nous avons

observé que la taille de la tumeur et la production d'Ang-1 étaient réduits dans de xénogreffes de tissu mammaire chez la souris dérivés de cellules ERα+, par rapport à ceux issus de cellules ERα–. De plus cet effet est inhibé lorsque les souris sont ovariectomisées.

Comme une grande partie du génome est sous le contrôle des microARNs (miARN), nous avons émis l'hypothèse que l'Ang-1 induit l'expression des miARNs pour protéger l'endothélium contre l'inflammation induite par le lipopolysaccharide (LPS) d'*E. Coli*. Nous avons constaté que le traitement de CEVOH pendant 24 heures avec Ang-1 réduit la phosphorylation de p38 et SAPK/JNK, ainsi que l'activation du facteur nucléaire kappa B (NF-κB) induite par le LPS. Ang-1 a également diminué l'expression de cytokines pro-inflammatoires, des molécules d'adhésion et l'adhérence des leucocytes *in vitro*. Nos résultats suggèrent que miR-146b-5p est induit par l'Ang-1 comme un mécanisme pour le contrôle de la signalisation des récepteurs de type toll (TLR) et l'expression de médiateurs pro-inflammatoires, en ciblant les protéines de signalisation IRAK1 et TRAF6.

Nous concluons que l'Ang-1 induit l'expression de DSP1, DSP4 et DSP5 à fin de coordonner son action anti-apoptotique et sa réponse migratoire et angiogénique. Ang-1 est également un modulateur important de la croissance et de la progression des cancers du sein ERα-. Enfin, le traitement avec Ang-1 antagonise les voies de signalisation pro-inflammatoires par l'induction de l'expression de miR-146-5p, qui cible des protéines de la voie de signalisation TLR: IRAK1 et TRAF6.

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IV. LIST OF PUBLICATIONS

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2- Echavarria R. and Hussain SN. Regulation of angiopoietin-1 signaling in endothelial cells by dual-specificity phosphatases 1, 4 and 5. *Submitted*

3- Ismail H, Mofarrahi M, **Echavarria R**, Harel S, Verdin E, Lim HW, Jin ZG, Sun J, Zeng H, Hussain SN. Angiopoietin-1 and vascular endothelial growth factor regulation of leukocyte adhesion to endothelial cells: role of nuclear receptor-77. Arterioscler Thromb Vasc Biol. 2012 Jul;32(7):1707-16

4- Echavarria R[1], Harfouche R[1], Rabbani SA, Arakelian A, Hussein MA, Hussain SN. J. Estradiol-dependent regulation of angiopoietin expression in breast cancer cells. Steroid Biochem Mol Biol. 2011 Jan;123(1-2):17-24

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V. CONTRIBUTIONS OF AUTHORS

Dr. Sabah N. Hussain was instrumental in the interpretation of data and the generation of the manuscripts. I am responsible for most of the experiments and the analysis carried out in the works comprised in this thesis. Dominique Mayaki provided technical assistance for the real-time PCR experiments throughout the work presented in this thesis. The co-authors contributed in the following ways:

Manuscript 2: Estradiol-dependent regulation of angiopoietin expression in breast cancer cells

Harfouche R. – Contributed with some of the experiments *in vitro*

Rabbani SA- Provided breast cancer cell lines

Arakelian A. and Hussein MA – Worked on the *in vivo* experiments

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Manuscript 1: Regulation of angiopoietin-1 signaling in endothelial cells by dual-specificity phosphatases 1, 4 and 5

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VII. LIST OF ABBREVIATIONS

ABIN-2: A20 binding inhibitor of NF-κB activation 2
AF: activation function
Ago-2: argonaute 2
Ang: angiopoietin
AP-1: activator protein 1
ARE: adenylate-uridylate (AU)-rich elements
ASK1: apoptosis signal-regulating kinase 1
ATF: activating transcription factor
BAD: Bcl-2-associated death promoter protein
Bax: Bcl-2-associated X protein
BMDC: bone marrow-derived cell
BMP: bone morphogenetic protein
bp: base pair
BRCA1: breast cancer 1
BSA: bovine serum albumin
CD: common docking
CH2: Cdc25 homology
Clo: cloche
CMV: cytomegalovirus
cPLA2: cytosolic phospholipases A2
CSF-1: colony stimulating factor 1
CT: comparative threshold
CXCR4: (C-X-C motif) receptor 4
DAPK: death-associated protein kinase
DBD: DNA-binding domain
DGCR8: DiGeorge syndrome critical region gene 8
DLL4: delta-like ligand 4
DMEM: Dulbecco's Modified Eagle Medium
DNA: deoxyribonucleic acid
DN: dominant negative
DN-IKKβ: dominant-negative IkB kinase beta
dNTP: deoxyribonucleotide triphosphate
Dok-R: downstream of kinase-related
DUSP: dual-specificity phosphatase
E2: estradiol
EC: endothelial cell
ECL: enhanced chemiluminescence
ECM: extracellular matrix
EDTA: ethylenediaminetetraacetic acid
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
Egr-1: early growth response 1
ELISA: enzyme-linked immunosorbent assay
EMT: epithelial-mesenchymal transition
eNOS: endothelial nitric oxide synthase
EPC: endothelial precursor cell
Eph: ephrin
ER: estrogen receptor
ERα-: estrogen receptor alpha negative
ERα+: estrogen receptor alpha positive

ERK1/2: extracellular signal regulated kinase 1 and 2
ET-1: endothelin 1
FADD: Fas-associated protein with death domain
FAK: focal adhesion kinase
FBS: fetal bovine serum
FCS: fetal calf serum
FGF: fibroblast growth factor
FITC: fluorescein isothiocyanate
FOXO1: forkhead box protein O1
GAP: GTPase-activating protein
GAPDH: glyceraldehyde phosphate dehydrogenase
GARG16: glucocorticoid attenuated response gene 16
GDP: guanosine-5'-diphosphate
GEF: guanine exchange factor
GFP: green fluorescent protein
GnRH: gonadotropin-releasing hormone
GPCR: G protein coupled receptor
GR: glucocorticoid receptor
GRE: glucocorticoid responsive element
Grb2: growth factor receptor-bound protein 2
GSK3β: glycogen synthase kinase-3 beta
GTP: guanosine-5'-triphosphate
HB-EGF: heparin-bound epidermal
HBSS: Hank's balanced solution
HEPES: growth factor 4-(2-hydroxy ethyl)-1-piperazineethanesulfonic acid
HER2: human epidermal growth factor receptor 2
HMEC: human mammary epithelial cells
HGF: hepatocyte growth factor
HRP: horseradish peroxidase
HSPG: heparin sulfate proteoglycan
HUVEC: human umbilical vein endothelial cell
iNOS: inducible nitric oxide synthase
ICAM1: intercellular adhesion molecule 1
Ig: immunoglobulin
IκBα: IkappaBalpha
IKK: IkappaB kinase
IL-1β: interleukin 1 beta
IL-1R: interleukin 1 receptor
IL-2: interleukin 2
IL-6: interleukin 6
IL-8: interleukin 8
IL-33 : interleukin 33
INF: interferon
IP10: interferon gamma-induced protein 10
IRAK1: interleukin-1 receptor-associated kinase 1
IRF3: interferon regulatory factor 3
JNK1/2: c-jun nuclear kinase 1 and 2
KIM: kinase interaction motif
KLF2: Kruppel-like factor 2
LBD: ligand-binding domain
LBP: lipopolysaccharide binding protein
LHRH: luteinizing hormone-releasing hormone

LLC: Lewis lung carcinoma
LPS: lipopolysacharide
MAPK: mitogen-activated protein kinase
MAPKK: mitogen-activated protein kinase kinase
MAPKKK: mitogen-activated protein kinase kinase kinase
mDIA: mammalian diaphanous
MEF2: myocyte enhancer factor 2
MEMα: minimum essential medium alpha
miRNA: microRNA
MK2: mitogen activated protein kinase-associated protein kinase 2
MKP: mitogen-activated protein kinase phosphatase
MLC: myosin light chain
MLCK: myosin light chain kinase
MMP-2: matrix metalloproteinase 2
MMP-16: matrix metalloproteinase 16
MOI: multiplicity of infection
NFAT: nuclear factor of activated T cells
NF-κB: nuclear factor kappa b
NGF: nerve growth factor
NLS: nuclear localization signal
NO: nitric oxide
NOD: nucleotide-binding oligomerization domain
NOS: nitric oxide synthase
Nox4: NADPH oxidase 4
NSCLC: non-small-cell lung carcinoma
Nur77: nuclear receptor 77
OVX: ovariectomized
oxLDL: oxidized low-density lipoprotein
p85β: PI-3 kinase regulatory subunit 2
p120-RasGAP: p120-Ras GTPase activating protein
PACT: protein activator of PKR
PAF: platelet activating factor
PAI-1: plasminogen activator inhibitor-1
PAK1: p21-activating kinase
PAMPs: pathogen-associated molecular patterns
PAR: protease activated receptor
PBS: phosphate buffer saline
PCR: polymerase chain reaction
PDGF: platelet derived growth factor
PDGFR: platelet derived growth factor receptor
PGI_2: prostacyclin
PH: pleckstrin homology
PI: phosphoinositide
PI3-K: phosphoinositide 3-kinase
PKB: protein kinase B
PKCζ: protein kinase C zeta
PLCγ: phospholipase C gamma
PLZF: promyelocytic leukaemia zinc finger
PMA: phorbol 12-myristate 13-acetate
PMSF: phenylmethanesulfonylfluoride
***p*-NPP**: p-nitrophenyl phosphate
PPAR: peroxisome proliferator-activated receptor
PR: progesterone receptor

PRR: pattern recognition receptors
Pro: proline
Pre-miRNA: precursor microRNA
Pri-miRNA: primary microRNA
Pro: proline
PTB: phosphotyrosine binding
PTP: protein tyrosine phosphatase
PVDF: polyvinylidene difluoride
RGD: Arginine-Glycine-Aspartic acid
RISC: RNA-induced silencing complex
RIG-I: retinoid acid inducible gene I
RLU: relative luminescence units
RNA: ribonucleic acid
RNA Pol II: RNA polymerase II
ROS: reactive oxygen species
RTK: receptor tyrosine kinase
S1P: sphingosine-1-phosphate
SAPK/JNK: stress-activated protein kinase/Jun-amino-terminal kinase
SDS: sodium dodecyl sulfate
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE: standard error
Ser: serine
sFBS: charcoal-stripped FBS
SFK: Src family kinase
SH2: Src homology 2
SNP: short nucleotide polymorphism
shRNA: short hairpin ribonucleic acid
siRNA: small interfering ribonucleic acid
SOS: son of sevenless
STAT: signal transducer and activator of transcription
sTie2: soluble Tie-2
TAK1: TGF-beta activated kinase 1
TAM: tamoxifen
TF: tissue factor
TGF-β: transforming growth factor beta
Thr: threonine
TIR: Toll-IL-1 receptor
TLR: toll-like receptor
TNF-α: tumor necrosis factor alpha
TRAF: TNF receptor associated factor
TSC2: tuberous sclerosis protein 2
Tyr: tyrosine
UTR: untranslated region
UV: ultraviolet
VCAM1: vascular cell adhesion molecule 1
VEGF: vascular endothelial growth factor
VEGFR: vascular endothelial growth factor receptor
VE-cadherin: vascular endothelial cadherin
VE-PTP: vascular endothelial protein tyrosine phosphatase
vSMC: vascular smooth muscle cell
WM: wortmannin
WPB: Weibel-Palade bodies
WT: wild-type
XPO5: exportin 5

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Chapter 1

Literature review

1.1 Vascular endothelium: structure, function and development

1.1.1 Introduction

The complex design of vertebrates requires an efficient transport of gases, nutrients, signaling molecules, cells and metabolic waste between tissues and organs. These functions are carried out by the cardiovascular system, comprised of the heart and a highly branched network of vessels that circulate blood throughout the body [1]. The luminal surface of every vessel is lined with a monolayer formed by endothelial cells (ECs), also known as endothelium, that separates the blood from the extravascular space and actively regulates the exchange between these compartments [1, 2]. Several physiological functions are controlled by the endothelium and include vascular tone, blood flow, coagulation, platelet and leukocyte interactions, wound healing and angiogenesis [2-4].

Angiogenesis refers to the formation of new blood vessels from pre-existing ones [5]. It occurs physiologically during embryonic development, bone morphogenesis, the menstrual cycle and pregnancy [6]. Angiogenesis is also a hallmark of several pathologies including intraocular neovascular disorders, rheumatoid arthritis, psoriasis and cancer [6]. For this reason, understanding the molecular mechanisms that regulate EC biology and blood vessel formation is important to identify new therapeutic strategies for diseases that involve vessel growth. Vascular endothelial growth factor (VEGF) is one of the most important regulators of blood vessel formation and it exerts its actions through EC-specific receptor tyrosine kinases (RTKs), namely VEGF receptor 1, 2 and 3 (VEGFR-1, VEGFR-2 and VEGFR-3) [7, 8].

In the case of cancer several anti-angiogenic agents that target VEGF signaling pathways, including *bevacizumab*, *sunitinib* and *sorafenib*, have been used to treat the disease [9]. However, they result in only transient responses mainly due to the presence of redundant angiogenic factors, the heterogeneity of tumor cells and the inflammatory nature of the tumors [9, 10]. It has been suggested that therapeutic strategies in which multiple angiogenic and inflammatory signaling pathways are simultaneously targeted could translate into better clinical responses [10, 11]. It is in this context that the angiopoietin-Tie family of proteins, another EC-specific RTK system, emerges as an alternative target for therapeutical intervention not only in tumor progression, but also in other vascular and inflammatory pathologies [12]. The angiopoietins (angiopoietin-1,-2,-3 and -4) and Tie receptors (Tie-1 and Tie-2) are not only essential regulators of angiogenesis and vascular homeostasis, but they also provide a link between angiogenesis and inflammation [12, 13].

Angiopoietin-1 (Ang-1), an agonist of Tie-2 receptors, promotes vessel growth in sites of active remodeling while maintaining quiescence in adult tissues by promoting vessel integrity [13]. Important advances have been made in understanding the molecular mechanisms responsible for Ang-1 functions in the vasculature. However, little is yet known about the intracellular signaling pathways activated by Ang-1 in ECs. The general objective of this thesis is then to characterize the molecular mechanisms through which Ang-1 regulates EC function and to study its role in breast cancer progression and inflammation.

1.1.2 Structure and function of the vascular endothelium

The vasculature delivers oxygen and nutrients to all the tissues in the body in response to the rhythmic contractions of the heart [14]. The oxygenated blood is first carried away from the heart by the arteries, thick-walled vessels able to expand when the blood enters under pressure, and then circulates from the arteries to the arterioles [14]. The arterioles, small arteries able to contract or relax to regulate the amount of flow that reaches individual tissues, direct the blood into the capillaries [14]. The capillaries are very small vessels that interact closely with the tissues and serve as connectors between arteries and veins [14]. Finally, the deoxygenated blood from the capillary beds is returned to the heart through venules and veins [14]. The vasculature depends on both, the structural composition of each vessel and the heterogeneity of ECs to perform its physiological functions [15]. The structure of a vessel not only reflects its function but also the amount of pressure to which it is subjected [15]. In general, blood vessels are formed by three distinct layers: tunica intima, tunica media and tunica externa. The tunica intima is the internal layer of a vessel, mainly comprised of ECs. The tunica media is formed by concentric layers of smooth muscle and connective tissue that surround ECs and control vascular tone. The tunica externa is an outer layer of connective tissue that anchors the vessel to the adjacent tissues [14, 15].

The blood vessels are formed by two different types of cells: ECs and mural cells [16]. ECs are generally flat, quiescent cells able to form tight monolayers due to their ability to establish adherens and tight junctions with neighboring ECs [15, 17]. Although their structure is determined by their particular function and anatomical location, some of their characteristic features include the presence of Weibel–Palade bodies (WPB), fenestrae and large amounts of caveolae that mediate the vesicular transport of proteins across the endothelial barrier [15, 18]. Moreover, the type of permeability required for each tissue largely

determines the characteristics of the ECs lining their capillaries. For example, the blood brain barrier is a very specialized endothelium that requires low permeability and is formed by microvascular ECs with a large number of tight and adherens junctions; whereas the sinusoidal capillaries of the liver form pores or fenestrae to facilitate the exchange of fluid and proteins [15, 19, 20]. Mural cells are mesenchymal cells that closely associate with ECs and are required for homeostasis and organ function [16]. They are commonly subdivided into vascular smooth muscle cells (vSMCs) and pericytes based on their morphology, location and the expression of specific markers [16, 21, 22]. In general vSMCs form multiple concentric layers around arteries and veins, while pericytes usually interact with ECs in smaller vessels such as arterioles, capillaries and venules [16, 21].

1.1.2.1 The vascular endothelium at rest

The main functions of a healthy endothelium are to maintain blood flow, regulate vascular tone, control coagulation and modulate vascular permeability [2-4]. The inability of ECs to correctly perform any of these functions is considered endothelial dysfunction and associates with various types of cardiovascular disease [23]. The endothelium regulates blood flow and vascular tone by releasing vasodilators such as nitric oxide (NO) and prostacyclin (PGI_2), or vasoconstrictors like endothelin-1 (ET-1) to increase or decrease the tone of the surrounding layers of vSMCs [24-29]. NO is an important vasorelaxant generated by the oxidation of l-arginine to l-citruline by a family of NO synthases (NOS) [25]. The endothelial isoform of NOS (eNOS) can be activated by VEGF, shear stress and agonists of heterotrimeric G-protein-coupled receptors (GPCRs) like bradykinin and estrogen [24, 30]. The effects of NO on the vasculature are pleiotropic and include vSMC relaxation, regulation of platelet aggregation and inhibition of leukocyte adhesion [25, 26]. PGI_2 is a byproduct of arachidonic acid metabolism released by ECs in response to disturbances in vascular

function [29]. Similarly to NO, PGI_2 limits vasoconstriction and inhibits platelet activation by stimulating cell surface prostacyclin receptors and intracellular peroxisome proliferator-activated receptors (PPAR) β/δ on platelets and vSMCs [27, 29]. In contrast, ET-1 is produced by ECs and vSMCs as a rapid mechanism to reduce vascular tone. At low concentrations ET-1 can also act as a pro-inflammatory mediator [28]. ET-1 binds to Type A endothelin receptors and this interaction mainly mediates the effect of ET-1 on vasoconstriction [28].

Thrombin is a serine protease particularly important in the coagulation cascade due to its role in the cleavage of fibrinogen to fibrin [31]. In ECs thrombin can also affect permeability, vasomotor tone, leukocyte trafficking, migration, angiogenesis and hemostasis; and some of these effects are mediated by the family of protease-activated receptors (PARs) [31, 32]. The endothelium inhibits coagulation by preventing the activation of thrombin through the expression of tissue factor pathway inhibitor, thrombomodulin and heparan sulphate proteoglycans (HSPGs) [32, 33]. However, in response to injury the endothelium can acquire pro-coagulant activity and in this case the expression of tissue factor (TF) is critical. TF promotes pro-coagulant effects on ECs through the activation of factor IX, factor X and pro-thrombinase [32].

1.1.2.2 The vascular endothelium during inflammation

During acute inflammation ECs are required to recruit immune cells into the sites of injury and need to become activated. EC activation leads to an increase in blood flow, adhesiveness and secretion of pro-inflammatory cytokines; characteristics that enhance the interaction of leukocytes with the vasculature [34]. EC activation can be divided into type I activation, rapid responses independent of new gene expression; and type II activation, slower responses that require new gene expression [34, 35].

Inflammatory mediators such as histamine and leukotrienes activate type I responses on ECs via heterotrimeric GPCRs [34, 36]. Type I activation generates NO and PGI_2 which synergize to create a vasodilator effect that increases blood flow and leukocyte delivery [37]. The release of Ca^{2+} from the endoplasmic reticulum into the cytosol also plays an important role in this response. Calmodulin-bound Ca^{2+} activates myosin light chain kinase (MLCK) which then phosphorylates myosin light chain (MLC) to induce contraction of actin filaments, disruption of tight and adherens junctions, and vascular leakage [38]. Phosphorylated MLC also initiates the exocytosis of P-Selectin from WPB which further promotes leukocyte extravasation [39]. Type I activation has a very limited effect on immune cell extravasation, particularly because after a very short period of time the receptors that mediate this response become desensitized [40].Type II activation also increases blood flow, vascular permeability and leukocyte recruitment [34, 35]. In contrast with type I activation, type II activation is necessary for a more sustained inflammatory response and requires a longer time to be initiated [34]. Pro-inflammatory cytokines secreted by activated leukocytes, such as tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL1-β), stimulate signaling pathways on ECs able to activate the transcription factors nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1) that initiate the transcription of pro-inflammatory proteins [34, 41, 42]. Among these proteins are cytokines and adhesion molecules that promote leukocyte rolling and extravasation, including E-selectin, intracellular adhesion molecule 1 (ICAM-1) and vascular cell-adhesion molecule 1 (VCAM-1) [43].

In the case of chronic inflammation, angiogenesis also occurs within the sites of sustained injury to support the survival of infiltrated inflammatory cells [34]. Angiogenesis promotes the transformation of the initial matrix into a more permanent stroma comprised

of connective tissue, a characteristic feature of certain chronic diseases such as rheumatoid arthritis and atherosclerosis [44].

1.1.3 The formation of blood vessels

The heart is the first functional organ that forms during development, ensuring that the embryo will receive enough oxygen and nutrients as it develops [45]. The heart and the blood vessels originate independently from the mesoderm and it is only at later stages of development that they connect to each other to create a functional circulatory system [45]. Two distinct processes, vasculogenesis and angiogenesis, are responsible for the formation of blood vessels [45].

1.1.3.1 Vasculogenesis

Vasculogenesis is the formation of new blood vessels from EC precursors or angioblasts [46]. During gastrulation mesodermal cells are induced by morphogens such as bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs) to become hemangioblasts, which are precursors of two distinct cell lineages: the angioblast and the pluripotent hematopoietic stem cell [46, 47]. Vasculogenesis occurs in the blood islands, located in the distal part of the yolk sac, where hemangioblasts aggregate and differentiate into hematopoietic stem cells (inner cells) and angioblasts (outer cells) [47]. Signals from the endoderm and mesoderm including VEGF, retinoic acid and BMP4 determine the commitment of hemangioblasts to an either endothelial or hematopoietic fate [46, 47]. The angioblasts further differentiate into ECs, expand throughout the entire yolk sac and form a primitive vascular network called the primary capillary plexus [45]. The primary capillary plexus eventually interconnects with the dorsal aorta and the cardinal vein in the embryo proper to create a functional network that allows blood flow as the heart begins to beat [45]. After vasculogenesis the capillary plexus is actively remodeled into a highly branched

network containing capillaries, arteries and veins [48]. Vascular remodeling involves the generation of new capillaries as well as changes in vessel diameter, lumen formation and stabilization of the vasculature through the recruitment of mural cells [45, 48, 49].

1.1.3.2 Angiogenesis

Angiogenesis is the formation of new capillaries from pre-existing ones. It is required during embryogenesis and for physiological and pathological processes of vascular remodeling that occur in the adult organism [7, 45]. There are two types of angiogenesis involved in the vascularization of tissues: sprouting and non-sprouting angiogenesis, also known as intussusception [5, 50].

During sprouting angiogenesis some ECs within the vessel wall, called the tip cells, are selected by a VEGF-A gradient to lead the growth of the sprout [51, 52]. VEGF-A together with notch receptors and their Delta-like-4 (DLL4) ligand control vessel morphogenesis [53]. The tips of the sprouts express high levels of VEGFR-2 and respond to spatial concentration gradients of VEGF-A that guide the growth of the sprout [53]. Meanwhile, DLL4 expression is induced in selected tip cells in response to VEGF-A and activates the notch signaling pathway in neighboring ECs, making them less responsive to pro-angiogenic stimuli by suppressing VEGFR-2 expression [54, 55]. In addition to VEGF-A other molecules including semaphorins, plexins and netrins contribute to sprout guidance [56].

Non-sprouting angiogenesis, or intussusception, is the process of generating new vessels by splitting pre-existing ones [50]. In capillaries intussusception occurs when opposing vascular walls protrude into the lumen creating a bridge between ECs that will later become a transluminal pillar with an interstitial core [50]. Vessels formed by this type of angiogenesis are less leaky [50]. Newly formed sprouts transform into functional blood vessels via sprout extension and the formation of new vascular connections [57]. Sprout

extension occurs through the migration and proliferation of ECs located behind the tip. Upon finding tips of other sprouts or capillaries, they suppress their motile behavior, establish adhesive interactions and form a lumen to allow blood flow [56, 57].

1.1.3.3 Vessel maturation

Vessel maturation occurs when the vasculature changes from a state of active remodeling into a fully functional, quiescent network [49]. Maturation requires the suppression of proliferation and sprouting, followed by the stabilization of existing structures through interaction with mural cells [16, 49]. One mechanism of paracrine regulation between mural cells and ECs is the platelet-derived growth factor (PDGF) β/platelet-derived growth factor receptor (PDGFR) β signaling pathway. PDGFβ secreted by the endothelium acts as a chemoattractant to pericytes who express PDGFRβ, induces differentiation of mesenchymal cells into mural cells and promotes proliferation of vSMCs [16, 58, 59]. Sphingosine-1-phosphatase (S1P) signaling also regulates the recruitment of mural cells through the activation of a group of endothelial differentiation gene receptors [60, 61]. In addition, transforming growth factor beta (TGF-β) induces the differentiation of mesenchymal cells into pericytes and stimulates them to produce VEGF [62, 63]. Importantly, the angiopoietin/Tie receptor system is another paracrine signaling pathway associated with pericyte recruitment [64]. Activation of Tie-2 receptors induces the expression of endothelial heparin-binding epidermal-like growth factor (HB-EGF) and hepatocyte growth factor (HGF) which stimulate migration of vSMC and induce their association with ECs [65, 66]. Additionally, serotonin has also been described as a mediator of vSMCs recruitment to ECs by Ang-1 in the context of pulmonary hypertension [67].

1.2 The angiopoietin-Tie system

1.2.1 Biological roles of receptor tyrosine kinases in the vasculature

The formation of a functional vascular network in the embryo and vascular remodeling in adult tissues are tightly controlled processes regulated by several growth factor families that act via EC-specific receptor tyrosine kinases (RTKs) [68]. Three different growth factor families with distinct roles in vascular function have been described: VEGFs/VEGF receptors, ephrins/ephrin (Eph) receptors and angiopoietins/Tie receptors [68, 69].

The VEGF/VEGF receptor family was the first endothelial-specific RTK system to be identified [8, 69]. Five ligands have been described (VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factor) that differentially interact with three related RTKs (VEGFR-1/Flt-1, VEGFR-2/KDR/Flk-1 and VEGFR-3/Flt-4) [8]. Additional receptor components, such as neuropilins and HSPGs, are also involved in the activation and modulation of VEGF signaling pathways [8]. Studies in knockout mice have demonstrated that vascular development is highly dependent on VEGFR signaling [70-73]. Mouse embryos lacking either VEGF-A or VEGFR-2 fail to develop a functional vasculature and even the loss of a single VEGF allele results in embryonic lethality [70-72]. VEGF-A and VEGFR-2 are critical not only during the early stages of vasculogenesis, but also at later stages when they regulate proliferation, migration, capillary tube formation, sprouting and survival of ECs [70-72]. In contrast, mice lacking VEGFR-1 do form a vascular network but its organization is abnormal due to excessive cellular growth. This suggests that VEGFR-1 might be an inhibitor of endothelial expansion rather than a promoter of EC development [73]. The interplay between different receptors, co-receptors and ligands that occurs in VEGF signaling provide the complexity necessary to make this family a crucial regulator of EC

biology not only during vascular development and maintenance, but also in pathological disease states such as tumor progression [8, 69, 74].

The Eph receptors and their membrane-bound ligands, ephrins, represent a large subfamily of RTKs that mediate bi-directional signals between adjacent cells and modulate cytoskeleton dynamics affecting cell motility and adhesion during vascular development and angiogenesis [68, 75]. Eph receptors have been divided into two sub-groups, nine EphA receptors (A1–A9) and six EphB (B1–B6) receptors, which preferentially bind to ephrinA and ephrinB ligands [69, 75]. The importance of this family of growth factors extends to neural development, cancer progression and metastasis [69, 75].

The Angiopoietin-Tie system is comprised by two type I tyrosine kinase receptors expressed primarily in ECs (Tie-1 and Tie-2) and three secreted ligands (Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2) and Angiopoietin-3 (Ang-3) in mice, and its human orthologue Angiopoietin-4 (Ang-4)), see Figure 1.1 [57, 69]. Several years after the discovery of VEGF, Tie-1 and Tie-2 receptors were described as orphan receptors critically involved in the formation of the vasculature and the search for their ligands led to the discovery of a growth factor family consisting of both, receptor agonists (Ang-1) and receptor antagonists (Ang-2) [64, 76-79]. This section focuses on the Angiopoietin-Tie system, with a special emphasis on Ang-1 and its regulation of vascular biology.

1.2.2 Tie-1 and Tie-2 receptors

Tie-1 and Tie-2, also known as Tek, are type I transmembrane RTKs first identified as orphan receptors predominantly expressed in ECs, but also found in primitive hematopoietic cells [80-82]. The name Tie, tyrosine kinase with immunoglobulin (Ig) and epidermal growth factor (EGF) homology domains, describes the structural properties of their extracellular domain comprised of two amino-terminal Ig-like loops, followed by

three EGF homology motifs, a third Ig-like loop and three fibronectin type III repeats close to the transmembrane region [80, 83]. Both receptors also contain a carboxyl-terminal tyrosine kinase domain responsible for the activation of intracellular signaling pathways (Figure 1.1) [80, 83].

Studies in mice carrying mutations on the Tie1 and Tie2 genes revealed that these receptors have non-redundant roles in the vasculature. In mice disruption of the Tie1 gene leads to embryonic lethality by E13.5 due to edema, haemorrhage and defects on vascular integrity [77, 78, 80]. Furthermore the phenotype of $Tie1^{-/-}$ mice also depends on their genetic strain [77, 78] . $Tie1^{-/-}$ mice also show severe defects in the lymphatic vasculature at very early stages of development, suggesting that Tie1 is not only required for normal vascular development but also for embryonic lymphangiogenesis [84]. In contrast, mice carrying targeting null mutations on the Tie2 gene have an earlier lethal phenotype and die by E10.5 [76, 78]. These mice have less ECs and distended blood vessels, exhibit abnormal heart development and fail to remodel the primary capillary plexus into a functional vascular network [76, 78].

To date, Tie-1 has no known ligands and evidence of its function in ECs is just beginning to emerge. Studies using chimeric receptors formed by the ectodomain of the RTK TrkA fused to the transmembrane and intracellular domains of Tie-1 or Tie-2 have shown that in contrast to TrkA/Tie-2, the TrkA/Tie-1 receptor is unable to autophosphorylate or phosphorylate other intracellular proteins [85]. However, Ang-1 and Ang-4 have been shown to stimulate Tie-1 phosphorylation in EA.hy926 cells and the use of a chimeric receptor formed by the extracellular domain of colony stimulating factor-1 (CSF-1) receptor fused to the intracellular domain of Tie-1 demonstrated that activated Tie-

1 can associate with phosphoinositide 3-kinase (PI3-K), phosphorylate AKT and protect cells from apoptosis [86, 87].

Current evidence suggests that Tie-1 does not signal via ligand-induced kinase activation, but instead Tie-1 associates with Tie-2 and modulates its activity [85, 86, 88-90]. First it was hypothesized that Tie-1 and Tie-2 are present as pre-formed complexes in ECs and their association is mediated by their intracellular domains [85]. However, the formation of Tie-1/Tie-2 heteromeric complexes upon treatment with COMP-Ang1, a soluble Ang-1 chimeric protein more potent than native Ang-1 has also been described [86]. More recently, it has been proposed that Tie-1 and Tie-2 are associated on the cell surface prior to ligand binding and that the ability of individual angiopoietins to effectively destabilize Tie-1/Tie-2 complexes define their respective agonistic or antagonistic roles [90]. Strikingly, reducing Tie-1 protein levels in human umbilical vein endothelial cells (HUVECs) using small hairpin RNA (shRNA) increased Ang-1 mediated Tie-2 phosphorylation and allowed Ang-2 to strongly activate Tie-2 receptors [90].

Tie-2 receptors are able to bind to all angiopoietins [64, 79, 91]. Mutagenesis and binding studies originally identified the first Ig loop and EGF repeats of the Tie-2 extracellular domain as necessary for angiopoietin recognition and binding [92, 93]. However, more recent crystallographic analysis of the Tie-2 receptor ectodomain and Ang-2/Tie-2 complexes revealed that the ligand binding site is the second Ig domain [94]. Despite being able to bind all angiopoietins with similar affinities, the effects of these ligands on Tie-2 receptor activation are different [64, 79]. Ang-1 consistently activates Tie-2 receptors, whereas Ang-2 seems to act in a context-dependent manner as either agonist or antagonist of Tie-2 [64, 79, 88, 95, 96]. The less studied Ang-4 seems to activate Tie-2 similarly to Ang-1 [91]. Tie-2 activation requires ligand multimerization and the formation of ligand-receptor clusters at

sites of cell-cell or cell-extracellular matrix (ECM) contacts, which induce Tie-2 receptor autophosphorylation in tyrosine (Tyr) residues near its catalytic carboxyl-terminal end and create high affinity binding sites for signaling molecules and adaptor proteins that recognize phosphotyrosine motifs [97, 98].

1.2.3 Angiopoietin-1

Angiopoietin-1 (Ang-1) is an agonist of Tie-2 receptors [64, 99]. Ang-1 was first cloned using a secretion-trap expression strategy with cDNA libraries constructed from human neuroepithelioma SHEPI-1 cells and from the mouse myoblast cell line C2C12ras [99]. The DNA sequences of both human and mouse Ang-1 cDNA clones share 97.6% identity and encode a glycosylated protein of 498 aminoacids that specifically binds to Tie-2 receptors in ECs and induces their Tyr phosphorylation [99]. Ang-1 is constitutively expressed in many adult tissues by mural cells, fibroblasts and other non-vascular cells [64, 99].

Mice lacking Ang-1 expression ($Ang1^{-/-}$) die during embryonic development (E12.5) and have a phenotype similar to Tie-2 receptor deficient mice [64, 76, 78]. The most prominent defect of $Ang1^{-/-}$ mice involves the heart, which has an immature and less intricate endocardium, and lacks myocardial trabeculae [64]. Loss of Ang-1 also leads to defects in vascular organization, including the formation of immature and dilated vessels, reduced branching and the inability to generate large and small vessels [64]. Tie-2 mRNA levels are substantially reduced in $Ang1^{-/-}$ embryos, suggesting that Ang-1 modulates the expression of its own receptor [64]. Further analysis of $Ang1^{-/-}$ vessels at the ultrastructural level revealed important defects on the interaction between ECs, mural cells and ECM that could help to explain the phenotype observed in these mice [64].

Structurally, Ang-1 is a glycoprotein comprised of a carboxyl-terminal fibrinogen-like domain, a central coiled-coiled domain, a short amino-terminal superclustering domain

and a secretory signal sequence [99-101]. The fibrinogen-like domain is responsible for receptor binding, whereas the coiled-coiled domain is responsible for oligomerization of the fibrinogen-like domains and the superclustering domain allows these oligomers to organize into multimers of variable size [98, 100, 101]. The proper formation of Ang-1 oligomers and multimers by disulfide links involving the cysteines 41 and 54, located in the superclustering domain, is crucial for Tie-2 receptor binding and activation [98]. The minimal unit able to activate Tie-2 receptors is a tetrameric form of Ang-1, and oligomers unable to form multimers have a reduced activation of Tie-2 receptors [98, 101]. Binding of Ang-1 to Tie-2 receptors induces their autophosphorylation and allows them to interact with different protein effectors and activate several downstream signaling pathways [13, 57]. Ang-1 has several distinct functions in the vasculature of the embryo and in adult tissues [13]. The intracellular signaling pathways activated by Ang-1 (Figure 1.2), as well as their functional significance for EC biology are discussed in detail in the following section.

1.2.4 Intracellular signaling pathways activated by Ang-1

Ang-1 is able to differentially induce angiogenic responses in sites of vascular remodeling while maintaining stability in mature, quiescent vessels. These opposing responses seem to originate from the differential activation of signaling pathways downstream of Tie-2 receptors between sparse and confluent cells [97, 102]. It has been demonstrated that Tie-2 localizes to cell–matrix contacts in sparse cells and to cell–cell junctions in contacting cells upon Ang-1 stimulation [97, 102]. In the absence of cell-cell junctions Ang-1/Tie-2 complexes accumulate at the cell edges and in sites of contact with the ECM [102]. Ang-1 co-localizes with Tie-2 at the rear end of sparse cells and preferentially promotes migration through the activation of the downstream of kinase related (Dok-R) signaling pathway [97, 102]. In contrast, confluent cells form Ang-1/Tie-2 complexes in cell-cell contacts allowing Tie-2 receptors

to interact with other Tie-2 receptors located in neighboring ECs. Localization of Tie-2 at cell-cell contacts leads to activation of a particular set of downstream proteins, mainly the pro-survival PI3-K/AKT signaling pathway [97]. Ang-1 stimulation also increases the expression of vascular endothelial protein tyrosine phosphatase (VE–PTP) at cell–cell junctions and, through the formation of Tie-2/VE–PTP complexes, inhibits vascular permeability [97].

The fact that Ang-1 is able to elicit various cellular responses depending on the context makes it a very interesting regulator of EC biology. Thus, the purpose of this section is to explain what we know about the activation of intracellular signaling pathways by Ang-1 and their differential contribution to survival, angiogenesis, vascular quiescence, inflammation and permeability.

1.2.4.1 The phosphoinositide 3-kinase/AKT signaling pathway

Phosphoinositide 3-kinases (PI3-Ks) are a family of heterodimeric lipid kinases comprised of a catalytic subunit and a regulatory subunit able to phosphorylate membrane-bound phosphatidylinositol to generate phosphoinositides (PIs) [103, 104]. PIs serve as docking sites for signaling molecules containing a pleckstrin homology (PH) domain, particularly protein kinase B (PKB) also known as AKT [104]. AKT is a multifunctional serine/threonine kinase involved in the regulation of various cellular responses including survival, growth, proliferation, metabolism, migration and angiogenesis [105].

In ECs the activation of Tie-2 receptors by Ang-1 leads to the recruitment of the p85 regulatory subunit of PI3-K mainly to the phosphorylated residue tyrosine (Tyr) 1101 on the cytoplasmic tail of Tie-2, and induces the activation of AKT [106]. The activation of the PI3-K/AKT signaling pathway is largely responsible for the anti-apoptotic effect of Ang-1 in ECs [107-109]. AKT promotes survival by suppressing mediators of apoptosis,

including caspase-9 and Bcl-2 associated death promoter (BAD) [110, 111]. In ECs activation of the PI3-K/AKT pathway by Ang-1 prevents apoptosis caused by serum deprivation through the induction of survivin and the inhibition of caspase-3, -7 and -9 activities [108, 109]. Additionally, Ang-1 activation of Tie-2 in confluent ECs induces the expression of Krüppel-like factor 2 (KLF2), a zinc finger transcription factor associated with vascular quiescence, downstream of PI3-K/AKT pathway [112-115]. Activation of the PI3-K/AKT pathway stimulates transcriptional activity of myocyte enhancer factor 2 (MEF2) to induce the expression of KLF2 in ECs [115]. Additionally, Ang-1 further stabilizes the vasculature by inhibiting Ang-2 production [116, 117]. Activation of PI-3K/AKT leads to phosphorylation and subsequent inactivation of the forkhead transcription factor 1 (FOXO1) that transcribes the Ang-2 gene [116, 117].

Although the activation of PI3-K in quiescent cells favors survival, its activation can also contribute to the angiogenic cascade in activated ECs. Ang-1 is able to induce endothelial sprouting, an initial step in angiogenesis, partly through cytoskeletal changes and proteinase secretion dependent on the activation of PI3-K [118, 119]. Ang-1 treatment in porcine pulmonary artery ECs induces phosphorylation of focal adhesion kinase (FAK), and secretion of plasmin and matrix metalloproteinase-2 (MMP-2); both inhibited by pharmacological inhibitors of PI3-K [119]. Another study done in porcine coronary artery endothelial cells also highlights the importance of the PI3-K/AKT pathway on Ang-1-induced angiogenesis. Ang-1 treatment promotes migration, capillary tube formation and sprouting partly through eNOS phosphorylation and NO production [120]. Pharmacological inhibition of the PI3K/AKT pathway reduces Ang-1-stimulated eNOS phosphorylation and NO production; and completely abrogates the effect of Ang-1 on angiogenesis [120].

1.2.4.2 The downstream of kinase related signaling pathway

The downstream of kinase related (Dok-R) protein belongs to a group of docking proteins termed the Dok family [121, 122]. The proteins from the Dok family have a similar structure characterized by an amino terminal PH domain, a central phosphotyrosine-binding (PTB) domain and a carboxyl-terminal tail rich in proline (Pro) and Tyr residues [122]. In ECs Dok-R has been described as a protein associated with Tie-2 receptors involved in further amplifying Ang-1 signaling by providing various docking sites for downstream signaling proteins [123]. Furthermore, Dok-R colocalizes with Tie-2 receptors at the rear of migrating cells stimulated with Ang-1 and mediates cell motility [97, 122].

Activated Tie-2 receptors recruit Dok-R through their PTB domain which results in its subsequent Tyr phosphorylation and the formation of high affinity binding sites for downstream signaling proteins including the adapter protein Nck and p120-Ras GTP-Activating protein (p120 RasGAP) [123]. The phosphorylation of Dok-R on Tyr351 facilitates its association with Nck and leads to the recruitment and activation of the serine/threonine kinase Pak [122, 123]. The recruitment of Pak to the receptor results in the reorganization of the actin cytoskeleton and is required for Ang-1-mediated EC migration [122]. Interestingly, the interaction between Nck and Pak, followed by Pak activation, is also involved in VEGF-induced EC migration [124]. Additionally, the recruitment of p120 RasGAP to Dok-R reduces the activation of the downstream extracellular related kinase 1 and 2 (ERK1/2) partially due to its function as a negative regulator of the small GTPase Ras [125-127]. In the case of EGF signaling, Dok-R attenuates ERK1/2 and AKT activation downstream of the epidermal growth factor receptor (EGFR) independently of its association with RasGAP, through the recruitment of the kinases Src and Csk, a Src family kinase (SFK)-inhibitory kinase [128].

Experiments in mice lacking Dok-R further support its role in the negative regulation of ERK1/2 [129].

Other than the role of Dok-R in cell migration, the function of this signaling pathway in relation to the Ang-1/Tie-2 signaling in ECs remains largely unknown. However, it has been suggested that Dok-R activation could be switching the EC response more towards a migratory state, rather than a pro-survival and quiescent state; which correlates with its localization at rear end of migrating cells alongside activated Tie-2 receptors [97].

1.2.4.3 The mitogen activated protein kinase signaling pathways

The mitogen activated protein kinases (MAPKs) are a family of evolutionarily conserved protein kinases that control a large number of cellular processes including proliferation, migration, survival and differentiation in response to extracellular stimuli [130, 131]. Protein kinases are enzymes able to covalently attach phosphate groups to serine (Ser), threonine (Thr) or Tyr residues on their target proteins and thus control their enzymatic activity, subcellular localization, protein interactions and proteolytic degradation [130-132]. MAPK signaling cascades are modules comprised of three kinases sequentially activated by phosphorylation: MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK [130, 131]. These MAPK modules can be activated upon stimulation of extracellular receptors or in response to physical stimuli by small guanosine triphosphate (GTP)-binding proteins, such as Ras and Rho GTPases, or by STE20 kinases [130, 131, 133]. The spatiotemporal activation of MAPK signaling largely determines the type of cellular response obtained, stressing the importance of the molecular mechanisms involved in the inactivation of these signaling cascades [134]. MAPK signaling can be terminated by inhibiting Ras or Rho activity through GTPase-activating proteins (GAPs) which catalyze the exchange of GTP for

guanosine diphosphate (GDP) [135]. Another mechanism through which MAPK signaling can be terminated is the dephosphorylation of Tyr and Ser/Thr residues mediated by phosphatases [136].

There are four typical MAPK signaling pathways in mammals: extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase 1, 2 and 3 (SAPK/JNK), p38 (α,β,γ,δ) and ERK5 [130, 131]. ERK1 and ERK2 were the first mammalian MAPKs to be cloned and characterized, and they share 83% aminoacid identity [137, 138]. ERK1 and 2 are activated mainly by cell surface receptors, such as RTKs and GPCRs, in response to growth factors and cytokines [138, 139]. In the case of RTKs, ligand binding induces receptor clustering and autophosphorylation of Tyr residues located in their cytoplasmic domain. These phosphorylated residues act as specific binding sites for scaffolding and signaling proteins containing Src homology 2 (SH2) or PTB domains including growth factor receptor-bound protein 2 (Grb2) which recruits son of sevenless (SOS) to the plasma membrane [132]. SOS is a guanine nucleotide exchange factor (GEF) that stimulates the exchange of GDP bound to the small GTPase Ras to GTP, which is required for a positive regulation of Ras activity [135]. Activated Ras is able to interact directly with its effectors, the MAPKKKs A-Raf, B-Raf, and Raf-1, to induce their activation [130]. Activated MAPKKKs phosphorylate the MAPKKs MEK1 and MEK2, which in turn phosphorylate the MAPKs ERK1/2 within a conserved Threonine-Glutamate-Tyrosine (TEY) motif located in their activation loop [130-132]. Phosphorylated ERK1/2 accumulates in the nucleus, where it can phosphorylate a large number of transcription factors such as nuclear factor of activated T cells (NFAT), Elk-1, MEF2, c-Fos, c-Myc and signal transducer and activator of transcription (STAT) 3 [132, 140, 141]. ERK1/2 can also phosphorylate cytoplasmic substrates

such as death-associated protein kinase (DAPK) and tuberous sclerosis complex 2 (TSC2); and substrates associated with the cell membrane like Syk, and calnexin, or the cytoskeleton such as paxillin [132, 141]. ERK1/2 mediates cell growth, proliferation, differentiation, survival and cytoskeletal changes through the activation of its multiple substrates [132, 141].

Unlike ERK1/2, the MAPKs p38 and SAPK/JNK are mainly activated by inflammatory cytokines and environmental stresses such as DNA damage, hypoxia, oxidative stress and ultraviolet (UV) light [132, 142, 143]. These kinases are activated by Rac and Cdc42, GTPases of the Rho family, downstream of RTKs and GPCRs; and by the recruitment of TNF receptor associated factor (TRAF) proteins in response to pro-inflammatory cytokines such as TNF-α and IL-1β [132, 142, 144]. The recruitment of TRAF proteins promotes the activation of downstream MAPKKKs, many of which are shared by the p38 and SAPK/JNK pathways [132].

Four genes encode the different isoforms of the p38 family of MAPKs [142]. p38α and p38β are widely expressed in cell lines and tissues, whereas p38γ and p38δ show a more restricted pattern of expression [132]. The major MAPKKs upstream of p38 are MKK3 and MKK6 [132, 145]. MKK3 and MKK6 are activated by several MAPKKKs, including MEKK1-3, ASK1 and TAK1 [142]. Upon stimulation, p38 isoforms phosphorylate a large number of substrates including calcium-dependent phospholipase A2 (cPLA2), MNK1/2 and Bcl-2-associated X protein (Bax) in the cytoplasm; and activating transcription factor (ATF) 1 and 2, MEF2, Elk-1, Ets1 and p53 in the nucleus [142]. Activation of p38 plays a key role in immune and inflammatory responses, cell cycle progression and apoptosis [142, 146].

There are three known isoforms of SAPK/JNK encoded by three distinct genes: JNK1/SAPKγ, JNK2/SAPKα and JNK3/SAPKβ [132, 143]. JNK1 and JNK2 exhibit a wide tissue distribution, whereas JNK3 localizes mainly in neurons, testis, and cardiac myocytes [147]. The major MAPKKs that activate SAPK/JNK isoforms are MKK4 and MKK7 [132, 143]. MKK4/7 are phosphorylated and activated by several MAPKKKs, including MEKK1, ASK1/2 and TAK1 [132]. Upon activation SAPK/JNK accumulates in the nucleus where it can interact with its substrates [148]. Some of the transcription factors that have been shown to be phosphorylated by SAPK/JNK include c-Jun, p53, ATF2, NFAT, Elk-1, STAT3, c-Myc and JunB [132, 149]. SAPK/JNK plays an important role in the control of cell proliferation via c-Jun and the transcription of genes that control the cell cycle, such as cyclin D1 [150]. SAPK/JNK phosphorylation of c-Jun on Ser63 and/or Ser73 increases c-Jun-dependent transcription of genes containing AP-1-binding sites [132, 150]. SAPK/JNK has also been implicated in the differentiation of hematopoietic cells and in apoptosis induced by cellular stresses [151, 152].

Several pro-angiogenic factors including VEGF, FGF and PDGF are known to activate ERK1/2 and induce proliferation in ECs [153, 154]. VEGFR signaling can also activate the p38 and SAPK/JNK pathways to promote endothelial migration in various types of ECs including microvascular, aortic and umbilical vein [155]. Similarly, Ang-1 signaling activates the MAPK signaling pathways ERK1/2, p38, and SAPK/JNK simultaneously in ECs [13, 107, 156-160]. In contrast to VEGF signaling, the mitogenic effect of ERK1/2 activation by Ang-1 is relatively modest [156]. In Ang-1 signaling ERK1/2 activation is more a pro-survival pathway involved in the anti-apoptotic effect of Ang-1 on ECs, rather than an inducer of proliferation [157]. Ang-1 also elicits activation of p38 and the inhibition of this pathway using pharmacological inhibitors of its α and β isoforms results in a potentiated effect of

Ang-1 on apoptosis, which confirms the pro-apoptotic role of p38 in this context [157]. The activation of SAPK/JNK by Ang-1 is also a pro-apoptotic pathway [159]. However, despite the activation of the pro-apoptotic pathways p38 and SAPK/JNK, Ang-1 strongly inhibits endothelial apoptosis induced in response to serum deprivation This suggests that the effect of the anti-apoptotic pathways ERK1/2 and PI-3K predominate over the pro-apoptotic MAPKs [157, 159].

In addition to its effects on cell survival, MAPK activation in response to Ang-1 affects angiogenic responses [160, 161]. Ang-1-induced activation of ERK1/2 and SAPK/JNK, in combination with PI3-K activation, stimulates production of interleukin 8 (IL-8) in ECs [160]. IL-8 is a chemokine not only associated with the infiltration of inflammatory cells at sites of injury, but also with angiogenesis [162]. Ang-1 signaling promotes the transcription of the IL8 gene through the AP-1 transcription factor by inducing phosphorylation of c-Jun on both Ser63 and Ser73 downstream of ERK1/2 and SAPK/JNK. IL-8 production partly mediates the migratory and proliferative effect of Ang-1 on ECs [160]. In addition to AP-1, MAPK activation in response to Ang-1 also leads to the induction of another transcription factor termed early growth response-1 (Egr-1) [161]. Egr-1 is a zinc finger transcription factor and an immediate early response gene rapidly induced by various stimuli including growth factors, cytokines, hypoxia and shear stress [163, 164]. The Egr-1 transcription factor has been involved in regulating angiogenesis by inducing expression of various growth factors including FGF [165, 166]. In the case of Ang-1, Egr-1 is transiently induced in ECs downstream from ERK1/2 and PI3-K signaling pathways and plays a role in Ang-1-mediated endothelial migration and proliferation of ECs, albeit the molecular mechanisms behind these effects remain largely unknown [161].

1.2.4.4 Regulation of vascular permeability by Ang-1

Vascular permeability refers to the ability of small molecules such as ions, water and nutrients to flow across the vessel wall [167]. The permeability of the endothelium relies upon the adherens and tight junctions between ECs, which are strictly regulated depending on the function and physiological state of the tissue that lies beneath them [17, 167]. Ang-1 signaling reduces vascular permeability by decreasing the number and size of gaps that form at EC junctions, and mice that overexpress Ang-1 in their vasculature have less leaky vessels [168-170]. Furthermore, systemic expression of Ang-1 in transgenic mice or by adenoviral gene delivery inhibits vascular leakage induced by pro-inflammatory stimuli including bradykinin, lipopolysacharide (LPS) and VEGF [168-170]. Although the regulation of endothelial junctions by Ang-1 remains largely unexplored, a few molecular mechanisms involved in the antagonistic function of Ang-1 on VEGF-induced permeability have been described [171, 172].

Some of the inhibitory effects of Ang-1 on vascular permeability are mediated by the sequestration of Src, a non-receptor tyrosine kinase, downstream of Tie-2 [171]. VEGF signaling in ECs increases permeability thorough the phosphorylation of the junctional protein vascular endothelial cadherin (VE-cadherin) by Src, which triggers its internalization and thus promotes the disassembly of adherens junctions [173]. The sequestration of Src is promoted by Ang-1-induced activation of Tie-2 and occurs through mammalian diaphanous (mDia), a downstream target of the small GTPase RhoA [171]. In this context, mDia blocks the activation of Src downstream of VEGFRs by making it unavailable to induce a permeability response [171].

Another mechanism through which Ang-1 limits endothelial permeability induced by VEGF is through the inhibition of NO release [172]. In ECs, the activation of the PI3-

K/AKT pathway downstream of VEGF signaling results in the phosphorylation of eNOS on Ser1177, which leads to an increase in eNOS activity and NO release [172, 174]. The release of NO in response to VEGF increases vessel permeability, highlighting the importance of the activation state of eNOS for vascular function. The phosphorylation status of eNOS on different aminoacid residues contributes to the regulation of its activity and thus affects the amount of NO that is released [172]. Ang-1 activation of Tie-2 receptors leads to phosphorylation of eNOS on Thr497 by atypical protein kinase C zeta (PKCζ), which inactivates the enzyme, limits NO production and inhibits endothelial permeability stimulated by VEGF [172].

1.2.4.5 Molecular mechanisms responsible for the anti-inflammatory effects of Ang-1 in the vasculature

In addition to its role in angiogenesis and vascular homeostasis, Ang-1 also functions as an anti-inflammatory agent [13]. The inflammatory activation of ECs leads to the recruitment of leukocytes to the sites of injury due to an increase in pro-inflammatory mediators and adhesion molecules expressed largely in response to the activation of the NF-κB signaling pathway [34]. A mechanism that has been proposed through which Ang-1 decreases inflammation, involves the recruitment of the intracellular protein A20 binding inhibitor of NF-κB activation 2 (ABIN-2) to Tie-2 receptors, which interferes with the activation of the NF-κB pathway through the zinc-finger protein A20 [175].

Besides its pro-angiogenic effects on the vasculature, VEGF also promotes leukocyte adhesion through the induction of E-selectin, VCAM1 and ICAM1 [176]. In contrast to the pro-inflammatory effects of VEGF, Ang-1 inhibits leukocyte adhesion, the expression of adhesion molecules and, as mentioned earlier, VEGF-induced vascular

leakage [115, 171, 172, 177]. Some of the mechanisms through which Ang-1 exerts these effects are beginning to emerge, particularly the role of nuclear receptor-77 (Nur77) and the transcription factor KLF2 in mediating this anti-inflammatory response [115, 178].

Nur77, a member of the nuclear receptor subfamily of ligand-independent nuclear receptors, is rapidly expressed in response to growth factors and cytokines [179-181]. In Jurkat cells, an immortalized T lymphocyte cell line, Nur77 interacts with the p65 subunit of NF-κB and interferes with its ability to bind promoters of pro-inflammatory cytokines, particularly interleukin 2 (IL-2) [182]. In ECs the expression of Nur77 increases in response to TNF-α as a negative feedback mechanism of the pro-inflammatory response and leads to inhibition of NF-κB activation by transcriptionally upregulating IkappaB alpha (IκBα), a protein that sequesters NF-κB dimers in the cytoplasm by masking their nuclear localization signal (NLS) [183]. Furthermore, inhibition of Nur77 increases TNF-α induced adhesion molecule expression and leukocyte adhesion [183]. In ECs Ang-1 also induces Nur77 expression and when combined with VEGF, its expression is potentiated [178, 184]. Ang-1 induction of Nur77 is mediated by the PI3-K and ERK1/2 signaling pathways, which differs from the mechanism described for VEGF-induced Nur77 [178, 184]. The inhibition of VEGF-induced NF-κB, VCAM1 and E-selectin expression, and leukocyte adhesion by Ang-1 disappears when Nur77 expression is disrupted, stressing the role of this protein in the anti-inflammmatory effects of Ang-1 on the vasculature [178].

Ang-1 induces the expression of the transcription factor KLF2 in ECs [115]. KLF2 is not only involved in vascular quiescence but also mediates the inhibitory effect of Ang-1 on VEGF-induced expression of VCAM1 and monocyte adhesion to ECs [115].

1.2.5 Angiopoietin-2

Angiopoietin-2 (Ang-2) has been described as an antagonist of Tie-2 receptors, but it can also act as an agonist depending on the context [79, 88, 95, 96]. The angiopoietins are structurally similar and Ang-2 shares ~60% amino acid identity with Ang-1 [79]. Both proteins have an amino-terminal coiled-coil domain and a carboxyl-terminal fibrinogen-like domain, although Ang-2 lacks a cysteine between the coiled-coil and fibrinogen-like domains [79, 99-101]. Ang-2 is usually found in dimeric form, and the differences in protein structure compared to Ang-1 restrict the protein to the formation of less order multimers [101]. Ang-2 is secreted by ECs and in the normal adult its expression is restricted to sites of active vascular remodeling such as the ovary, placenta and uterus [79].

Embryos of transgenic mice that specifically overexpress Ang-2 in their vasculature die at E9.5–10.5 and exhibit an abnormal vascular phenotype similar to mice lacking Ang-1 or Tie-2, although their defects appear to be more severe [64, 78, 79]. Ang-2 expression leads to heart abnormalities and to the formation of a discontinuous vascular network [79]. The defects observed in these mice suggest that Ang-2 has antagonistic functions to Ang-1, which is further supported by the pattern of expression of Ang-2 in adult tissues at sites of active vascular remodeling and by the fact that Ang-2 can also block Ang-1-induced activation of Tie-2 receptors [79, 185]. However, Ang-2 can also act as an agonist and activate Tie-2 receptors to induce similar signaling pathways and cellular responses as Ang-1 depending on the context [88, 95, 96].

In addition, Ang-2 promotes destabilization of the vasculature by antagonizing Ang-1-induced recruitment of mural cells to ECs [186, 187]. In diabetic retinopathy Ang-2 causes loss of pericyte coverage and destabilizes the retinal capillaries in the absence of VEGF [187]. Furthermore, in a transgenic model in which Ang-2 is expressed in ECs the restoration of

blood flow after limb ischemia is drastically impaired, an effect mainly attributed to defective recruitment of vSMC in Ang-2 transgenic mice [186].

Ang-2 also primes the vasculature to potentiate its response to angiogenic and inflammatory stimuli. The local cytokine environment largely determines the outcome of Ang-2 signaling [188-190]. In the presence of VEGF, Ang-2 induces migration, proliferation and sprouting of blood vessels; while in the absence of VEGF Ang-2 favors cell death and vessel regression [188-190]. In the case of inflammation, Ang-2 deficient mice are unable to elicit a rapid pro-inflammatory response in response to TNF-α and fail to induce adhesion molecule expression [191]. These findings stress the antagonistic mode of action of Ang-2 on the vasculature, which clearly oppose the quiescent and anti-inflammatory effects of Ang-1 in ECs [169, 191].

1.2.6 Angiopoietin-3/4

Angiopoietin-4 (Ang-4) and its mouse orthologue angiopoietin-3 (Ang-3) share all the structural characteristics of angiopoietins and are able to bind to Tie-2 receptors, but not to Tie-1 [91]. Ang-4 was first identified as an agonist of Tie-2 receptors, whereas Ang-3 was described as an antagonist [91, 192]. Evidence later demonstrated that Ang-3 can also phosphorylate Tie-2 receptors on ECs of its own species [192]. Ang-4 and Ang-3 phosphorylate AKT on Serine 473, promote survival of ECs and induce corneal angiogenesis *in vivo* [192]. Additionally, Ang-4 has been found to protect against serum deprivation-induced apoptosis, to increase migration and to promote capillary tube formation in cultured ECs through the activation of Tie-2 receptors [193]. Studies carried out in Lewis lung carcinoma (LLC) cells have demonstrated that Ang-3 can bind to the cell surface or localize to the basement membrane via perlecan, a HSPG [194]. Furthermore, cell

bound Ang-3 is able to induce retraction and loss of integrity in bovine pulmonary artery EC monolayers [194]. Ang-3 and Ang-4 are expressed in response to hypoxia, highlighting their role in vascular remodeling. Moreover, it has been proposed that Ang-4 functions similarly to Ang-1 during hypoxia-induced angiogenesis [193].

1.2.7 The role of integrins in angiopoietin signaling

In addition to Tie receptors, Ang-1 and Ang-2 can also bind to different integrins including α2β1, α5β1, αvβ3, and αvβ5 [195-198]. Integrins are heterodimeric membrane glycoproteins responsible for outside-in and inside-out signaling that modulate cell adhesion and migration [199, 200]. The integrin family is comprised of structurally related receptors for ECM proteins and Ig superfamily proteins [200]. Eighteen α and eight β subunits associate to form 24 distinct pairs of integrins able to bind a single ligand, such as α5β1, or several ligands [200]. Various integrins recognize the tripeptide Arginine-Glycine-Aspartate (RGD) in their ligands, whereas others recognize alternative short peptide sequences like Glutamate-Isoleucine-Leucine-Aspartate-Valine (EILDV) and Arginine-Glutamate-Aspartate-Valine (REDV) [201, 202]. Some integrins like α4β1 can also bind cell surface receptors like VCAM1 to promote cell–cell adhesion and the activation of intracellular signaling pathways [203, 204].

Ang-1 can bind directly to α5β1 integrins through its fibrinogen-like domain, although the precise peptide sequence mediating this interaction remains elusive [197]. Interestingly, Ang-1 interaction with α5β1 is inhibited by RGD peptides despite the fact that the fibrinogen-like domain of Ang-1 lacks a RGD motif, or any known binding sites to integrin [195, 196]. It has been suggested that the conserved peptide sequence Glutamine-Histidine-Arginine-Glutamate-Aspartate-Glycine-Serine (QHREDGS) within the fibrinogen-like domain of Ang-1 could mediate its binding to integrins due to its similarity

to integrin-binding sequences found in fibrinogen and fibronectin [196]. Ang-2 also binds and activates integrins, particularly in non-vascular cells such as myocytes, glioma and breast cancer cells [195, 205, 206]. In ECs, Ang-2 has been found to co-immunoprecipitate with $\alpha_5\beta_1$ integrin after stimulation with TNF-α [207]. Furthermore, in activated endothelial tip cells Ang-2 induces FAK phosphorylation on Tyr397 in an integrin-dependent manner and stimulates EC migration and sprouting [198].

1.3 Dual-specificity phosphatases as modulators of mitogen activated protein kinase signaling pathways

The proper activation of intracellular signaling cascades largely depends on the reversible phosphorylation of proteins, lipids and other small molecules [208]. Phosphatases are enzymes with the ability to hydrolyse the phosphoester bonds on their substrates [209, 210]. Phosphatases were discovered several years after protein kinases and although less studied, they are also powerful controllers of intracellular signaling cascades [209-211]. Dual-specificity phosphatases (DUSPs) are a subclass of phosphatases able to specifically dephosphorylate both serine/threonine and tyrosine residues on their targets, which include MAPKs [212, 213]. Previously, we described how Ang-1 mediates some of its biological effects through the activation of ERK1/2, p38 and SAPK/JNK including survival, migration and proliferation of ECs [13, 157-159]. Therefore, understanding the precise mechanisms of activation and inactivation of the ERK1/2, p38 and SAPK/JNK signaling pathways downstream of Ang-1 represents an important challenge to find new ways to manipulate MAPK-dependent responses in the context of angiogenesis.

1.3.1 Dual-specificity phosphatases- An overview

The outcomes of MAPK signaling are largely determined by the magnitude and duration of MAPK phosphorylation, suggesting that the mechanisms of signaling inactivation are just as important as the activation of the signaling cascades themselves [131, 214]. MAPKs can be inactivated completely by dephosphorylation of only one residue, either Thr or Tyr, or both residues in their active kinase domains [215]. The kinetics of dephosphorylation and inactivation of MAPKs vary from minutes to several hours depending on the cell type and the nature of the stimulus [214]. Several types of phosphatases have been described that are able to dephosphorylate MAPKs on their phosphorylated active sites and include serine/threonine phosphatases and protein tyrosine phosphatases (PTPs) [213, 214, 216].

PTPs belong to the largest family of phosphatases characterized by the presence of the consensus motif $HC(X)_5R$ in their active site, in which the cysteine is necessary for the catalytic activity of the phosphatase [210]. In humans, approximately one hundred genes encode PTPs and several of them have been found to possess additional alternative splicing variants and/or to suffer post-translational modifications; which suggests that the regulation of signal transduction by PTPs is highly complex [210]. PTPs have been divided into phosphotyrosine-specific phosphatases and DUSPs [217, 218].

DUSPs constitute a highly heterogeneous family of phosphatases with little sequence similarity outside of the PTP consensus motif [210, 213]. In the human genome there are approximately 65 genes that encode DUSPs, all of which share the same catalytic mechanism as the classical PTPs [210]. However, the conformation of the DUSP active site allows them to fit not only the phosphotyrosine residues but also the phosphoserine and phosphothreonine residues of their substrates [210]. In addition to their phosphatase activity, DUSPs can also control the subcellular localization of MAPKs [212, 213, 219-221]. DUSPs have

been subdivided into CH2 (CDC25 homology)-motif-containing MAPK phosphatases (MKPs), JSP1-like phophatases, MKP6-like, VHR-like, slingshot-like and SKPR1/hyVH1 [212, 213, 216]. To date, sixteen mammalian DUSPs that dephosphorylate MAPKs have been identified, of which eleven belong to the subfamily of MKPs [213].

The MKPs are a subfamily of DUSPs comprised of ten phosphatases and one protein, DUSP24/MK-STYX, that shares many characteristics of a MKP but lacks the cysteine in the catalytic site necessary for their enzymatic activity [212]. These MKPs contain a motif in their active site that shares high sequence similarity with the PTP VH1 from *vaccinia virus* and an amino-terminal kinase interactive motif (KIM) that contributes to their substrate specificity [212, 213]. MKPs have been grouped into three major subfamilies based on their sequence similarity, substrate specificity and subcellular localization (Figure 1.3) [213]. The largest group includes four inducible nuclear phosphatases: DUSP1 (MKP-1), DUSP2 (PAC-1), DUSP4 (MKP-2) and DUSP5 [212, 216]. The second group is comprised of ERK-specific phosphatases that are found in the cytoplasm: DUSP6 (MKP-3), DUSP7 (MKP-X) and DUSP9 (MKP-4). The third group contains phosphatases that selectively inactivate p38 and SAPK/JNK: DUSP8 (hVH5), DUSP10 (MKP-5) and DUSP16 (MKP-7) [212].

1.3.1.1 Dual-specificity phosphatase 1

Dual-specificity phosphatase 1 (DUSP1) is an early response gene that encodes a 40 kDa protein mainly expressed in the nucleus and induced by serum growth factors, oxidative stress, heat shock, inflammatory stimuli and UV irradiation [222-226]. DUSP1 was first described as an ERK-specific phosphatase, although later studies have shown that DUSP1 can also efficiently inactivate the stress-activated MAPKs p38 and SAPK/JNK [225-230].

The transcription of the DUSP1 gene is under the control of the glucocorticoid receptor and various transcription factors including as Sp1, Sp3, and AP-1 [225, 231-233]. In addition, chromatin remodeling also plays a critical role in the regulation of the DUSP1 gene [234]. DUSP1 is transcriptionally induced by glucocorticoids as a mechanism to counteract inflammatory responses. Glucocorticoids activate the intracellular glucocorticoid receptor (GR) and activate DUSP1 transcription via glucocorticoid response elements (GREs) found upstream of the human DUSP1 gene [231, 232]. Interestingly, glucocorticoid treatment also modifies the chromatin structure of the DUSP1 gene increasing DNase I accessibility [232].

Several immediate early genes such as c-jun and c-fos, encoded by short-lived mRNA, are regulated post-transcriptionally by RNA-binding proteins, which can stabilize or destabilize the transcripts [235, 236]. The 3' untranslated (UTR) region of the DUSP1 mRNA contains several evolutionally conserved AU-rich elements (AREs) that could potentially act as binding sites for various RNA-binding proteins, such as TTP and HuR [222, 237, 238]. The mRNA stability of DUSP1 is affected by hydrogen peroxide and heat shock [224, 239]. In HeLA cells treated with hydrogen peroxide the half-life of DUSP1 mRNA increases dramatically as a result of the interaction between the RNA-binding proteins HuR and NF90 with the 3´ UTR of DUSP1 [239].

The post-translational modifications of DUSP1 also play an important role in regulating the activity of this phosphatase; and include phosphorylation, oxidation and acetylation [240]. DUSP1 can be phosphorylated by ERK1/2 in two different regions, and each has a different effect on its stability without affecting its phosphatase activity [241-243]. The transient activation of ERK1/2 phosphorylates DUSP1 at two carboxyl-terminal

residues, Ser359 and Ser364, enhancing DUSP1 stability [241, 242]. Meanwhile, the sustained activation of ERK1/2 phosphorylates DUSP1 at Ser296 and Ser323, which targets the phosphatase for proteasomal degradation by facilitating its interaction with the Skp-cullin-F-box ubiquitin ligase [243, 244]. Additionally, DUSP1 possesses a redox sensitive motif in its active site making it a target of oxidation by reactive oxygen species (ROS) which results in the loss of phosphatase activity [240, 245]. DUSP1 is acetylated by p300 on the lysine 57 located within its substrate-binding domain and this acetylation does not affect its protein stability or its phosphatase activity, but it increases its affinity for p38 and indirectly increases its activity [246]. The acetylation of DUSP1 has been linked to the mechanisms through which DUSP1 inhibits the MAPKs and decreases pro-inflammatory signaling downstream of Toll-like receptors (TLRs) [246].

In ECs the expression of DUSP1 is induced by thrombin, VEGF and hypothermia [247-249]. The activation of the ERK1/2 pathway is necessary for thrombin-induced DUSP1 expression, whereas SAPK/JNK activation downstream of VEGFR-2 is required for VEGF-induced transcription of DUSP1 [247]. Furthermore, DUSP1 induction plays a role in VEGF-stimulated EC migration [248]. In the case of hypothermia, DUSP1 induction in ECs protects against endothelial barrier dysfunction and apoptosis caused by TNF-α [249].

1.3.1.2 Dual-specificity phosphatase 4

Dual-specificity phosphatase 4 (DUSP4) is an early response gene induced by various stimuli including phorbol-ester, IBMX-forskolin and serum [250, 251]. The signaling pathways that mediate DUSP4 induction vary with the nature of the stimulus and the cell type. Serum induces the expression of DUSP4 through activation of the ERK1/2 pathway and the induction of DUSP4 in pituitary cells upon activation of the gonadotropin receptor (GnRH)

depends on ERK1/2 and SAPK/JNK activation, but not on p38 [252, 253]. Multiple transcription factors regulate the expression of the DUSP4 gene, including Egr-1, E2F-1, p53 and the homeobox gene HoxA10 [253-257].

The human DUSP4 gene is comprised by 6 exons and through alternative splicing encodes two proteins with a similar level of phosphatase activity *in vitro* termed long MKP2-L (42.9 kDa) and short MKP2-S (32.9 kDa) [258]. Although most studies on DUSP4 have focused on its long isoform, the MKP2-S variant is endogenously expressed in different cell types and in prostate tumors [258]. MKP2-S is homologous to the carboxyl-terminal catalytic region found in MKP2-L, but it lacks the KIM domain necessary for its interaction with MAPKs and a proximal NLS [258]. MKP-2-S is unable to bind to ERK, p38 and SAPK/JNK regardless of its activation state, which correlates with its lack of KIM domain. However, MKP2-S is able to decrease SAPK/JNK activity and COX-2 expression in HUVECs treated with TNF-α, although not as effectively as MKP2-L. Furthermore, MKP-2-S is also able to inhibit ERK1/2 activation and EC proliferation in response to fetal calf serum (FCS) [258].

MKPs have different substrate preferences despite their similar structure and their catalytic activity can be modulated upon binding to their substrates [212]. Initially the ability of DUSP4 to dephosphorylate ERK1/2 and SAPK/JNK was thought to be higher than its ability to dephosphorylate p38 [252, 258]. However, evidence points to an important role of DUSP4 in p38 inactivation [259, 260]. DUSP4 is able to dephosphorylate all three MAPKs in co-transfection experiments and inhibits the activation of myelin basic protein, ATF-2 and c-Jun, downstream targets of ERK1/2, p38 and SAPK/JNK respectively [259]. Additionally, DUSP4 has been found to be a potent inhibitor of p38 activity in PC-12 cells in response to

nerve growth factor (NGF) [260]. Moreover, macrophages derived from DUSP4 knockout mice have enhanced SAPK/JNK and p38 activation but not increased ERK activation in response to LPS [261]. DUSP4 catalytic activity increases in a dose dependent manner preferentially through its interaction with ERK1 and JNK1, while p38 had a very small effect [54]. This provides a possible explanation on earlier findings in which DUSP4 preferentially dephosphorylated ERK and SAPK/JNK and in a lesser degree p38 [252, 258]. Interestingly, DUSP4 binding affinity is higher for ERK and p38 than for SAPK/JNK, which does not correspond with the levels of activation of the phosphatase upon substrate binding [262]. Also, SAPK/JNK has a stronger interaction to the catalytically inactive form of DUSP4 [258]. These findings point to a possible extra level of regulation of MAPKs activity in which DUSP4 could be sequestered by ERK and p38, who have higher binding affinity for the phosphatase, leading to SAPK/JNK activation [262].

In various systems, DUSP4 has been described as a promoter of pro-apoptotic signals through inhibition of the ERK1/2 signaling pathway. DUSP4 is a transcriptional target of p53 and E2F-1 and its expression is required for apoptosis in response to oxidative stress [255, 256]. In contrast DUSP4 has been shown to inhibit apoptosis by limiting SAPK/JNK activation, an important regulator of cell death in response to pro-inflammatory cytokines, UV irradiation and cellular stress [151, 263]. In a model of apoptosis induced in HUVECs by repressing NF-κB signaling using a dominant-negative IκB kinase beta (DN-IKKβ), adenoviral expression of MKP-2 was able to rescue the cells from TNF-α-induced apoptosis by selectively dephosphorylating SAPK/JNK [264]. Also, in a model of apoptosis induced by UV and cisplatin in HEK-293 cells the overexpression of MKP-2 was able to partially rescue cells from apoptosis by decreasing SAPK/JNK activation [265]. Moreover, in

undifferentiated myeloid cells HoxA10 inhibits apoptosis by inducing MKP-2 transcription and thus impairing SAPK/JNK activation [257]. These studies suggest that DUSP4 is an anti-apoptotic molecule able to limit SAPK/JNK-mediated apoptosis.

Additionally, DUSP4 is involved in the regulation of cellular growth and senescence. DUSP4 is considered to be a tumor suppressor due to its growth suppressive activity through its ability to negatively regulate ERK1/2 activation [255]. In human fibroblasts the induction of DUSP4 during senescence or in contact inhibition seems to be a mechanism to induce growth arrest also through the regulation of the ERK1/2 pathway [266, 267].

1.3.1.3 Dual-specificity phosphatase 5

Dual-specificity phosphatase 5 (DUSP5/hVH3) encodes a protein of 42 kDa expressed in human brain, heart, lung, liver, pancreas, skeletal muscle, kidney and placenta [250, 268]. DUSP5 is an ERK-specific phosphatase that not only causes the inactivation and nuclear translocation of ERK2, but also allows its sequestration in the nucleus [221, 250, 268]. The structure of the DUSP5 protein includes a conserved KIM domain and a functional NLS required for its functions *in vivo*. The KIM allows DUSP5 to bind ERK1/2 by interacting directly with their common docking domain (CD), while the NLS localizes DUSP5 in the nucleus [221].

Several MKPs suffer changes in their activity and/or stability upon binding to their substrates [136]. Interestingly, DUSP5 interaction with ERK2 does not affect its catalytic activity and evidence from the crystal structure of the protein suggests that this is possibly because the catalytic domain of DUSP5 is in an active conformation [221, 269]. Nonetheless, this interaction promotes stabilization of the phosphatase independently of ERK2 kinase activity [270].

DUSP5 expression can be induced by serum, heat shock and mitogenic stimuli [248, 250, 268]. DUSP5 is also a direct transcriptional target of p53 and its transcription has been found to be dependent on sustained ERK1/2 activation and c-jun [270, 270-272, 272, 273]. Post-translational modifications can affect the stability of DUSP proteins [136, 213]. DUSP5 can be phosphorylated by ERK1/2 in 3 different sites within its carboxyl-terminal domain, Thr321, Ser346 and Ser376, and the KIM domain in DUSP5 is required for ERK-mediated phosphorylation [270]. Interestingly the phosphorylation of DUSP5 in any of these sites does not modify its stability, nuclear localization or ability to bind and inactivate ERK2 [270].

Several studies highlight the role of DUSP5 in modulating immune responses through ERK1/2 inactivation [274-277]. Moreover, expression of DUSP5 in human cancers has been shown to have both, tumor suppressor and tumor promoter effects [271, 272, 278]. Interestingly, DUSP5 expression is important for the development and maintenance of the vasculature [279, 280]. DUSP5 first appeared as a candidate gene involved in vascular development in a microarray analysis of the zebrafish cloche (*clo*) mutation that affects the earliest known steps in differentiation of blood and ECs in vertebrates. In wild-type embryos DUSP5 was expressed in all vascular ECs and their precursors, while in $clo^{-/-}$ mutants DUSP5 was significantly downregulated [279]. In a loss-of-function study using DUSP5 knockdown embryos, it was demonstrated that DUSP5 is also required to maintain the angioblast populations in zebrafish embryos. Embryos that lacked DUSP5 had higher amounts of phosphorylated ERK and interestingly HUVECs in which DUSP5 has been knocked down using siRNA had increased cell death by apoptosis [280]. VEGF controls proliferation of ECs by inducing ERK1/2 phosphorylation, and in HUVECs VEGF-induced transcription of DUSP5 regulates proliferation by modulating ERK1/2 activity [248].

1.4 The angiopoietin-Tie system in tumor progression and metastasis

1.4.1 Breast cancer- An overview

Breast cancer is the most prevalent hormone-dependent cancer in women and its ability to form metastasis in different organs accounts for its high mortality rate. Estrogen and the estrogen receptor (ER) are implicated in breast cancer progression and approximately 70% of human breast tumors express hormone receptors, ER and/or progesterone receptor (PR) [281]. Breast tumors are highly heterogeneous and distinct tumor subtypes have been classified by their differences in gene expression to better reflect clinical tumor types and prognosis [282-284]. The ER is an important marker to discriminate between breast cancers and divides tumors into two major subtypes ER-positive (ER+) and ER-negative (ER-) [282]. ER+ subtypes include luminal A, luminal B and luminal C tumors, whereas ER-negative subtypes include normal like, human epidermal growth factor receptor 2 positive (HER2+), basal and claudin-low tumor types [282-286]. Luminal ER+ tumors express high amounts of cytokeratins and genes commonly found in luminal epithelial cells of normal breast tissue [287]. Tumors from the luminal A subtype are less heterogeneous than those characterized as luminal B, and they have the highest expression of genes from the ER cluster including ERα, GATA binding protein 3, hepatocyte nuclear factor 3 α and LIV-1, a zinc transporter regulated by estrogen [282]. Tumors from the luminal B and C subtypes have low to moderate expression of the luminal-specific genes including the ER cluster, and tumors classified as luminal subtype C additionally express genes found in the basal-like and HER2+ subtypes [282].

Patients with ER+ breast tumors are commonly treated with adjuvant therapy to antagonize ER signaling. The therapeutic strategies include the use of the estrogen

antagonist tamoxifen, aromatase inhibitors that block estrogen biosynthesis, luteinizing hormone–releasing hormone (LHRH) agonists and fulvestrant to decrease ER levels [281]. However, despite the adjuvant therapy several patients relapse and those with metastatic disease often become resistant to anti-estrogen treatment [281]. For these reasons, understanding the molecular mechanisms through which ERs contribute to breast cancer progression will lead to the generation of targeted therapies able to improve survival rates in patients with breast cancer.

The most studied mechanism through which estrogen promotes tumorigenesis is the proliferation of ER+ cells within the tumors. However, estrogen is also an important regulator of angiogenesis, a marker of poor prognosis in patients with breast cancer [283, 288, 289]. Angiogenesis is required for tumor growth and metastasis dissemination, and represents a promising alternative for therapeutic intervention in breast cancer [290]. Estrogen can act directly on ECs from the tumor microenvironment, which express ERs, to promote survival, proliferation and vessel growth [291]. However, activation of ER signaling in tumor cells can also affect the expression of pro-angiogenic factors such as VEGF and angiopoietins, thus modifying the microenvironment of the tumor and stimulating the formation of new vessels [292-294].

1.4.1.1 Estrogen receptor signaling and its role in breast cancer

Estrogen can bind to distinct ERs from the nuclear receptor family of transcription factors, ERα (NR3A1) and ERβ (NR3A2), in order to mediate its biological effects [295]. Both ERs have similar affinities for estrogen, bind the same DNA response elements and share a high degree of sequence homology [295]. ERs possess a DNA-binding domain (DBD) required for DNA recognition and binding, a carboxyl-terminal ligand-binding domain (LBD) and an

amino-terminal domain that varies in sequence and length [295, 296]. Two different activation functions (AF) domains facilitate transcriptional activation by recruiting various co-regulatory protein complexes to the DNA-bound receptor. AF-1, located at the amino-terminal part of the receptor, is constitutively active; whereas AF-2 situated in the carboxyl-terminal LBD is ligand-dependent [296]. The major ER subtype in the mammary epithelium is ERα and it regulates the development of the mammary gland after puberty, as well as breast cancer progression [297, 298].

Estrogen signaling begins with the binding of estrogen to ERs, which subsequently induces the transcription of estrogen responsive genes in cooperation with co-regulatory proteins [295, 296]. The interactions of ERs with DNA can be direct or indirect [295]. Estrogen-activated ERs can directly bind to estrogen-responsive elements in cis-regulatory DNA sequences to promote the expression of genes that regulate the cell cycle, such as the transcription factor E2F1 [299, 300]. Transcriptional activation of E2F1 and its downstream target genes by ERα induces proliferation of breast cancer cells and has been associated with a higher breast cancer grade and poor survival [300-302]. ERs can also interact indirectly with the DNA through other transcription factors, such as AP-1 [303, 304]. AP-1 regulates proliferation of breast cancer cells and the crosstalk between ERα and AP-1 has been associated with invasiveness and resistance to tamoxifen in ERα+ breast tumors [305-307].

1.4.2 Tumor angiogenesis in control of cancer progression

In order for tumors to develop they need to gain access to the host vasculature [308]. In primary and distant metastatic sites small lesions of less than 2 mm in diameter can stay dormant indefinitely in an avascular phase [309]. However, small subsets of these lesions are able to enter into a vascular phase characterized by exponential growth [290, 309]. In tumors

lacking blood vessels, hypoxia and nutrient deprivation trigger an "angiogenic switch" that allows them to grow in size and metastatic potential [290, 309-311]. The angiogenic switch consists mainly on altering the balance between pro-angiogenic and anti-angiogenic factors to promote vascular growth within the microenvironment of the tumor [290, 310-312]. Tumor cells frequently express pro-angiogenic factors such as VEGF, FGF, and angiopoietins [310, 311, 313]. Additionally, tumors are able to activate other neighboring cells from their microenvironment to release growth factors and cytokines, which initiate angiogenic and inflammatory cascades that further contribute to tumor progression [310, 314].

The role of ECs in cancer progression has been highlighted by several studies demonstrating that ECs actively recruit bone marrow–derived cells (BMDCs) to the tumor microenvironment [315]. Tumor-associated ECs secrete Ang-2 and thus promote recruitment of Tie-2-expressing monocytes that interact with the vasculature and stimulate ECs to secrete additional angiogenic factors to amplify the angiogenic cascade [316, 317]. Furthermore, blocking the infiltration of BMDCs into the tumor microenvironment with anti-angiogenic drugs reduces angiogenesis, makes the tumor more responsive to therapy and inhibits metastasis [318-321]. Angiogenesis is also necessary for metastasis dissemination since larger numbers of vessels increase the opportunity for tumor cells to enter the circulation [322]. Moreover, microvessel density often correlates with the degree of aggressiveness in different types of tumors [323].

In general the tumor vasculature is disorganized, discontinuous and often dilated; characteristics that cause irregular blood flow to the tumors and further increase hypoxia [324]. Tumor-associated vessels are often leaky and in a constant state of remodeling mainly due to an excess of VEGF and a lack of association of ECs with pericytes [312, 325-327]. In addition to angiogenesis, vasculogenesis is also an important mechanism for the development of

tumor vasculature [328]. The mobilization of circulating endothelial progenitor cells (EPCs) derived from the bone marrow into the tumor site is involved in the formation of new blood vessels and inhibiting EPC recruitment impairs tumor growth [328-330].

1.4.3 Angiopoietin-Tie signaling in tumor angiogenesis and metastasis

Tie-2 receptors have been found to be up-regulated in the tumor vasculature and their role in tumor progression has been highlighted by *in vivo* experiments in which a soluble extracellular domain of Tie-2 (sTie2) applied locally to the tumor site or systemically delivered using adenoviral vectors inhibited tumor growth and metastasis, respectively [11, 60, 331]. However, the individual contributions of angiopoietin ligands to cancer progression remain to be elucidated.

The role of Ang-1 in tumor growth and metastasis; as well as its potential as a therapeutic target remain controversial. Studies in mice have shown that ectopic expression of Ang-1 reduces xenograft tumor growth of MCF-7 breast cancer cells, A431 squamous cell carcinoma cells and HT-29 colon cancer cells due to increased pericyte coverage and reduced angiogenesis [332-334]. These results are contradicted by studies in which Ang-1 overexpression increases the growth of human cervical cancers in mice and in a rat glioma model by promoting angiogenesis [335, 336]. Additionally, systemic adenoviral delivery of Ang-1 into mice with LNM35/Luc lung cancer cells implanted subcutaneously promoted metastasis dissemination to the lungs due to vessel enlargement [337].

Ang-2 expression is increased in the tumor vasculature under hypoxic conditions, where it acts as an antagonist of Tie-2 receptors and inhibits Ang-1 signaling to promote primary tumor growth and angiogenesis [338-340]. In various tumors xenograft models Ang-2 inhibition using monoclonal antibodies and fusion proteins results in suppression of tumor growth and reduced vascularization [340-342]. Ang-2 from the host was found to be required

for the initial stages of tumor growth, but not for later stages in experimental models of LLC, MT-ret and B16F10 melanomas [343]. The tumor vasculature of mice lacking Ang-2 exhibited an altered pattern of pericyte recruitment and maturation, while no significant changes in microvascular density were observed [343]. In contrast, in a study in which Ang-2 was ectopically expressed in a breast cancer cell line, the tumor vasculature was characterized by non-functional vessels and hemorrhage associated with loss of pericytes and EC apoptosis [344]. These findings support the notion that Ang-2 has a dual function in tumor angiogenesis. On one hand Ang-2 can induce robust angiogenesis in the presence of VEGF, while the expression of Ang-2 in the absence of VEGF can lead to vessel regression [339, 344, 345]. Interestingly, Ang-2 also increases tumor cell invasion and promotes metastasis formation. Ang-2 overexpression in breast cancer cells promoted epithelial to mesenchymal transition and increased their invasive potential by an autocrine effect mediated by integrin $\alpha5\beta1$ and independent of Tie-2 [206]. Systemic overexpression of Ang-2 in mice using adenoviruses not only promoted growth of LNM35 primary tumors but it also increased metastasis to lymph nodes and lung [345]. Similar results have been observed in an endothelium-specific conditional transgenic mouse model overexpressing Ang-2 (VEC-tTA/Tet-OS-Ang2) [345]. The use of an Ang-2 blocking antibody in both of these models inhibited tumor growth, decreased homing of tumor cells into the lungs and reduced metastasis [345]. Additionally, Ang-2 is able to modify the inflammatory and immune responses in tumors [317, 346]. It has been reported that Ang-2 stimulates monocytes that express Tie-2 to suppress T-cell activation [346]. Interestingly, Ang-2 blocking antibodies have been shown to not only reduce angiogenesis and inhibit metastasis but also to interfere with Tie-2 expression in a subpopulation of pro-angiogenic myeloid cells [317].

1.4.4 The angiopoietin-Tie system in malignancy – Evidence from epidemiological studies

Tie-2 receptors have been found to be upregulated in the tumor vasculature of several human cancers, although in most cases there has been no correlation between Tie-2 expression and clinical outcomes [333, 347-352]. However, a high level of Tie-2 in the plasma of patients with neuroendocrine tumors has been proposed as a predictor of metastasis, and in colorectal carcinoma Tie-2 expression has been associated with venous and lymphatic invasion [353, 354]. In contrast, Tie-2 receptor expression in bladder cancer has been described as an independent favorable prognostic factor for both metastasis and disease specific survival [355].

Studies of solid tumors in humans including colorectal carcinoma, non-small cell lung cancer (NSCLC), pancreatic cancer and bladder cancer have found no direct correlation between Ang-1 expression and prognosis [256, 356, 357]. However, a study in patients with NSCLC revealed that high Ang-1 expression was linked to poor outcomes, and in colorectal carcinoma Ang-1 expression correlated with the presence of lymphatic invasion, but not with lymph node metastasis [354, 358]. Furthermore, in clinical specimens of breast cancer Ang-1 expression has been found to be low despite being upregulated in many human breast cancer cell lines, and Ang-1 expression has been associated with reduced microvessel densities [294, 333, 347].

In contrast, Ang-2 has been found to be highly expressed in tumor vasculature and in many cases Ang-2 expression also correlates with VEGF expression in solid tumors including breast cancer, glioblastomas, hepatocellular carcinomas, gastric cancer and ovarian cancer [359-364]. These studies support the idea that Ang-2 and VEGF coordinate to promote angiogenesis in several cancers. Additionally, Ang-2 overexpression, as well as

higher Ang-2/Ang-1 ratios compared to normal tissues, correlates with poor prognosis in many cancers [256, 350, 360, 365-367]. Interestingly, elevated levels of Ang-2 in the plasma of patients with neuroendocrine tumors has been associated with the extent of lymphatic metastasis [353]. Additionally, Ang-2 has been found to be expressed in several human breast cancer samples and its presence correlates with lymph node invasion and short survival rates [62, 254, 368, 369].

Several human cancers including NSCLC, colorectal adenocarcinoma, glioblastoma and breast cancer express Ang-4 [348, 354, 370-372]. However, the levels of Ang-4 not always correlate with clinical outcomes [348]. In breast cancer Ang-4 expression correlated with estrogen receptor and in NSCLC Ang-4 expression in tumor or stromal cells has been associated with survival [294, 370].

1.5 MicroRNAs in control of the inflammatory response

In the early 1990s the studies of Victor Ambros, Gary Ruvkun and colleagues described the first microRNA (miRNA) genes, lin-4 and let-7, which control development in *Caenorhabditis elegans* by regulating gene expression at the post-transcriptional level [373, 374]. Since then, our knowledge of miRNAs and their mechanisms of regulation quickly expanded to plants and animals [375, 376]. Furthermore, it has been estimated that miRNAs could potentially regulate a very large portion of the human genome, emphasizing their importance as regulators of gene expression [377].

miRNAs are small, non-coding RNA molecules of approximately 22 nucleotides in length that can function as post-transcriptional repressors of gene expression [378]. miRNAs use short regions of complementarity, around 6-8 nucleotides within the 3' UTR of their target mRNAs, to mediate their repressor functions [378]. The extent of pairing between the 3'UTR of the target mRNA and the miRNA "seed", a 5′ region of the miRNA located on nucleotides 2–7, is important for miRNA target recognition and determines the mechanism of repression [377, 378]. Two mechanisms through which miRNAs regulate gene expression at the post-transcriptional level have been described [378, 379]. The first occurs when the base pairing between miRNAs and mRNA targets is by precise or nearly precise complementarity, and leads to direct cleavage and destruction of the target mRNA through a process involving the machinery of RNA interference [378, 379]. The second mechanism occurs when miRNAs are only partially complementary to their mRNA targets, and results in inhibition of protein synthesis while the stability of the mRNA target is maintained [378, 379].

The evolutionary plasticity of miRNAs and the sensitivity of their repression make them ideal regulators of inflammatory responses in organisms that are constantly being challenged by pathogens [380]. The first evidence on the role of miRNAs in the regulation of immunity came from studies of mice deficient in genes required for miRNA biogenesis and silencing [381, 382]. Knocking down the enzyme Drosha in a T-cell specific manner causes autoimmune disease in mice, and mice that lack the enzyme Dicer fail to produce B cells [381, 382]. More recently, several families of miRNAs have been described that regulate innate and adaptive immune responses. These include the miR-146 family of miRNAs that interferes with NF-κB, a key regulator of inflammation [380].

1.5.1 Mechanisms of microRNA biogenesis and silencing by the RNA-induced silencing complex

The biogenesis of miRNAs starts in the nucleus, where miRNA genes are transcribed by RNA polymerase II or III into primary miRNA transcripts (pri-miRNA), see Figure 1.4 [383, 384]. The expression of miRNAs can be modulated by transcription factors such as NF-κB, c-Myc and p53, or by epigenetic changes in their promoter sequences [385-389]. Often the genes of different miRNAs with multiple mRNA targets can be found clustered in the genome in tandem and in many cases these miRNAs can be coordinately transcribed as a common polycistronic precursor transcript [390, 391].

The pri-miRNA is then cleaved by a nuclear microprocessor complex comprised of Drosha, a RNase III enzyme, and the DiGeorge critical region 8 (DRGCR8) protein (Figure 1.4) [392]. In general the structure of a pri-miRNA includes a double-stranded hairpin stem of 33 nuceotides, a terminal loop and two single-stranded regions upstream and downstream of the hairpin [393]. The hairpin stem and the single-stranded regions flanking the pri-miRNA are critical for binding to DGCR8 and subsequent cleaving by Drosha at a particular site located 11 nucleotides away from the single-stranded/double-stranded junction at the base of the hairpin stem to generate the precursor miRNA (pre-miRNA) [393, 394]. Interestingly, Drosha-mediated processing of pri-miRNAs into pre-miRNAs is not required by miRNAs derived from introns, called mirtrons, that have been released from their transcripts after splicing and their size resembles a pre-miRNA (Figure 1.4) [395].

Subsequently the pre-miRNAs are exported from the nucleus into the cytoplasm by exportin-5 (XPO5) in a complex with Ran-GTP (Figure 1.4) [396]. Once in the cytoplasm, the pre-miRNAs are processed by Dicer, a RNAse III enzyme, to generate a short RNA duplex

that will give origin to a mature, single-stranded miRNA of around 20–30 nucleotides in length [379, 393]. Although the miRNA duplex could give rise to two different mature miRNAs, only one strand is usually incorporated into the RNA-induced silencing complex (RISC) to guide the repression of its target mRNAs, while the other strand is degraded [397]. Based on the thermodynamic stability of the base pairs at the two ends of the duplex, the miRNA strand with the less stable base pair at its 5' end is loaded into the RISC [398]. The RISC is comprised of the RNase Dicer, the double-stranded RNA-binding domain protein TRBP, the protein activator of PKR (PACT) and the core component Argonaute-2 (Ago-2) [379]. The RISC silences the expression of its target genes at the post-transcriptional level, which are selected through interactions between the miRNA loaded into the RISC and the mRNA target [378, 379]. Then, depending on their degree of complementarity, partial or full, the RISC proceeds to repress translation or induces mRNA degradation, respectively (Figure 1.4) [378, 379].

1.5.2 MicroRNAs as modulators of signaling pathways

Several biological processes including inflammation, angiogenesis and cardiovascular development require miRNA regulation [399-402]. miRNAs are often found as families of redundant genes with numerous possible targets, which makes it difficult to dissect their physiological functions [403]. Furthermore, the degree of target repression by miRNAs is often low, suggesting that few miRNA targets could be relevant enough to exert a phenotypical change [403, 404]. Signaling pathways are excellent candidates for miRNA regulation due to their dynamic nature and their sensitivity to slight protein variations [403, 405]. Various miRNAs that target modulators of key signaling pathways have been identified and some of their functions include signal amplification, crosstalk between different pathways and signal robustness [403].

In the case of inflammation, the activation of signaling pathways must be tightly controlled to prevent an excessive activation of the inflammatory response, as is the case in bacterial sepsis [406]. Sepsis occurs as a result of an exaggerated systemic inflammatory response to Gram-negative bacteria and their cell wall component LPS; and is characterized by an increase in the production of pro-inflammatory cytokines, vascular permeability and lung edema [406, 407]. Interestingly, systemic expression of Ang-1 using adenoviruses, or mesenchymal cells overexpressing Ang-1, exerts a protective role by reducing lung damage and improving overall survival in a mouse model of endotoxin shock induced by *E. Coli* LPS [408-411]. *E. Coli* LPS elicits this response through the activation of Toll-like receptor 4 (TLR4), a cellular receptor responsible for innate immune responses that plays a crucial role in inflammatory diseases [412, 413].

miRNAs can have a significant impact on the magnitude of the inflammatory response and in the onset of sepsis by targeting signal transduction proteins involved in the transmission of intracellular signals following initial pathogen recognition by TLRs and by directly targeting mRNAs that encode inflammatory cytokines [414]. In the following sections we will address in more detail the influence of miRNAs on inflammation, particularly on TLR signaling pathways.

1.5.2.1 Influence of miRNAs on Toll-like receptor signaling pathways

There are two types of immunity, innate and adaptive [415]. The innate immunity is the first protective mechanism against pathogens and acts as an initiator of the inflammatory responses [416]. Innate immunity is a cellular response that involves macrophages, granulocytes and dendritic cells; and it is initiated by the activation of pattern recognition receptors (PRRs) encoded in the germ line that recognize pathogen-associated molecular patterns (PAMPs) expressed by viruses, bacteria, fungi and parasites [416, 417]. PAMPs

include lipoproteins, LPS, double- and single-stranded RNA, flagellin and DNA [416, 417]. LPS is a component of the outer membrane of Gram-negative bacteria formed by a core oligosaccharide, an O-specific chain made up of repeating sequences of polysaccharides and a lipid A component responsible for its inflammatory properties [407, 412]. The diversity of molecular structures found on LPS from different bacterial strains provides antigenic specificity to the immune response of the host [417]. The activation of PRRs by PAMPs initiates intracellular signaling cascades. PRRs are comprised of several types of receptors including TLRs, retinoid acid-inducible gene I (RIG-I)-like receptors and nucleotide-binding oligomerization domain (NOD)-like receptors [416, 418]. TLRs were the first PRRs to be identified in mammals based on their homology to the Toll protein found in *Drosophila melanogaster* [416, 417, 419]. TLRs are type I transmembrane receptors that contain a leucine-rich ectodomain responsible for the recognition of PAMPs, a transmembrane domain, and cytosolic Toll-IL-1 receptor (TIR) domains which activate intracellular signaling pathways and are homologous to the cytoplasmic domain of the interleukin-1 receptor (IL-1R) family [416, 417]. In humans ten TLRs have been described to date and each one displays a different specificity to PAMPs [416].

The interaction of TLRs with their ligand initiates intracellular signaling cascades that provide specific immunological responses specific to the pathogen [416]. The cytoplasmic TIR domain recruits TIR domain-containing adaptor protein such as MyD88, TIRAP, TRIF or TRAM [416]. TLR4 is the main TLR activated by bacterial LPS, although LPS from other strains such as *Porphyromonas gingivalis* activate TLR2 [420]. TLR4 activates two distinct signaling pathways, the MyD88-dependent and the TRIF-dependent pathways [417]. LPS interacts with TLR4 via LPS-binding protein (LBP), which brings LPS to the cell surface and forms a ternary complex with the LPS receptor molecule CD14 [421]. Upon binding to

LPS, TLR4 in complex with MD2 recruits MyD88 through its interaction with TIRAP, an adaptor molecule located in the plasma membrane [422] [423]. MyD88 recruits interleukin-1 receptor-associated kinase (IRAK) 1 and 4, TNF receptor-associated factor 6 (TRAF6) and the TGF-beta activated kinase 1 (TAK1) complex, leads to an early-phase activation of NF-κB and MAPKs, and promotes transcription of pro-inflammatory genes [416, 417]. The TRIF-dependent pathway is activated in intracellular vesicles after the TLR4 is endocytosed, where it forms a complex with TRAM and TRIF [416]. The recruitment of TRAF3 and the protein kinases TBK1 and IKKi to the complex then catalyzes the phosphorylation of interferon (IFN) regulatory factor 3 (IRF3) [416]. The activation of IRF3 leads to the expression of type I IFN [416]. Additionally, TRAM-TRIF recruits TRAF6 and TAK1 to mediate the late activation of NF-κB and MAPKs. The MyD88-dependent pathway is largely responsible for controlling the expression of inflammatory cytokines, such as TNF-α, interleukin 6 (IL-6) and interleukin 12 (IL-12) whereas the MyD88-independent pathway induces expression of IFN-inducible genes, such as interferon gamma-induced protein (IP10) and glucocorticoid-attenuated response gene 16 (GARG16) [412, 424]. In the endothelium activation of TLR4 by LPS mainly leads to the activation of MyD88-dependent pathways; although some studies have shown LPS can induce MyD88-independent genes like IFNβ, IP10 and iNOS in ECs [425].

Evidence that miRNAs control innate immune responses comes from studies of miRNAs like miR-125b and let-7. These miRNAs target the mRNA of the pro-inflammatory cytokines TNF-α and IL-6, respectively and are downregulated by TLR signaling [426-428]. Likewise, TLR signaling also induces the expression of various miRNAs that regulate immune responses including miR-155, miR-21 and miR-146a. miR-155 is a

pro-inflammatory miRNA, whereas miR-21 and miR-146a act as negative feedback regulators that attenuate the immune response [385, 426, 429]. In the following section we will explore the role of the family of miRNAs miR-146 in inflammation and immunity.

1.5.2.2 The miR-146 family

The miR-146 family is comprised of two genes: miR-146a and miR-146b (Figure 1.5) [385]. The mature sequences of miR-146a (miR-146a-5p) and miR-146b (miR-146b-5p) differ by two nucleotides in their 3' region but they share the same "seed", suggesting that they might regulate the same mRNA targets [385, 430, 431]. miR-146a has been implicated in the modulation of TLR signaling [380].

The miR-146a gene is located in an intergenic region on the long arm of chromosome 5 in humans and it is widely expressed in hematopoietic cells [432, 433]. The promoter of miR-146a contains binding sites for the transcription factor NF-κB which are required for its expression in response to LPS, IL-1β, TNF-α and viral infection [385, 434-436]. In myeloid cells the basal transcription of the miR-146a gene is regulated by the transcription factor PU.1; whereas in lymphoid cells Ets1, a member of the E-26 transcription factor family, controls miR-146a expression [435, 437]. Additionally, transcription of the miR-146a gene can also be suppressed by the oncogene *c*-myc and by the promyelocytic leukemia zinc finger (PLZF) protein [438, 439]. Some of the mRNA targets of miR-146a include TRAF6 and IRAK1, proteins that belong to the TLR signaling cascade and are essential for the activation of NF-κB [385]. Additional mRNA targets of miR-146a have been described and include TLR4, IRAK2, IRF5, Fas-Associated protein with Death Domain (FADD), breast cancer 1 (BRCA1), SMAD4, (C-X-C motif) receptor 4 (CXCR4) and STAT1 [440-444 445, 446]. miR-146a acts as a negative feedback mechanism of NF-κB

activation and reduces the expression of pro-inflammatory cytokines like IL-6 and TNF-α in response to LPS [385, 447]. Mice carrying a deletion on the miR-146a locus are hypersensitive to bacterial challenge and die of sepsis faster than their wild type counterparts [447]. Furthermore, miR-146a deficient mice develop an autoimmune disorder as they age and exhibit severe inflammation, increased basal production of cytokines and autoantibodies [447]. In addition, miR-146a contributes to the establishment of endotoxin tolerance, an adaptive mechanism of the host in response to bacterial infection; plays a role in the determination of T cell fate in mice; and regulates the development and function of the hematopoietic and lymphoid cell lineages [380, 433, 437, 446, 448].

The miR-146b gene is located on the long arm of the human chromosome 10 [380, 449]. The expression of the miR-146b gene appears to be regulated differently than miR-146a, and so far mir-146b has not been detected in immune cells [447, 450]. Furthermore, the strong immune phenotype displayed by miR-146a knockout mice suggests that the functions of miR-146a and miR-146b are not redundant [447]. The proteins TRAF6 and IRAK1 are also direct mRNA targets of miR-146b, highlighting the potential role of this miRNA in inflammation [385]. However, the role of miR-146b as a regulator of immune responses has not yet been explored. Several reports have detected miR-146b in tumors and in human lung carcinoma A549 cells IL-1β induced the expression of miR-146b through the ERK1/2 and SAPK/JNK signaling pathways [451-454]. In the particular case of papillary thyroid carcinoma the expression of the 5′ strand of miR-146b, miR-146b-5p, is high and is considered a diagnostic marker for this type of cancer [451, 455]. The miRNA miR-146b-5p contributes to tumorigenesis by repressing its target SMAD4 and thus modulating the TGF-β pathway [456]. In human papillary carcinoma cell lines suppression of miR-146b-5p

sensitizes the cellular response to the anti-proliferative signal of TGF-β [456]. Additionally, the activation of the oncogenes RET and BRAF increases miR-146b-5p expression, highlighting the role of this miRNA in cancer progression [456]. In human glioblastoma cell lines miR-146b-5p suppresses the expression of the EGFR and the matrix metalloproteinase 16 (MMP16), and affects cell migration and invasion *in vitro* [452, 457]. Moreover, miR-146b is a miRNAs significantly deregulated in human glioblastoma [452].

1.6 General objective and specific aims

The family of angiopoietins and Tie receptors regulates vascular homeostasis, angiogenesis and inflammation under both, physiological and pathological conditions [12, 13]. Hence, unraveling the molecular mechanisms through which angiopoietins differentially mediate their effects represents a unique opportunity to find new targets for therapeutical intervention against vascular and inflammatory pathologies such as tumor progression and bacterial sepsis [12]. Although important advances have been made in understanding the various biological functions of Ang-1, the main agonist of Tie-2 receptors, little is yet known about the regulation of the intracellular signaling pathways that it activates in ECs and its expression in breast cancer. In general, the objective of this thesis is to characterize the molecular mechanisms through which Ang-1 regulates EC biology as well as to study its role in breast cancer and inflammation.

To that end, we have the following specific aims:

1- Characterize the mechanisms of inactivation of MAPK signaling pathways activated downstream of Ang-1 signaling by DUSPs, as well as to elucidate the importance of these phosphatases in Ang-1-induced survival and migration of ECs.
2- Study the influence of estrogen and ERα on the expression of angiopoietins 1, 2 and 4 in breast cancer cells, as well as to examine their contribution to tumor progression.
3- Understand the mechanisms through which Ang-1 affects the pro-inflammatory TLR4 signaling pathway activated by *E. Coli* LPS in ECs, and study the role of the miR-146 family of miRNAs in mediating these effects.

Throughout this work we used human umbilical vein endothelial cells (HUVECs) as our *in vitro* model to study endothelial biology and a panel of human breast cancer cell lines to help us understand the role of ERα and angiopoietins in cancer. Additionally, we also included an *in vivo* study using a murine xenograft model of breast cancer to gain insight on the effect of estrogen in the progression of primary mammary tumors.

1.7 Figures and figure legends

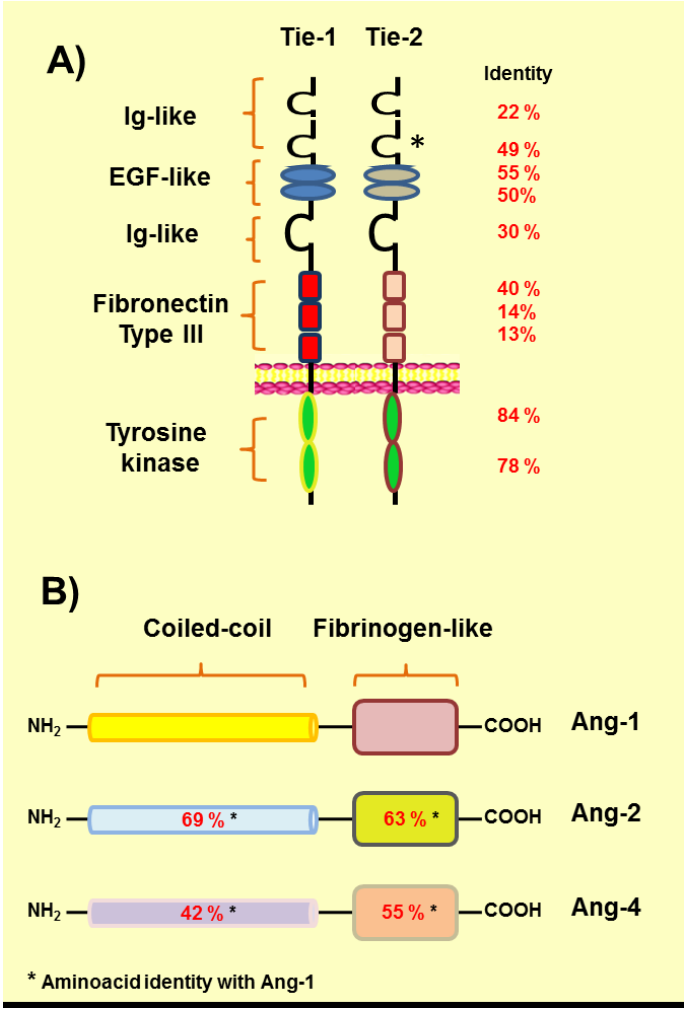

Figure 1.1 The family of angiopoietins and Tie receptors

A) Tie-1 and Tie-2 tyrosine kinase receptors contain extracellular Ig-like domains, three EGF-like domains and fibronectin type III domains; followed by an intracellular tyrosine kinase domain. **B)** Angiopoietins contain a coiled-coil domain and fibrinogen-like domains. Adapted from *Sato et al.*, 1993 and *Jones N et al.,* 2001 [458, 459].

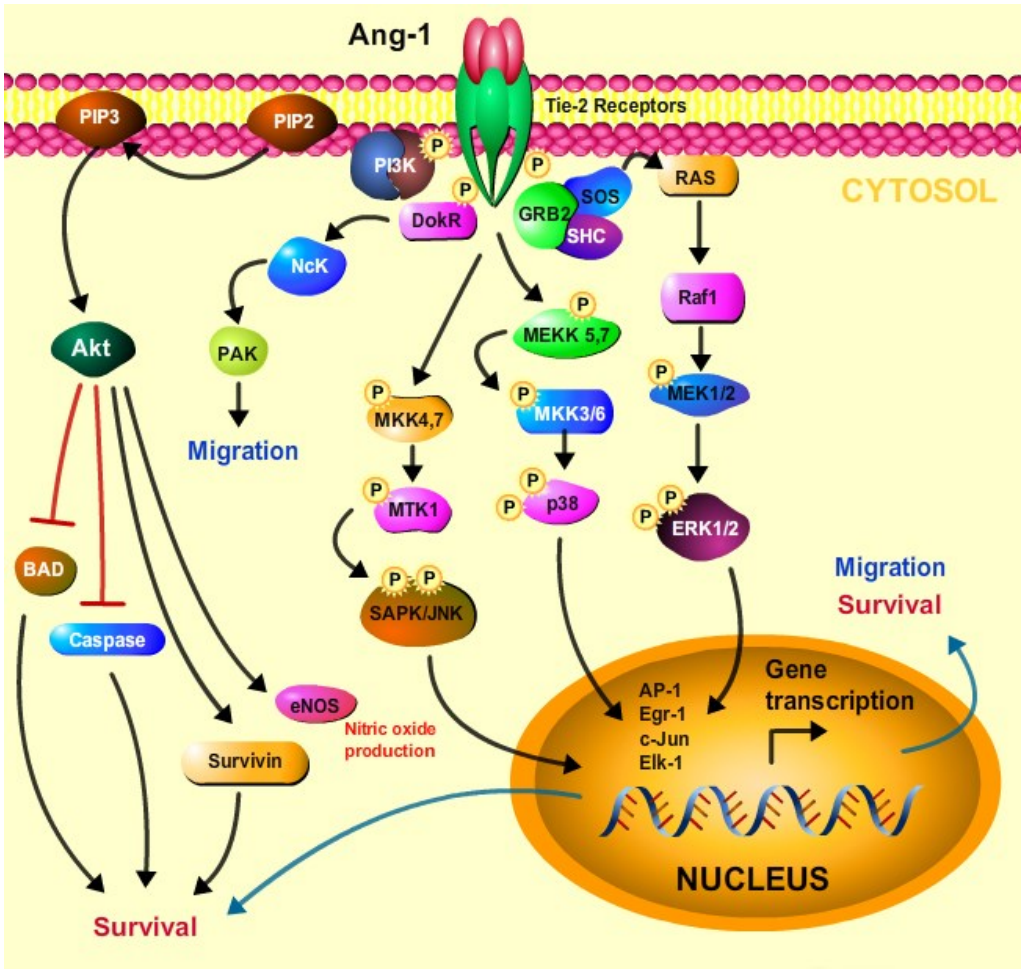
Ang-1
Tie-2 Receptors
PIP3
PIP2
PI3K
DokR
GRB2
SOS
SHC
RAS
CYTOSOL
NcK
Raf1
Akt
PAK
MEKK 5,7
MEK1/2
Migration
MKK4,7
MKK3/6
MTK1
p38
ERK1/2
BAD
Caspase
SAPK/JNK
Migration
Survival
eNOS
Nitric oxide
production
AP-1
Egr-1
c-Jun
Elk-1
Gene
transcription
Survivin
NUCLEUS
Survival

Figure 1.2

Figure 1.2 Intracellular signaling pathways activated by angiopoietin-1

Ang-1 induces the oligomerization of Tie-2 receptors and the autophosphorylation of their tyrosine kinase domain. The phosphorylated receptor facilitates the interaction of adaptor and signaling proteins, and triggers the activation of various signaling pathways. The signaling cascades activated by Ang-1 play distinct roles in the regulation of survival, migration and differentiation of ECs, and include PI3-K/AKT, Dok-R/PAK and MAPKs [13].

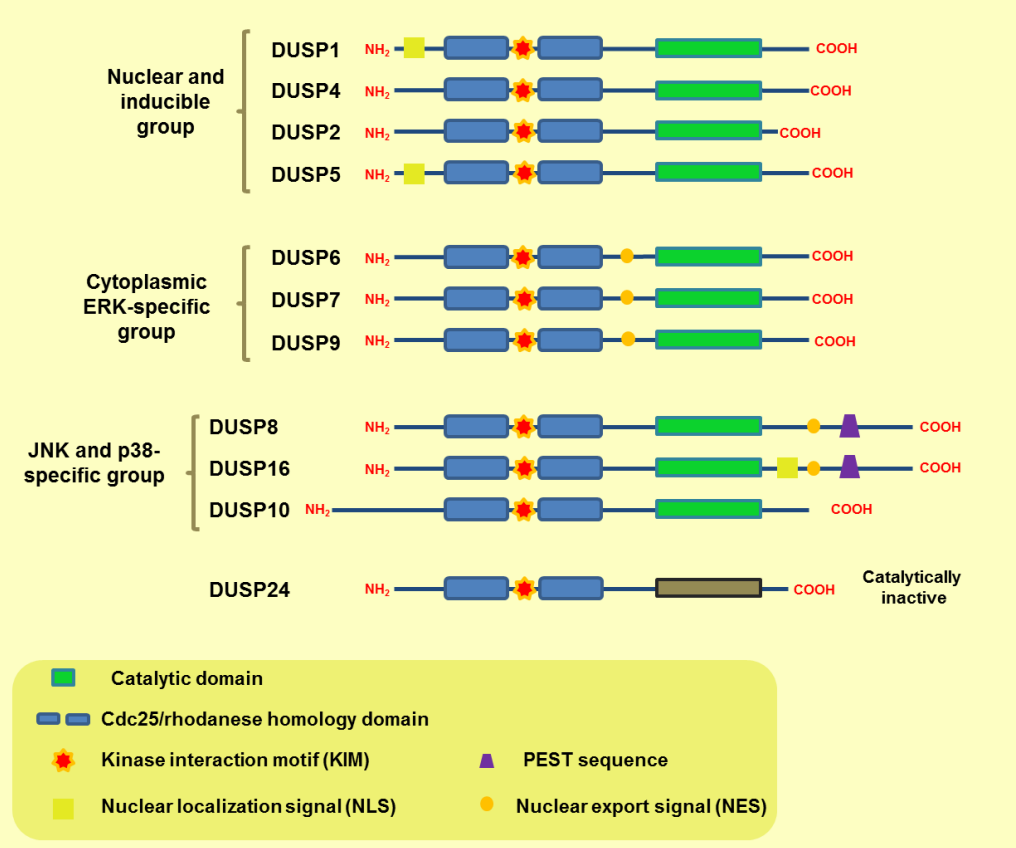
Nuclear and inducible group
DUSP1
DUSP4
DUSP2
DUSP5
Cytoplasmic ERK-specific group
DUSP6
DUSP7
DUSP9
JNK and p38-specific group
DUSP8
DUSP16
DUSP10
DUSP24
NH2
COOH
Catalytically inactive
Catalytic domain
Cdc25/rhodanese homology domain
Kinase interaction motif (KIM)
PEST sequence
Nuclear localization signal (NLS)
Nuclear export signal (NES)

Figure 1.3

Figure 1.3 Structure and classification of dual-specificity phosphatases that inactivate mitogen activated protein kinases

In general the structure of DUSPs comprises an amino terminal domain with homology to Cdc25/rhodanese domain, a conserved kinase-interaction motif (KIM) and a carboxyl terminal catalytic site. Additionally, some DUSPs contain nuclear localization signals (NLS), nuclear export signals (NES) and PEST sequences. There are eleven DUSPs able to dephosphorylate MAPKs, all catalytically active except for DUSP24, which have been further subdivided into three subgroups based on their substrate preference. Adapted from *Dickinson RJ* and *Keyse SM*, 2006 [212].

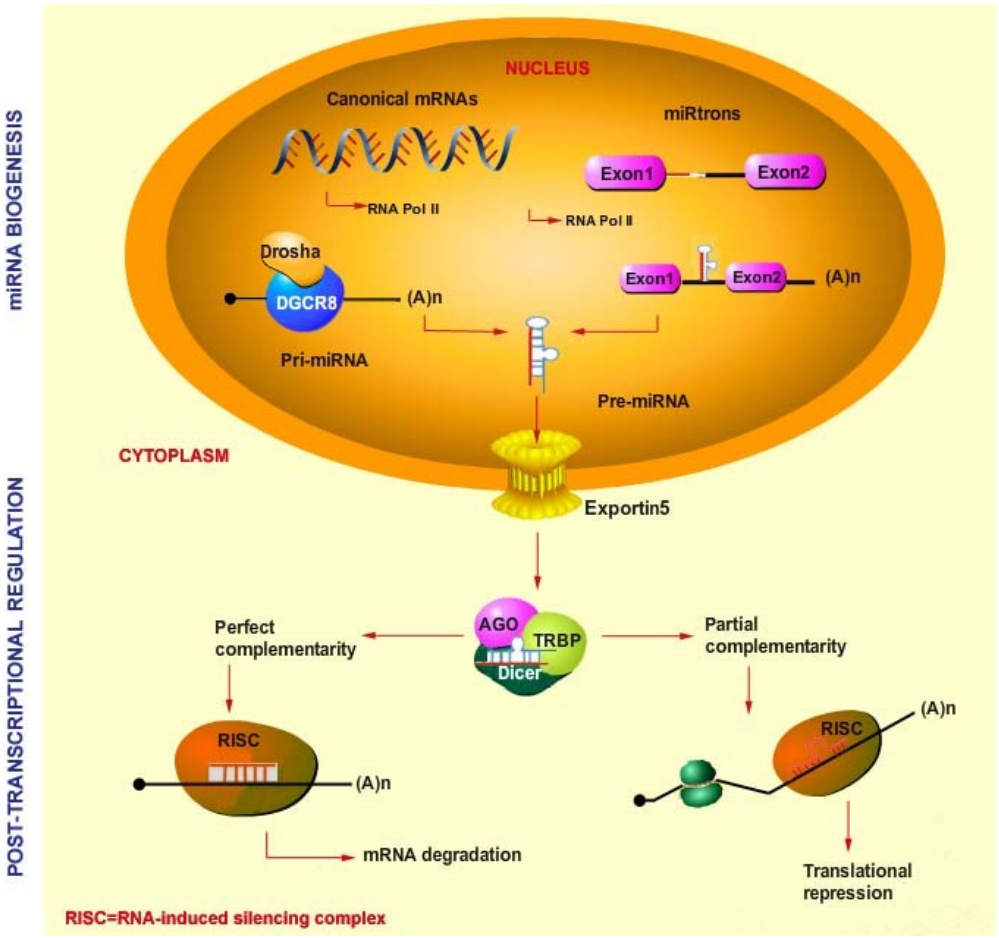
miRNA BIOGENESIS
POST-TRANSCRIPTIONAL REGULATION
NUCLEUS
Canonical mRNAs
miRtrons
Exon1
Exon2
RNA Pol II
RNA Pol II
Drosha
DGCR8
(A)n
Exon1
Exon2
(A)n
Pri-miRNA
Pre-miRNA
CYTOPLASM
Exportin5
AGO
TRBP
Dicer
Perfect complementarity
Partial complementarity
RISC
(A)n
RISC
(A)n
mRNA degradation
Translational repression
RISC=RNA-induced silencing complex

Figure 1.4

Figure 1.4 MicroRNA biogenesis and mechanisms of post-transcriptional repression

miRNAs are transcribed in the nucleus as primary miRNAs (Pri-miRNAs) by RNA polymerase II (RNA Pol II) from independent portions of the genome. Pri-miRNAs are transformed to precursor miRNAs (pre-miRNAs) by Drosha and DGCR8. Some pre-miRNAs are generated by splicing from short introns, miRtrons, and do not require processing by Drosha. Pre-miRNAs are exported to the cytoplasm by exportin 5 where they are processed by Dicer to produce a mature miRNA duplex (~20 bp). A strand of the miRNA duplex preferentially associates with a RNA-induced silencing complex (RISC) and guides the complex to the 3′UTR of its target mRNAs. Interaction with the RISC leads to translational repression or mRNA degradation, depending on the degree of complementarity between the miRNA and the mRNA target [460].

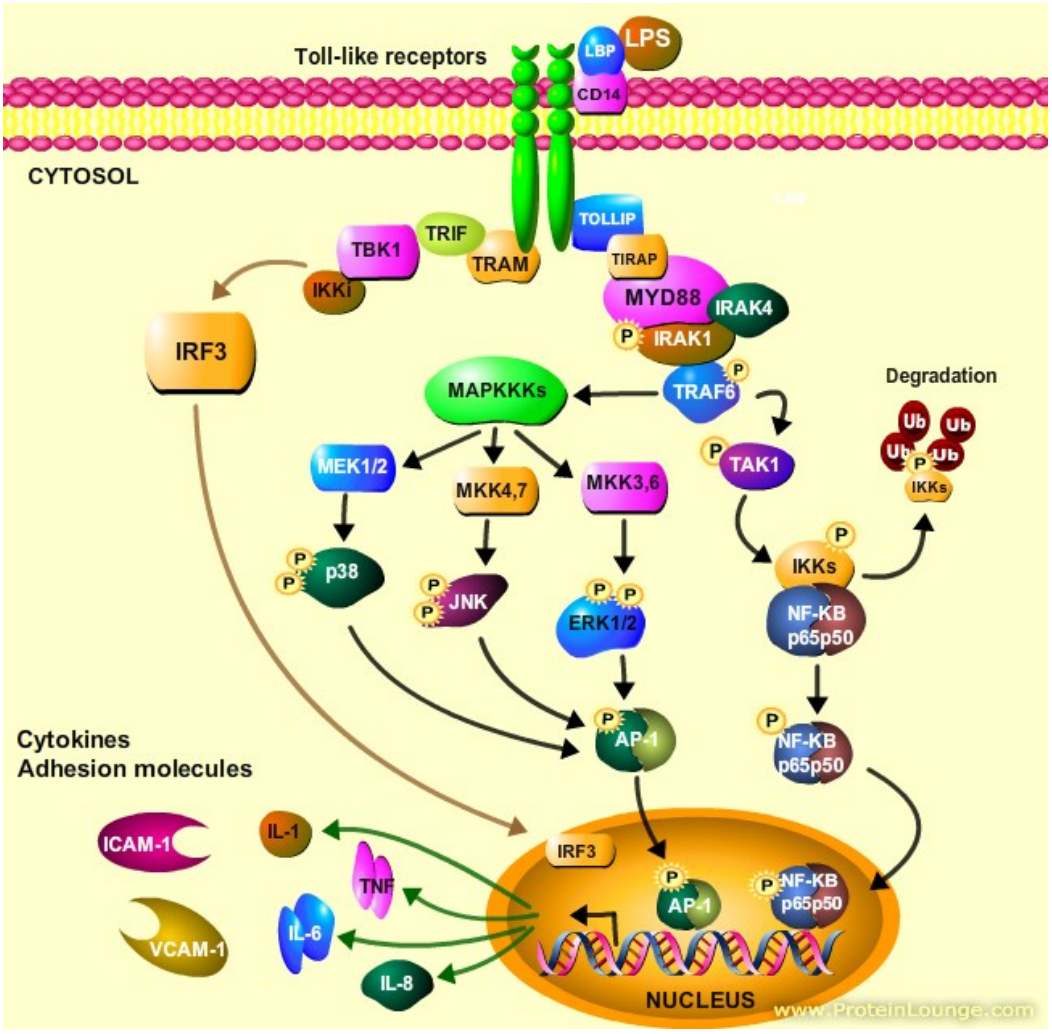
Toll-like receptors
LPS
LBP
CD14
CYTOSOL
TOLLIP
TRIF
TBK1
TRAM
TIRAP
IKKi
MYD88
IRAK4
IRF3
P
IRAK1
MAPKKKs
TRAF6
P
Degradation
Ub
Ub
Ub
Ub
P
IKKs
MEK1/2
MKK4,7
MKK3,6
P
TAK1
P
IKKs
P
P
p38
P
P
JNK
P
P
ERK1/2
NF-KB
p65p50
P
AP-1
P
NF-KB
p65p50
Cytokines
Adhesion molecules
ICAM-1
IL-1
IRF3
TNF
P
AP-1
P
NF-KB
p65p50
VCAM-1
IL-6
IL-8
NUCLEUS
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Figure 1.5

Figure 1.5 Signaling pathways activated by Toll-like receptor 4

LPS activation of TLR4 activates both MyD88-dependent and independent signaling pathways. MyD88-dependent signaling requires IRAK1 and TRAF6 activation, leads to the downstream activation of MAPKs and the transcription factors AP-1 and NF-κB, and promotes transcription of pro-inflammatory mediators. The MyD88-independent pathway involves the activation of IRF3 and transcription of IRF3-dependent genes. Adapted from *Dauphinee S.M.* and *Karsan A.*, 2006 [412].

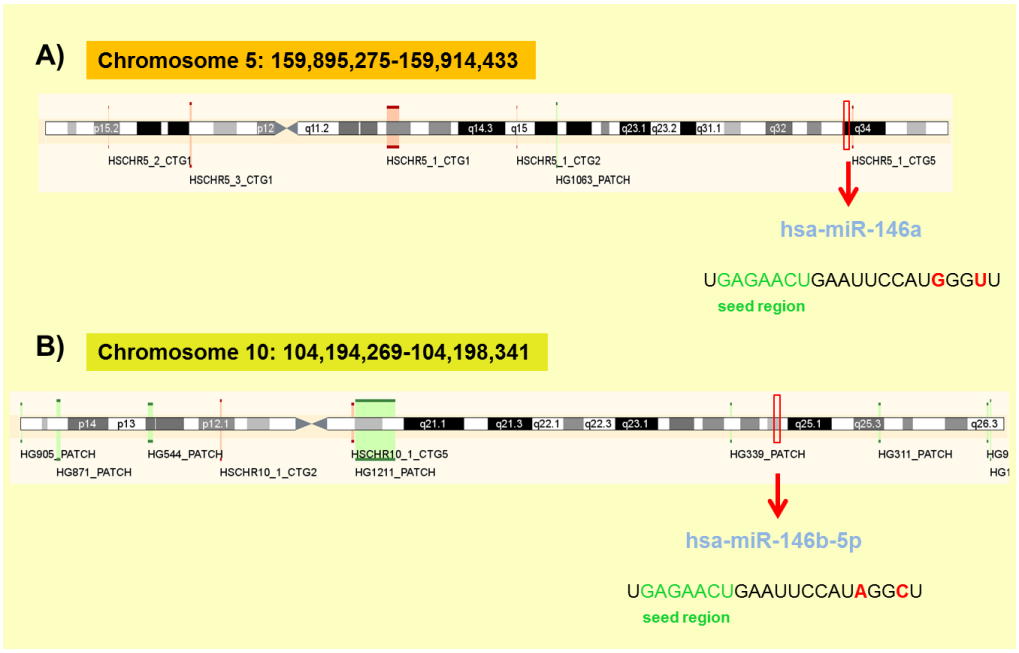
A)
Chromosome 5: 159,895,275-159,914,433
HSCHR5_2_CTG1
HSCHR5_3_CTG1
HSCHR5_1_CTG1
HSCHR5_1_CTG2
HG1063_PATCH
HSCHR5_1_CTG5
hsa-miR-146a
UGAGAACUGAAUUCCAUGGGUU
seed region
B)
Chromosome 10: 104,194,269-104,198,341
HG905_PATCH
HG871_PATCH
HG544_PATCH
HSCHR10_1_CTG2
HSCHR10_1_CTG5
HG1211_PATCH
HG339_PATCH
HG311_PATCH
hsa-miR-146b-5p
UGAGAACUGAAUUCCAUAGGCU
seed region

Figure 1.6

Figure 1.6 The miR-146 family

A) In humans the miR-146a gene is located in an intergenic region on the long arm of chromosome 5. **B)** The miR-146b gene is located on the long arm of the human chromosome 10. Mature sequences of both miR-146a and miR-146b share the same "seed" sequence, suggesting that they might share the same mRNA targets. Figure generated from information obtained in the miRBase database and the Ensembl genome browser [461, 462].

Preface to Chapter 2

Ang-1 promotes survival, migration and proliferation of ECs through the activation of the MAPK signaling pathways ERK1/2, p38 and SAPK/JNK [13, 156-159] [107, 160]. The outcomes of MAPK signaling are largely determined by the magnitude and duration of MAPK phosphorylation, highlighting the importance of the molecular mechanisms involved in the inactivation of these pathways [131, 214]. Previous studies carried out in our lab on gene expression profiling of ECs exposed to Ang-1 revealed that various immediate-early response genes, including the phosphatases DUSP4 and DUSP5, are induced by Ang-1 signaling [463]. These findings suggest that the induction of these phosphatases could contribute to the regulation of MAPK signaling pathways activated by Ang-1 and thus control the endothelial response to this angiogenic factor. Therefore, we hypothesized that several DUSPs could be induced by Ang-1 signaling as a negative feedback mechanism to control MAPK signaling activation ehich differentially affect Ang-1-dependent survival and migration of ECs.

Chapter 2

Regulation of angiopoitetin-1 signaling in endothelial cells by dual-specificity phosphatases 1, 4 and 5

Regulation of Angiopoitetin-1 signaling in endothelial cells by dual-specificity phosphatases 1, 4 and 5

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Running Title: DUSP1, 4 and 5 and Tie-2 receptor signaling

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2.1 ABSTRACT

Angiopoietin-1 (Ang-1) promotes survival and migration of endothelial cells (ECs), in part through the activation of mitogen-activated protein kinase (MAPK) pathways downstream of Tie-2 receptors. Dual-specificity phosphatases (DUSPs) dephosphorylate phosphotyrosine and phosphoserine/phosphothreonine residues on target MAPKs. The mechanisms by which DUSPs modulate MAPK activation in Ang-1/Tie-2 receptor signaling are unknown in ECs. Expressions of various DUSPs in human umbilical vein endothelial cells (HUVECs) exposed to Ang-1 were measured. The functional roles of DUSPs in Ang-1-induced regulation of MAPK activation, endothelial cell survival, migration, differentiation and permeability were measured using selective siRNA oligos. Ang-1 differentially induces DUSP1, DUSP4, and DUSP5 in HUVECs through activation of the PI-3 kinase, ERK1/2, p38, and SAPK/JNK pathways. Lack-of-function siRNA screening revealed that DUSP1 preferentially dephosphorylates p38 protein and is involved in Ang-1-induced cell migration and differentiation. DUSP4 preferentially dephosphorylates ERK1/2, p38, and SAPK/JNK proteins and, under conditions of serum deprivation, is involved in Ang-1-induced cell migration, several anti-apoptotic effects, and differentiation. DUSP5 preferentially dephosphorylates ERK1/2 proteins and is involved in cell survival and inhibition of permeability. DUSP1, DUSP4, and DUSP5 differentially modulate MAPK signaling pathways downstream of Tie-2 receptors, thus highlighting the importance of these phosphatases to Ang-1-induced angiogenesis.

2.2 INTRODUCTION

Angiopoietin-1 (Ang-1) is an agonist of Tie-2 receptors and promotes migration, proliferation, and differentiation of endothelial cells (ECs) [1]. Mice lacking Ang-1 do not survive early development and exhibit major defects in vascular organization [2]. In adult tissues, Ang-1 exerts a dual role by stimulating angiogenesis at sites of active vascular remodeling and by promoting vascular quiescence in mature vessels through the inhibition of apoptosis and inflammation [1]. Ang-1 induces autophosphorylation of Tie-2 receptors and activates downstream signaling pathways such as the mitogen-activated protein kinase (MAPK) pathways, which include ERK1/2, p38, and SAPK/JNK [3, 4]. MAPK activation is dependent on phosphorylation of threonine and tyrosine residues by dual-specificity MAPK kinases (MAPKKs), which are activated through phosphorylation of serine/threonine residues by upstream MAPKK kinases (MAPKKKs) [5]. In ECs, the ERK1/2 pathway mediates anti-apoptotic properties of Ang-1 while the p38 pathway promotes apoptosis [3]. Ang-1-induced ERK1/2 and SAPK/JNK phosphorylation, in combination with phosphatidylinositol 3-kinase (PI3-K) activation, induces IL-8 production, which is essential for EC migration and proliferation [6]. The transcription factors activating protein-1 (AP-1) and early growth response-1 (Egr-1) are activated downstream from Tie-2 receptors in cells exposed to Ang-1. They also play a role in migration and proliferation [6]. Outcomes of MAPK signaling are determined by the magnitude and duration of MAPK phosphorylation, suggesting that mechanisms of signaling inactivation are just as important as the activation of cascades themselves [5]. Specific dephosphorylation of MAPKs on phosphoserine/phosphothreonine and phosphotyrosine residues is mediated by dual-specificity phosphatases (DUSPs), a family of cystein-dependent protein tyrosine phosphatases (PTPs) [7]. To date, sixteen mammalian DUSPs that dephosphorylate MAPKs

have been identified, of which eleven belong to a sub-family of CH2 (CDC25 homology)-motif-containing MAPK phosphatases (MKPs) [8]. These MKPs contain a motif in their active site that shares high sequence similarity with the protein tyrosine phosphatase VH1 from the *Vaccinia* virus and an NH_2-terminal kinase interactive motif that contributes to substrate specificity [8]. They have been grouped into three major subfamilies based on their sequence similarity, substrate specificity, and subcellular localization [7, 8]. The largest group includes four inducible nuclear phosphatases: DUSP1 (MKP-1), DUSP2, DUSP4 (MKP-2), and DUSP5 [9].

In ECs, recent studies have identified DUSP1 and DUSP5 as important negative modulators of those MAPK signaling pathways that are activated by angiogenesis factors like vascular endothelial growth factor (VEGF) [10]. There is also evidence that DUSP4 regulates TNF-α–induced apoptosis in human umbilical vein endothelial cells (HUVECs) [11]. However, despite their known importance to the regulation of MAPK signaling pathways downstream from angiogenic factors, no information is as yet available regarding the involvement of DUSPs in Ang-1/Tie-2 receptor signaling. The main focus of this study, therefore, is to characterize the ways in which DUSPs negatively regulate MAPK signaling and to investigate how they influence Ang-1–induced EC survival and migration.

2.3 MATERIALS AND METHODS

Materials: HUVECs were harvested from umbilical cords kindly donated by the birthing centre at the Royal Victoria Hospital (Montreal, Quebec). Recombinant human angiopoietin-1 and angiopoietin-2 proteins were purchased from R&D Systems (Minneapolis, MN). Protein A/G PLUS-Agarose, DUSP4 and DUSP5 antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Antibodies for p-Erk1/2 (Thr^{202}/Tyr^{204}), p-SAPK/JNK (Thr^{183}/Tyr^{185}), p-p38 (Thr^{180}/Tyr^{182}), Erk1/2, SAPK/JNK, p38 and AKT were obtained from Cell Signaling Technology (Danvers, MA). Antibodies for DUSP1 and histone H3 were obtained form Millipore (Billerica, MA). The pharmacological inhibitors PD1843, SB203580 and SP600125 were purchased from Calbiochem (San Diego, CA), and Birb0796 was a gift from Dr. Simon Rousseau (McGill University). The GenElute Mammalian Total RNA Miniprep Kit, actinomycin D, wortmannin and *p*-nitrophenyl phosphate were purchased from Sigma-Aldrich (St. Louis, MO). The recombinant adenovirus Ad-CMV-c-JUN (DN) containing a transactivation domain deletion mutant of c-Jun (TAM67) [12] was purchased from Vector Biolabs (Philadelphia, PA); while the GFP adenovirus (Ad-GFP) was obtained from Gene Transfer Vector Core (University of Iowa). The pGL3-erp7 luciferase reporter plasmid driven by the -1716/+88 bp segment of mouse DUSP1 promoter [14] and the luciferase reporter plasmid (MKP-2-Luc) [15] containing the rat MKP-2 sequence from nucleotides −1664 to +123 was obtained from Dr. Mark S. Roberson (Cornell University). Alexa Fluor 568-goat-anti-rabbit, Alexa Fluor 488-goat anti-mouse and Lipofectamine RNAiMAX reagent were purchased from Invitrogen (Burlington, ON). siCONTROL siRNA and siGENOME SmartPool siRNAs specific for DUSP1, DUSP4 and DUSP5 were obtained from Dharmacon (Lafayette, CO). Egr-1-specific or scrambled negative control Dicer-substrate siRNA

duplexes were purchased from Integrated DNA Technologies. Amaxa Nucleofector System was purchased from LONZA (Walkersville, MD). The Dual-Luciferase Reporter Assay System and the ApoTox-Glo Triplex Assay kit were obtained from Promega (Madison, WI).

Cell culture: Human umbilical vein endothelial cells (HUVECs) were isolated as previously described, with a few modifications. Briefly, donated umbilical cords of approximately 25cm in length were placed in Hank's Balanced Salt Solution (HBSS) containing penicillin (100U/ml), streptomycin (100mg/ml), and amphotericin B (0.25mg/ml). Both ends of the umbilical vein were cannulated, washed with HBSS and then filled with 7ml of 0.1% collagenase type 1 (Bioshop, Burlington, Canada). The cord was then placed in a beaker containing HBSS and incubated for 9min at 37°C. The umbilical vein was flushed three times with 10ml of HBSS into a tube containing HUVEC growth media. The liquid was centrifuged at 1000rpm for 10min and the pellet was re-suspended and plated on cell culture plates coated with 0.1% gelatin. HUVECs were grown in MCDB131 medium (Wisent, St. Bruno, Canada) supplemented with 20% fetal bovine serum (FBS), endothelial mitogen growth factor (Biomedical Technologies Inc. Stoughton, MA), 2mM glutamine, heparin, and gentamicin (Invitrogen, Burlington, ON); and incubated at 37 °C in 5% CO_2. After reaching confluence in 100-mm dishes, the cells were sub-cultured following trypsin (0.25%) and EDTA (0.01%) treatment and plated at a density of 2500 cells/cm^2. HUVECs were used for experiments between passages 3 and 7. HUVEC-MSCV and HUVEC-MSCV-JNK-APF cells that express a dominant negative SAPK/JNK in which the phosphorylation site Thr-Pro-Tyr has been mutated to Ala-Pro-Phe (JNK-APF) were described previously [3]. These cells were maintained in the same conditions as HUVECs, except penicillin/streptomycin was also added to the medium.

Real-time PCR: Total RNA was extracted using GenElute Mammalian Total RNA Miniprep Kit according to the manufacturer's instructions. Quantification and purity of total RNA was assessed by A260/A280 absorption. SuperScript II RNase H-Reverse Transcriptase enzyme (Invitrogen, Burlington, ON) was used to reverse transcribe 2µg of RNA for 50min at 42°C and then for 5min at 90°C. Specific primers (Table 2.1) and the Real Time PCR System 7500 from Applied Biosystems (Foster City, CA) were used to carry out the real-time PCR reactions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-actin expression were monitored as control genes throughout the analysis. SYBR Green PCR Master Mix (25µl) from Qiagen (Hilden, Germany) was added to 1µl of the reverse transcriptase reaction and 3.5µl of primers (10µM). The thermal profile used with the Real Time PCR System 7500 was: 10min at 95°C, 40 cycles of 15s at 95°C, 30s at 57°C, and 34s at 72°C. Primer-dimer formation and contamination were assessed with a melt analysis for each PCR experiment and a single melt peak for each set of primers was used to confirm that a single PCR reaction product was generated. The comparative threshold (C_T) cycle method (also referred to as the $2^{-\Delta\Delta CT}$ method) was used to analyze the results. All real-time PCR experiments were done in triplicate.

Subcellular fractionation of cytosolic and nuclear extracts: Cytosolic and nuclear extracts were prepared from cells harvested by scraping 100-mm cell culture dishes using 1ml of ice cold phosphate-buffered saline (PBS) and recovered by centrifugation at 2500g for 5min. The cell pellets were then re-suspended in 150µl of ice-cold hypotonic buffer (20mM Tris pH 7.4, 10mM NaCl, 3mM $MgCl_2$, 0.5mM phenylmethylsulfonyl, 0.1mM sodium orthovanadate, 1µg/ml leupeptin and 1µg/ml aprotinin), and incubated on ice for 15min. 7.5µl of 10% Nonidet P-40 substitute were added to the cell pellets followed by 10s

vortexing and centrifugation at *960 x g* for 10min at 4°C. The supernatants were recovered as cytosolic fractions, while the remaining pellets were further re-suspended in 50µl of extraction buffer (100mM Tris, pH 7.4, 2mM Na_3VO_4, 100mM NaCl, 1% Triton X-100, 1mM EDTA, 10% glycerol, 1mM EGTA, 0.1% SDS, 1mM NaF, 0.5% deoxycholate, 20mM $Na_4P_2O_7$) and maintained on ice for 30min. Finally, the pellet was centrifuged at 14,000 x g for 30min at 4°C and the supernatant was separated as the nuclear fraction. Nuclear and cytosolic proteins were stored at -80°C until used for immunoblotting.

Immunoblotting: HUVECs were lysed in RIPA buffer (Santa Cruz Biotechnologies, Ca) and protein concentration was measured by the Bio-Rad protein assay using bovine serum albumin (BSA) as a standard (Bio-Rad Laboratories, Mississauga, ON). Cell lysates were diluted in *Laemmli* sample buffer, boiled for 5 min and loaded onto Tris-glycine SDS-polyacrylamide gels. After SDS-PAGE the proteins were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes, blocked with 5% non-fat dry milk and then incubated with the specific primary antibodies overnight at 4ºC. Predetermined molecular weight standards were used as markers, and the proteins were detected using horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies and ECL reagents (Chemicon, Temecula, CA).

Imaging of DUSP1, DUSP4 and DUSP5 proteins: HUVECs were plated onto Lab-Tek eight chamber slides (Thermo Fisher Scientific, Waltham, MA) pre-coated with 10µg/ml of fibronectin (Sigma, St. Louis, MO). Confluent cells were serum starved for 4h prior to treatment with vehicle or Ang-1 (300ng/ml) for 1h. Then, the media was aspirated and the cells were washed gently three times with PBS, fixed with 4% paraformaldehyde for 20min at room temperature and permeabilized with 0.2% Triton X-100. The cells were blocked

using 10% FBS in PBS for 1h at room temperature before incubation with anti-DUSP1 (1:200), anti-DUSP4 (1:50) and anti-DUSP5 (1:100) antibodies overnight in a humidified chamber at 4°C. The next day the cells were washed three times with PBS and incubated for 1h with the appropriate secondary antibodies, either Alexa Fluor 568-goat-anti-rabbit or Alexa Fluor 488 goat anti-mouse, in a humidified chamber. Finally, mounting media with DAPI (VectaShield, Vector Laboratories) was added to each slide before sealing and the cells were visualized with an Olympus 1X70 inverted fluorescence microscope.

Assay of DUSP1, DUSP4 and DUSP5 phosphatase activity: Phosphatase activity was measured using immunoprecipitated DUSP1, DUSP4 and DUSP5 proteins from HUVECs treated with vehicle or Ang-1 (300ng/ml). Immunoprecipitation was performed as described previously [13]. Briefly, cells were harvested by scraping using 1ml of ice cold PBS and recovered by centrifugation at 250 x *g* for 5min. The cell pellets were then re-suspended in 200µl ice-cold NP-40 lysis buffer (1% Nonidet P-40 substitute, 150mM NaCl and 50 mM Tris·Cl, pH 8.0, 10µg/ml leupeptin, 1µg/ml pepstatin A and 20µg/ml phenylmethylsulfonyl fluoride), gently rocked for 30min at 4°C and centrifuged at 20,000× *g* for 30min at 4°C. 200µg of supernatant protein were incubated with 2µg of antibody specific for DUSP1, DUSP4 or DUSP5 at 4°C for 2h, followed by incubation with protein A/G PLUS-Agarose for 1h. The immunoprecipitates were washed three times and re-suspended in 20ul phosphatase buffer (25mM HEPES, pH 7.4, 0.1 mM EDTA, 5mM DTT, 100µg/ml BSA, 0.01% Brij 35). Phosphatase activity of immunoprecipitated DUSPs was analyzed using *p*-nitrophenyl phosphate (*p*-NPP) as substrate. 20µL of immunoprecipitate were added to 130µl of *p*-NPP (10mM) in phosphatase buffer and incubated at 30°C for 45min. The reaction was stopped with 50µl of 1N NaOH and the

absorbance was measured at 405nm using a microplate reader. Non-specific hydrolysis of *p*-NPP was measured in immunoprecipitates obtained from proteins incubated only with protein A/G PLUS-Agarose, which served as a control and was subtracted from the data.

mRNA stability Assay: HUVECs grown to confluence were treated with Ang-1 (300ng/ml) for 1h. Then the cells were washed with PBS and maintained in either serum-free medium, medium containing actinomycin D (5μg/ml) or medium containing actinomycin D (5μg/ml) plus Ang-1 (300ng/ml). Total RNA was extracted at different time points (0, 30, 60 and 90min) and the transcripts for DUSP1, DUSP4 and DUSP5 were analyzed by real-time PCR.

Analysis of promoter activity: In this study, we analyzed a firefly luciferase reporter plasmid driven by the –1716/+80 bp segment of mouse DUSP1 (pGL3-erp7) promoter and DUSP4 promoter (MKP-2-Luc) containing the rat MKP-2 sequence from nucleotides −1664 to +123. HUVECs were transiently co-transfected with 0.05μg of renilla luciferase plasmid (pRL-TK) and 0.5μg of either DUSP1 or DUSP4 reporter plasmids by electroporation using the Amaxa Nucleofector System (Lonza, Walkersville, MD) according to the manufacturer's instructions. Cells were left to recover in HUVEC medium for 30h at 37ºC, serum-starved for 12h and maintained in medium with vehicle or Ang-1 (300ng/ml) for another 6h. Then, the cells were lysed and the luciferase activity (RLU) was quantified using the Dual-Luciferase Reporter (DLR) Assay System. Normalized values are expressed as the mean of the ratios of Luc1(Firefly)/Luc2 (Renilla).

Adenoviral infection: HUVECs were transduced for 6h using serum-free media containing 100 multiplicity of infection (MOI) virus units of either Ad-CMV-c-JUN (DN), Ad-ExTek

^{7} or Ad-GFP as control. Then, the virus containing medium was replaced with HUVEC growth medium and the cells were allowed to recover for 48h before the experiments.

siRNA transfections: HUVECs were transfected with Egr-1-specific or scrambled negative control Dicer-substrate siRNA duplexes (Integrated DNA Technologies, Coralville, IA); siRNA (siGENOME SMARTpool) directed against DUSP1, DUSP4, and DUSP5; or a non-targeting siRNA pool (siCONTROL) (Dharmacon, Lafayette, CO), using Lipofectamine™ RNAiMAX (Invitrogen, Burlington, ON). Briefly, HUVECs plated at a density of 30,000 cells/cm^2 in antibiotic-free medium were transfected with 10nM siRNA using Lipofectamine RNAiMAX. All the experiments were performed 48h after siRNA transfection.

Wound healing assay: 250,000 HUVECs were plated into 12-well tissue culture plates 24h after being transfected with siGENOME SMARTpool siRNAs specific for DUSP1, DUSP4 and DUSP5, or with siCONTROL scrambled siRNA. 48h after transfection the cell monolayers were carefully wounded using a 200µl pipette tip and the cellular debris was removed by washing once with PBS. The wounded cells were maintained in MCBD131 media containing 2% FBS with or without Ang-1 (300ng/ml) for 8h. Wound healing was visualized in an Olympus inverted microscope (40X). The wounded area was quantified using the Image Pro-Plus software (Media Cybernetics) and reported as percentage wound healing using the following equation: %Wound Healing = [1 – (wound area at t_{8h}/wound area at t_0)] x 100, where t_0 is the time immediately after wounding.

Cytotoxicity and caspase 3/7 activation assays: 24,000 HUVECs were plated in opaque-walled 96-well plates 24h after being transfected with siGENOME SMARTpool siRNAs specific for DUSP1, DUSP4 and DUSP5, or with siCONTROL scrambled siRNA. 48h after transfection, the cells were washed with PBS and maintained in medium with 20%

FBS, 0.2% FBS or 0.2%FBS plus Ang-1 (300ng/ml). The viability, cytotoxicity and caspase 3/7 activation were analyzed 24h later using the ApoTox-Glo Triplex Assay kit (Promega, Madison, WI).

Capillary tube formation: 200,000 HUVECs transfected with scrambled, DUSP1, DUSP4 and DUSP5 siRNA oligos were seeded onto 24-well plates pre-coated with growth factor-reduced Matrigel in MCDB131 medium plus 1% FBS. Images of tube formation were captured 24h later using an Olympus inverted microscope (40x). Images from a total of 10 fields per well were analyzed using the Image Pro software (Media Cybernetics). Angiogenic activity was determined by measuring the average tube length and the number of branching points.

Vascular permeability assay: Permeability across endothelial monolayers was assessed using Transwell inserts coated with collagen type I (6.5 mm diameter, 3.0 μm pore size polycarbonate filter; Corning Costar). HUVECs were transfected with scrambled, DUSP1, DUSP4 and DUSP5 siRNA (10nM). After 24h, cells were plated at a density of 100,000 cells per well and cultured for 1 day to form a tight monolayer. Cells were serum-starved for 1h in MCDB131 medium containing 1% bovine serum albumin (BSA) before Ang-1 (300ng/ml) or VEGF (40ng/ml) were added to the upper chambers in the presence of 1mg/ml FITC-labeled dextran (molecular mass: 40 kDa; Invitrogen, Burlington ON). After 30min the permeability was assessed by measuring the fluorescence of 20μl of sample from the lower compartment diluted with 180μl PBS at 492nm excitation/520nm emission with a SpectraMax M2 microplate reader (Molecular Devices).

2.4 RESULTS

Ang-1 induction of DUSP expression: To identify DUSP induction in response to Ang-1 exposure, HUVECs were treated with Ang-1 (300 ng/ml) for 1 h. The mRNA expression of DUSP1, DUSP3, DUSP4, DUSP5, DUSP6, DUSP7, DUSP11, DUSP12, DUSP14, and DUSP22 was measured using real-time PCR. Ang-1 induced the expression of DUSP1, DUSP4, and DUSP5 but exerted no significant effect on the expression of any other DUSPs (Figure 2.1A). In comparison, VEGF significantly induced DUSP1 and DUSP5 mRNA expression but had no effect on DUSP4 (Supplementary Figure S2.1). Exposure for 1 hr to a combination of Ang-1 and VEGF did not result in any additional increases in DUSP1 or DUSP5 mRNA levels, compared to those measured in response to Ang-1 alone (Supplementary Figure S2.2). Ang-1-induced expression of DUSP1, DUSP4 and DUSP5 mRNA was dose- (Figure 2.1B) and time-dependent, with peak levels detected 1 hr after Ang-1 exposure (Figure 2.1C). Exposure to Ang-2 (300 ng/ml) elicited time-dependent declines in DUSP1 and DUSP5 mRNA expression while DUSP4 expression remained unchanged as compared to control values (Figure 2.1D).

DUSP1, DUSP4, and DUSP5 protein levels and sub-cellular localization were evaluated by separating cell lysates into cytosolic and nuclear fractions. In PBS-treated cells (control), DUSP1, DUSP4, and DUSP5 were mainly detected in the nuclear fraction and Ang-1 exposure increased their levels within 1 to 3h (Figure 2.1D). Immunostaining confirmed that DUSP1, DUSP4, and DUSP5 were present in the cell nuclei of control and Ang-1-treated cells (Supplementary Figure S2.3). Measurements of *p*-nitrophenyl phosphate hydrolysis revealed that the phosphatase activity of DUSP1, DUSP4, and DUSP5 was transiently elevated within 1 to 3h of Ang-1 exposure (Figure 2.1F).

Ang-1 regulation of DUSP transcription and mRNA stability: To identify mechanisms through which Ang-1 induces DUSP expression in ECs, luciferase reporter assays were used to measure DUSP1 and DUSP4 promoter activities in response to Ang-1 exposure. Ang-1 significantly induced DUSP1 and DUSP4 promoter activities, suggesting that increased transcription is a mechanism of upregulation (Supplementary Figure S2.4). To determine whether Ang-1 increases DUSP1, DUSP4 and DUSP5 mRNA levels through alterations in mRNA stability, transcription was inhibited by actinomycin D and the decline rates of DUSP1, DUSP4 and DUSP5 mRNA were measured by real time PCR. Our results show that Ang-1 has no significant influence on the decline rates of DUSP1, DUSP4, or DUSP5, indicating that Ang-1 does not affect mRNA stability (Supplementary Figure S2.4).

Mechanisms of DUSP induction: Ang-1 activates the ERK1/2, p38, SAPK/JNK, and PI-3 kinase/AKT signaling pathways. Their individual roles in DUSP1, DUSP4 and DUSP5 expression by Ang-1 were assessed using pharmacological inhibitors of ERK1/2 (PD184352, 2μM), p38 (BIRB0796, 0.01μM), SAPK/JNK (SP600125, 10μM), and PI-3 kinase (wortmannin, 50nM). Ang-1 did not induce DUSP1 in HUVECs pre-treated with p38 or PI-3 kinase inhibitors for 1 h (Figure 2.2A). Ang-1 did not induce DUSP4 in the presence of p38, ERK1/2, SAPK/JNK, or PI-3 kinase inhibitors (Figure 2.2B). Ang-1 did not induce DUSP5 expression in the presence of ERK1/2 or PI-3 kinase inhibitors (Figure 2.2C). To further assess the role of the SAPK/JNK pathway in DUSP1, DUSP4 and DUSP5 mRNA expression by Ang-1, HUVECs were transduced with empty retroviruses (MSCV) or retroviruses expressing a dominant-negative form of SAPK/JNK (MSCV-JNK-APF) [3]. Ang-1 was unable to induce DUSP4 mRNA expression in MSCV-JNK-APF cells, as compared to MSCV cells (Figure 2.2B). In contrast, Ang-1 induced DUSP1 (Figure 2.2A)

and DUSP5 (Figure 2.2C) mRNA in MSCV-JNK-APF cells. These results suggest that Ang-1 induction of DUSP1 is mediated by the p38 and PI-3 kinase pathways; Ang-1 induction of DUSP4 induction is mediated by the ERK1/2, p38, SAPK/JNK, and PI-3 kinase pathways; and Ang-1 induction of DUSP5 is mediated by the ERK1/2 and PI-3 kinase pathways. To assess the importance of Tie-2 receptors in Ang-1-induced in DUSP1, DUSP4, and DUSP5 mRNA expression, HUVECs were transduced with adenoviruses expressing green fluorescent protein (Ad-GFP) or adenoviruses expressing a recombinant soluble Tie-2 receptor capable of blocking Tie-2 receptor activation [17] (Ad-Ex Tek). Ang-1 induces DUSP1, DUSP4, and DUSP5 expression in HUVECs transduced with Ad-GFP (Figure 2.2), but fails to induce their expression in HUVECs transduced with Ad-Ex Tek, suggesting that Tie-2 receptor activation is important for DUSP1, DUSP4 and DUSP5 induction by Ang-1 (Figure 2.2).

Activating protein-1 (AP-1) and early growth response-1 (Egr-1) are transcription factors activated by Ang-1 signaling which contribute to Ang-1-induced migration and proliferation of ECs [6, 18]. The protein c-Jun, the main subunit of AP-1, is activated by Tie-2 receptors. To assess the role of AP-1 in Ang-1-induced DUSP expression, the activity of c-Jun was inhibited using adenoviruses expressing a dominant-negative form of the protein (Ad-TAM67) [12]. In HUVECs transduced with Ad-TAM67 Ang-1 was unable to induce DUSP4 and DUSP5 mRNA expression; while DUSP1 induction by Ang-1 remained unaffected (Supplementary Figure S2.5). These results suggest that AP-1 is critically involved in Ang-1-induced DUSP4 and DUSP5 expression. To assess the role of Egr-1 in Ang-1-induced expression of DUSPs, HUVECs transfected with Egr-1 siRNA oligos were treated with Ang-1 (Supplementary Figure S2.5). In Egr-1-depleted cells, Ang-1 was unable to induce DUSP1 mRNA expression, while still being able to induce DUSP4 and

DUSP5 expression (Supplementary Figure S2.5). These results suggest that the transcription factor Egr-1 is critically important to Ang-1-induced DUSP1 expression.

Functional roles of DUSPs: MAPK phosphorylation, cytotoxicity, caspase 3/7 activity, migration, capillary tube formation, and vascular permeability were evaluated in cells transfected with scrambled, DUSP1, DUSP4, or DUSP5 siRNA oligos. Initially, DUSP1, DUSP4, and DUSP5 levels were confirmed as being significantly attenuated using their corresponding siRNA oligos (Supplementary Figure S2.6).

DUSP1: In cells transfected with scrambled siRNA oligos, Ang-1 transiently increases ERK1/2, p38, and SAPK/JNK phosphorylation (Figure 2.3A). Knockdown of DUSP1 expression increased Ang-1-induced p38 phosphorylation and attenuated Ang-1-induced SAPK/JNK phosphorylation, but had no influence on Ang-1-induced ERK1/2 phosphorylation (Figure 2.3A-B). These results suggest that DUSP1 plays an important role in the regulation of p38 and SAPK/JNK activation by Ang-1. In HUVECs transfected with scrambled siRNA oligos 24 h serum deprivation increases cytotoxicity and caspase 3/7 activity, while simultaneously decreasing cell numbers, and Ang-1 attenuates these effects (Figure 2.3C-D). Knockdown of DUSP1 expression had no effect on serum deprivation-induced cytotoxicity, caspase 3/7 activity and cell numbers (Figure 2.3C-D). Moreover, Ang-1 was still able to reduce cytotoxicity, caspase 3-7 activity and increase cell numbers in the presence of DUSP1 knockdown; suggesting that DUSP1 does not play a major role in the anti-apoptotic and pro-survival effects of Ang-1 in ECs (Figure 2.3C-D).

In HUVECs transfected with scrambled siRNA oligos, Ang-1 significantly enhanced migration as measured by wound healing assays *in vitro* (Figure 2.3E). Knockdown of DUSP1 expression eliminated Ang-1-induced migration (Figure 2.3E); suggesting tha DUSP1 plays an important role in the regulation of cell migration by Ang-1. Moreover, in

HUVECs transfected with scrambled siRNA oligos Ang-1 promotes capillary tube formation *in vitro,* as indicated by increases in the number of branching points and capillary tube length (Figure 2.4A-C). Knockdown of DUSP1 eliminated the Ang-1 effect on capillary tube formation, indicating that DUSP1 is essential to Ang-1-induced EC differentiation (Figure 2.4A-C). Additionally, in HUVECs transfected with scrambled siRNA oligos Ang-1 reduces vascular permeability, assesed by the passage of FITC-dextran across an endothelial monolayer (Figure 2.4D). The effect of Ang-1 on vascular permeability was not affected by DUSP1 knockdown (Figure 2.4D).

DUSP4: Knockdown of DUSP4 expression resulted in augmentation and prolongation of Ang-1-induced ERK1/2, p38, and SAPK/JNK phosphorylation, suggesting that DUSP4 plays a major role in the inactivation of these pathways (Figure 2.5A-B). Knockdown of DUSP4 expression decreased serum deprivation-induced cytotoxicity and caspase 3/7 activity (Figure 2.5C). Moreover, the effect of Ang-1 on serum deprivation-induced cytotoxicity, caspase 3/7 activity and cell number were not observed in when DUSP4 was knockdown. These results suggest that DUSP4 plays a major role in the anti-apoptotic and pro-survival effects of Ang-1 (Figure 2.5C-D). Knockdown of DUSP4 also eliminated Ang-1-induced migration, indicating that DUSP4 also plays an important role in migration (Figure 2.5E). Furthermore, knockdown of DUSP4 eliminated Ang-1-induced capillary tube formation, indicating that DUSP4 is essential to Ang-1-induced EC differentiation (Figure 2.4A-C). In contrast, the inhibitory effect of Ang-1 on vascular permeability was not affected by DUSP4 knockdown indicating that DUSP4 does not play a major role in this effect (Figure 2.4D).

DUSP5: Knockdown of DUSP5 expression resulted in augmentation and prolongation of Ang-1-induced ERK1/2 phosphorylation but had no influence on Ang-1-induced p38 and

SAPK/JNK phosphorylation; suggesting that DUSP5 plays a significant role in the inactivation of the ERK1/2 pathway (Figure 2.6A-B). Knockdown of DUSP5 significantly increased basal and serum deprivation-induced cytotoxicity and caspase 3/7 activity (Figure 2.6C-D). However, the effect of Ang-1 on serum deprivation-induced cytotoxicity, caspase 3/7 activity and cell number were unaffected by DUSP5 knockdown (Figure 2.6C-D). Our results suggest that DUSP5 does not play a major role in the anti-apoptotic and pro-survival effects of Ang-1. Knockdown of DUSP5 had no effect on Ang-1-induced cell migration (Figure 2.6E) or capillary tube formation (Figure 2.4A-C). In contrast, knockdown of DUSP5 completely eliminated the inhibitory effect of Ang-1 on vascular permeability (Figure 2.4D).

2.5 DISCUSSION

The principal findings of this study are: 1) In HUVECs, Ang-1 transiently increases the expression of DUSP1, DUSP4, and DUSP5 through the activation of the PI-3 kinase, ERK1/2, p38, and SAPK/JNK signaling pathways; 2) Ang-1 induction of DUSP1 and DUSP4 expression occurs through enhanced transcription; (3) DUSP1 and DUSP5 regulate Ang-1-induced p38 and ERK1/2 phosphorylation, respectively; while DUSP4 regulates Ang-1-induced ERK1/2, p38, and SAPK/JNK phosphorylation; 4) DUSP4 plays an important role in Ang-1-induced cell survival and inhibition of apoptosis; 5) DUSP1 and DUSP4 play important roles in Ang-1-induced cell migration and differentiation; and 6) DUSP5 is essential to HUVEC survival and Ang-1-induced inhibition of vascular permeability.

Regulation of DUSP1, DUSP4 and DUSP5: We report here that exposure of HUVECs to Ang-1 induces DUSP1, DUSP4, and DUSP5 expression and phosphatase activity. Previous studies in ECs have demonstrated that DUSP1 and DUSP5 are early response genes that are upregulated in response to angiogenic growth factors, including VEGF and thrombin [10, 19]. In this study we demonstrate that DUSP1 and DUSP5 mRNA and protein expressions are transiently increased by Ang-1 in a fashion similar to that elicited by VEGF. Moreover, our results also indicate that DUSP4 is induced by Ang-1 but not by VEGF, suggesting that the regulatory functions of DUSP4 are unique to Ang-1 signaling and are unlikely to contribute to VEGF signaling.

MAPK signaling pathways are well-known regulators of cell survival, migration, and proliferation. Our group and others have demonstrated that Ang-1 exposure triggers simultaneous and significant increases in ERK1/2, p38, and SAPK/JNK phosphorylation [3, 20]. It has also been well established that MAPKs induce DUSPs as a

transcriptional negative feedback mechanism to control their activities [7, 8]. The results of this study indicate that the Ang-1/Tie-2 axis uses the ERK1/2, p38, and SAPK/JNK pathways to upregulate DUSP1, DUSP4, and DUSP5. Specifically, we found that while the ERK1/2 and p38 pathways separately regulate DUSP5 and DUSP1 expression, they, along with the SAPK/JNK pathway, also regulate DUSP4 expression (Figure 2.2).

Previously, we described that in HUVECs stimulated with Ang-1 the PI-3 kinase pathway activates the transcription factors AP-1 and Egr-1 in a MAPK-independent fashion [6, 18]. We suggest that these transcription factors also participate in PI-3 kinase pathway regulation of DUSPs. Specifically, we found that inhibition of the c-Jun subunit of AP-1 using a dominant-negative form of the protein resulted in elimination of Ang-1-induced DUSP4 and DUSP5 expression, but had no effect on Ang-1-induced DUSP1 expression. We have previously reported that Ang-1 induces transactivation of AP-1 in ECs by increasing c-Jun phosphorylation on Ser^{63} and Ser^{73} and that this effect is mediated through the ERK1/2, SAPK/JNK, and PI-3 kinase pathways [6]. These findings suggest that Ang-1 triggers activation of these pathways which, in turn, induce transactivation of c-Jun, enhanced binding of c-Jun to DUSP4 and DUSP5 promoters, and transcription upregulation of both DUSPs. This scenario is supported by observations in cancer cells that implicate c-Jun activation in the regulation of DUSP5 [21].

We also report here that attenuation of Egr-1 expression by selective siRNA oligos results in the elimination of Ang-1-induced DUSP1 expression, while DUSP4 and DUSP5 expression remains unaffected. These findings suggest that Egr-1 is involved in the regulation of DUSP1 expression in HUVECs exposed to Ang-1 [22]. Recently, we have observed that Egr-1 expression and activity are rapidly induced in HUVECs exposed to Ang-1 and that Egr-1 is essential to the pro-angiogenic effects of Ang-1 in ECs [18]. DUSP1

is a transcriptional target of several factors including p53, c- Jun, ATF2, CREB, E2F, and SAP-1 [23-26]. To our knowledge, no information is as yet available regarding Egr-1 regulation of DUSP1 expression. It is possible that Egr-1 may bind directly to the DUSP1 promoter although DUSP1 promoter analyses have not revealed the presence of any distinct Egr-1 binding domains [26]. It is also possible that Egr-1 may indirectly act on DUSP1 transcription by interacting with other transcription factors, specificity factor 1 (Sp-1) for example, which directly binds to several domains on the DUSP1 promoter [26]. This is plausible since the Egr-1 DNA-binding domain shares a high degree of homology with that of Sp1. However, further studies are needed to elucidate the exact mechanisms through which Egr-1 regulates DUSP1 expression in the context of Ang-1/Tie-2 receptor signaling.

It has been well established that the PI-3 kinase pathway is critical to the pro-angiogenic and anti-apoptotic effects of Ang-1 in ECs [27, 28]. Our finding that wortmannin markedly reduces Ang-1 inductions of DUSP1, DUSP4, and DUSP5 implies that the PI-3 kinase pathway plays a major role in the regulation of these DUSPs downstream from Tie-2 receptors. The PI-3 kinase pathway is known to regulate DUSP1 and DUSP4 expressions in a variety of cells, including smooth muscles, macrophages, and cancer cells [29-31]; although little is known regarding DUSP5 regulation by this pathway. The mechanisms through which it regulates DUSP expression are likely to involve multiple factors, including those which are dependent on MAPK and MAPK-independent mechanisms. In the context of the Ang-1/Tie-2 receptor axis, we have reported that the PI-3 kinase pathway activates the ERK1/2 pathway in ECs exposed to Ang-1[3]. Thus, it is likely that the PI-3 kinase pathway effects on DUSP4 and DUSP5 that were observed in our experiments are also mediated through ERK1/2.

Yet another mechanism through which the PI-3 kinase pathway is known to regulate DUSP1 expression involves the production of nitric oxide (NO) [30]. However, we believe that this is not important in the context of DUSP regulation of the Ang-1/Tie-2 axis since Ang-1 exposure is known to inhibit rather than stimulate NO release in ECs [32].

Regulation of MAPK signaling by DUSPs: Despite their structural similarities, different DUSPs exhibit different substrate preferences and their activities can be modulated upon binding to these substrates [33]. We found that DUSP1 inactivates p38 downstream of the Ang-1/Tie-2 axis and DUSP5 negatively regulates ERK1/2 (Figures 2.3 and 2.6). These findings, that DUSP1 and DUSP5 are selective regulators of p38 and ERK1/2, respectively, are in accordance with those described in ECs exposed to VEGF [10, 19]. Surprisingly though, we found that DUSP4 has the ability to inactivate ERK1/2, p38, and SAPK/JNK in response to Ang-1 exposure (Figure 2.5). While some *in vitro* studies have demonstrated that this ability is more marked in relation to ERK1/2 and SAPK/JNK than it is in relation to p38 [34, 35], others have identified an important role for DUSP4 in p38 inactivation. Indeed, DUSP4 is able to dephosphorylate all three MAPKs in CCL-38 cells [36] and can also function as a potent inhibitor of p38 activity in PC-12 cells in response to nerve growth factor [37]. These differences in DUSP4 substrate specificity can be explained in part by differential levels of phosphatase expression, since DUSP4 at very high concentrations loses its selectivity for determined MAPKs [35]. We should also emphasize that our study does not rule out the participation of DUSPs other than DUSP1, DUSP4, or DUSP5 in the regulation of MAPK signaling downstream of Tie-2 receptors.

DUSP regulation of Ang-1-induced migration and differentiation: It has been well established that Ang-1 stimulates cell migration and that Tie-2 receptors are essential to this response [38-40]. The signaling pathways through which Ang-1 stimulates migration involve

PI-3 kinase and the adaptor protein Dok-R, which is recruited to activated Tie-2 receptors and, in turn, creates binding sites for Nck and the serine kinase p21-activating kinase (PAK1) [41]. Enhanced Ang-1-induced migration also involves the GTPases RhoA and Rac1 and the adaptor protein ShcA [39, 42]. Moreover, Ang-1-induced migration requires the release of reactive oxygen species (ROS) from NADPH oxidase. ROS modulate the activities of PI-3 kinase/AKT and the MAPKs, leading to enhanced cell migration [43, 44].

Selective contributions of various MAPK members to Ang-1-induced cell migration have as yet to be fully explored, but indirect evidence suggests that ERK1/2 activation is involved [6]. Our study indicates that Ang-1-induced cell migration is strongly influenced by DUSP1 and DUSP4, since attenuation of their expressions completely abrogates the pro-migratory effect of Ang-1 (Figures 2.5 and 2.6). This observation, along with our findings that DUSP1 and DUSP4 regulate the intensities and kinetics of the ERK1/2, p38, and SAPK/JNK pathways in cells exposed to Ang-1, suggests that MAPKs are important mediators of the pro-migratory effects of Ang-1. To test this hypothesis, we measured Ang-1-induced EC migration in the presence of selective inhibitors of MAPKs. Our results indicate that inhibition of the ERK1/2 and p38 pathways, but not of the SAPK/JNK pathway, abrogated Ang-1-induced cell migration, thereby confirming the importance of ERK1/2 and p38 as mediators of migration. These results, which demonstrate the regulatory importance of DUSP1 and DUSP4 in Ang-1-induced migration, are similar to those that indicate that they also play important roles in VEGF-induced migration [10, 19].

ECs are able to form capillary-like structures or tubes when maintained in a three-dimensional matrix. Using a model that mimics the steps of tube formation during angiogenesis [45], we found that DUSP1 and DUSP4 knockdown results in significant reductions in Ang-1-induced tube formation, indicating that both have a strong effect on

tube formation in HUVECs (Figure 2.4). We speculate that these effects may be mediated by the excessive p38 activation that is associated with their knockdown (Figures 2.3 and 2.5). This is based on observations that growth factor-induced angiogenesis is negatively affected by the p38 pathway [46]. It is also possible that attenuation of SAPK/JNK pathway activation in DUSP1 knockdown cells may contribute to poor tube formation since this pathway has been shown to play a central role in capillary tube formation in ECs [47].

DUSP contributions to Ang-1-induced EC survival: It has been well established that the Ang-1/Tie-2 receptor axis promotes cell survival through activation of the ERK1/2 and PI-3 kinase/AKT pathways, reduction in cytosolic levels of the mitochondrial caspase activator Smac, and upregulation of survivin [27]. In HUVECs, Ang-1 attenuates serum deprivation-induced cytotoxicity and caspase 3/7 activity, while significantly increasing cell number (Figures 2.3, 2.5 and 2.6). Ang-1 does not attenuate serum deprivation-induced cytotoxicity and caspase 3/7 activity in DUSP4 knockdown cells (Figure 2.5). This observation suggests that DUSP4 serves as an anti-apoptotic protein in cells exposed to Ang-1. However, DUSP4 knockdown reduced cytotoxicity and caspase 3/7 activity in HUVECs grown in medium containing 0.2% FBS alone, indicating that, under serum-deprivation conditions, DUSP4 promotes apoptosis in the absence of Ang-1. These observations suggest that the degree and direction of DUSP4 regulation of EC survival and apoptosis are context-dependent and are influenced by such factors as the presence or absence of nutrients or exposure to growth factors such as Ang-1.

We speculate that he anti-apoptotic effects of DUSP4 in ECs exposed to Ang-1 may be mediated through selective inhibition of DUSP4-induced SAPK/JNK activation. This is based on several reports indicating that that inactivation of SAPK/JNK by Ang-1 represents an important mechanism through which the Ang-1 pathway promotes EC survival [43, 48] and

the observation that overexpression of DUSP4 rescues HEK-293 cells from UV and cisplatin-induced apoptosis, also as a result of SAPK/JNK inhibition [49]. The pro-apoptotic role of DUSP4 in serum-deprived cells is more difficult to explain, but might be the result of an imbalance between pro- and anti-apoptotic MAPK pathways and the nature of interactions between MAPKs and other signaling pathways that are activated under conditions of serum deprivation.

This study also reveals that DUSP5 knockdown significantly increases both basal and serum deprivation-induced cytotoxicity and caspase 3/7 activity and decreases cell number. Ang-1-induced inhibitory effects on these parameters remain unaffected. These findings suggest that DUSP5 is not an important mediator of the anti-apoptotic and pro-survival effects of Ang-1 in ECs despite the fact that it is a negative regulator of ERK1/2 signaling, which is pro-survival and anti-apoptotic. One study has demonstrated that sustained ERK1/2 activation in MCF-7 cells stimulated with phorbol 12-myristate 13-acetate (PMA), a consequence of diminished DUSP5 and MKP-3 levels, results in growth arrest and differentiation, while transient ERK/12 activation leads to a proliferative and migratory cell phenotype [21]. Our findings lend support to that study and to the fact that DUSP5 is essential to maintain optimal activation of the ERK1/2 pathway since, in the absence of functional DUSP5, sustained ERK1/2 activation does not potentiate EC survival, even in the presence of Ang-1.

Role of DUSPs in vascular permeability: Ang-1 is a strong inhibitor of vascular permeability, as shown in studies *in vitro* and *in vivo* [32, 50, 51]. Our observation that significant reduction in basal vascular permeability occurs in response to Ang-1 in HUVECs transfected with scrambled siRNA oligos (Figure 2.4) is in agreement with these studies. Ang-1 inhibited vascular permeability in DUSP1 and DUSP4 knockdown cells but

not in DUSP5 knockdown cells, indicating that DUSP5 is essential to this Ang-1 effect (Figure 2.4).

Multiple signaling pathways modulate the effect of Ang-1 on vascular permeability. For instance, Ang-1 antagonizes VEGF-induced permeability through sequestration of Src by mammalian diaphanous (mDia) and through the inhibition of nitric oxide release that results from phosphorylation of endothelial NO synthase (eNOS) by protein kinase C zeta (PKCζ) [32]. Sphingosine kinase-1 has also been shown to mediate the inhibitory effect of Ang-1 on vascular permeability [52]. Furthermore, many reports have confirmed that MAPKs in general and ERK1/2 in particular, are important regulators of vascular permeability. The ERK1/2 role is mediated in part through activation of myosin light-chain kinase (MLCK), which promotes EC contractility and disruption of cell–cell junctions [53-55]. Based on these observations and on the fact that DUSP5 regulates ERK1/2 activation, we suggest that the role of DUSP5 in Ang-1 inhibition of vascular permeability is mediated primarily by regulation of the intensity and kinetics of ERK1/2 activation.

In summary, we report here that Ang-1 induces the expressions and activations of DUSP1, DUSP4, and DUSP5 and that these effects are mediated through the PI-3 kinase, ERK1/2, p38, and SAPK/JNK pathways. We found that at least two transcription factors, AP-1 and Egr-1, play important roles in the regulation of DUSPs. We also report that DUSP1, DUSP4, and DUSP5 differentially contribute to Ang-1-induced EC migration, differentiation, vascular permeability, and survival.

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AUTHORSHIP CONTRIBUTIONS:

R. Echavarria: Performed experiments, analyzed results, interpreted results and wrote and edited the manuscript.

S. Hussain: Designed experiments, analyzed results, interpreted and edited the final manuscript

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DISCLOSURE OF CONFLICTS OF INTEREST

All authors declare no conflicts of interest or financial interests.

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2.8 FIGURES

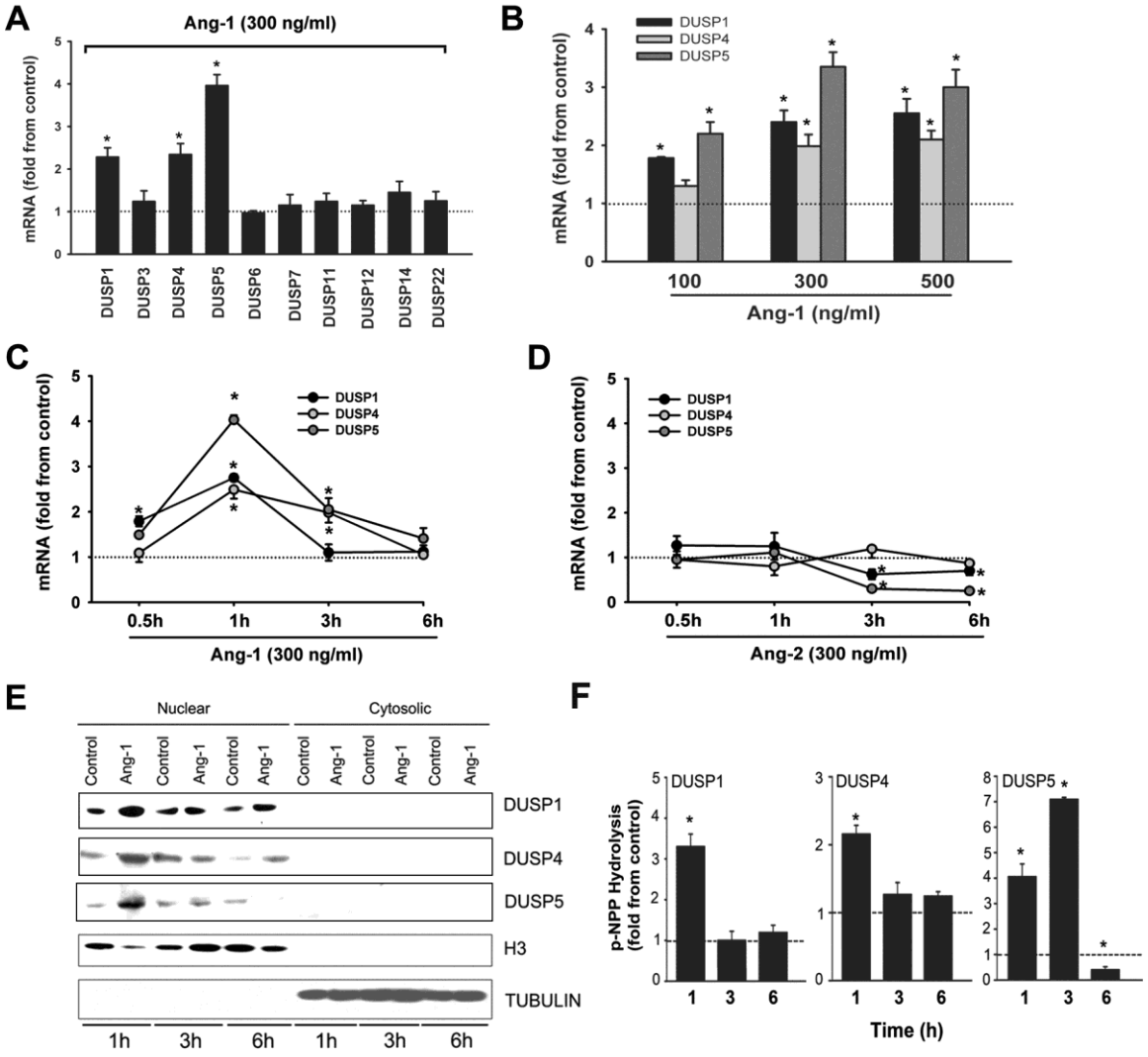


Figure 2.1

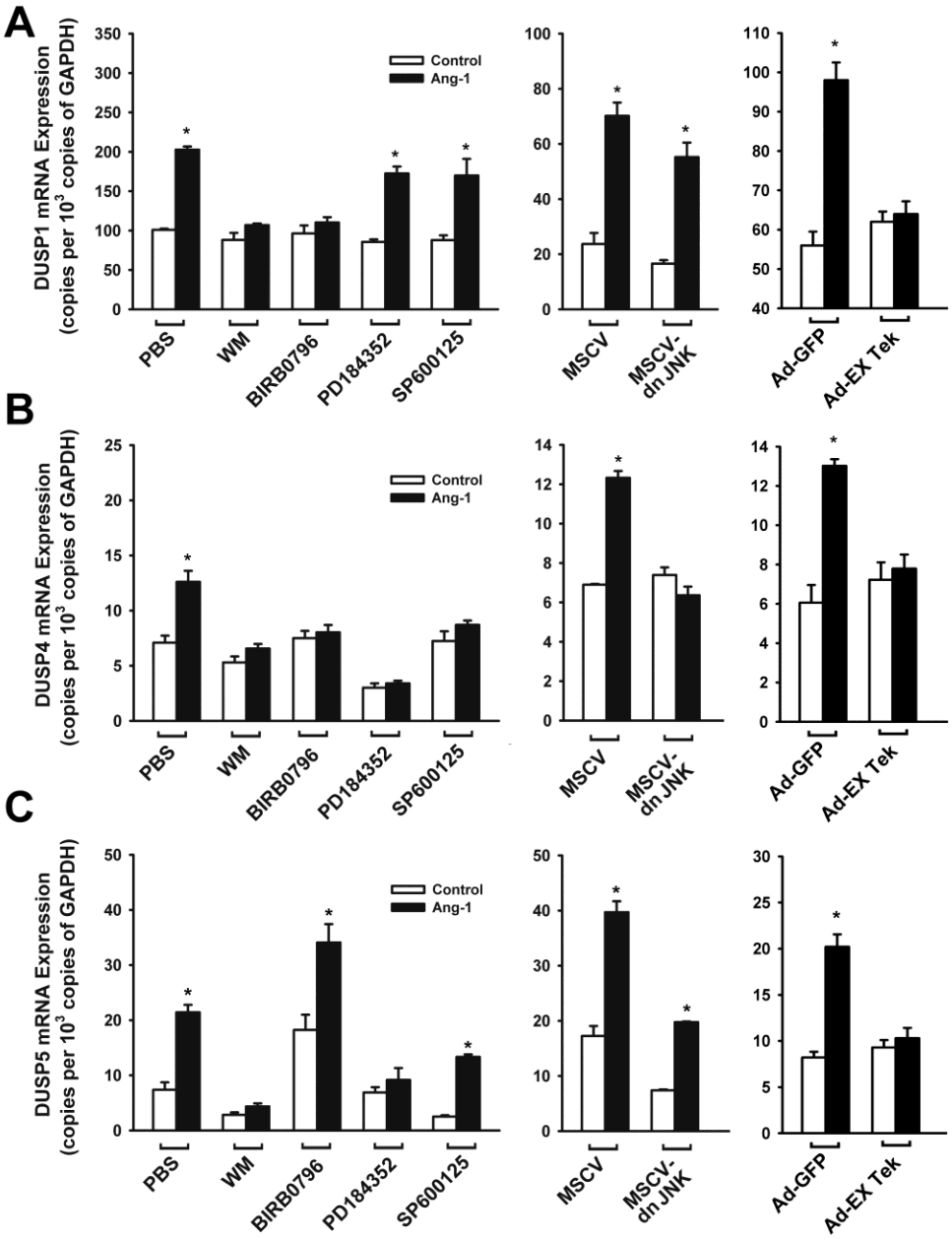

A
DUSP1 mRNA Expression
(copies per 10^3 copies of GAPDH)
Control
Ang-1
PBS
WM
BIRB0796
PD184352
SP600125
MSCV
MSCV-dn JNK
Ad-GFP
Ad-EX Tek
B
DUSP4 mRNA Expression
(copies per 10^3 copies of GAPDH)
C
DUSP5 mRNA Expression
(copies per 10^3 copies of GAPDH)

Figure 2.2

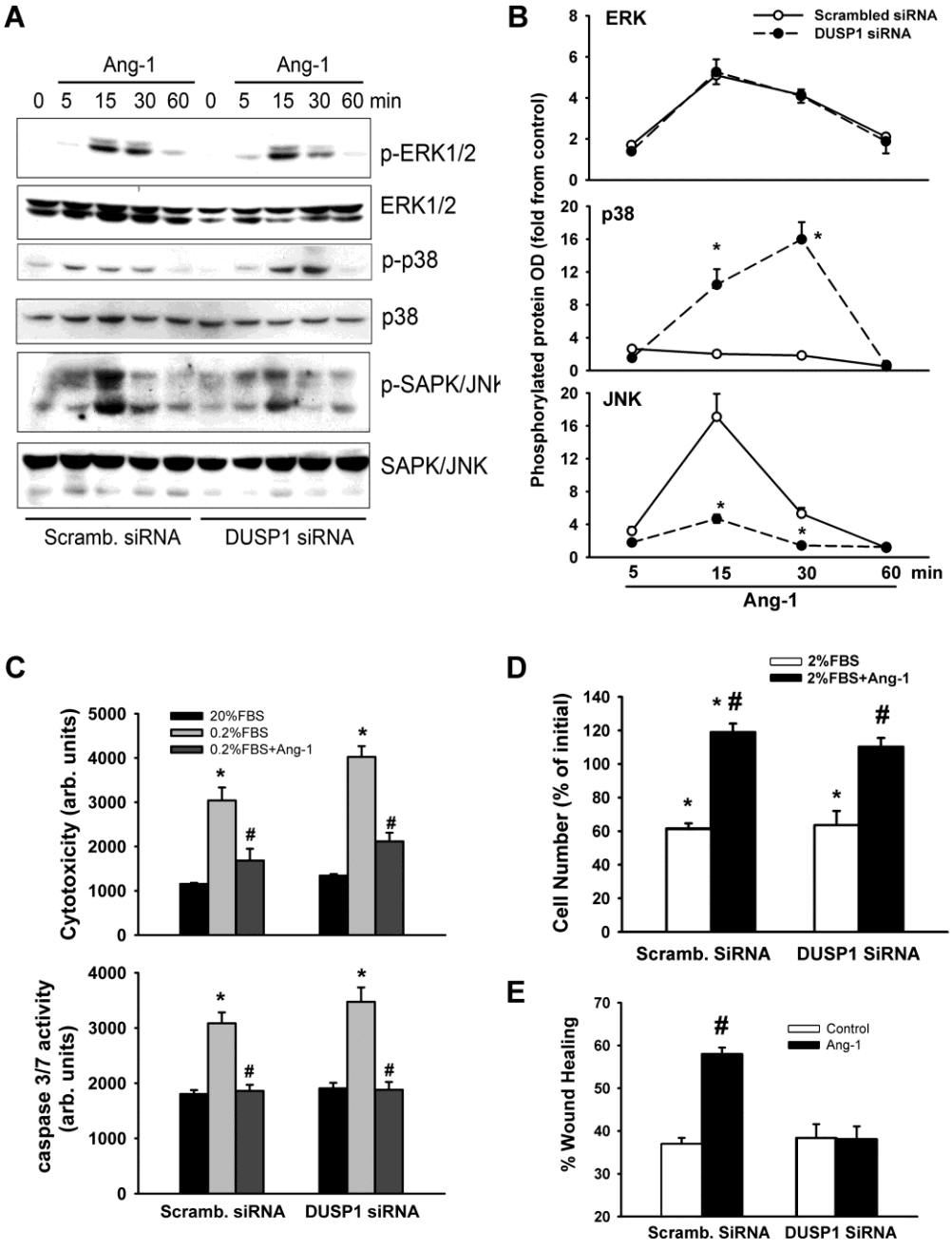

A
Ang-1
Ang-1
0 5 15 30 60 0 5 15 30 60 min
p-ERK1/2
ERK1/2
p-p38
p38
p-SAPK/JNK
SAPK/JNK
Scramb. siRNA
DUSP1 siRNA
B
ERK
Scrambled siRNA
DUSP1 siRNA
p38
JNK
Phosphorylated protein OD (fold from control)
5 15 30 60 min
Ang-1
C
20%FBS
0.2%FBS
0.2%FBS+Ang-1
Cytotoxicity (arb. units)
caspase 3/7 activity (arb. units)
Scramb. siRNA
DUSP1 siRNA
D
2%FBS
2%FBS+Ang-1
Cell Number (% of initial)
Scramb. SiRNA
DUSP1 SiRNA
E
Control
Ang-1
% Wound Healing
Scramb. SiRNA
DUSP1 SiRNA

Figure 2.3

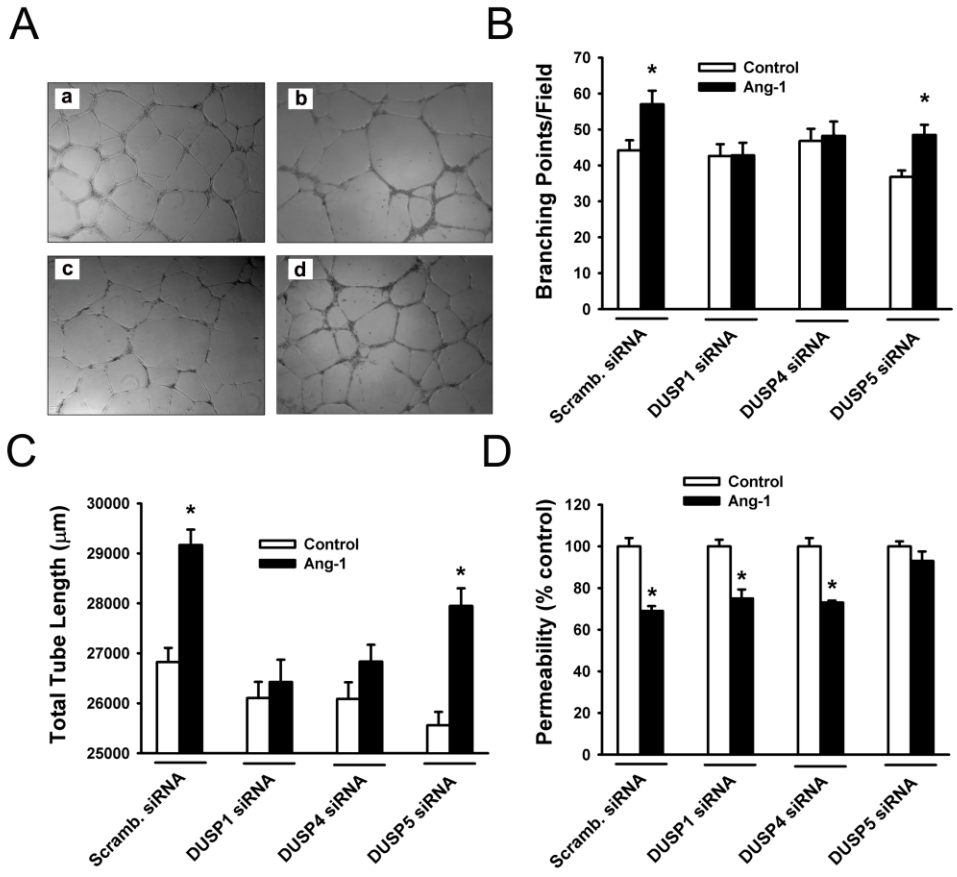

A
a
b
c
d
B
Branching Points/Field
Control
Ang-1
Scramb. siRNA
DUSP1 siRNA
DUSP4 siRNA
DUSP5 siRNA
C
Total Tube Length (μm)
D
Permeability (% control)

Figure 2.4

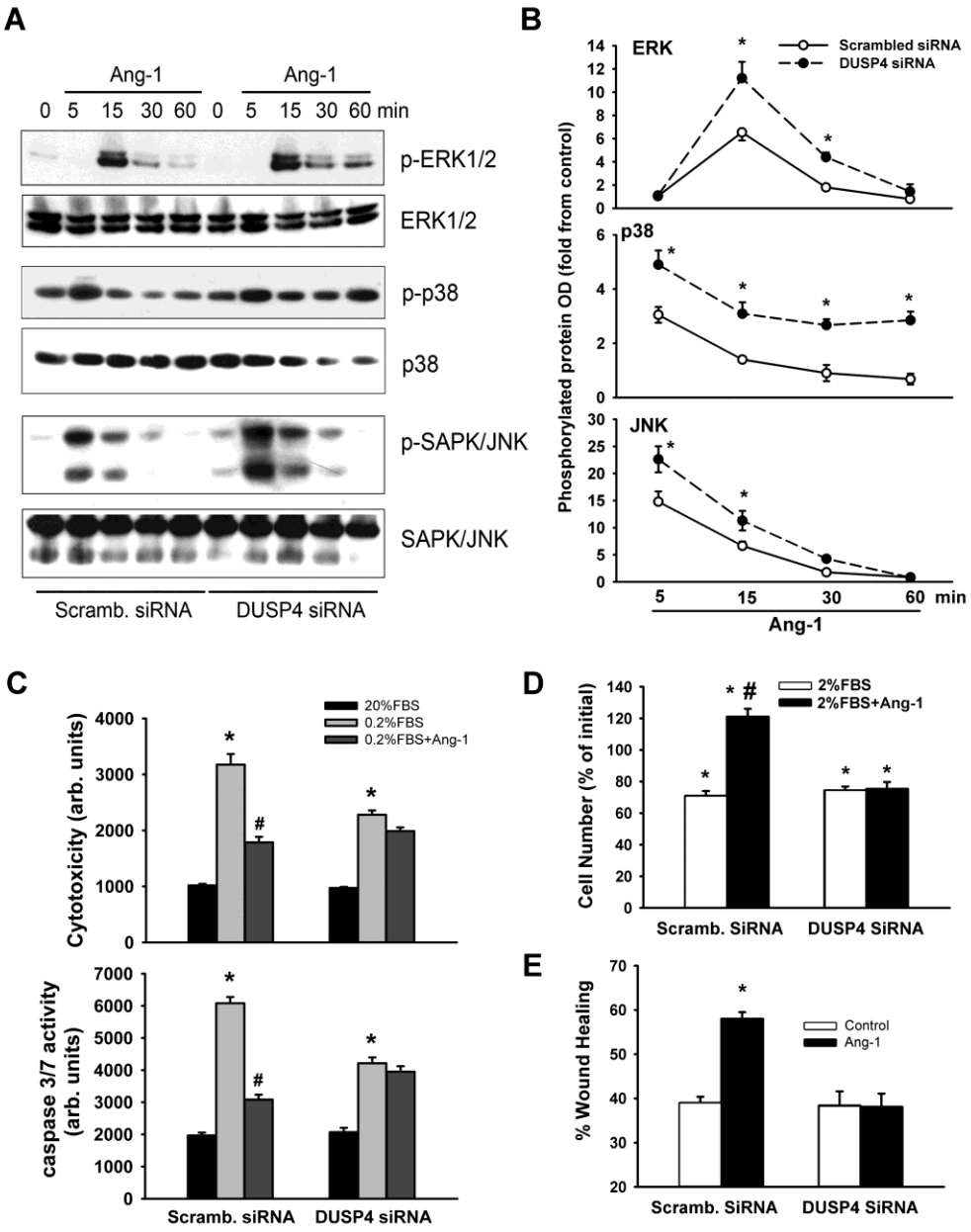

A
Ang-1
Ang-1
0 5 15 30 60 0 5 15 30 60 min
p-ERK1/2
ERK1/2
p-p38
p38
p-SAPK/JNK
SAPK/JNK
Scramb. siRNA
DUSP4 siRNA
B
ERK
Scrambled siRNA
DUSP4 siRNA
p38
JNK
Phosphorylated protein OD (fold from control)
5 15 30 60 min
Ang-1
C
20%FBS
0.2%FBS
0.2%FBS+Ang-1
Cytotoxicity (arb. units)
caspase 3/7 activity (arb. units)
Scramb. siRNA
DUSP4 siRNA
D
2%FBS
2%FBS+Ang-1
Cell Number (% of initial)
Scramb. SiRNA
DUSP4 SiRNA
E
Control
Ang-1
% Wound Healing
Scramb. SiRNA
DUSP4 SiRNA

Figure 2.5

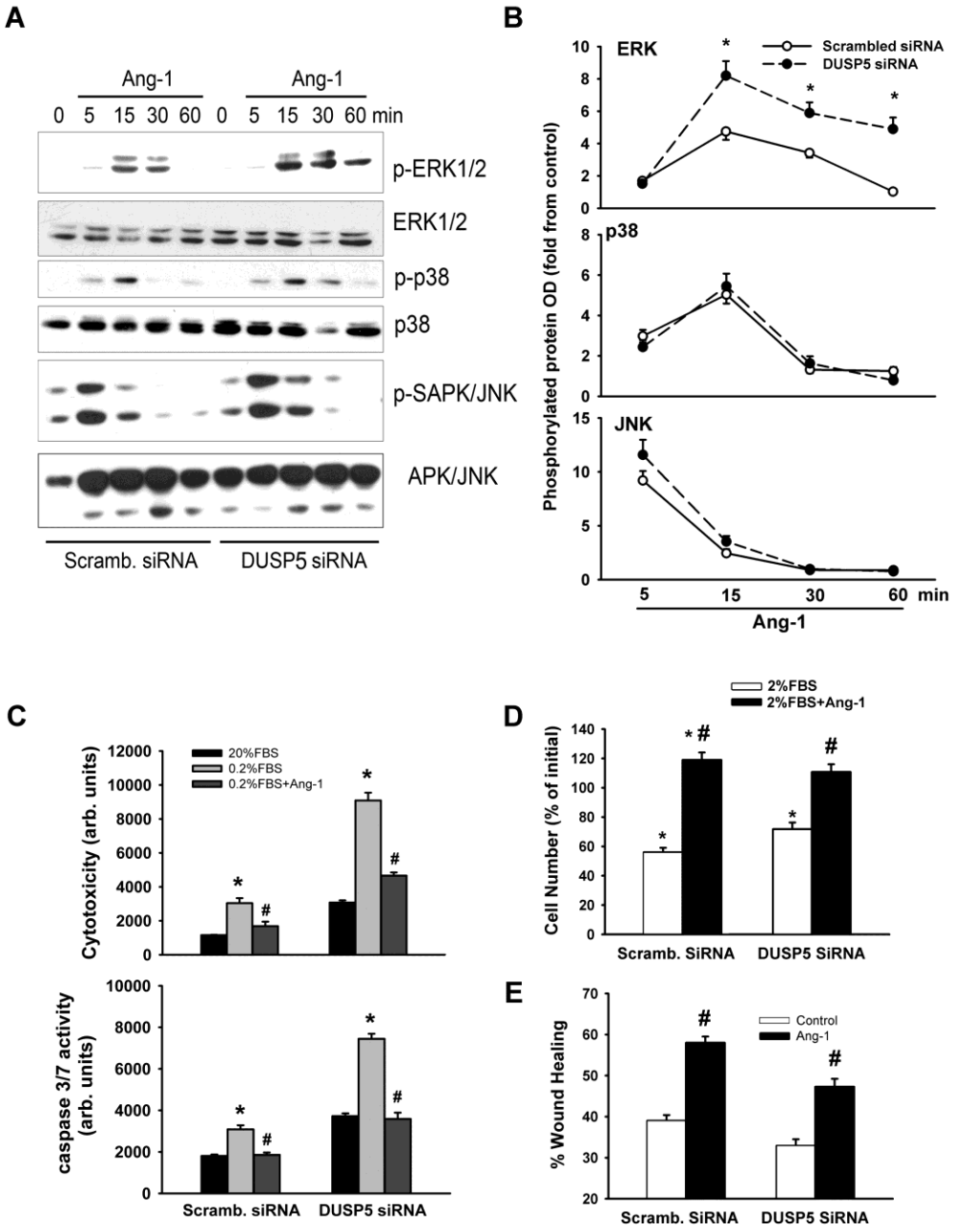
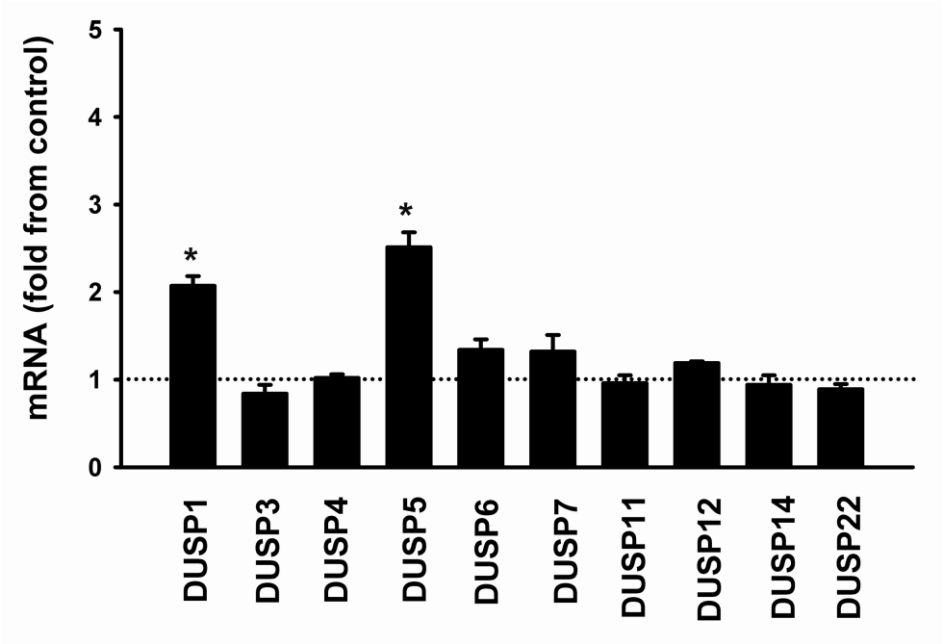

A
Ang-1
Ang-1
0 5 15 30 60 0 5 15 30 60 min
p-ERK1/2
ERK1/2
p-p38
p38
p-SAPK/JNK
APK/JNK
Scramb. siRNA
DUSP5 siRNA
B
ERK
Scrambled siRNA
DUSP5 siRNA
p38
JNK
Phosphorylated protein OD (fold from control)
5 15 30 60 min
Ang-1
C
20%FBS
0.2%FBS
0.2%FBS+Ang-1
Cytotoxicity (arb. units)
caspase 3/7 activity (arb. units)
Scramb. siRNA
DUSP5 siRNA
D
2%FBS
2%FBS+Ang-1
Cell Number (% of initial)
Scramb. SiRNA
DUSP5 SiRNA
E
Control
Ang-1
% Wound Healing
Scramb. SiRNA
DUSP5 SiRNA

Figure 2.6



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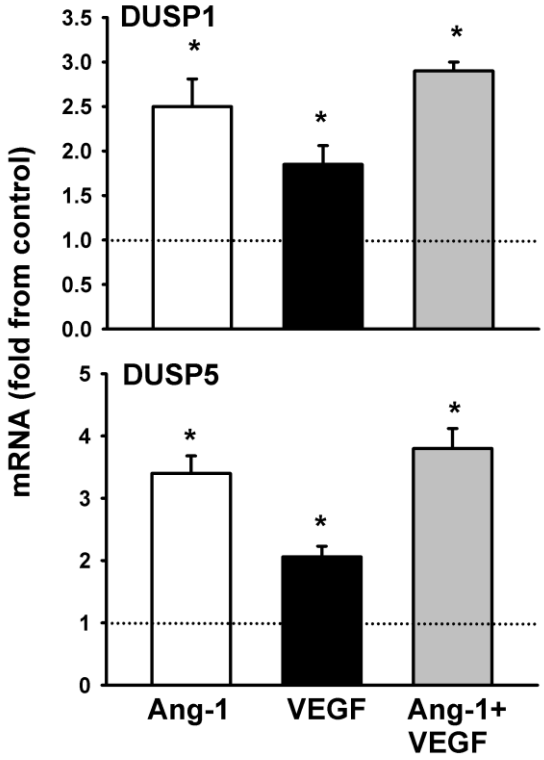

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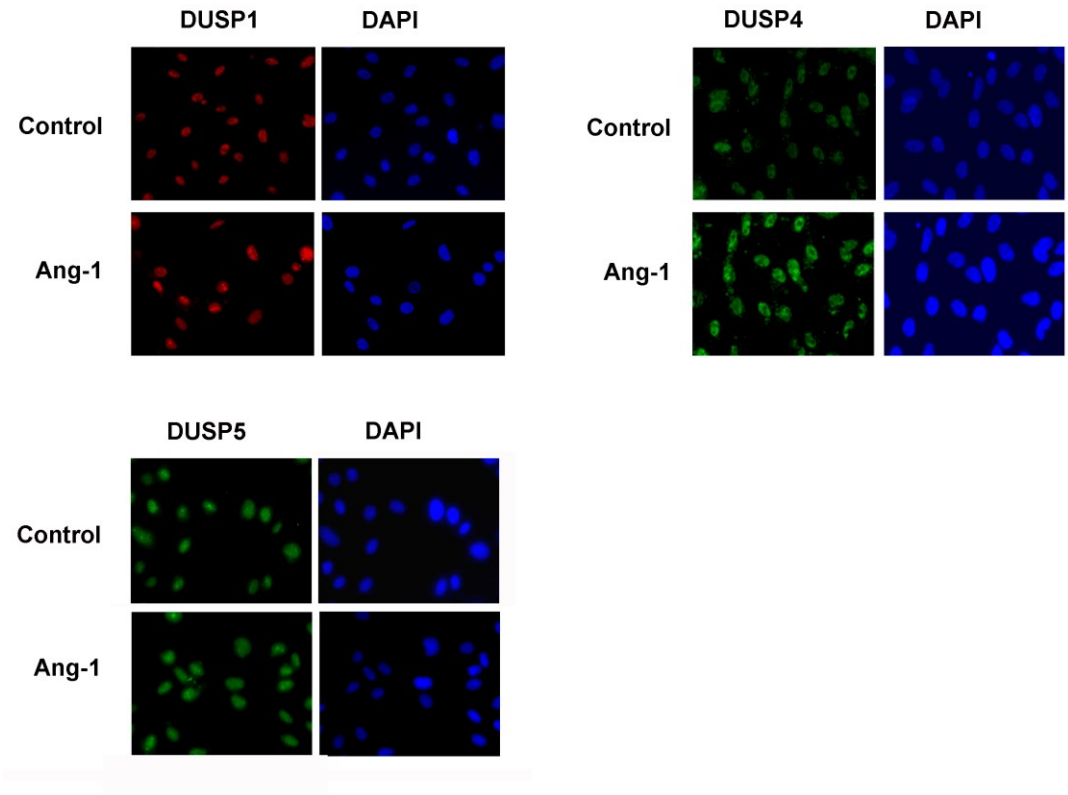

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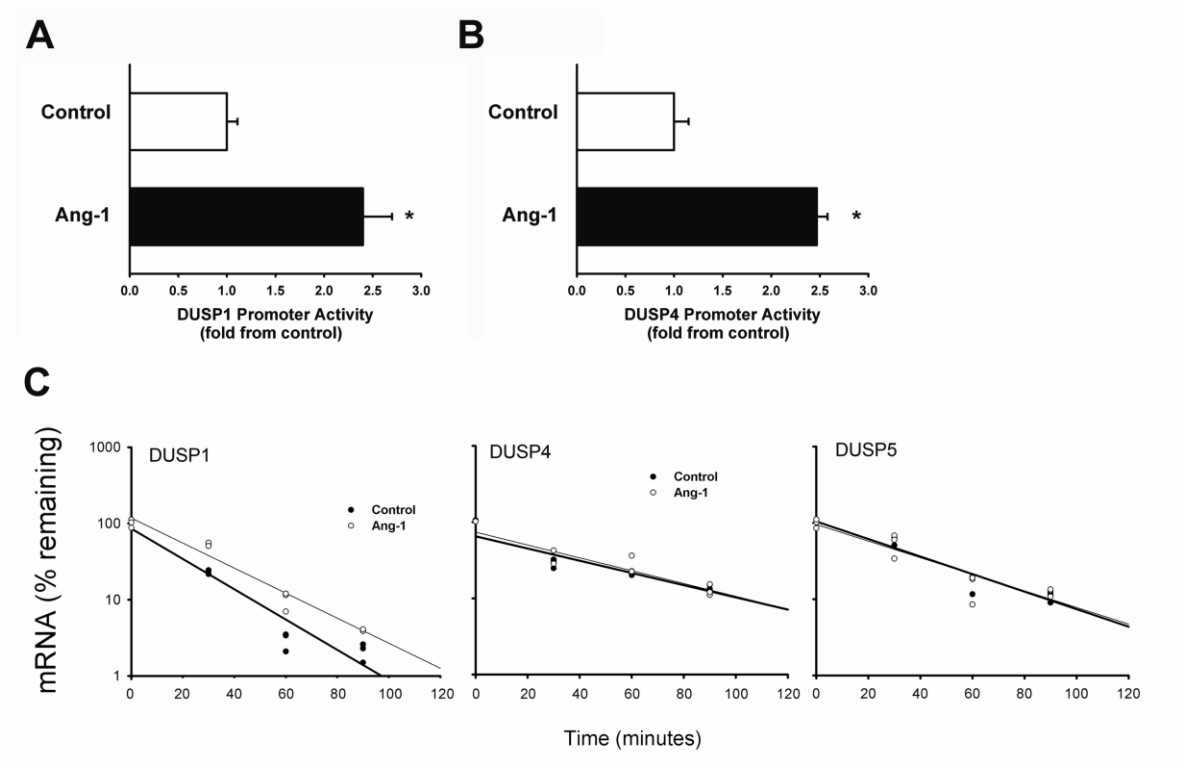
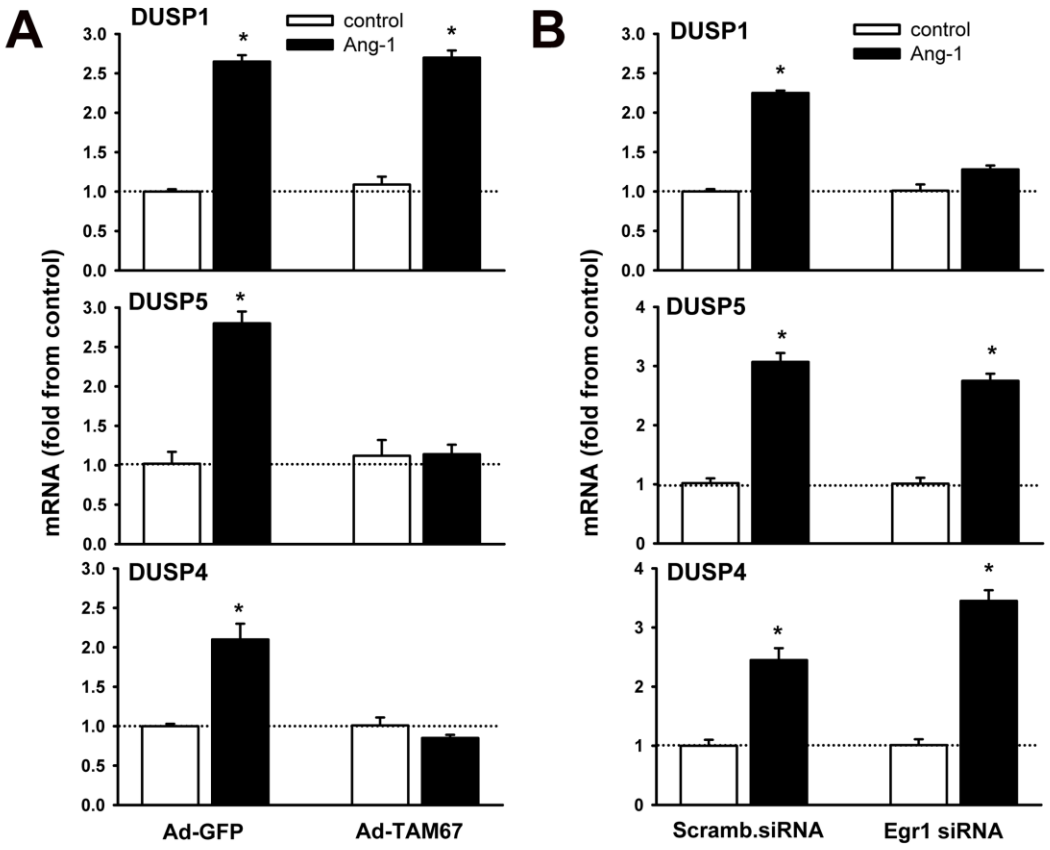
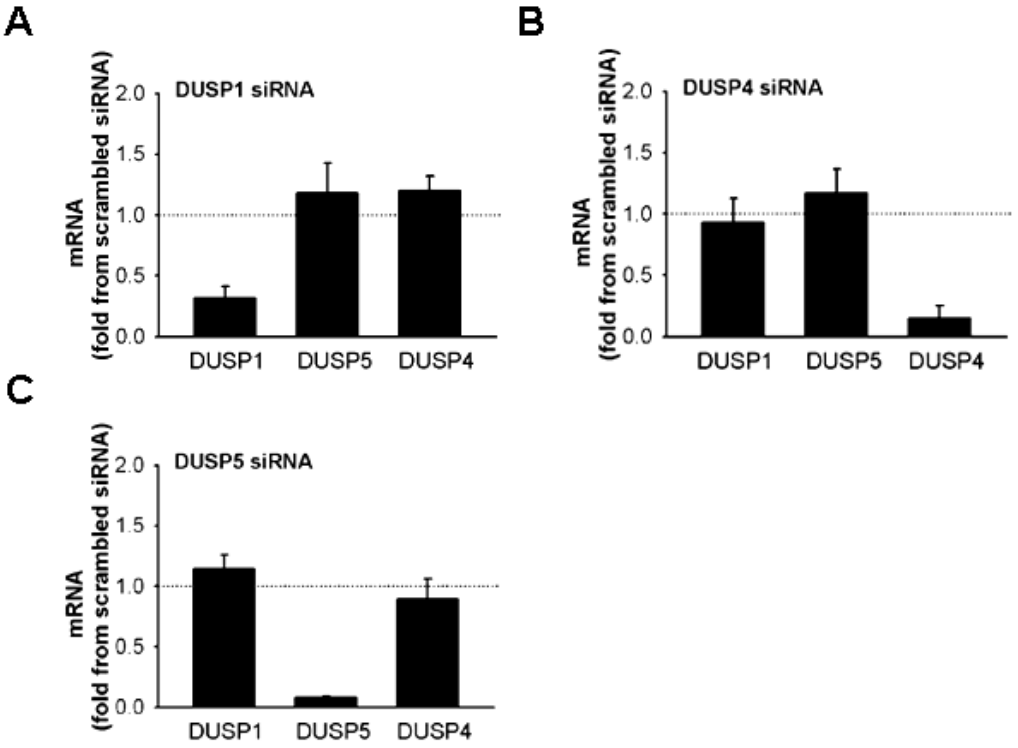


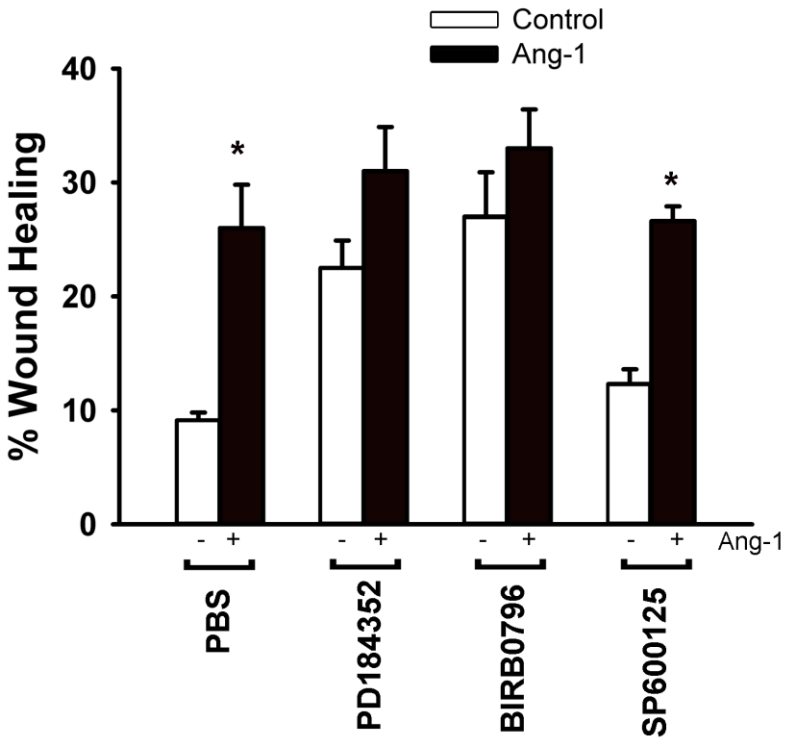
Supplementary Figure S2.4



Supplementary Figure S2.5



Supplementary Figure S2.6



Supplementary Figure S2.7

2.9 FIGURE LEGENDS

Figure 2.1: Ang-1 induces DUSP1, DUSP4 and DUSP5 expression

A) mRNA expressions of DUSPs in HUVECs exposed to PBS (control) or Ang-1 (300 ng/ml) for 1 hr. Values expressed as means ± SE. *P<0.05 compared to control. N=6. **B)** mRNA expressions of DUSP1, DUSP4, and DUSP5 in HUVECs exposed to PBS (control) or varying concentrations of Ang-1 for 1 hr. Values expressed as means ± SE. *P<0.05 compared to control. N=6. **C)** mRNA expressions of DUSP1, DUSP4, and DUSP5 in HUVECs exposed to PBS (control) or Ang-1 (300 ng/ml) for varying durations. Values expressed as means ± SE. *P<0.05 compared to control. N=6. **D)** mRNA expressions of DUSP1, DUSP4, and DUSP5 in HUVECs exposed to PBS (control) or Ang-2 (300 ng/ml) for varying durations. Values expressed as means ± SE. *P<0.05 compared to control. N=6. **E)** Representative immunoblots of DUSP1, DUSP4, and DUSP5 proteins in nuclear and cytosolic fractions of HUVECs exposed to PBS (control) for 1 hr or Ang-1 (300 ng/ml) for 1, 3, or 6 hr. H3 refers to histone 3, a marker of nuclear proteins. Tubulin was used as a marker for cytosolic proteins. **F)** Phosphatase activities of DUSP1, DUSP4, and DUSP5 in HUVECs exposed to PBS (control) for 1 hr or Ang-1 (300 ng/ml) for varying durations. Values expressed as means ± SE. *P<0.05 compared to control. N=5.

Figure 2.2: Role of MAPKs, PI3-K and Tie-2 in the induction of DUSPs by Ang-1

A-C) Left: Effects of pre-treatment with PBS, wortmannin (WM) (PI-3 kinase inhibitor), BIRB0796 (p38 inhibitor), PD18435 (ERK1/2 inhibitor), or SP600125 (SAPK/JNK

inhibitor) on mRNA expressions of DUSP1, DUSP4, and DUSP5 in HUVECs exposed to PBS (control) or Ang-1. Values expressed as means ± SE. *P<0.05 compared to own control. N=6. **A-C) Middle:** mRNA expressions of DUSP1, DUSP4, and DUSP5 in HUVECs stably expressing empty vector (MSCV) or a dominant-negative form of JNK (MSCV-dn JNK) and exposed to PBS (control) or Ang-1. Values expressed as means ± SE. *P<0.05 compared to own control. N=6. **A-C) Right:** mRNA expressions of DUSP1, DUSP4, and DUSP5 in HUVECs infected with Ad-GFP or Ad-Ex Tek and exposed 48 hr post-infection to PBS (control) or Ang-1 (300 ng/ml). Values expressed as means ± SE. *P<0.05 compared to own control. N=6.

Figure 2.3: DUSP1 inactivates p38 and is involved in EC migration

A) Representative immunoblots of ERK1/2, p38, and SAPK/JNK protein expressions in HUVECs transfected with scrambled or DUSP1 siRNA, and exposed 48 hr post-transfection to PBS (control) for 1 hr or Ang-1 (300 ng/ml) for varying durations. **B)** Relative protein optical densities of phosphorylated ERK1/2, p38, and SAPK/JNK in HUVECs transfected with scrambled or DUSP1 siRNA and exposed 48 hr post-transfection to PBS (control) for 1 hr or Ang-1 (300 ng/ml) for varying durations. Values expressed as means ± SE. *P<0.05 compared to scrambled siRNA. **C)** Cytotoxicity and caspase 3/7 activity in HUVECs transfected with scrambled or DUSP1 siRNA and maintained for 24 hr in medium containing 20% FBS, 0.2% FBS, or 0.2% FBS plus Ang-1. Values expressed as means ± SE. *P<0.05 compared to 20% FBS. $^{\#}$P<0.05 compared to corresponding 0.2% FBS values. **D)** Cell number of HUVECs transfected with scrambled or DUSP1 siRNA and maintained for 24 hr in medium containing 2% FBS (control) or 2% FBS plus Ang-1. Cell

numbers expressed as % of initial cell count. *P<0.05 compared to initial cell count. #P<0.05 compared to corresponding 2% FBS values. **E)** Percent wound healing in HUVECs transfected with scrambled or DUSP1 siRNA and maintained for 8 hr in medium containing 2% FBS (control) or 2% FBS plus Ang-1. #P<0.05 compared to corresponding control values.

Figure 2.4: Role of DUSP1, DUSP4 and DUSP5 in capillary tube formation and vascular permeability

A) Representative micrograph of capillary tube formation in HUVECs transfected with a) scrambled, b) DUSP1, c) DUSP4, or d) DUSP5 siRNA and exposed 48 hr post-transfection for 24 hr to Ang-1 **B-C)** Branching points/field and total tube length in HUVECs transfected with scrambled, DUSP1, DUSP4 or DUSP5 siRNA and exposed 48 hr post-transfection for 24 hr to PBS (control) or Ang-1.Values expressed as means ± SE.*P<0.05 compared to corresponding control values. **D)** Permeability changes in HUVECs transfected with scrambled, DUSP1, DUSP4 or DUSP5 siRNA and exposed 48 hr post-transfection for 30 min to PBS (control) or Ang-1. Values expressed as means ± SE.*P<0.05 compared to corresponding control values.

Figure 2.5: DUSP4 inactivates ERK1/2, p38 and SAPK/JNK and is required for Ang-1-dependent survival and migration of ECs

A) Representative immunoblots of ERK1/2, p38, and SAPK/JNK protein expressions in HUVECs transfected with scrambled or DUSP4 siRNA and exposed 48 hr post-transfection to PBS (control) for 1 hr or Ang-1 (300 ng/ml) for varying durations. **B)** Relative protein

optical densities of phosphorylated ERK1/2, p38, and SAPK/JNK in HUVECs transfected with scrambled or DUSP4 siRNA and exposed 48 hr post-transfection to PBS (control) for 1h or Ang-1 for varying durations. Values expressed as means ± SE. *P<0.05 compared to scrambled siRNA. **C)** Cytotoxicity and caspase 3/7 activity in HUVECs transfected with scrambled or DUSP4 siRNA and maintained for 24 hr in medium containing 20% FBS, 0.2% FBS, or 0.2% FBS plus Ang-1. *P<0.05 compared to 20% FBS. $^{\#}$P<0.05 compared to corresponding 0.2% FBS values. **D)** Cell number of HUVECs transfected with scrambled or DUSP4 siRNA and maintained for 24 hr in medium containing 2% FBS (control) or 2% FBS plus Ang-1. Cell numbers expressed as % of initial cell count. *P<0.05 compared to initial cell count. $^{\#}$P<0.05 compared to corresponding 2% FBS values. **E)** Percent wound healing in HUVECs transfected with scrambled or DUSP4 siRNA and maintained for 8 hr in medium containing 2% FBS (control) or 2% FBS plus Ang-1. $^{\#}$P<0.05 compared to corresponding control values.

Figure 2.6: DUSP5 is an ERK-specific phosphatase

A) Immunoblots of ERK1/2, p38, and SAPK/JNK protein expressions in HUVECs transfected with scrambled or DUSP5 siRNA and exposed to PBS (control) for 1 hr or Ang-1 for varying durations. **B)** Relative protein optical densities of phosphorylated ERK1/2, p38, and SAPK/JNK in HUVECs transfected with scrambled or DUSP5 siRNA and exposed 48 hr post-transfection to PBS (control) for 1 hr or Ang-1 for varying durations. Values expressed as means ± SE. *P<0.05 compared to scrambled siRNA. **C)** Cytotoxicity and caspase 3/7 activity in HUVECs transfected with scrambled or DUSP5 siRNA and maintained for 24 hr in medium containing 20% FBS, 0.2% FBS, or 0.2% FBS

plus Ang-1. Values expressed as means ± SE. *P<0.05 compared to 20% FBS. #P<0.05 compared to corresponding 0.2% FBS values. **D)** Cell number of HUVECs transfected with scrambled or DUSP5 siRNA and maintained for 24 hr in medium containing 2% FBS (control) or 2% FBS plus Ang-1. *P<0.05 compared to initial cell count. #P<0.05 compared to corresponding 2% FBS values. **E)** Percent wound healing in HUVECs transfected with scrambled or DUSP5 siRNA and maintained for 8 hr in medium containing 2% FBS (control) or 2% FBS plus Ang-1. #P<0.05 compared to corresponding control values.

Supplementary Figure S2.1: Expression of DUSPs by VEGF

Means ± SE of mRNA expressions of various DUSPs in HUVECs exposed to PBS (control) or VEGF (40 ng/ml) for 1h. N=6. *P<0.05 compared to control.

Supplementary Figure S2.2: DUSP1 and DUSP5 expression by Ang-1 and VEGF

Means ± SE of mRNA expressions of DUSP1 and DUSP5 in HUVECs exposed for 1h to PBS (control), Ang-1 (300 ng/ml), VEGF (40 ng/ml) or combination of Ang-1 and VEGF. N=6. *P<0.05 compared to control.

Supplementary Figure S2.3: Cellular localization of DUSP1, DUSP4 and DUSP5 proteins

Immunostaining for DUSP1, DUSP4 and DUSP5 proteins in HUVECs exposed to PBS (control) or Ang-1 (300 ng/ml) for 1h.

Supplementary Figure S2.4: Promoter activity and mRNA stability of DUSPs

A) DUSP1 and DUSP4 promoter activities in HUVECs exposed to PBS (control) or Ang-1 (300ng/ml) for 6h. Values expressed as means ± SE. *P<0.05 compared to control. N=5. **B)** Ang-1 regulation of DUSP1, DUSP4, and DUSP5 mRNA stability in HUVECs exposed to Ang-1 (300ng/ml) for 1h. Cells were washed post-exposure with PBS and maintained in serum-free medium containing actinomycin D (control) or medium containing actinomycin D plus Ang-1 (300ng/ml). Total RNA was extracted after varying durations (0, 30, 60 and 90min) of exposure. DUSP1, DUSP4, and DUSP5 transcripts were analyzed using real-time PCR.

Supplementary Figure S2.5: Role of Ap-1 and Egr-1 in the induction of DUSPs by Ang-1

A) DUSP1, DUSP4, and DUSP5 mRNA expressions in HUVECs infected with adenoviruses expressing GFP (control) or dominant-negative c-Jun (TAM67) and exposed 48h post-infection to PBS (control) or Ang-1 (300ng/ml) for 1h. Values expressed as means ± SE. *P<0.05 compared to own control group. **B)** DUSP1, DUSP4, and DUSP5 mRNA expressions in HUVECs transfected with scrambled siRNA or Egr-1 siRNA oligos and exposed 48h post-transfection to PBS (control) or Ang-1 (300ng/ml) for 1h. Values expressed as means ± SE. *P<0.05 compared to own control group.

Supplementary Figure S2.6: Effect of DUSP1, DUSP4 and DUSP5 siRNA on DUSP expression

A) DUSP1, DUSP4 and DUSP5 mRNA expression measured with real time PCR after 48h of transfection with scrambled siRNA and DUSP1 siRNA. N=3. **B)** DUSP1, DUSP4 and DUSP5 mRNA expression measured with real time PCR after 48h of transfection with scrambled siRNA and DUSP4 siRNA. N=3. **C)** DUSP1, DUSP4 and DUSP5 mRNA expression measured with real time PCR after 48h of transfection with scrambled siRNA and DUSP5 siRNA. N=3.

Supplementary Figure S2.7: Influence of selective inhibitors of the ERK1/2, p38 and SAPK/JNK pathways on Ang-1-induced wound healing

Influence of PBS and selective inhibitors of the ERK1/2, p38 and SAPK/JNK pathways on Ang-1-induced wound healing in HUVECs. Cells were pre-incubated for 1h with PBS or selective inhibitors and were then exposed to PBS (control) or Ang-1 (300 ng/ml) for 8h. *$P<0.05$ compared to control.

2.10 TABLES

Table 2.1 Primers used for real-time PCR experiments

Type	Sequence (5'to 3')	Accession Number	Expected Size (bp)
DUSP1	F: TGTGGAGGACAACCACAAGG R: AAACTCAAAGGCCTCGTCCA	NM_004417	190
DUSP3	F: AGCTCTCGGTGCAGGATCTC R: AGTTGGCATTGGTGTTGACG	NM_004090	213
DUSP4	F: AGCATCATCTCGCCCAACTT R: AGACCGGAAAGCTGAAGACG	NM_001394 NM_057158	169
DUSP5	F: GGATCCCTGTGGAAGACAGC R: GACCATGCTCCTCCTCTGCT	NM_004419	221
DUSP6	F: AGCTCGACCCCCATGATAGA R: CGATGTGCGACGACTCGTAT	NM_001946 NM_022652	163
DUSP7	F: CTCGGCAAGTATGGCATCAA R: GGTCAGAGATGGGGATCTGC	X93921	106
DUSP11	F: CAGCACATCTCATGCAACCA R: TGGAAATGTCGAGGAGCTGA	NM_003584	100
DUSP12	F: TGCAGGCGATCATTATTTCG R: CCATCACTCCCAACAAAGCA	NM_007240	184
DUSP14	F: CCACAGTGTGAGCAGGAAGC R: TACGTTGGGCCTGATGACAG	NM_007026	166
DUSP22	F: GGGGAATGGGATGAACAAGA R: GCCTGGCACTATCATGGACA	NM_020185	125
β-Actin	F: AGAAAATCTGGCACCACACC R: GGGGTGTTGAAGGTCTCAAA	NM_001101	123

Preface to Chapter 3

Breast cancer is the most common hormone-dependent cancer in women and has a high mortality rate. Tumors of the breast are highly heterogeneous and differences in their gene expression often reflect clinical tumor subtypes and prognosis [282-284]. The expression of ERα divides tumors into two major subtypes ERα+ and ERα- [282]. It has been shown that the switch from an ERα+ to an ERα− phenotype correlates with increased invasiveness and poor outcomes [298]. Furthermore, the presence of ERα also affects the angiogenic profile of these tumors [292-294]. We hypothesized that the activation of ERα by estrogen in human breast cancer cell lines could modulate the expression of angiopoietins, Ang-1, Ang-2 and Ang-4, and affect tumor progression in a murine model of breast cancer.

Chapter 3

Estradiol-dependent regulation of angiopoietin expression in breast cancer cells

Estradiol-dependent regulation of angiopoietin expression in breast cancer cells

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Key words: Estrogen receptor, estradiol, angiopoietin, breast cancer, angiogenesis

3.1 ABSTRACT

Angiopoietin-1 (Ang-1) is a ligand for Tie-2 receptors and a promoter of angiogenesis. Angiogenesis plays an important role in breast cancer, as it is one of the critical events required for tumors to grow and metastasize. In this study, we investigated the influence of estradiol (E2) on the expression of angiopoietins in breast cancer cell lines. Ang-1 mRNA and protein expressions were significantly higher in estrogen receptor-negative (ERα−) breast cancer cells than in estrogen receptor-positive (ERα+) cells. Exposure of ERα+ cells to E2 resulted in further reductions of Ang-1 levels. In mouse mammary pads inoculated with breast cancer cells, both tumor size and Ang-1 production were significantly lower in ERα+ cell-derived xenografts, as compared to those derived from ERα− cells. Reduction of circulating levels of E2 by ovariectomy eliminated this response. Overall, these results indicate that Ang-1 mRNA and protein expressions: (1) negatively correlate with the level of ERα in breast cancer cell lines; (2) are downregulated by E2 in an ERα dependent manner; and (3) positively correlate with the degree of angiogenesis in vivo. We conclude that Ang-1 is an important modulator of growth and progression of ERα− breast cancers.

3.2 INTRODUCTION

Breast cancer is the most common hormone-dependent cancer in women, resulting in very high rates of mortality [1]. The hormone estrogen stimulates the growth of breast cancer tumors and women who develop the disease tend to have higher levels of circulating estrogen than those that do not. The biological effects of estradiol (E2), the most potent form of estrogen, are primarily mediated by estrogen receptor α (ERα) [2]. E2 is initially required for the survival and proliferation of normal breast epithelia, but prolonged E2 exposure promotes breast carcinogenesis [2] and [3]. As ERα levels gradually increase during the early stages of breast cancer, activation promotes cell survival, but also inhibits angiogenesis [4] and [5]. One of the mechanisms by which ERα activation inhibits angiogenesis is through downregulation of the pro-angiogenic factor vascular endothelial growth factor (VEGF) [4], [5], [6] and [7], although invasive breast cancers also express many other pro-angiogenic factors, including the recently identified angiopoietins.

The angiopoietins, including Ang-1, Ang-2 and Ang-4 in humans, are soluble ligands of endothelial cell (EC)-specific tyrosine kinase receptors, Tie-2 [8], [9] and [10]. Ang-1 is the main agonist for Tie-2, hence it elicits strong angiogenic effects, including promotion of EC differentiation, tube formation, sprouting, migration and EC survival [11], [12] and [13]. Ang-2 can also activate Tie-2 and promote angiogenic potentials within cells, but at relatively higher concentrations than for Ang-1 [9] and [14]. Ang-4, the least well-characterized angiopoietin, exerts qualitatively similar yet quantitatively weaker effects than Ang-1 [10] and [15]. Accumulating evidence suggests that Ang-1, Ang-2 and Ang-4 are all expressed in human breast cancer samples [16] and that angiopoietin expression correlates with lymph node invasion and short survival rates in patients with breast cancer [17]. However, despite these newly emerging roles for angiopoietins in breast cancer

progression and angiogenesis, little is known about the specific role of E2 in modulating angiopoietin expression. Our group has previously reported that E2 stimulates Ang-2 mRNA expression while inhibiting Ang-1 expression in non-reproductive organs of normal rats [18]. In comparison, brain Ang-1 expression is significantly induced by E2 in overiectomized female mice [19].

In this study, we investigated the direct influence of E2 and ERα on the expression of Ang-1, Ang-2 and Ang-4 in various human breast cancer cell lines. We also examined the association between Ang-1 expression and tumor progression in a murine xenograft model of breast cancer. Our results indicate that Ang-1 mRNA and protein expressions negatively correlate with levels of ERα in breast cancer cell lines and are downregulated by E2 in an ERα dependent manner. Ang-1 mRNA and protein expressions also positively correlate with the degree of angiogenesis *in vivo*.

3.3 MATERIALS AND METHODS

Cell cultures: Human primary mammary epithelial cells (HMECs) were obtained from Cambrex Corporation (San Diego, CA) and maintained in MEGM medium supplemented with bovine pituitary extract, human epidermal growth factor, insulin and hydrocortisone. All other cell lines and media were purchased from the American Type Culture Collection (ATCC) (Rockville, MD) and Invitrogen (Burlington, ON). MDA-MB-468, BT549 and ZR-75-1 cells were grown in RPMI 1640 supplemented with 1 mM sodium pyruvate and 10% fetal bovine serum (FBS). MCF7, BT474 and MDA-MB-231 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. Mock transfected MDA-MB-231 cells and those transfected with a functional ERα (S30 cells) were kindly provided by Dr. C. Jordan (Northwestern University, Chicago, IL) and were maintained in Minimum Essential Medium Alpha (MEMα) and 5% FBS, under selection for neomycin resistance with geneticin (G418 sulfate) (Life Technologies, Grand Island, NY), as previously described [20]. MDA-MB-468 cells were a generous gift from Dr. B. Jean-Claude (McGill University, Montréal, QC). After reaching first confluence in 100 mm dishes, cells were subcultured following trypsin (0.25%) and ethylenediaminetetraacetic acid (EDTA) (0.01%) application at a ratio of 1:3 to 1:6, depending on cell type. For all estradiol experiments, cells were grown in stripped medium (phenol-red-free media (Invitrogen)) and charcoal-stripped FBS (sFBS (Biomeda, Foster City, CA)) to remove endogenous estrogens, and in the presence of 10 μM E2 or cyclodextrin solvent (E2 vehicle) and/or 10 μM anti-E2 tamoxifen (TAM). E2, cyclodextrin and TAM were purchased from Sigma–Aldrich Canada (Oakville, ON).

Cell proliferation assay: MDA-MB-231 and S30 cells were plated in triplicate at a density of 10,000 cells per well in six-well plates and grown in stripped medium. Where indicated,

cells were treated with E2 alone or in combination with TAM. Cells were trypsinized and counted at various time points using a Coulter counter Model ZF (Coulter Electronics, Harpenden, Hertfordshire, UK). Culture medium was replenished every second day.

Northern blotting: Total RNA (5 μg) was mixed with ethidium bromide and separated by electrophoresis on 1.2% agarose gels containing 2.2 M formaldehyde. RNA was transferred to nylon membranes and rRNA was visualized under ultraviolet light to verify equal loading. Membranes were probed with Ang-1 at 42 °C overnight in ULTRAhyb™ buffer (Ambion, Inc., Austin, TX), stripped and then re-probed with 18S. Probes were non-isotopically labeled DNA probes designed in our laboratory using RT-PCR and a PCR DIG Probe Synthesis Kit (Roche Diagnostics, Laval, QC). Ang-1 and 18S probes were designed using known sequences (accession number NM_001146, corresponding to sequences 537–935, and accession number M10098, corresponding to sequences 951–1458, respectively). Labeled DNA probes were added directly to the ULTRAhyb™ solution. Washes were performed twice at room temperature for 5 min (low stringency solution 2.0× SSC/0.1% SDS) and then twice at 42 °C for 15 min (high stringency solution 0.1× SSC/0.1% SDS). Signals were detected using a DIG Luminescent Detection Kit and CDP-*Star*, ready-to-use substrate (Roche Diagnostics). Membranes were exposed to BioMax™ Light film (Amersham Biosciences, Piscataway, NJ), at room temperature, using an intensifying screen for up to 2 h.

Real time PCR: Total RNA was collected from all cell types (90% confluent) under basal conditions. In addition, MDA-MB-231, S30, BT474 and MCF7 cells grown in stripped medium (see above) were also stimulated with E2. Prior to E2 experiments, sub-confluent cells were serum-starved for 12 h, then incubated with E2 or cyclodextrin (solvent) for 6 h. RNA was extracted with an RNeasy® kit (Qiagen, Mississauga, ON), reverse-transcribed

with Superscript™II (Invitrogen) and subjected to either reverse transcription PCR or real-time PCR. For RT PCR, one tenth of synthesized cDNA was used as a template in PCR reactions containing 2.5 units Taq DNA polymerase, 50mmol/l each dNTP, 1.5 mM MgCl2, buffer provided by the manufacturer and 0.4 μM primers. Amplification cycles consisted of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. RT PCR products were analyzed on a 2% agarose gel. Three different primer pairs were used to detect regions corresponding to full length Ang-1 (1.5 kb) and 1.3 and 0.9 kb isoforms. Full length Ang-1 was detected using forward primer 5′-GGAAGTCTAGATTTCCAAAGAGGC-3′ and reverse primer 5′-CTTTATCCCATTCAGTTTTCCATG-3′, corresponding to sequences 1306−1326 bp and 1711−1734 bp, respectively, designed to give a 429 bp product [21]. Amplification of the full-length coding region of Ang-1 was accomplished using forward primer 5′-GCTGGCAGTACAATGACAGGT-3′ (identical to 5′ end) and reverse primer 5′-TCAAAAATCTAAAGGTCGAAT-3′ (complementary to 3′ end), yielding a 312 bp product [21]. Evaluation of the 1.3 kb isoform was completed using forward primer 5′-GGAATATAAAATGGTTGTATTTAA-3′ (specific for Ang-1.3 kb) paired with reverse primer 5′-TCAAAAATCTAAAGGTCGAAT-3′. The 0.9 kb isoform was generated using forward primer 5′-GTGGCTGCAAAAAGTGTTTTGC-3′ (specific for Ang-0.9 kb) and reverse primer 5′-ATCGCTTCTGACATTGCGCTT-3′, to give an amplification fragment of 595 bp [21]. Forward primer 5′-TCAGCTTGCTCCTTTCTGGAACT-3′ and reverse primer 5′-TTTACGGGCCAGATTGTAAGC-3′ were used to amplify Tie-2. 18S primers were used as internal controls. Real-time PCR was performed using a 7500 real-time PCR system and TaqMan® reagents and primers designed to amplify 18S, Ang-1, -2 and -4

(Applied Biosystems, Carlsbad, CA). Absolute copy numbers were calculated using standard curves generated by plasmids containing full length coding sequences of human Ang-1, -2 and -4.Total RNA was collected from all cell types (90% confluent) under basal conditions. In addition, MDA-MB-231, S30, BT474 and MCF7 cells grown in stripped medium (see above) were also stimulated with E2. Prior to E2 experiments, sub-confluent cells were serum-starved for 12 h, then incubated with E2 or cyclodextrin (solvent) for 6 h. RNA was extracted with an RNeasy® kit (Qiagen, Mississauga, ON), reverse-transcribed with Superscript™II (Invitrogen) and subjected to either reverse transcription PCR or real-time PCR. For RT PCR, one tenth of synthesized cDNA was used as a template in PCR reactions containing 2.5 units Taq DNA polymerase, 50 mmol/l each dNTP, 1.5 mM $MgCl_2$, buffer provided by the manufacturer and 0.4 μM primers. Amplification cycles consisted of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. RT PCR products were analyzed on a 2% agarose gel. Three different primer pairs were used to detect regions corresponding to full length Ang-1 (1.5 kb) and 1.3 and 0.9 kb isoforms. Full length Ang-1 was detected using forward primer 5′-GGAAGTCTAGATTTCCAAAGAGGC-3′ and reverse primer 5′-CTTTATCCCATTCAGTTTTCCATG-3′, corresponding to sequences 1306–1326 bp and 1711–1734 bp, respectively, designed to give a 429 bp product [21]. Amplification of the full-length coding region of Ang-1 was accomplished using forward primer 5′-GCTGGCAGTACAATGACAGGT-3′ (identical to 5′ end) and reverse primer 5′-TCAAAAATCTAAAGGTCGAAT-3′ (complementary to 3′ end), yielding a 312 bp product [21]. Evaluation of the 1.3 kb isoform was completed using forward primer 5′-GGAATATAAAATGGTTGTATTTAA-3′ (specific for Ang-1.3 kb) paired with reverse primer 5′-TCAAAAATCTAAAGGTCGAAT-3′. The 0.9 kb isoform was generated using forward primer 5′-GTGGCTGCAAAAAGTGTTTTGC-3′ (specific for Ang-0.9 kb) and

reverse primer 5′-ATCGCTTCTGACATTGCGCTT-3′, to give an amplification fragment of 595 bp [21]. Forward primer 5′-TCAGCTTGCTCCTTTCTGGAACT-3′ and reverse primer 5′-TTTACGGGCCAGATTGTAAGC-3′ were used to amplify Tie-2. 18S primers were used as internal controls. Real-time PCR was performed using a 7500 real-time PCR system and TaqMan® reagents and primers designed to amplify 18S, Ang-1, -2 and -4 (Applied Biosystems, Carlsbad, CA). Absolute copy numbers were calculated using standard curves generated by plasmids containing full length coding sequences of human Ang-1, -2 and -4.

Immunoblotting: Adherent cells were washed twice with PBS and lysed using basic lysis buffer (pH 7.5) containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 100 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM EDTA, 1 mM phenylmethanesulfonylfluoride (PMSF), 2 μg/ml leupeptin, 5 μg/ml aprotinin and 0.5% Triton®-X 100. Cell debris and nuclei were separated by centrifugation at 14,000 × g at 4 °C for 5 min. Resulting supernatants (cell lysates) were used for immunoblotting. Conditioned media were concentrated 80 times using Centricon® filters with a molecular weight cut-off of 50 kDa. Cell lysates (25–80 μg protein) or concentrated media (15–100 μg protein) were heated at 100 °C for 5 min and loaded onto Tris–glycine SDS-polyacrylamide gels. Proteins were electrophoretically transferred onto polyvinylidene difluoride membranes, blocked for 1 h with 5% non-fat dry milk, and incubated overnight at 4 °C with primary monoclonal antibodies specific for Ang-1 (R&D Systems, Minneapolis, MN). Ang-1 protein was used as a positive control. Proteins were detected with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies and ECL reagents (Chemicon, Temecula, CA). Blots were scanned with an imaging densitometer and optical densities of protein bands were quantified with Image-Pro® Plus

software (MediaCybernetics, Carlsbad, CA). Predetermined molecular weight standards were used as markers. Protein concentration was measured by the Bradford method with bovine serum albumin as a standard.

Mice xenograft protocols: Normal and ovariectomized (OVX) 5-week-old BALB/c nu.nu female mice were obtained from Charles River, Inc. (St. Constant, QC). Before inoculation, MDA-MB-231 or S30 cells grown in serum-containing culture medium were washed with Hank's balanced buffer and centrifuged at 1500 × g for 5 min. Cell pellets (5 × 105 cells/mouse) were re-suspended in 100 µl of Matrigel® (BD Labware, Oakville, ON) and saline mixture (20% Matrigel®) and injected into the mammary fat pads of mice as previously described [22]. All animals were numbered and kept separately in a temperature-controlled room on a 12-h light/dark schedule with food and water ad libitum. All animal studies were approved by the University Animal Care Committee (UACC) of McGill University.

Immunocytochemistry and immunohistochemistry: MDA-MB-231 and S30 cells cultured in 24-well plates were stained for Ang-1. Paraffin-embedded tumor samples were cut into 5-µm thick sections and stained for Ang-1, VEGF and CD31 (index of vessel density), using the avidin–biotin-peroxidase complex. Polyclonal VEGF and CD31 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All steps were performed at room temperature. Cells and archived tissues were treated in the same manner except that tissue sections were initially de-waxed in xylene and rehydrated through a series of ethanol-to-water gradients prior to incubation for 30 min in 1% normal goat serum (Vector Laboratories, Burlingame, CA) then overnight with a 1:100 dilution of the corresponding primary antibody, followed by incubation with biotinylated secondary antibodies for 30 min. Samples were then treated with a Vectastain® ABC-AP kit (Abcam,

Cambridge, MA), developed with SIGMAFast™ Red TR (Abcam)/Naphthol AS-MX phosphate (Sigma–Aldrich) containing 1 mM levamisole, counterstained with hematoxylin (Fisher Scientific Ltd., Nepean, ON) and mounted with Kaiser's glycerol jelly. For negative controls, a non-specific immunoglobulin primary antibody was used. Images of stained cells and tissue sections were photographed with a Leica digital camera and processed using BioQuant image analysis software (Bio-Quant Image Analysis Corp., Nashville, TN).

Statistical analysis: All results were expressed as mean ± SEM of at least three samples. For immunocytochemistry and immunohistochemistry at least five slides per group were analyzed. Statistical comparisons were obtained using one-way analysis of variance. Probability (P) values less than 0.05 were considered significant.

3.4 RESULTS

Effect of E2 on breast cancer cell growth

Effects of E2 on cell number of ERα− MDA-MB-231 and ERα+ S30 cells were assessed. Under basal conditions, significant increases in the number of MDA-MB-231 cells were seen after 5 days, as compared with S30 cells, indicating that overexpression of ERα inhibits breast cancer cell proliferation (Figure 3.1). Specificity of this response was examined by culturing S30 cells in sFBS-containing stripped medium devoid of endogenous steroids. Under these conditions, a marked increase in S30 cell number was seen, where cell numbers are similar to those of MDA-MB-231 cells. Addition of E2 to S30 cells cultured in stripped medium reduced numbers to levels similar to those seen in unstimulated S30 cells. This inhibitory effect of E2 on S30 cell proliferation was reversed when cells were co-incubated with anti-E2 TAM (Fig. 3.1).

Expression of angiopoietins in breast cancer cells

To evaluate whether the presence of ERα correlates with angiopoietin expression, RT-PCR was performed to amplify Ang-1 mRNA in MDA-MB-231 and S30 cells, as these cells differ only by the presence of ERα in S30 cells (Figure 3.2A). Three sets of Ang-1 primers, corresponding to full length (1.5 kb), 1.3 kb and 0.9 kb isoforms, were used and the sequence-specificities of these primers were confirmed by direct sequencing of the amplified products. Expression levels of full length Ang-1 and the 0.9 kb isoform were markedly higher in MDA-MB-231 cells than they were in S30 cells. Primers designed to amplify the 1.3 kb isoform did not reveal any positive bands. However, full-length Ang-1 primers yielded a faint secondary band in MDA-MB-231, corresponding to 262 bp, which appears to be the 1.3 kb isoform [21]. Tie-2 mRNA was not detected in either cell line (Figure 3.2A). Northern blotting confirmed that full-length Ang-1 mRNA was significantly

more abundant in MDA-MB231 cells, as compared to amounts measured in ERα+ cells such as S30, MCF7 and HMEC (Figure 3.2B). Since significant Ang-2 and Ang-4 expression was not detected by northern blotting, the more sensitive real-time PCR assay was also used, which allowed for the detection of exact copy numbers of Ang-1, -2 and -4 mRNA transcripts in various breast cancer cell lines. Figure 3.2C shows absolute copy numbers of angiopoietin mRNA in ERα− cell lines (MDA-MB-231, MDA-MB-468 and BT549) and in ERα+ cell lines (S30, MCF7, ZR-75-1, BT474 and the parental HMEC). Although no relationships between ERα expression and Ang-2 or Ang-4 expressions were detected, the presence of ERα correlated inversely with the abundance of Ang-1 mRNA in various cell lines, with Ang-1 mRNA being more abundantly expressed in MDA-MB-231 and MB-MB-468 cells, as compared with MCF7, HMEC, and ZR75-1 cells (Figure 3.2C). In addition, absolute Ang-1 mRNA expression was relatively higher than that of Ang-2 and Ang-4 in each cell type, except for ZR75-1, where Ang-2 mRNA copy numbers were higher than for Ang-1. In all cell types except for MCF-7 cells, Ang-4 mRNA expression was negligible. In MCF-7 cells, more than 1 copy/ng of Ang-4 was detected (Figure 3.2C). Immunoblotting of the conditioned media of unstimulated breast cancer cells revealed that Ang-1 protein is robustly expressed in MDA-MB-231 cells, whereas it was almost undetectable in the ERα+ cell lines (HMEC, S30 and MCF7) (Figure 3.3A). Likewise, immunocytochemistry analysis showed 2-fold more Ang-1 expression in MDA-MB-231 cells, as compared to S30 cells (Figure 3.3B).

Regulation of angiopoietin expression by E2

Real-time PCR measurements revealed that treatment of S30 cells with E2 results in significant attenuation of Ang-1, -2 and -4 mRNA levels, indicating that expression of all angiopoietin members is E2-sensitive in ERα+ breast cancer cells (Figure 3.4A). We should

emphasize that treatment of MDA-MB231 cells with E2 demonstrated no significant effect on expressions of Ang-1, Ang-2 and Ang-4 mRNA (results not shown). Figure 3.4B shows that E2 treatment significantly attenuated Ang-1 protein expression in ERα+ cells (S30), but had no effect on Ang-1 protein levels in ERα− cells (MDA-MB-231). In addition to studying S30 cells, inhibition of angiopoietin expression by E2 in other ERα+ breast cancer cells was verified by treating BT474 and MCF7 breast cancer cells with E2. Figure 3.4C and D illustrates that E2 treatment significantly attenuated mRNA expression of the three angiopoietins in BT474 and that of Ang-1 and Ang-2 in MCF7 cells. Both of these cell lines are ERα+. These results confirm that E2 treatment exerts a negative influence on angiopoietin expression in ERα+ breast cancer cells.

Effects of E2 on in vivo tumor growth

The effects of E2 on tumor growth were examined using a xenograft model of breast cancer where both MDA-MB-231 and S30 cells were injected into the mammary fat pads of normal female BALB/c nu.nu mice. Animals that received MDA-MB-231 cells developed palpable tumors by week 5, which continued to grow for the following 4 weeks. However, experimental animals that were inoculated with S30 cells at various time points grew tumors of significantly lower volumes (approximately 2.5-fold), as compared with MDA-MB-231 xenografts, as previously reported [22]. The role of E2 in altering tumor growth in the S30 xenografts was further investigated by comparing tumor volumes between normal (non-OVX) and ovariectomized (OVX) animals. In OVX animals, tumor volume was significantly higher (approximately 2-fold), as compared to non-OVX animals that were inoculated with S30 cells. At the conclusion of experiments, animals were sacrificed and primary tumors from all groups were removed and embedded in paraffin. Archived samples from control and experimental animals were subjected to immunohistochemical analysis for

Ang-1, VEGF and CD31. Expression of Ang-1 protein was significantly higher in tumors derived from MDA-MB-231 recipients, as compared with S30-derived xenografts (Figure 3.5). Analysis of S30 tumors in OVX animal revealed that Ang-1 protein expression was partly restored to levels similar to those found in MDA-MB-231 xenografts, indicating that Ang-1 expression is inhibited by endogenous production of E2 (Figure 3.5). To assess whether the degree of tumor volume correlates with angiogenesis, tissues were also probed for two angiogenic markers, namely VEGF and CD31 (Figure 3.5). Similar expression patterns to those of Ang-1 were obtained for these markers, as shown by lower expressions in S30 xenografts, as compared with MDA-MB-231 tumors. More importantly, these effects were reversed following E2 ablation by ovariectomy (Figure 3.5).

3.5 DISCUSSION

In this study, we are the first to report that: (1) Ang-1 expression is inversely correlated with the presence of ERα in breast cancer cell lines; (2) mRNA expressions of Ang-1, Ang-2 and Ang-4 are inhibited by E2 treatment in ERα+ breast cancer cells; and (3) *in vivo* expression of Ang-1 is significantly elevated along with markers of angiogenesis in ERα− xenografts, as compared with ERα+ xenografts, and that reduction of circulating E2 levels by ovariectomy in animals with ERα+ xenografts results in elevated Ang-1 expressions.

It is has been well established that the switch from an ERα+ to an ERα− phenotype correlates with increased breast cancer invasiveness and lethality [2]. With this study, we demonstrate that cell growth and tumor volume are inhibited in breast cancer cells transfected with ERα (S30), as compared to ERα− counterparts (MDA-MB-231), both in culture and in murine mammary xenografts. In addition, these inhibitory effects of ERα are partly reversed upon E2 depletion, which is in accordance with several previous reports [4] and [22]. The anti-invasive role of E2 occurs mainly when levels of ERα reach relatively high levels, which leads to simultaneous upregulation of anti-angiogenic factors, such as α1-anti-chymotrypsin, and downregulation of pro-angiogenic factors, including VEGF [4], [5], [22] and [23].

There has been increasing evidence that E2 regulates angiopoietin expression, however, the differential influence of E2 on Ang-1 vs. Ang-2 expressions varies considerably between studies. In non-reproductive rat tissues, our group [18] has reported that E2 administration reduces Ang-1 expression while inducing Ang-2 expression. Subsequent studies have revealed that in primate endometrium, Ang-1 expression varies considerably during the menstrual cycle [24] and that acute administration of E2 induces endometrial Ang-1 expression [25]. In contrast, in pregnant baboons subjected to prolonged

exposure of the estrogen precursor androstenedione, placental Ang-1 expression is significantly attenuated but has no influence on Ang-2 levels [26]. In humans, Lee et al. [27] have described significant reductions in Ang-1 levels, as well as upregulation of Ang-2 expression, in endometrial biopsies that have been taken following chorionic gonadatrophin injection.

Little information is as yet available regarding the influence of E2 on angiopoietin expression in cancerous tissues. Currie et al. [16] have detected Ang-1, Ang-2 and Ang-4 expressions in malignant breast tissues and have described a significant correlation between Ang-4 expression and ER, and a negative correlation between Ang-1 expression and thymidine phosphorylase (promoter of angiogenesis) expression. These authors have also reported that E2 treatment of MCF7 cells has no influence on Ang-4 expression.

Our study is the first to investigate the expression of all three human angiopoietins in breast cancer, under both basal conditions and in response to E2. Our results reveal that under basal conditions, only Ang-1 expression is inversely correlated with ERα expression in breast cancer cell lines and tissues. We also found that following acute E2 exposure, Ang-1, -2 and -4 mRNA expressions are all downregulated in an ERα dependent manner, except for that of Ang-4 expression in MCF7 cells, which, as Currie et al. have previously reported, is unresponsive to E2 treatment.

We did not investigate the exact mechanisms through which E2 regulates angiopoietin expression in ERα+ breast cancer cells; however, we speculate that E2 may inhibit angiopoietin expression through decreased transcription of angiopoietin genes. Upon hormone binding, ERs exert their effects by interacting with DNA elements in target gene promoters, either directly or through interactions with other transcription factors [28]. Although molecular analyses of human Ang-1 and Ang-2 promoters have not uncovered

the presence of any of the abundant palindromic estrogen response elements that bind to ER with relatively high affinity and specificity [29] and [30], there is evidence that ER interacts directly with various transcription factors, particularly AP-1, which is composed of heterodimers of the Jun/Fos family of proteins [31]. Both Ang-1 and Ang-2 promoters possess abundant AP-1 binding sites. No information is as yet available regarding the molecular structure of Ang-4 promoter.

In ERα− cells lines, the most abundantly expressed angiopoietin is Ang-1, followed by Ang-2, while Ang-4 mRNA expression is very low. This suggests that Ang-1 is the primary angiopoietin that is expressed in ERα− cells. Unlike the situation for Ang-1, mRNA expressions of Ang-2 and Ang-4 do not correlate with ERα status. This is particularly true with respect to Ang-4, as its expression is extremely low in all cell types, save MCF7 cells. In addition, we observed that the basal Ang-4/Ang-1 mRNA ratio in MCF7 is much higher than in other breast cancer cells, suggesting that Ang-4 may function as the main Tie-2 receptor agonist in MCF7 cells. A similar suggestion has been made by Currie et al. [16].

Although we did not directly investigate the roles of Ang-1 in breast cancer, we report, for the first time, that expressions of Ang-1 and two angiogenic markers, VEGF and CD31, are significantly lower in S30 tumor xenografts as compared to those generated by MDA-MB-231 xenografts. Although several studies have recently been published which address the functional roles of Ang-1 in breast cancer, the main conclusions of these studies are contradictory. Hayes et al. [32], for instance, report that Ang-1 overexpression inhibits breast tumor growth by stabilizing the vasculature. Others, however, have used relatively lower concentrations of exogenous Ang-1 or have targeted inhibition of Ang-1 expression by siRNA to demonstrate that Ang-1 actually promotes tumor angiogenesis and that this

effect is potentiated by the presence of VEGF [33], [34] and [35]. These contradictory findings can be attributed, in part, to the possibility that overexpression of Ang-1 may mask the effects of other angiogenic factors, such as VEGF, thereby shifting the angiogenic balance toward greater vessel stabilization. This usually occurs in the late stages of breast cancers. In contrast, in earlier stages, enhanced VEGF production causes strong EC proliferation and migration. As such, the positive correlation that we observed between Ang-1 and VEGF expression in the larger tumors, along with the increased vessel density evidenced by CD31 staining, suggests that Ang-1 may cooperate in a positive manner with VEGF to enhance breast cancer angiogenesis. One major limitation of this study is worth noting, namely, that we have only assessed Ang-1 protein levels, but not those of Ang-2 and Ang-4, in cells and tissues. This is because protein levels of Ang-2 and Ang-4 were significantly lower than those of Ang-1. In addition, commercially available anti-Ang-2 and Ang-4 antibodies showed significant cross reactivity with Ang-1 protein when tested in several pilot experiments. This lack of Ang-2 and Ang-4 protein measurements limits our discussion solely to the data on mRNA expressions of these two angiopoietins. However, the relatively low levels of Ang-2 and Ang-4 proteins reinforce our conclusion that Ang-1 is the main angiopoietin under hormonal control in breast cancer.

In summary, we have demonstrated that Ang-1 expression inversely relates to ERα status in breast cancer cells, and positively correlates with VEGF and CD31 in breast cancer xenografts. These results suggest that the expression of Ang-1 by breast cancer cells is hormonally regulated. Finally, our results also suggest that targeted inhibition of Ang-1 expression, combined with VEGF blockade, might be more effective in inhibiting breast cancer angiogenesis and growth than would inhibition of the VEGF pathway alone. This proposal is supported by recent studies that reveal that inhibition of VEGF signaling alone

fails to enhance overall survival of breast cancer patients and that this is partly due to the upregulation of other angiogenic factors, including Ang-1 [36]. Given then, that Ang-1 appears to be an important modulator of growth and progression of ERα− breast cancers, simultaneous blockade of VEGF and Ang-1 signaling represents a new and promising therapeutic approach to the treatment of late stage breast cancer patients.

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3.8 FIGURES

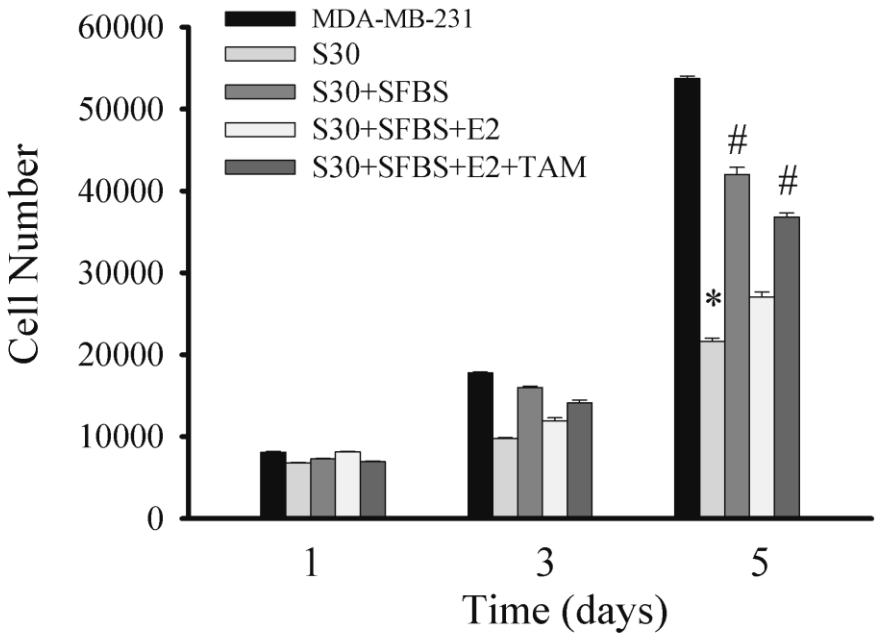
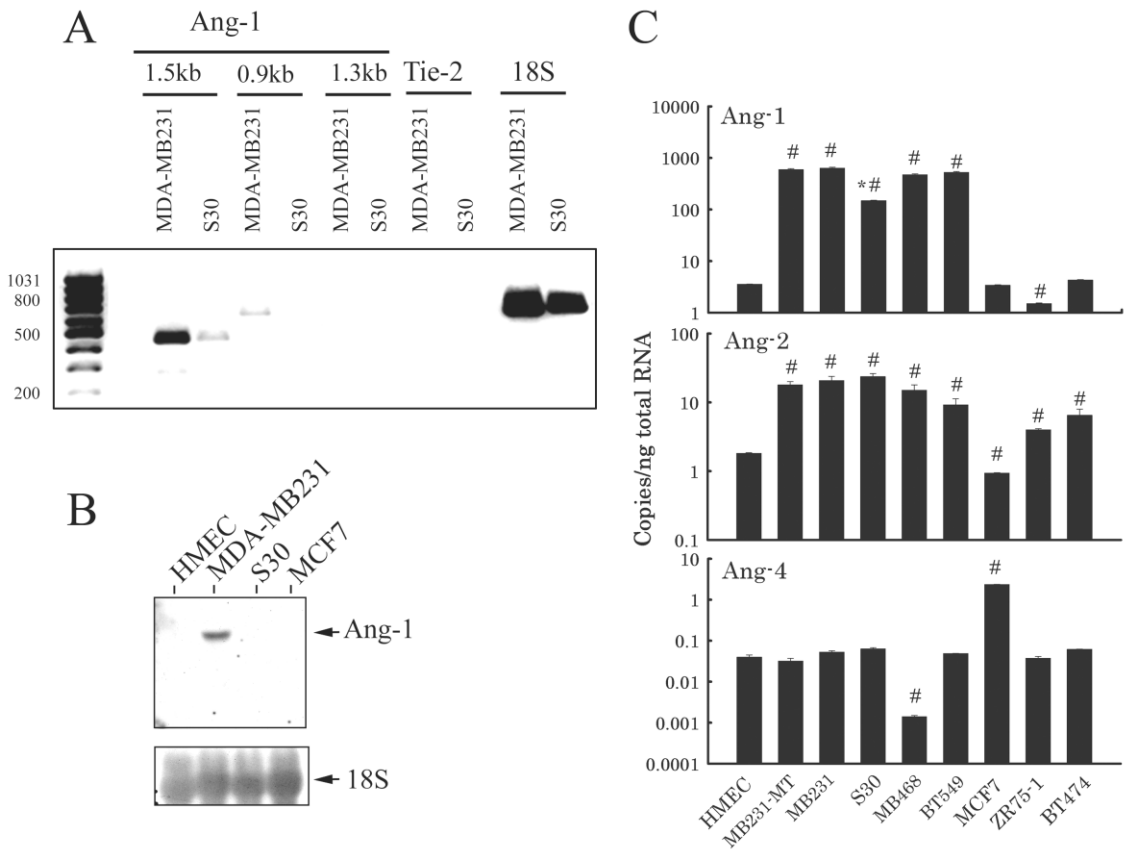


Figure 3.1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1031
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HMEC
MDA-MB231
S30
MCF7
Ang-1
18S
C
Ang-1
Ang-2
Ang-4
Copies/ng total RNA
10000
1000
100
10
1
0.1
0.01
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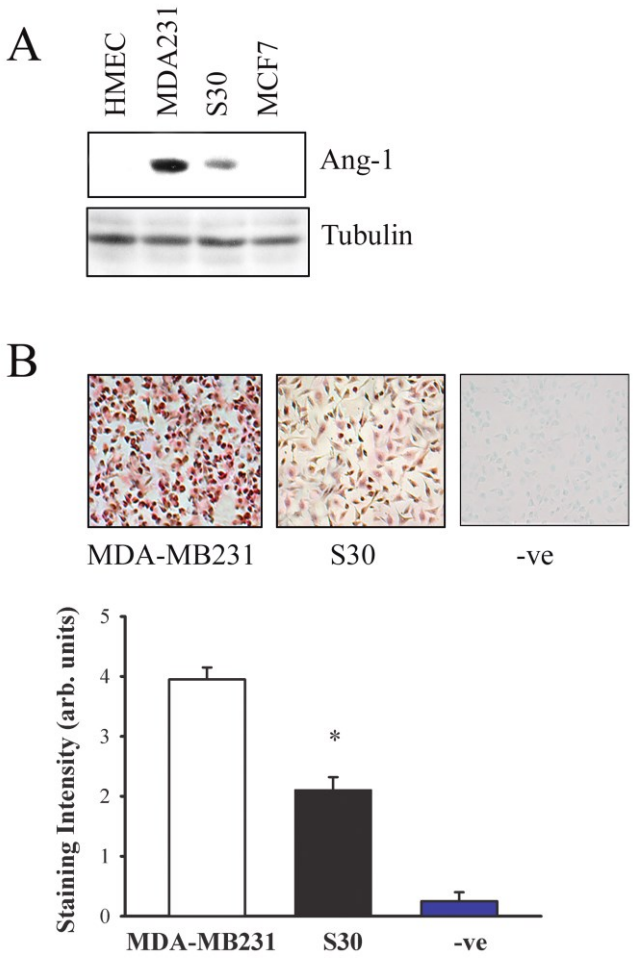
BT474

Figure 3.2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HMEC
MDA231
S30
MCF7
Ang-1
Tubulin
B
MDA-MB231
S30
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Staining Intensity (arb. units)
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3
2
1
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MDA-MB231
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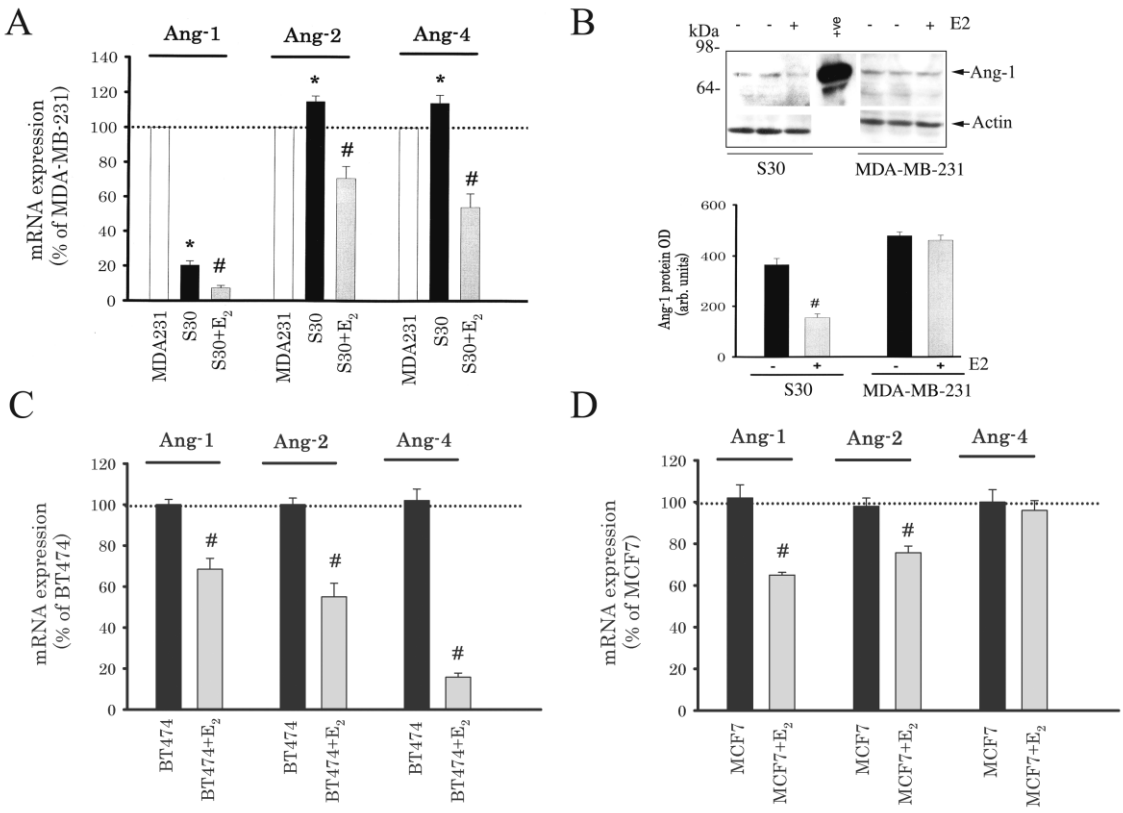
Figure 3.3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Ang-1
Ang-2
Ang-4
mRNA expression
(% of MDA-MB-231)
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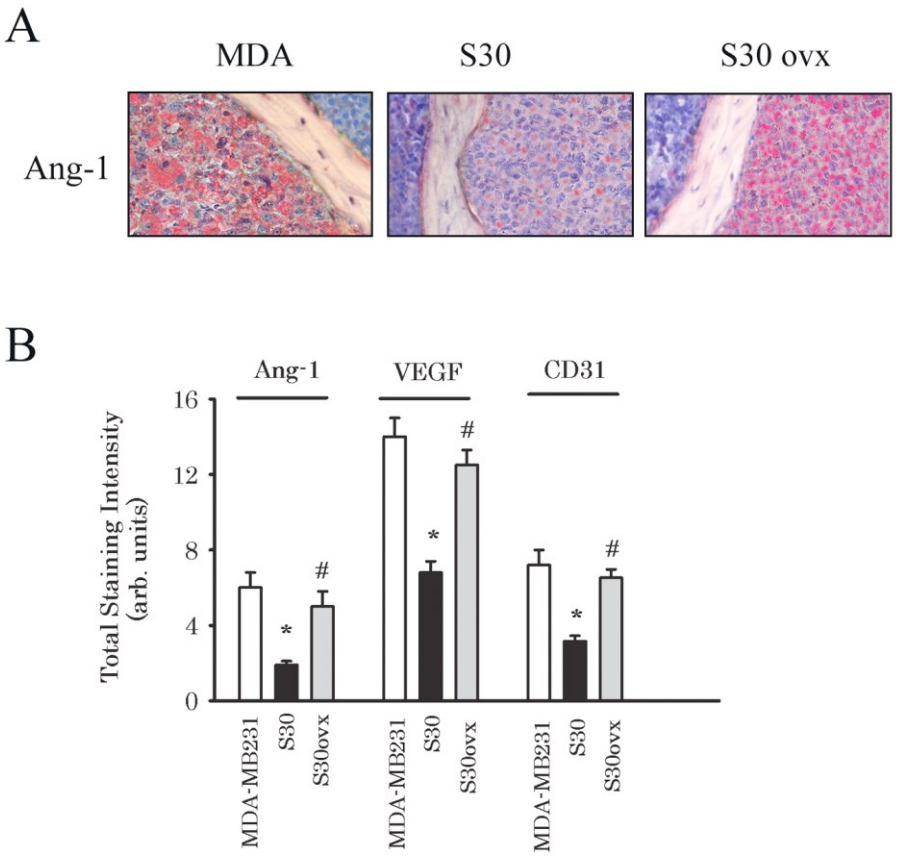
S30
S30+E2
B
kDa
98-
64-
-ve
+ve
E2
Ang-1
Actin
S30
MDA-MB-231
Ang-1 protein OD
(arb. units)
C
mRNA expression
(% of BT474)
BT474
BT474+E2
D
mRNA expression
(% of MCF7)
MCF7
MCF7+E2

Figure 3.4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MDA
S30
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Ang-1
VEGF
CD31
Total Staining Intensity (arb. units)
16
12
8
4
0
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MDA-MB231
S30
S30ovx

Figure 3.5

3.9 FIGURE LEGENDS

Figure 3.1 Effect of E2 on breast cancer cell growth

Results are representative of two independent experiments ± SEM. *P < 0.05 compared with MDA-MB-231 cells. #P < 0.05 vs. S30 cells. sFBS = stripped medium devoid of endogenous steroids.

Figure 3.2 Expressions of angiopoietin mRNA in breast cancer cells

A) Expressions of Ang-1 and Tie-2 transcripts in MDA-MB-231 and S30 cells determined by RT-PCR. Ang-1 mRNA amplified using three sets of primers. **B)** Northern blot detection of 1.5 kb Ang-1 mRNA transcripts in various breast cancer cells lines and primary human mammary epithelial cells (HMECs). **C)** Expressions of angiopoietin mRNA levels in unstimulated breast cancer cell lines and HMECs. Results represent three experiments ± SEM. MB231-MT refers to mock transfected MDA-MB-231 cells. $*P < 0.05$ represents a significant difference between S30 and MDA-MB-231 cells and $^{\#}P < 0.05$ represents a significant difference between all cell types relative to parental HMEC cells.

Figure 3.3 Expression of Ang-1 protein in breast cancer cells

A) Immunoblot detection of Ang-1 protein in concentrated culture media of HMEC and breast cancer cell lines. **B)** Immunocytochemical analysis of the expression of Ang-1 protein in MDA-MB-231 and S30 cells. −ve refers to negative control. Bars represent mean of three experiments ± SEM. $*P < 0.05$ vs. MDA-MB-231 cells.

Figure 3.4 Regulation of angiopoietin expressions by E2 in breast cancer cells

A: Change in angiopoietin mRNA levels in response to E2 treatment in S30 cells. *$P < 0.05$ compared with MDA-MB-231 cells. $^{\#}P < 0.05$ compared with S30 cells without E2. **B:** Immunoblots and mean values of Ang-1 protein optical densities in S30 and MDA-MB-231 cells with or without E2 treatment. Results are representative of three experiments ± SEM. $^{\#}P < 0.05$ compared with control (no E2 treatment) values. +ve refers to positive control. (**C and D**) Change in angiopoietin mRNA levels in response to E2 treatment in BT474 and MCF7 cells. #$P < 0.05$ vs. cells without E2.

Figure 3.5 Effects of E2 on in vivo tumor growth

A: Representative Ang-1 immunostaining (red color) and **B:** mean ± SEM of Ang-1, VEGF and CD31 staining intensity in tumor samples from animals inoculated with MDA-MB-231, S-30 and S-30 cells into OVX animals. *P < 0.05 vs. MDA-MB-231-derived tumors. #P < 0.05 vs. S30-derived tumors.

Preface to Chapter 4

There is increasing evidence of the anti-inflammatory effects of Ang-1 on the vasculature and the molecular mechanisms behind them [115, 171, 172, 175, 177, 178]. Most of the studies have focused mainly on the antagonistic actions of Ang-1 and VEGF on leukocyte adhesion and vascular permeability, which involve the expression of transcription factors such as Nur77 and KLF2 that directly impact gene expression, and the dynamic regulation of adherent junctions [115, 171, 172, 178]. It has been demonstrated that Ang-1 has a protective role in endotoxin shock induced by *E. Coli* LPS, albeit the intracellular effectors mediating this response remain to be identified [408-411]. Although it has been speculated that an important portion of the genome is under the control of miRNAs, their role as downstream effectors of the Ang-1 signaling pathway in ECs is unknown [377]. Because TLR signaling proteins are sensitive to miRNA regulation, we hypothesize that Ang-1 induces the expression of miRNAs, such as the miR-146 family, to act as its downstream effectors and thus protects the endothelium against LPS-induced inflammation.

Chapter 4

Angiopoietin-1 inhibits Toll-like receptor 4 signaling in endothelial cells: role of microRNA miR-146b-5p

Angiopoietin-1 inhibits Toll-Like Receptor 4 signaling in endothelial cells: role of miR-146b-5p

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Key words: angiopoietin-1, miRNA, endotoxin shock, inflammation

4.1 ABSTRACT

Angiopoietin-1 (Ang-1) is an angiogenic factor that inhibits vascular leakage, leukocyte adhesion and lipopolysaccharide (LPS)-induced inflammation in murine models of sepsis. *E. coli* LPS elicits inflammatory responses by activating Toll-like receptor 4 (TLR4) and downstream effectors including mitogen-activated protein kinases (MAPKs) and nuclear factor κB (NF-κB) transcription factor. Micro RNAs are small, non-coding RNA molecules that act as post-transcriptional repressors. Recently, various miRNAs such as the miR-146 family have been implicated in the control of inflammation. It is unknown if Ang-1 regulates the expression of these miRNAs and whether they play a role in the anti-inflammatory effects of Ang-1 in ECs. Here, we examined the effect of prolonged Ang-1 exposure on LPS-induced biological responses and molecular signaling in ECs. We found that in human umbilical vein endothelial cells (HUVECs) pre-treatment with Ang-1 (300ng/ml) for 24 h reduces LPS-induced leukocyte adhesion, expression of adhesion molecules (VCAM1, ICAM1 and E-SELECTIN) and pro-inflammatory cytokines (TNFα, IL1β, IL-8 and IL-6). Furthermore, Ang-1 pretreatment reduces LPS-induced p38 and SAPK/JNK phosphorylation and strongly attenuates LPS-induced NFκB reporter activity. Ang-1 pretreatment significantly attenuated the expressions of IRAK1 and TRAF6 proteins but had no influence on the expression of TLR4, IRAK4, MYD88 or TAK1. Ang-1 pretreatment also triggers a significant induction of miR-146b-5p expression after 12 and 24h exposure but has no influence on the expression of miR-146a and miR-146b-3p. Transfection of HUVECs with a mimic of miR-146b-5p strongly attenuates LPS-induced leukocyte adhesion, adhesion molecule expression, cytokine production and NFκB reporter activity. Furthermore, expression of miR-146b-5p mimics significantly attenuates IRAK1

and TRAF6 protein expression in a fashion similar to that elicited by Ang-1 pre-treatment. Transfection with a selective inhibitor of miR-146b-5p eliminates the inhibitory effect of Ang-1 pre-treatment on LPS-induced leukocyte adhesion, inflammatory cytokine production, and IRAK1 and TRAF6 expression. Ang-1 pre-treatment inhibits luciferase reporter activity containing wild type 3'UTRs of IRAK1 and TRAF6 but not those containing mutated miR-146b-5p targeting sites. Overall, our results suggest that miR-146b-5p is induced by Ang-1 as a mechanism to control TLR4 signaling and that the inhibitory effect of Ang-1 on TLR4 signaling in HUVECs is mediated through selective binding of miR-146b-5p to specific targeting sites located on the 3'UTRs of IRAK1 and TRAF6.

4.2 INTRODUCTION

Angiopoietin-1 (Ang-1) and its endothelial cell-specific receptor, Tie-2, are important modulators of angiogenesis and inflammation. In embryos, both Ang-1 and Tie-2 receptors are essential for vascular development [1, 2]. In mature vasculature, Ang-1 promotes vascular integrity and survival of endothelial cells (ECs) [3-6]. Ang-1 also stimulates EC migration and angiogenesis at sites of active vascular remodeling [7-9 10-12].

During acute inflammation EC becomes activated as manifested by an increase in permeability, secretion of pro-inflammatory cytokines and enhanced leukocyte adhesion to ECs [13, 14]. EC activation during acute inflammation proceeds in two phases, a rapid phase (type I activation) which is largely independent of the transcription processes and a slow phase (type II activation) which requires new gene expression [13, 14]. Recent studies have confirmed that Ang-1 regulates both phases of EC activation. The nature of this regulation varies depending in part on the kinetics of Ang-1 exposure. For instance, within minutes of Ang-1 exposure, an increase in the translocation of the adhesion molecule P-Selectin to the cell membrane is observed and is associated with a transient increase in leukocyte adhesion to ECs which lasts for 15 min [15]. This rapid and transient pro-inflammatory effect has been attributed to Ca^{2+} release and activation of phospholipase C gamma (PLCγ); and is similar to that observed in ECs stimulated with vascular endothelial growth factor (VEGF) [15, 16]. Ang-1 can also induce a rapid anti-inflammatory effect manifested as a strong attenuation of vascular leakage which has been attributed to sequestration of Src kinases by mammalian diaphanous (mDIA) and to inhibition of nitric oxide (NO) production [17-19].

Many reports have confirmed that when ECs are exposed to Ang-1 for several hours, anti-inflammatory effects become evident particularly when Ang-1 is combined with VEGF

or tumor necrosis factor (TNFα). Indeed, Ang-1 attenuates VEGF- and TNFα-induced leukocyte adhesion to ECs, trans-endothelial leukocyte migration, the expression of the adhesion molecules E-Selectin, intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) [20-23]. The mechanisms through which Ang-1 triggers these anti-inflammatory responses remain speculative. However, recent studies implicated the nuclear receptor 77 (Nur77), the transcription factor Kruppel-like factor 2 (KLF2); and direct inhibition of NFκB transcription factor as a result of recruitment of A20 binding inhibitor of NF-κB activation 2 (ABIN-2) to Tie-2 receptors [3, 24]. It should be emphasized that until recently the anti-inflammatory effects of Ang-1 were only monitored after few hours of exposure.

More recently, prolonged exposure to Ang-1 has been shown to be beneficial in the treatment of systemic and local inflammatory processes triggered by bacterial lipopolysascharide (LPS) (a cell wall component of gram-negative bacteria). Indeed, administration of Ang-1 *in vivo,* using adenoviruses or mesenchymal stem cells carrying Ang-1 triggers significant reduction of leukocyte infiltration into the lungs, attenuation of vascular leakage and significant improvement of survival in murine models of LPS-induced systemic sepsis and acute lung injury [25-27]. The molecular mechanisms behind these strong anti-inflammatory effects of Ang-1 on LPS-induced inflammatory responses remain unclear. It is possible that Ang-1 regulates these responses indirectly through secondary mediators. It is also possible that Ang-1 may exert a direct inhibitory effects on the function of the main transmembrane receptor receptors involved in transducing the biological responses to LPS, namely, toll-like receptor 4 (TLR-4) [28]. Activation of TLR4 receptors initiates pro-inflammatory intracellular signaling cascades that involve MyD88, interleukin

1 receptor associated kinase (IRAK1), IRAK4, TNF receptor associated factor 6 (TRAF6), mitogen activated protein kinases (MAPKs) and activation of several transcription factors including NFκB, activating protein 1 (AP1) and interferon regulatory factor 3 (IRF3) [28-30]. No information is available regarding the influence of Ang-1 on TLR-4 signaling in ECs. The first objective of the current study is to assess the effect of prolonged Ang-1 exposure on LPS-induced molecular signaling and biological responses in ECs. We propose that prolonged Ang-1 exposure triggers significant attenuation of LPS-mediated responses through selective inhibition of TLR4 signaling.

One possible mechanism through which Ang-1 could impair TLR4 signaling is by regulating the expression of micro RNAs (miRNAs). miRNAs are small, non-coding RNAs which act as post-transcriptional repressors of gene expression by binding to the 3′ un-translated (3′UTR) region of their mRNA targets to inhibit their translation or promote degradation of mRNA [31]. Recent studies have documented that TLRs and their downstream signaling proteins can be targeted by miRNAs including the miR-146 family as a way to control excessive inflammation [32-34]. The miR-146 family is comprised of two genes, miR-146a and miR-146b, whose mature forms share the same "seed" sequence suggesting they might target the same transcripts [35-37]. Expression of miR-146a but not miR-146b is regulated by pro-inflammatory stimuli including LPS [35] and several reports have confirmed that miR-146a acts as a negative feedback mechanism of NF-κB activation in response to LPS [35]. Moreover, recent studies indicate that IRAK1 and TRAF6 are direct targets of both miR-146a and miR-146b [35]. No information is available regarding whether Ang-1 regulate the expression of these miRNAs and whether these miRNAs play any role in the anti-inflammatory effects of Ang-1. The second objective of the current study is to assess the

regulation and the functional roles of miRNA-146a and miR-146b in the biological responses elicited by prolonged Ang-1 exposure in ECs. We propose that miR-146a and miR-146b play a significant role in the inhibitory effects of Ang-1 on LPS-mediated responses in ECs and that this role is mediated in part through selective inhibition of TRL4 signaling.

4.3 MATERIALS AND METHODS

Materials: HUVECs were harvested from umbilical cords donated by the birthing centre at Royal Victoria Hospital (Montreal, QC). Recombinant human Ang-1, Ang-2, FGF-2 and VEGF proteins, antibodies for E-Selectin and TLR4, and the DuoSet Development system ELISA for IL-8 were purchased from R&D Systems (Minneapolis, MN). Antibodies for phospho-Erk1/2 (Thr202/Tyr204), phospho-JNK1/2 (Thr183/Tyr185), phospho-p38 (Thr180/Tyr182), Erk1/2, JNK1/2, p38, phospho-p65, IRAK1, IRAK4, TRAF6, MyD88 and IκBα were obtained from Cell Signaling Technology (Danvers, MA). Antibodies for p65, ICAM1 and VCAM1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Adenoviruses containing a luciferase reporter driven by NF-κB (Ad5 HSV-NFk-b luc) and beta-tubulin antibodies were purchased from the Gene Transfer Vector Core (University of Iowa). pMIR-Report plasmids containing the wild-type and mutated 3'UTRs of IRAK1 and TRAF6 (IRAK1-UTR, IRAK1mut-UTR, TRAF6-UTR or TRAF6mut-UTR) were generated by Taganov *et al* [35] and purchased from Addgene (Cambridge, MA). The GenElute Mammalian Total RNA Miniprep Kit was purchased from Sigma-Aldrich (St. Louis, MO). Lipofectamine RNAiMAX reagent and the NCode miRNA First-Strand cDNA Synthesis and qRT-PCR Kits were obtained from Invitrogen (Burlington, ON). Synthetic mature miRNA mimics for mir-146b-5p and controls were purchased from Shanghai Genepharma (Shanghai, China). The miRNeasy Mini Kit and miScript miRNA Inhibitors for miR-146b-5p and controls were obtained from QIAGEN (Hilden, Germany). The Amaxa Nucleofector System was purchased from LONZA (Walkersville, MD). The Dual-Luciferase Reporter Assay System kit was obtained from Promega (Madison, WI). The

OptEIA cytokine kit for IL-6 ELISA was obtained from BD Biosciences (Franklin Lakes, NJ).

Cell Culture: Human umbilical vein endothelial cells (HUVECs) were isolated as previously described with a few modifications [38]. Briefly, umbilical cords of approximately 25 cm in length were placed in Hank's Balanced Salt Solution (HBSS) containing penicillin (100 U/ml), streptomycin (100 mg/ml), and amphotericin B (0.25 mg/ml). Both ends of the umbilical vein were cannulated, washed with HBSS and then filled with 7 ml of 0.1% collagenase type 1 (Bioshop, Burlington, Canada). The cord was then placed in a beaker containing HBSS and incubated for 9 min at 37°C. The umbilical vein was flushed three times with 10 ml of HBSS into a tube containing HUVEC growth media. The liquid was centrifuged at 1000 rpm for 10 min and the pellet was resuspended and plated on cell culture plates coated with 0.1% gelatin. HUVECs were grown in MCDB131 medium (Wisent, St. Bruno, Canada) supplemented with 20% fetal bovine serum (FBS), endothelial mitogen growth factor (Biomedical Technologies Inc. Stoughton, MA), 2mM glutamine, heparin, and gentamicin (Invitrogen, Burlington, ON); and incubated at 37 °C in 5% CO_2. After reaching confluence in 100-mm dishes, the cells were sub-cultured following trypsin (0.25%) and EDTA (0.01%) treatment and plated at a density of 2500 cells/cm^2. HUVECs were used for experiments between passages 3 and 7. The human leukemic monocyte lymphoma (U937) cell line was grown in suspension in RPM1 1640 (Invitrogen, Burlington ON) supplemented with 10% FBS and incubated at 37°C in 5% CO_2.

Experimental design for the analysis of prolonged Ang-1 exposure: HUVECs were serum-starved for 6 h in MCDB 131 medium and then maintained in medium with PBS or Ang-1 (300 ng/ml) for 24 h in 2 % FBS prior to LPS (10 μg/ml) stimulation. The

experiments using miRNA inhibitors were performed 36 h after transfection, when the cells were serum-starved in MCDB 131 medium for 6 h and then maintained in medium with PBS or Ang-1 (300 ng/ml) for 24 h prior to LPS (10 μg/ml) stimulation. In the cases when LPS stimulation was not required, HUVECs were serum-starved for 6h in MCDB 131 medium and then maintained in medium with PBS or Ang-1 (300 ng/ml) for 24h in 2% FBS before being processed for RNA extraction or immunoblotting.

U937 Monocyte Adhesion Assay: U937 cells (500,000) labeled with 3, 3'-dioctadecylindocarbocyanin-iodide (DiI) dye (Biotium Inc) were resuspended in 300 μl of MCDB131 medium and co-incubated for 1h at 37°C with HUVEC monolayers pre-treated with PBS or LPS (10 μg/ml) for 6 h. The media was then removed and the wells were washed three times with PBS and fixed in 4% paraformaldehyde. Ten fields per well were visualized with an Olympus 1X70 inverted microscope (60X) and adhered monocytes were quantified using the Image-Pro plus software.

Quantitative Real-Time PCR analysis: Total RNA was extracted using GenElute Mammalian Total RNA Miniprep Kit according to the manufacturer's instructions and the quantification and purity of total RNA was determined by A260/A280 absorption. SuperScript II RNase H-Reverse Transcriptase enzyme (Invitrogen, Burlington, ON) was used to reverse transcribe 2 μg of RNA for 50 min at 42°C and then for 5 min at 90°C. The Real Time PCR System 7500 from Applied Biosystems (Foster City, CA) was used to carry out the real-time PCR reactions. Specific primers for VCAM1, ICAM1, E-selectin, TLR4, MyD88, IRAK4, IRAK1, TRAF6 and TAK1 (Table 4.1) were used for SYBR-Green-based quantitative RT-PCR and the expression of beta-actin was monitored as a control gene throughout the analysis. SYBR Green PCR Master Mix (25 μl) from Qiagen (Hilden,

Germany) was added to 1 µl of the reverse transcriptase reaction and 3.5 µl of primers (10 µM). The thermal profile used with the Real Time PCR System 7500 was: 10 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 57°C, and 34 s at 72°C. To quantify the expression of IL1b, IL6, IL8, TNFA and 18S (endogenous control) transcripts, we used Applied Biosystems TaqMan gene expression assays (catalogue# Hs99999029_m1, Hs99999034, Hs99999034_m1 and Hs00174128_m1, respectively. The thermal profile was as follows: 50°C for 2 min, 95°C for 10 min and 40 cycles (95°C for 15 s and 60°C for 1 min). Primer-dimer formation and contamination were assessed with a melt analysis for each PCR experiment and a single melt peak for each set of primers was used to confirm that a single PCR reaction product was generated. The comparative threshold (C_T) cycle method (also referred to as the $2^{-\Delta\Delta CT}$ method) was used to analyze the results. All real-time PCR experiments were done in triplicate.

ELISA: the concentrations of interleukin 6 (IL6) and interleukin 8 (IL8) in cell culture supernatants were measured by ELISA using OptEIA cytokine kit (BD Biosciences, Franklin Lakes, NJ) and the DuoSet Development system (R&D Systems, Minneapolis, MN) respectively, following the manufacturers' instructions.

Immunoblotting: HUVECs were lysed in RIPA buffer (Santa Cruz Biotechnologies, Ca) and the protein concentration was measured with the Bio-Rad protein assay using bovine serum albumin (BSA) as a standard (Bio-Rad Laboratories, Mississauga, ON). Cell lysates were diluted in Laemmli sample buffer, boiled for 5 min and loaded onto Tris-glycine SDS-polyacrylamide gels. After SDS-PAGE the proteins were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes, blocked with 5% non-fat dry milk and then incubated with the specific primary antibodies overnight at 4ºC. Predetermined molecular weight standards were used as markers and the proteins were detected using

horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies and enhanced chemoluminescence (ECL) reagents (Chemicon, Temecula, CA). Loading of equal amounts of protein was confirmed by detection of beta-tubulin in the membranes. Quantification of the optical density (OD) for each protein band was carried out in scanned western blots using the Gel-Pro Analyzer software (Media Cybernetics Inc, Silver Spring, MD).

Analysis of NF-κB promoter activity: HUVECs were transduced with serum-free media containing 100 mutiplicity of infection (MOI) units of adenovirus containing a luciferase reporter driven by NF-κB (Ad5 HSV-NFk-b luc) [39] (Gene Transfer Vector Core, University of Iowa) for 6h and allowed to recover for 48h before the experiments. The luciferase activity (RLU) was quantified in a LMax II-384 luminometer (Molecular Devices) using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) and expressed as relative luminescence units (RLU).

Analysis of miRNA expression: Total RNA was isolated with TRIZOL (Invitrogen) followed by miRNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. For miRNA detection we used the NCode miRNA First-Strand cDNA Synthesis and qRT-PCR Kits (Invitrogen, Burlington ON). After extraction a poly(A) tail was added to the miRNA in the total RNA sample using Poly A Polymerase. Then, the polyadenylated miRNA was reversed-transcribed using SuperScript III RT and a Universal RT Primer (Invitrogen, Burlington, ON) for 50 min at 50°C and then for 5 min at 85°C to generate first-strand cDNA. The Real Time PCR System 7500 from Applied Biosystems (Foster City, CA) was used to carry out the SYBR-Green-based quantitative RT-PCR reactions. Specific primers for miR-146a and miR-146b-5p (Table 4.1) were used and the

miRNA expression data was normalized to U6 snRNA. SYBR Green PCR Master Mix (25µl) from Qiagen (Hilden, Germany) was added to 1µl of the reverse transcriptase reaction and 3.5 µl of primers (10 µM). The thermal profile used with the Real Time PCR System 7500 was: 2 min at 50°C, 2 min at 95°C and 40 cycles of 15 s at 95°C and 30 s at 60°C. Primer-dimer formation and contamination were assessed with a melt analysis for each PCR experiment and a single melt peak for each set of primers was used to confirm that a single PCR reaction product was generated. The comparative threshold (C_T) cycle method (also referred to as the $2^{-\Delta\Delta CT}$ method) was used to analyze the results. All real-time PCR experiments were done in triplicate.

Transfections with miRNA mimics and inhibitors: HUVECs were transfected with the indicated amount of synthetic mature miRNA mimic (Genepharma, Shanghai) or miScript miRNA Inhibitors (QIAGEN, Hilden, Germany) using Lipofectamine™ RNAiMAX (Invitrogen, Burlington, ON) according to the manufacturer's protocol. Briefly, HUVECs were plated at a density of 30,000 cells per cm^2 a day prior to the transfection and maintained in growth medium without antibiotics. For each transfection RNAi duplex-Lipofectamine™ RNAiMAX complexes were prepared by diluting the miRNA mimics or miRScript inhibitors to the desired concentration in Opti-MEM I Reduced Serum Medium without serum and adding it to a mix of Lipofectamine™ RNAiMAX and Opti-MEM I with gentle mixing. Then, the complexes are incubated for 10-20 min at room temperature before adding them to the cells maintained in growth medium without antibiotics. The experiments using miRNA mimics were performed 48 h after transfection.

3'UTR luciferase constructs: pMIR-Report plasmids containing the wild-type and mutated 3'UTRs of IRAK1 and TRAF6 were generated by Taganov *et al.* [35] (Addgene, Cambridge, MA). HUVECs ($1x10^6$) were transfected with 5 µg luciferase pMIR-Report

plasmids IRAK1-UTR, IRAK1mut-UTR, TRAF6-UTR or TRAF6mut-UTR, and 0.5 μg Renilla vector (pRL-TK). 24 h after transfection the cells were starved for 6h in MCDB131 medium and then maintained in medium with or without Ang-1 (300 ng/ml) in 2% FBS for 24 h. Firefly and Renilla luciferase were quantified in a LMax II-384 luminometer (Molecular Devices) using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). The results are expressed as a ratio of Firefly luciferase relative to Renilla luciferase.

Data analysis: Data are expressed as means $\pm$ standard errors (SE). Differences between experimental groups were determined by One-Way Analysis of Variance and were considered statistically significant at $p<0.05$.

4.4 RESULTS

Ang-1 attenuates LPS-induced leukocyte adhesion, adhesion molecule expression and cytokine production: We first examined the influence of prolonged (24h) exposure to PBS or Ang-1 (300 ng/ml) on LPS-induced leukocyte adhesion to ECs. Figure 4.1 shows that *E. coli* LPS (10 μg/ml) triggers within 4 to 6h significant increases in the number of adhered U937 cells, significant induction of VCAM1, ICAM1 and E-SELECTIN adhesion molecule expression, significant upregulation in mRNA levels of TNFα, IL1β, IL6 and IL8, and upregulation of IL6 and IL8 release into the media in HUVECs pre-treated 24h earlier with PBS. LPS fails to induce an increase in leukocyte adhesion in HUVECs pre-treated 24h earlier with Ang-1 (Figure 4.1). In addition, the extent of LPS-induced upregulation of VCAM1, ICAM1, E-SELECTIN and cytokine mRNA and protein levels in HUVECs pre-treated with Ang-1 is significantly lower than that observed in HUVECs pre-treated with PBS (Figure 4.1).

Ang-1 attenuates LPS-induced TLR4 signaling: Activation of TLR4 by LPS exposure triggers a cascade of signaling events involving activation of mitogen-activated protein kinases particularly SAPK/JNK and p38 and activation of the NF-κB transcription factor. In this study, we evaluated the time course and extent of MAPK and NFκB activation in response to *E. coli* LPS exposure in HUVECs pre-treated with PBS or Ang-1. LPS triggers a significant increase in the phosphorylation of p38, SAPK/JNK and ERK1/2 in HUVECs pre-treated 24h with PBS (Figure 4.2). LPS-induced phosphorylation of p38, SAPK/JNK and ERK1/2 is also observed in HUVECs pre-treated 24h earlier with Ang-1, however, the relative increase in p38 and SAPK/JNK phosphorylation in response to LPS in these cells is significantly lower than that observed in cells pre-treated with PBS (Figure 4.2). LPS-

induced ERK1/2 phosphorylation is similar in magnitude in HUVECs pre-treated with PBS and those pre-treated with Ang-1 (Figure 4.2).

Activation of NFκB transcription in response to LPS exposure was assessed by measuring protein levels of IκBα, phosphorylation of the p65 subunit of NF-κB and by monitoring the activity of a NF-κB luciferase reporter (Ad5 HSV-NFκB-luc). LPS triggers within 60 min a significant decline in IκBα protein levels in HUVECs pre-treated 24h earlier with PBS, a response which lasts for 120 min after LPS exposure (Figure 4.2). LPS in these cells also elicits a significant increase in p65 phosphorylation and robust augmentation of NF-κB luciferase reporter activity (Figure 4.2). LPS-induced decline in IκBα protein is significantly lower and has a shorter duration in HUVECs pre-treated 24h earlier with Ang-1 when compared with that observed in cells pre-treated with PBS (Figure 4.2). Similarly, LPS-induced p65 phosphorylation and NF-κB reporter activity are significantly attenuated in HUVECs pre-treated with Ang-1 when compared to those pre-treated with PBS (Figure 4.2).

To investigate whether the attenuation of LPS-induced responses in HUVECs pre-treated with Ang-1 is due to abnormalities in the expression of proteins involved in TLR4 signal transduction, we measured mRNA and protein levels of TLR4, MYD88, IRAK1, IRAK4, TRAF6 and TAK1. Pre-treatment with Ang-1 for 24 h has no influence on the mRNA levels of these genes, but it induces a significant reduction in IRAK1 and TRAF6 protein levels (Figure 4.3). These results suggest that exposure of HUVECs to Ang-1 selectively inhibits the expression of two proteins that are critical for TLR4 signaling cascade, namely, IRAK1 and TRAF6.

Regulation of miRNA expression by Ang-1: To assess the influence of Ang-1 on the expression of miR-146 family members, HUVECs were treated with PBS or Ang-1 for 12, 24 and 48 h. Expression of miR-146a, miR-146b-3p and miR-146b-5p was measured using real-time PCR. Ang-1 has no effect on the expression of miR-146a and miR-146b-3p, but significantly induces the expression of miR-146b-5p after 12 h and 24 h of exposure (Figure 4.4). To evaluate whether these changes in miR-146b-5p are selective to Ang-1, we assessed the influence of two important angiogenesis factors, namely, vascular endothelial growth factor (VEGF, 40ng/ml) and fibroblast growth factor 2 (FGF-2, 10ng/ml) in addition to the other member of the angiopoietin family, angiopoietin-2 (Ang-2). A significant decline in the expression of miR-146a is observed after 24h of exposure to VEGF, FGF-2 or Ang-2 (Figure 4.4). Additoonally, VEGF and FGF-2 have no influence on the expression of miR-146b-5p, but Ang-2 significantly reduces miR-146b-5p expression at 24 h (Figure 4.4). These results indicate that Ang-1 and Ang-2 exert opposing effects on miR-146b-5p expression in HUVECs and that that miR-146a expression is inhibited by VEGF, FGF-2 and Ang-2 but not by Ang-1.

miR-146b-5p attenuates LPS-induced leukocyte adhesion, adhesion molecule expression and cytokine production: To study the effect of miR-146b-5p on TLR4 signaling, HUVECs were transfected with mature single-stranded miR-146b-5p mimic or control mimic and later exposed to LPS. Transfection of miR-146b-5p mimic significantly increases miR-146b-5p expression in HUVECs as compared to control mimic (100nM) as verified by real-time PCR (Supplementary Figure S4.1). In HUVECs transfected with control mimic, LPS significantly enhances U937 cell adhesion to these cells and triggers significant increases in the expression of VCAM1, ICAM1, E-SELECTIN and pro-inflammatory cytokine production (TNFα, IL1β, IL6 and IL8) (Figure 4.5). By

comparison, in HUVECs transfected with miR-146b-5p mimic, LPS fails to enhance U937 cell adhesion to these cells (Figure 4.5). In addition, the extent of LPS-induced upregulation of VCAM1, ICAM1, E-SELECTIN and cytokine mRNA and protein levels in HUVECs transfected with miR-146b-5p mimic is significantly lower than that observed in HUVECs transfected with control mimic (Figure 4.5).

miR-146b-5p attenuates LPS-induced TLR4 signaling: LPS triggers a significant increase in the phosphorylation of p38, SAPK/JNK and ERK1/2 in HUVECs transfected with control mimic (Figure 4.6). LPS-induced phosphorylation of p38, SAPK/JNK and ERK1/2 is also observed in HUVECs transfected with miR-146b-5p mimic, however, the relative increase in p38 and SAPK/JNK phosphorylation in response to LPS in these cells is significantly lower than that observed in cells transfected with control mimic (Figure 4.6). LPS-induced ERK1/2 phosphorylation is similar in magnitude in HUVECs transfected with control mimic and those transfected with miR-146b-5p mimic (Figure 4.6). LPS triggers a significant increase in NF-κB luciferase reporter activity in HUVECs transfected with control mimic (Figure 4.6). LPS-induced NF-κB luciferase activity in HUVECs transfected with miR-146b-5p mimic is significantly lower than that observed in HUVECs transfected with control mimic (Figure 4.6).

The proteins IRAK1 and TRAF6 amplify signaling cascades initiated by TLRs and are known targets of miR-146a and miR-146b-5p [35]. We assessed the influence of miR-146b-5p mimic on the expression of IRAK1 and TRAF6 in HUVECs. No differences in IRAK1 and TRAF6 mRNA levels are observed in HUVECs transfected with control mimic or 146b-5p mimic (100nM) (Figure 4.7). However, significantly lower IRAK1 and TRAF6 protein levels are observed in HUVECs transfected with miR-146b-5p mimic compared to

those measured in cells transfected with control mimic (Figure 4.7). To verify whether the effect of miR-146b-5p mimic on the expression of IRAK1 and TRAF6 is mediated through selective binding to specific targeting sites located at the 3'UTR regions of IRAK1 and TRAF6 mRNA transcripts, we co-transfected HUVECs with control mimic or miR-146b-5p mimic along with luciferase reporters expressing wild type 3' UTR of IRAK1 (IRAK1-wt) or TRAF6 (TRAF6-wt) as previously described [35]. Relative luciferase activities of IRAK1-wt and TRAF6-wt measured in HUVECs transfected with miR-146b-5p are significantly lower than those measured in HUVECs transfected with control mimic (Figure 4.7). These results suggest that miR-146b-5p inhibits the expression of IRAK1 and TRAF6 by selectively targeting sequences in the 3'UTRs of these genes, which leads to impaired translation of these transcripts.

Inhibition of miR-146b-5p expression eliminates the effects of Ang-1 on TLR4 signaling: To confirm that the inhibitory effects of pre-treatment with Ang-1 on LPS-induced TLR4 signaling is mediated through miR-146b-5p, HUVECs are transfected with control miScript® miRNA inhibitor or miScript®146b-5p inhibitor. After 36 h recovery period, cells were then pre-treated with PBS or Ang-1 for 24h followed by exposure to vehicle (control) or LPS for 4 to 6h. miScript miRNA inhibitors are chemically synthesized, single-stranded, modified RNAs designed to specifically inhibit endogenous miRNA function after transfection into cells. In HUVECs transfected with control miRNA inhibitor and pre-treated with PBS, LPS induces a significant increase in U937 adhesion and upregulation of IL6 and IL8 production (Figure 4.8). In HUVECs transfected with control miRNA inhibitor and pre-treated with Ang-1, LPS fails to induce an increase in U937 cell adhesion and the extent of LPS-induced IL6 and IL8 production is significantly

lower than that measured in cells pre-treated with PBS (Figure 4.8). In HUVECs transfected with miR-146b-5p inhibitor, the degree of LPS-induced U937 cell adhesion, and IL6 and IL8 production is similar when these cells are pre-treated with PBS or Ang-1 (Figure 4.8). These results indicate that miR-146b-5p is critical for the inhibitory effect of Ang-1 on LPS-induced responses in HUVECs. To verify that this inhibitory role of miR-146b-5p on LPS-induced responses is mediated through selective targeting of IRAK1 and TRAF6 by this miRNA, we transfected HUVECs with luciferase reporters of wild type or mutated IRAK1 and TRAF6 3'UTRs. The mutated reporters contain 3'UTR regions of IRAK1 and TRAF6 in which the selective targeting sequences of miRNA-146b-5p have been mutated (Figure 4.9). In HUVECs transfected with wild type IRAK1 and TRAF6 3'UTRs, pre-treatment with Ang-1 for 24h results in significantly lower luciferase reporter activities compared to those measured in cells pre-treated with PBS, thereby confirming that Ang-1 pre-treatment results in inhibition of IRAK1 and TRAF6 expression (Figure 4.9). The inhibitory effect of Ang-1 on luciferase reporter activities is not observed when the binding site for miR-146b-5p in the 3'UTR of IRAK1 and TRAF6 has been mutated (Figure 4.9). These results confirm that the inhibitory effects of Ang-1 pretreatment on IRAK1 and TRAF6 expression is mediated through selective binding of miR-146b-5p to specific sequences in the 3' UTRs of these mRNAs. To confirm the importance of miR-146b-5p in the inhibitory effects of Ang-1 on IRAK1 and TRAF6 levels, we measured IRAK1 and TRAF6 protein expression in HUVECs transfected with control inhibitor or miR-146b-5p inhibitor (50nM). In HUVECs transfected with control miRNA inhibitor and pre-treated with Ang-1 for 24h, the levels of IRAK1 and TRAF6 proteins are significantly lower than those detected in cells pre-treated with PBS (Figure 4.9). In HUVECs transfected with miR-146b-5p inhibitor and pre-treated with Ang-1 for 24h, IRAK1 and

TRAF6 protein levels are similar to those measured in cells pre-treated with PBS, thereby confirming the importance of miR-146b-5p in the inhibitory effect of Ang-1 on IRAK1 and TRAF6 expression (Figure 4.9).

4.5 DISCUSSION

The main findings of this study are as follows: 1) Pre-treatment of HUVECs with Ang-1 for 24h results in significant attenuation of LPS-induced leukocyte adhesion, adhesion molecule expression, pro-inflammatory cytokine production, p38 and SAPK/JNK phosphorylation, and NF-κB activation; 2) pre-treatment of HUVECs with Ang-1 for 24h results in significant attenuation of IRAK1 and TRAF6 protein expressions; 3) transfection of HUVECs with miR-146b-5p mimic results in significant attenuation of LPS-induced leukocyte adhesion, adhesion molecule expression, pro-inflammatory cytokine production, p38, SAPK/JNK and NFκB activation; and significant attenuation of IRAK1 and TRAF6 protein expression; and 4) inhibition of miR-146b-5p with a selective miRNA inhibitor reverses the inhibitory effect of Ang-1 on LPS-induced leukocyte adhesion, cytokine production and IRAK1 and TRAF6.

Inhibition of LPS-induced TLR4 signaling in ECs by Ang-1: ECs play an important role in initiating and sustaining the host inflammatory response during sepsis [40]. In the present study, we show that prolonged Ang-1 exposure inhibits LPS-induced leukocyte adhesion to ECs (Figure 4.1). Several reports have demonstrated that Ang-1 reduces basal and VEGF-induced vascular leakage, and strongly attenuates leukocyte adhesion in response to TNF-α and VEGF by decreasing the expression of VCAM1, ICAM1 and E-selectin [21, 22, 41]. Ang-1 exposure also promotes a rapid and transient increase in neutrophil adhesion by inducing the translocation of P-selectin [15, 42]. The effect of prolonged Ang-1 signaling on LPS-induced EC inflammation was not addressed in these studies. Several reports have indicated that sustained expression of Ang-1, or a Tie-2 agonist, in models of murine sepsis improves hemodynamic function, increases survival and reduces the

infiltration of immune cells to the lungs [25-27, 43]. However, the present study is the first to demonstrate that prolonged Ang-1 exposure acts directly on ECs to reduce LPS-induced leukocyte adhesion. The induction of the adhesion molecules VCAM1, ICAM1 and E-selectin is an essential step in the recruitment of immune cells to ECs at sites of injury [44, 45]. Our results show that Ang-1 reduces the expression of LPS-induced VCAM1, ICAM1 and E-selectin. We propose that this reduction in adhesion molecule expression could be largely responsible for the decline in LPS-induced leukocyte adhesion observed after Ang-1 pre-treatment.

It has been well-established that LPS is a powerful regulator of pro-inflammatory cytokines in ECs through activation of TLR4 signaling [46-48]. In the present study, we report that prolonged Ang-1 exposure also attenuates LPS-induced TNFα, IL1β, IL6 and IL8 expression. IL6 and IL8 are essential for the recruitment of leukocytes and neutrophils to sites of injury, and their circulating serum levels are increased in septic patients [49, 50]. We found that prolonged Ang-1 exposure attenuates the expression of pro-inflammatory mediators elicited by LPS in ECs. We believe that these effects are largely mediated through attenuation of SAPK/JNK phosphorylation and NF-κB transcriptional activation (Figure 4.2). An interesting observation in our study is that pretreatment with Ang-1 attenuates LPS-induced p38 and SAPK/JNK activation, but not ERK1/2 phosphorylation (Figure 2). The reasons behind this effect are unclear, primarily because the mechanisms that regulate the activation of these pathways in ECs have not been studied in detail. There is evidence that the phosphorylation of individual MAPKs by LPS requires different upstream signaling molecules. For instance, MEKK3 is an essential signaling protein of MyD88-dependent TLR4 signaling that forms a complex with TRAF6 and is required for

the LPS-induced activation of the p38 and SAPK/JNK signaling pathways, but not ERK1/2 [51]. Hull *et al* have implicated TRAF6 in LPS-induced activation of SAPK/JNK in ECs, but not in ERK1/2 and p38 phosphorylation [52]. Nonetheless, other studies have found that TRAF6 is a key activator of p38 in response to LPS [51, 53]. Further studies are needed to understand the molecular mechanisms through which Ang-1 differentially modulates the activation of MAPK signaling pathways by LPS.

In the present study, we also demonstrate that prolonged stimulation of ECs with Ang-1 decreases NFκB activation in response to LPS. The activation and nuclear translocation of NF-κB downstream of TLR4 require the phosphorylation of IκB kinase (IKK) complex by TAK1 and the degradation of IkBα [515][219]. Our results demonstrate that Ang-1 pre-treatment not only reduces LPS-induced degradation of IκBα, but also attenuates the phosphorylation of the NFκB p65 subunit and NF-κB activity, measured using a luciferase reporter gene (Figure 4.2). We believe that the failure of LPS exposure to trigger strong NF-κB activation in HUVECs pre-treated with Ang-1 is due to impaired upstream TLR4 signaling mechanisms because of reduction in IRAK1 and TRAF6 expression (see below). However, our study does not rule out other yet to be determined mechanisms through which Ang-1 pretreatment may influence LPS-induced NFκB activation in HUVECs. One of these mechanisms is the direct binding of Tie-2 receptors to ABIN-2, a protein that binds A20 and directly inhibits NFκB activation [24, 54]. ABIN-2 interacts with phosphorylated Tie-2 receptors [54]. Tadros *et al* have reported that introduction of an exogenous form of ABIN-2, unable to inactivate NF-κB, prevents Ang-1 from rescuing ECs from apoptosis induced by growth factor deprivation [24]. Nevertheless,

the particular role of ABIN-2 as a mediator of the anti-inflammatory effects of Ang-1 remains to be elucidated.

Activation of MAPKs and NF-κB downstream of TLR4 depends on MYD88 to recruit IRAK1, IRAK4, TRAF6 and the TAK1 complex [28]. In the present study, we report that Ang-1 pretreatment reduces the expression of IRAK1 and TRAF6 protein levels but had no influence on mRNA and protein levels of TLR4, MYD88, IRAK4 and TAK1 (Figure 4.3). IRAK1 and TRAF6 are fundamental components of signal transduction cascades activated by TLRs [28]. We propose that prolonged Ang-1 exposure attenuates LPS-induced TLR4 signaling through downregulation of IRAK1 and TRAF6 protein expression. This conclusion is consistent with studies in IRAK1 knockout mice in which activation of NFκB, p38 and SAPK/JNK, and the expression of IL6 and TNFα are attenuated downstream of TLR4 and IL-1 receptor (IL-1R) [55-57]. ECs deficient in TRAF6 also exhibit defective expression of pro-inflammatory cytokines and adhesion molecules after stimulation with oxidized low-density lipoprotein (oxLDL), a modified lipid able to initiate TLR signaling in a model of atherosclerosis [58]. In addition, mice deficient in IRAK1 are less susceptible to the lethal effects of LPS when compared to their wild-type counterparts, and an intronic single-nucleotide polymorphism (SNP) in the TRAF6 gene that increases its mRNA expression has been linked to a higher risk of sepsis-induced acute lung injury [56, 59]. Overall, our data suggests that Ang-1-induced attenuation of IRAK1 and TRAF6 protein levels in HUVECs can affect the magnitude of the inflammatory response to LPS downstream of TLR4 in ECs.

Induction of miR-146b-5p by Ang-1: Members of the miR-146 family, miR-146a and miR-146b, play important roles in inflammation and innate immune responses [33-35, 60]. The

present study demonstrates that Ang-1 exposure in HUVECs induces the expression of miR-146b-5p, a mature product of the miR-146b gene generated from the 5' arm of the double stranded stem region in the precursor miRNA [34], but not miR-146a (Figure 4.4). We also found that VEGF and FGF-2 had no significant effect on miR-146b-5p expression, suggesting that upregulation of this mRNA is selective to Ang-1 signaling and is not shared by these important pro-angiogenesis factors. The miR-146a gene, located on the human chromosome 5, is induced by LPS, IL-1β, TNFα and viral infection [35, 61-63]. In contrast, the miR-146b gene is located on the long arm of human chromosome 10 and is transcribed independently from miR-146a [34]. Although miR-146b-5p is upregulated in thyroid [64], breast [65] and non-small cell lung cancer [66], little is known about the mechanisms that regulate its expression. Perry *et al* [67] reported that miR-146b is upregulated by IL-1β in A549 human lung carcinoma cells and its expression requires the activation of the ERK1/2 and SAPK/JNK signaling pathways.

Despite the similarities shared by miR-146a and miR-146b, the biological functions of these miRNAs are distinct and non-redundant [34, 68 35]. The roles of miR-146a in inflammation, endotoxin tolerance, myeloid cell proliferation and cancer has been well established [34, 68, 69]. miR-146b-5p has been associated with various cellular processes other than inflammation. Indeed, it has been shown that miR-146b-5p regulates the TGF-β signaling pathway by targeting SMAD4 and modulates cell migration through its targets epidermal growth factor receptor (EGFR) and matrix metalloproteinase 16 (MMP16) [64, 70]. Recent studies by Taganov *et al* proposed that miR-146b-5p may be a potential regulator of TLR signaling [35], however, the particular function of this miRNA in innate immunity and inflammation remains to be elucidated. In the present study, we used a mimic of mature

miR-146b-5p to demonstrate that this micro RNA has a powerful inhibitory effect on LPS-induced leukocyte adhesion, adhesion molecule expression and cytokine secretion in ECs (Figure 4.5). We also found that transfection with miR-146b-5p mimic results in strong attenuation of LPS-induced p38 and SAPK/JNK activation and reduction in NF-κB reporter activity. A previous report demonstrated that NFκB induces the transcription of the miR-146a and miR-146b genes after LPS stimulation, but only the mature form of miR-146a functions as a negative feedback mechanism of NF-κB activation [35]. Our findings demonstrate that miR-146b-5p can also attenuate LPS-induced NFκB activation in ECs and suggest that one of the functions of this miRNA is to limits the activation of TLR4 signaling by LPS in the vasculature.

Regulation of IRAK1 and TRAF6 by miR-146b-5p: The mature forms of miR-146a and miR-146b share the same "seed" sequence, which suggest that they can bind to the same mRNA targets [35-37]. We found that transfection of miR-146b-5p mimic in ECs results in reduced IRAK1 and TRAF6 protein levels and a decrease in luciferase expression containing 3'UTR of IRAK1 and TRAF6 (Figure 4.7). Our results confirm previous studies identifying IRAK1 and TRAF6 as direct targets of miR-146b-mediatd regulation [35] and implicate these proteins in the regulation of LPS-induced signaling in ECs. More importantly, we propose here that the inhibitory effect of Ang-1 pre-treatment on IRAK1 and TRAF6 protein levels is mediated through upregulation of miR-146b-5p and binding of this micro RNA to specific targeting sites located at the 3'UTR of these genes. This proposal is supported by two observations. First, we found that the activities of luciferase reporters containing the wild type 3'UTRs of IRAK1 and TRAF6 are strongly inhibited in HUVECs pre-treated with Ang-1, and this inhibitory effect is not present when the miR-

146b-5p targeting sites on the 3'UTR of IRAK1 and TRAF6 have been mutated (Figure 4.9). Second, we transfected HUVECs with a selective inhibitor of miR-146b-5p and re-assessed the influence of Ang-1 pretreatment on LPS-induced responses. We found that transfection with a miR-146b-5p inhibitor reversed the effect of Ang-1 on LPS-induced leukocyte adhesion, cytokine production and eliminated the inhibitory effect of Ang-1 pre-treatment on IRAK1 and TRAF6 protein expressions (Figure 4.9). The present study also raises the question as to whether Ang-1-induced miR-146b-5p affects other signaling pathways that depend on IRAK1 and/or TRAF6 proteins. It was recently described that IL-33 promotes angiogenesis and vascular permeability through TRAF6-mediated activation of phosphoinoside-3-kinase, AKT and endothelial nitric oxide synthase (eNOS) [71], and IL-1β assembles a pro-angiogenic signaling complex in ECs formed by caveolin-1, TRAF6, p38 and MAPK-activated protein kinase 2 (MK2) [72]. These studies suggest that miR-146b-5p could potentially be involved in the modulation of angiogenesis through its target TRAF6, and more studies are needed to understand the function of this miRNA in ECs.

An interesting observation in our study is that, unlike Ang-1, exposure to Ang-2 dramatically decreases miR-146b-5p expression, suggesting that Ang-1 and Ang-2 have opposing roles in the expression of this miRNA (Figure 4.4). Moreover, Ang-2 treatment also decreases miR-146a expression. Ang-2 is an antagonist of Ang-1-mediated Tie-2 receptor activation and can also act as a weak agonist at relatively high concentrations [73-76]. Recent studies have revealed that Ang-2 destabilizes the vasculature [77, 78] and potentiates the responses to pro-inflammatory stimuli [79]. Indeed, Ang-2 deficient mice are unable to elicit a rapid pro-inflammatory response to TNF-α and fail to induce adhesion molecule expression [79]. The inhibitory effect of Ang-1 on miR-146a and miR-146b-5p expressions in

HUVECs suggests that the pro-inflammatory effect of Ang-2 in these cells may mediated in part through attenuation of the expression of these miRNAs. The functional importance of miR-146a and miR-146b-5p in regulating the pro-inflammatory effects of Ang-2 in ECs need to be fully investigated.

We should emphasize that post-transcriptional repression is generally regulated by multiple and cooperative miRNAs, and we do not exclude possible roles for other miRNAs in the modulation of IRAK1 and TRAF6 expression in response to Ang-1 pre-treatment.

In summary, we report here that pre-treatment of HUVECs for 24h with Ang-1 attenuates LPS-induced leukocyte adhesion, expression of pro-inflammatory mediators and activation of p38, SAPK/JNK and NFκB. The present study also demonstrates that the anti-inflammatory effects of Ang-1 are mediated in part through the induction of miR-146b-5p and the repression of its targets, the signaling proteins IRAK1 and TRAF6. Our results propose a novel mechanism through which prolonged Ang-1 exposure limits inflammatory responses in the vasculature during bacterial infection.

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AUTHORSHIP CONTRIBUTIONS

R. Echavarria: Performed experiments, analyzed results, interpreted results and wrote and edited the manuscript.

S. Hussain: Designed experiments, analyzed results, interpreted and edited the final manuscript

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DISCLOSURE OF CONFLICTS OF INTEREST

All authors declare no conflicts of interest or financial interests.

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4.8 FIGURES

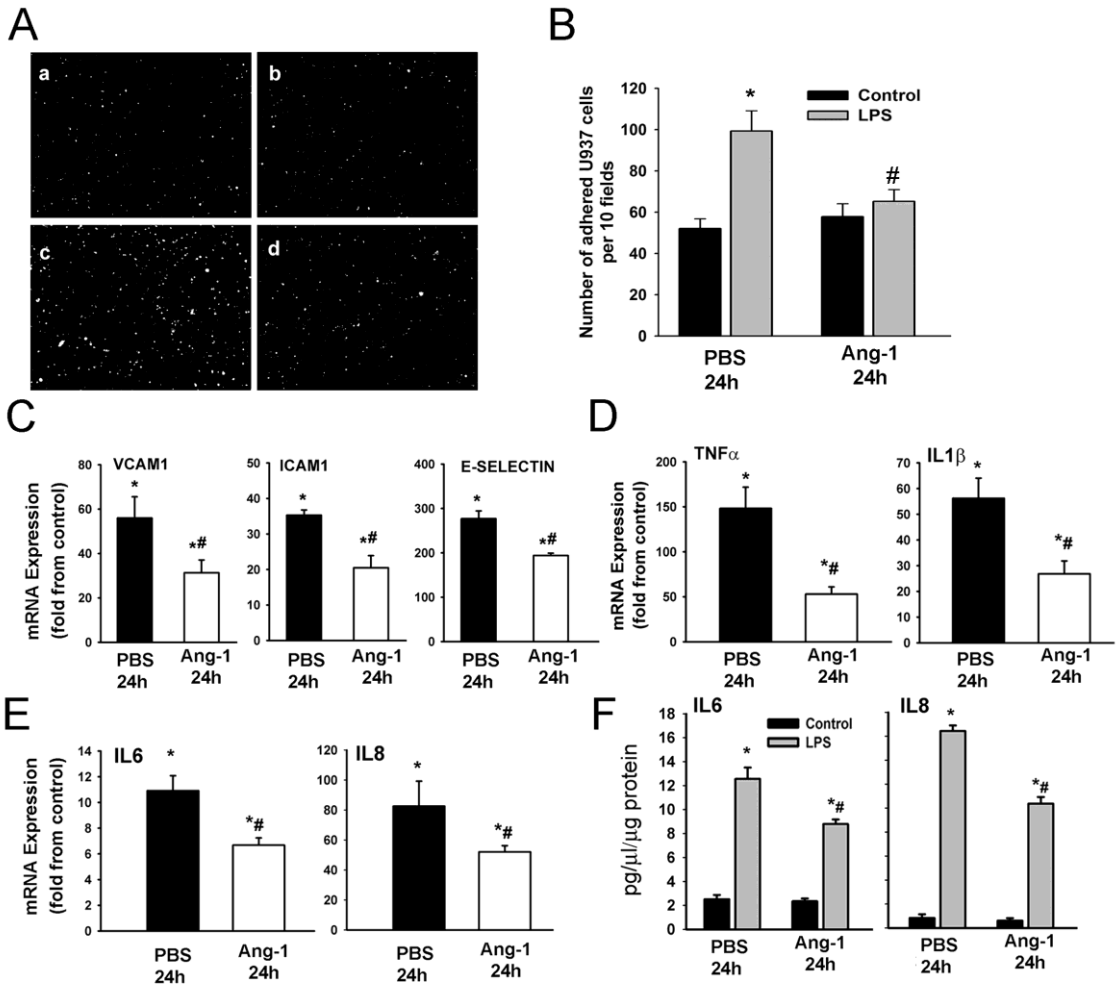
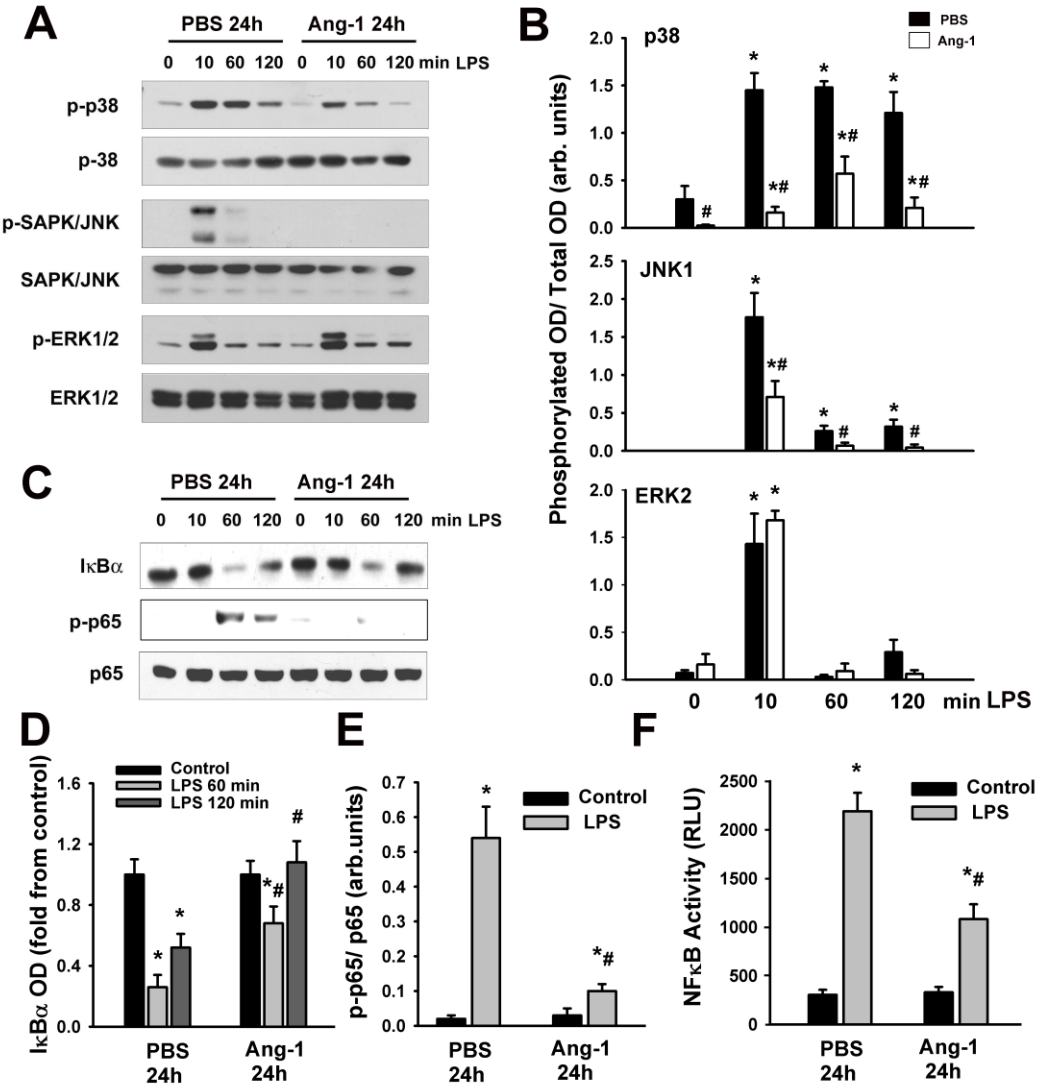


Figure 4.1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PBS 24h
Ang-1 24h
0 10 60 120 0 10 60 120 min LPS
p-p38
p-38
p-SAPK/JNK
SAPK/JNK
p-ERK1/2
ERK1/2
B
PBS
Ang-1
p38
JNK1
ERK2
Phosphorylated OD/ Total OD (arb. units)
0 10 60 120 min LPS
C
PBS 24h
Ang-1 24h
0 10 60 120 0 10 60 120 min LPS
IκBα
p-p65
p65
D
Control
LPS 60 min
LPS 120 min
IκBα OD (fold from control)
PBS 24h
Ang-1 24h
E
Control
LPS
p-p65/ p65 (arb.units)
PBS 24h
Ang-1 24h
F
Control
LPS
NFκB Activity (RLU)
PBS 24h
Ang-1 24h

Figure 4.2

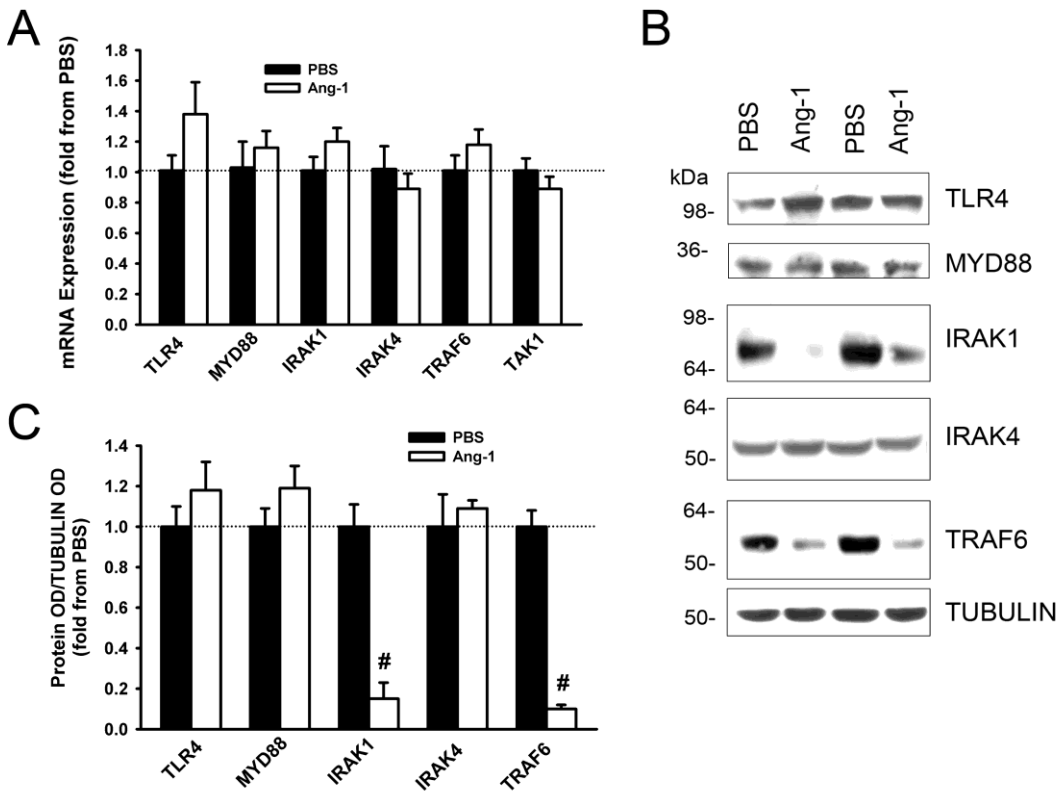

A
mRNA Expression (fold from PBS)
PBS
Ang-1
TLR4
MYD88
IRAK1
IRAK4
TRAF6
TAK1
B
PBS
Ang-1
PBS
Ang-1
kDa
98-
TLR4
36-
MYD88
98-
64-
IRAK1
64-
50-
IRAK4
64-
50-
TRAF6
50-
TUBULIN
C
Protein OD/TUBULIN OD
(fold from PBS)
PBS
Ang-1
#
#
TLR4
MYD88
IRAK1
IRAK4
TRAF6

Figure 4.3

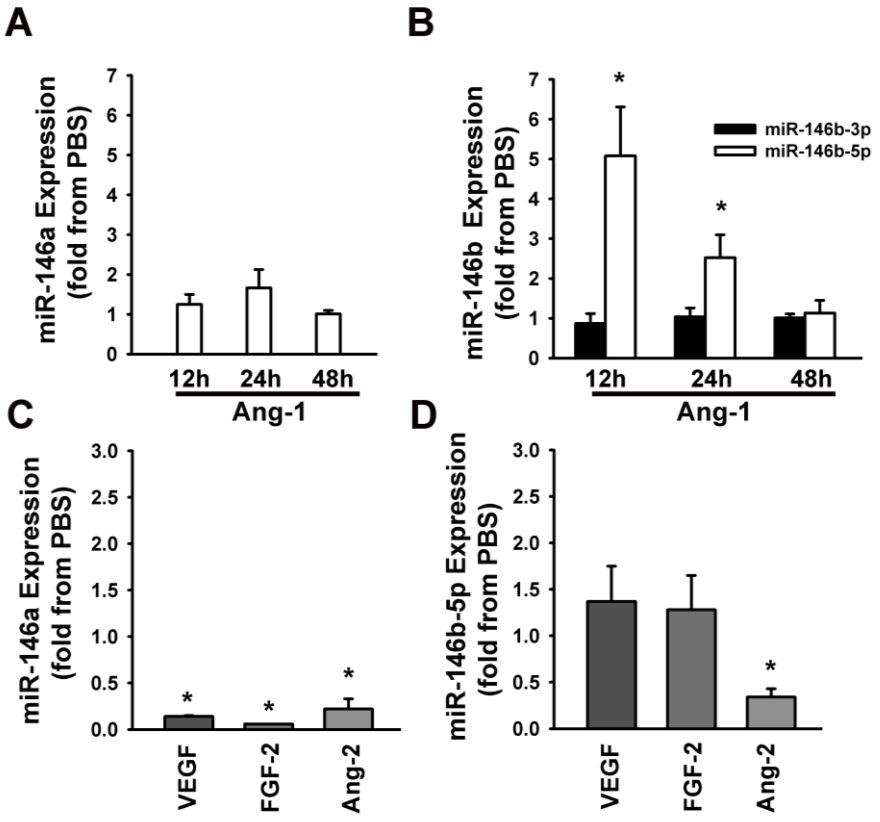

A
B
C
D
miR-146a Expression (fold from PBS)
miR-146b Expression (fold from PBS)
miR-146b-5p Expression (fold from PBS)
miR-146b-3p
miR-146b-5p
12h
24h
48h
Ang-1
VEGF
FGF-2
Ang-2
*

Figure 4.4

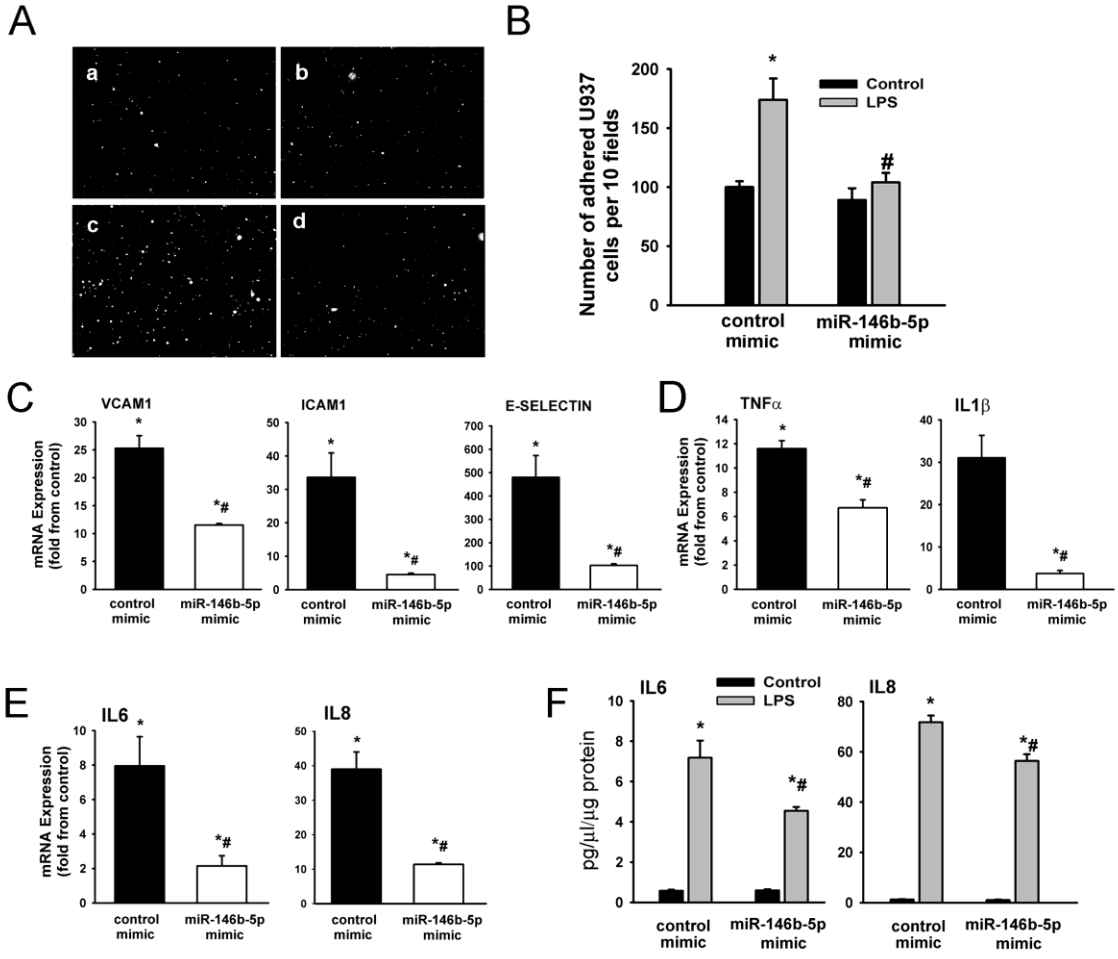
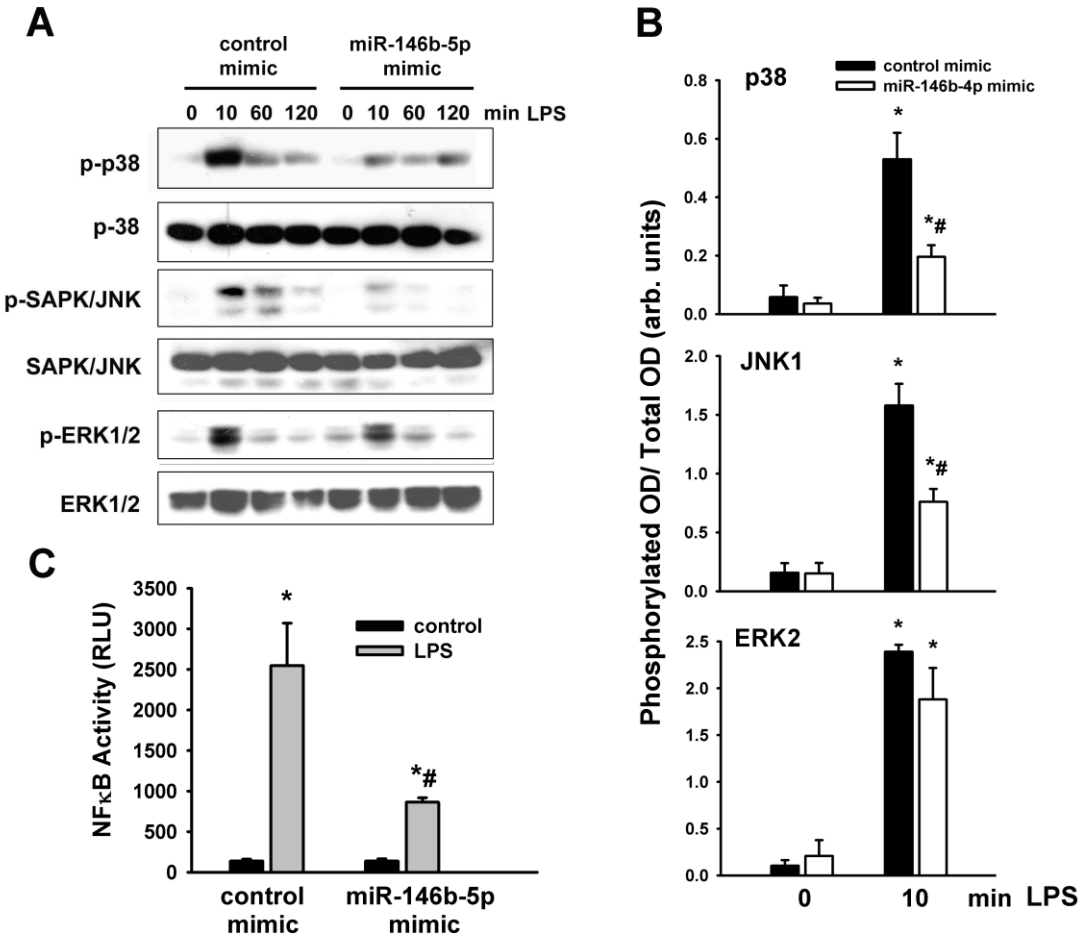

A
a
b
c
d
B
Number of adhered U937 cells per 10 fields
Control
LPS
control mimic
miR-146b-5p mimic
C
VCAM1
ICAM1
E-SELECTIN
mRNA Expression (fold from control)
D
TNFα
IL1β
E
IL6
IL8
F
IL6
IL8
pg/μl/μg protein

Figure 4.5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control mimic
miR-146b-5p mimic
0 10 60 120 0 10 60 120 min LPS
p-p38
p-38
p-SAPK/JNK
SAPK/JNK
p-ERK1/2
ERK1/2
B
p38
control mimic
miR-146b-4p mimic
JNK1
ERK2
Phosphorylated OD/ Total OD (arb. units)
0 10 min LPS
C
NFκB Activity (RLU)
control
LPS
control mimic
miR-146b-5p mimic

Figure 4.6

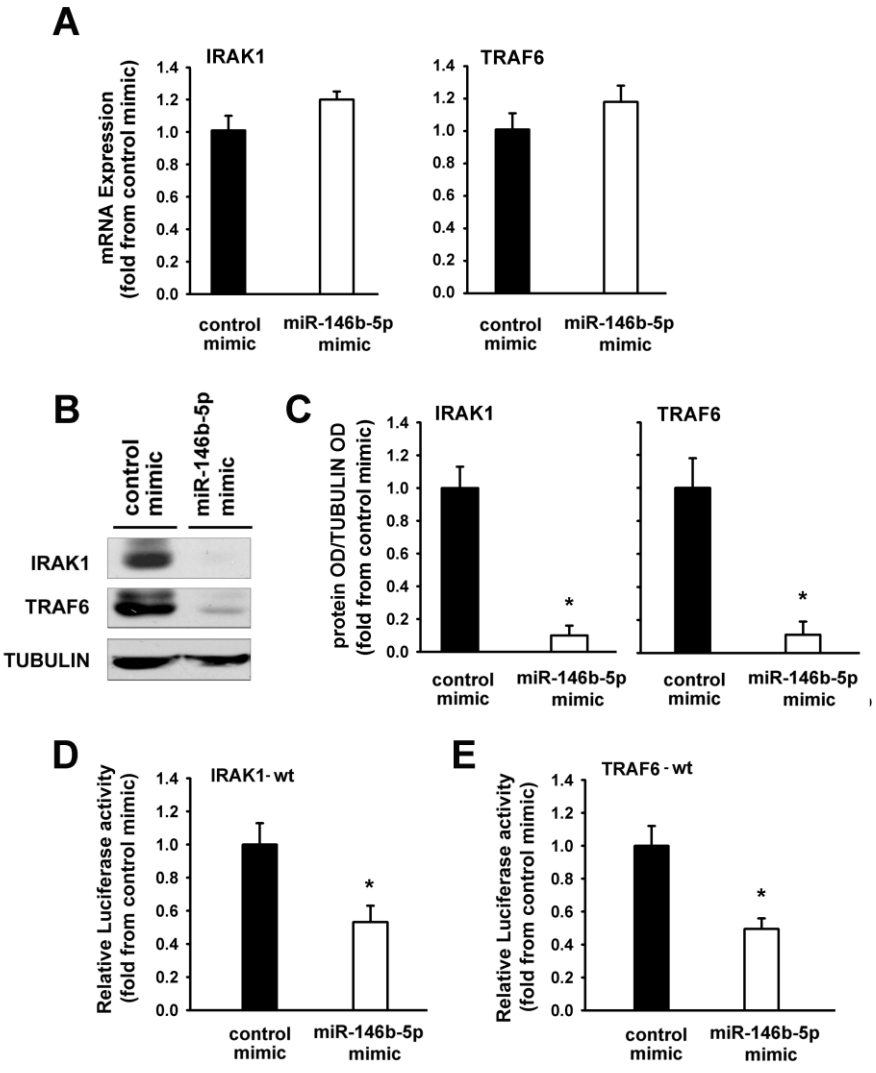

A
IRAK1
TRAF6
mRNA Expression (fold from control mimic)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
control mimic
miR-146b-5p mimic
B
control mimic
miR-146b-5p mimic
IRAK1
TRAF6
TUBULIN
C
IRAK1
TRAF6
protein OD/TUBULIN OD (fold from control mimic)
*
D
IRAK1- wt
Relative Luciferase activity (fold from control mimic)
E
TRAF6 - wt

Figure 4.7

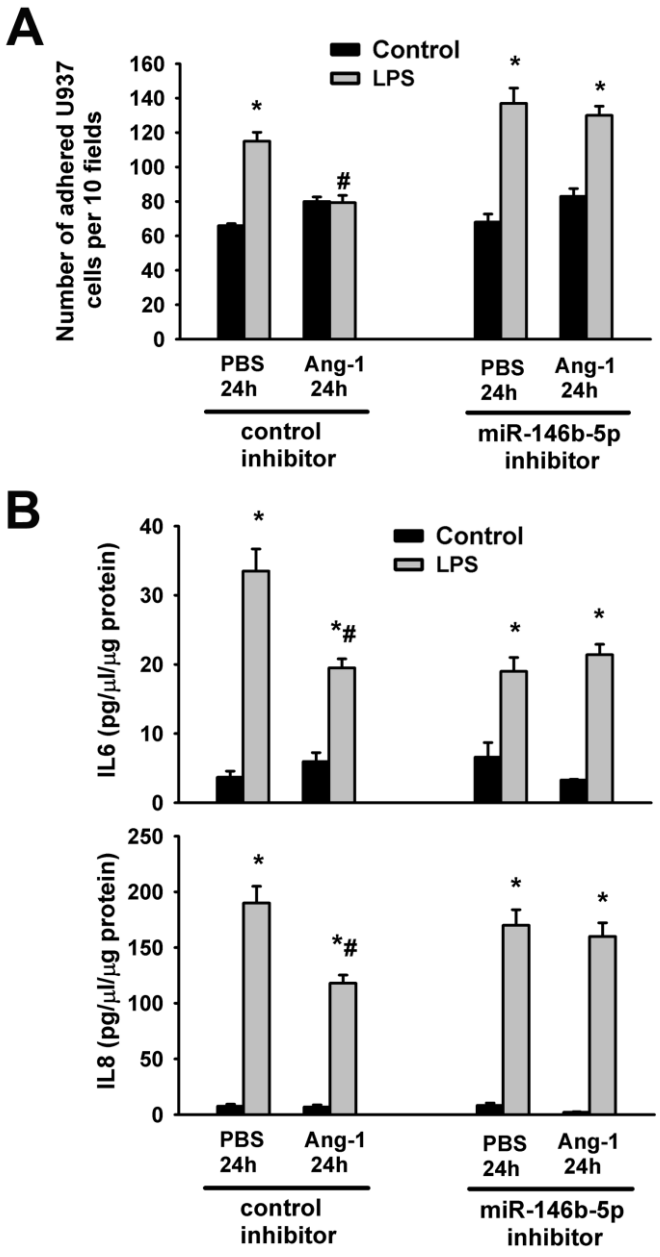
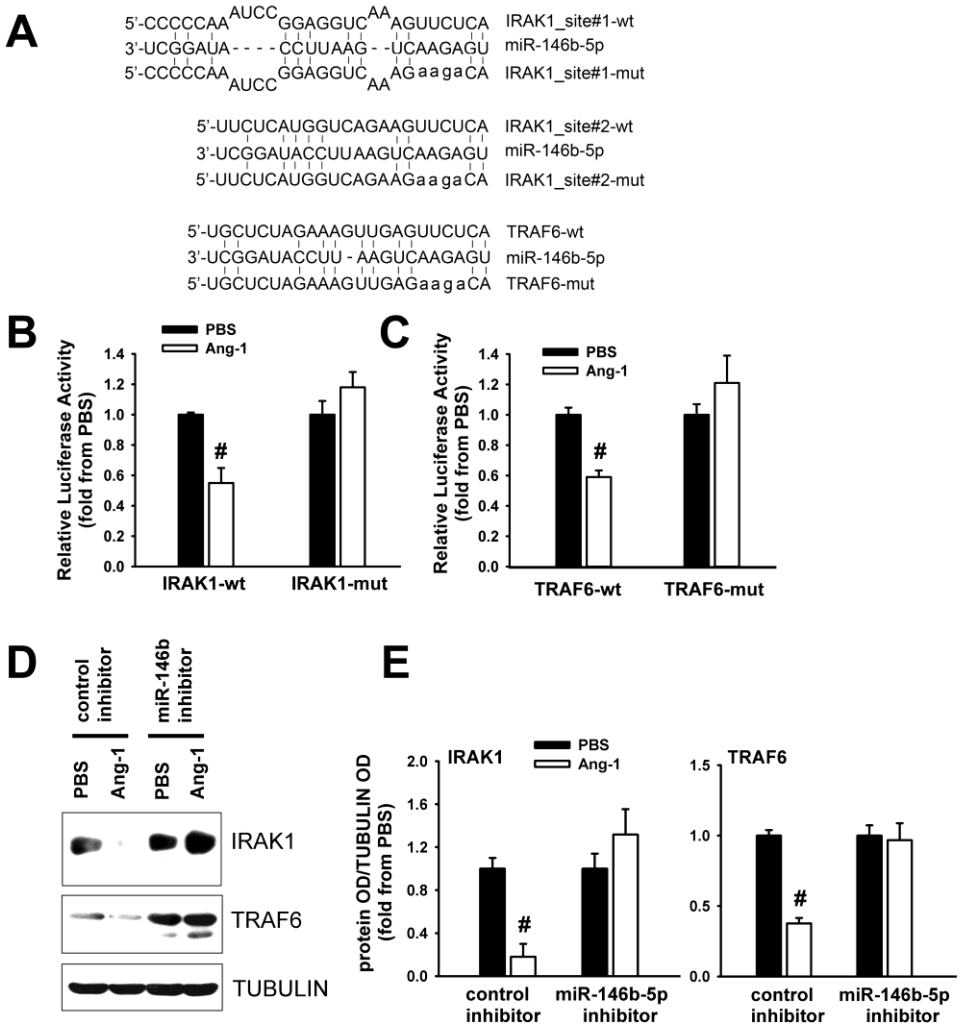

A
Control
LPS
Number of adhered U937 cells per 10 fields
160
140
120
100
80
60
40
20
0
*
#
*
*
PBS 24h
Ang-1 24h
PBS 24h
Ang-1 24h
control inhibitor
miR-146b-5p inhibitor
B
Control
LPS
IL6 (pg/μl/μg protein)
40
30
20
10
0
*
*#
*
*
IL8 (pg/μl/μg protein)
250
200
150
100
50
0
*
*#
*
*
PBS 24h
Ang-1 24h
PBS 24h
Ang-1 24h
control inhibitor
miR-146b-5p inhibitor

Figure 4.8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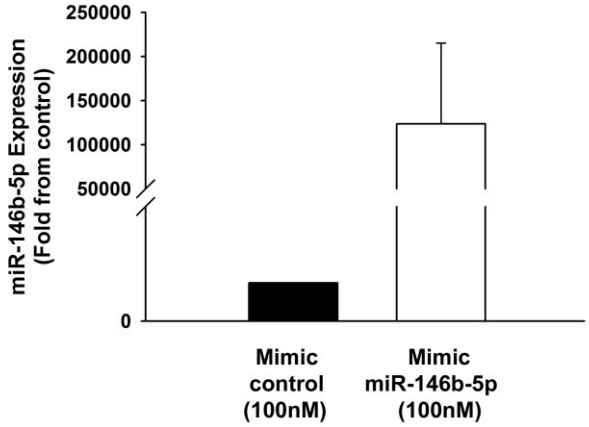
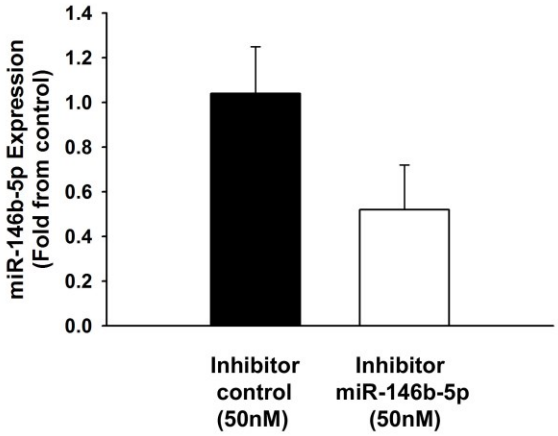
5'-UUCUCAUGGUCAGAAGaagaCA IRAK1_site#2-mut
5'-UGCUCUAGAAAGUUGAGUUCUCA TRAF6-wt
3'-UCGGAUACCUU - AAGUCAAGAGU miR-146b-5p
5'-UGCUCUAGAAAGUUGAGaagaCA TRAF6-mut
B
PBS
Ang-1
Relative Luciferase Activity (fold from PBS)
IRAK1-wt
IRAK1-mut
C
TRAF6-wt
TRAF6-mut
D
control inhibitor
miR-146b inhibitor
PBS
Ang-1
IRAK1
TRAF6
TUBULIN
E
protein OD/TUBULIN OD (fold from PBS)
IRAK1
TRAF6
control inhibitor
miR-146b-5p inhibitor

Figure 4.9



Supplementary Figure S4.1



Supplementary Figure S4.2

4.9 FIGURE LEGENDS

Figure 4.1: Pretreatment with Ang-1 attenuates LPS-induced leukocyte adhesion, adhesion molecule expression and cytokine production

A) Representative image of adhered U937 cells to HUVECs. HUVECs are pre-treated for 24h with PBS (a, c) or Ang-1 (b, d). Cells are then treated with vehicle (control)(a, b) or LPS (c, d) for 6h following which labelled U937 cells were added for 1h. **B)** Mean ± SEM of number of adhered U937 cells to HUVECs in response to 6h exposure to vehicle (control) or LPS in HUVECs pre-treated 24h earlier with PBS or Ang-1. *$P<0.05$, compared to control. $^{\#}P<0.05$ compared to cells pre-treated with PBS. **C)** VCAM1, ICAM and E-selectin mRNA expressions measured in response to 4h exposure to vehicle (control) or LPS in HUVECs pre-treated for 24h with PBS or Ang-1. *$P<0.05$, compared to control. $^{\#}P<0.05$ compared to cells pre-treated with PBS. **D-E)** TNFα, IL1β, IL6 and IL8 mRNA expressions measured in response to 6h exposure to vehicle (control) or LPS in HUVECs pre-treated for 24h with PBS or Ang-1. *$P<0.05$, compared to control. $^{\#}P<0.05$ compared to cells pre-treated with PBS. **F)** IL6 and IL8 protein levels measured with ELISAs in response to 6h exposure to vehicle (control) or LPS in HUVECs pre-treated for 24h with PBS or Ang-1. *$P<0.05$, compared to control. $^{\#}P<0.05$ compared to cells pre-treated with PBS.

Figure 4.2: Pretreatment with Ang-1 attenuates LPS-induced TLR4 signaling

A-B) Representative immunoblots and means ±SEM of optical densities of phosphorylated/total p38, SAPK/JNK and ERK1/2 in response to vehicle (control, 0 time) and LPS exposure in HUVECs pre-treated for 24h with PBS or Ang-1. *$P<0.05$, compared

to 0 time. #P<0.05 compared to cells pre-treated with PBS. **C)** Representative immunoblots of IκBα, phosphorylated and total p65 subunit of NFκB in response to vehicle (0 time) and LPS exposure in HUVECs pre-treated for 24h with PBS or Ang-1. **D)** Means ± SEM of optical densities of IκBα (expressed as fold change from 0 time, control) measured after 60 and 120 min of LPS exposure in HUVECs pre-treated for 24h with PBS or Ang-1. *P<0.05, compared to control. #P<0.05 compared to cells pre-treated with PBS. **E)** Means ± SEM of optical densities of phosphorylated/total p65 subunit of NFκB measured after 60 min of vehicle (control) or LPS exposure in HUVECs pre-treated for 24h with PBS or Ang-1. *P<0.05, compared to control. #P<0.05 compared to cells pre-treated with PBS. **F)** Means ± SEM of NFκB reporter activity (expressed as relative light units) measured after 6h of vehicle (control) or LPS exposure in HUVECs pre-treated for 24h with PBS or Ang-1. *P<0.05, compared to control. #P<0.05 compared to cells pre-treated with PBS.

Figure 4.3: Regulation of TLR4 signaling by Ang-1

A) Means ±SEM of TLR4, MYD88, IRAK1, IRAK4, TRAF6 and TAK1 mRNA expressions measured in HUVECs in response to 24h pretreatment with PBS or Ang-1 (300 ng/ml). **B-C)** Representative examples and means± SEM of TRL4, MYD88, IRAK1, IRAK4 and TRAF6 protein levels in HUVECs in response to 24h pretreatment with PBS or Ang-1. #P<0.05, compared to PBS pretreament.

Figure 4.4: Regulation of miR-146a and miR-146b-3p and 5p by angiogenesis factors

A-B) miR-146a, miR-146b-3p and miR-146b-5p expression measured with real-time PCR in HUVECs treated for 12, 24 and 48h with PBS or Ang-1. Values are means ± SEM and

are expressed as fold change from values measured in cells treated with PBS. *P<0.05, compared to cells treated with PBS. **C-D)** miR-146a and miR-146b-5p expression measured with real-time PCR in HUVECs treated for 24h with PBS, VEGF, FGF-2 or Ang-2. Values are means ± SEM and are expressed as fold change from values measured in cells treated with PBS. *P<0.05, compared to cells treated with PBS.

Figure 4.5: miR-146b-5p attenuates LPS-induced leukocyte adhesion, adhesion molecule expression and cytokine production

A) Representative images of adhered U937 cells to HUVECs. HUVECs are transfected with control mimic (a, c) or miR-146b-5p mimic (b, d). Cells are then treated with vehicle (control)(a, b) or LPS (c, d) for 6h following which labelled U937 cells are added for 1h. **B)** Mean ± SEM of number of adhered U937 cells to HUVECs in response to 6h exposure to vehicle (control) or LPS in HUVECs transfected with control mimic or mi-R146b-5p mimic. *P<0.05, compared to control. $^{\#}$P<0.05 compared to cells transfected with control mimic. **C)** VCAM1, ICAM and E-selectin mRNA expressions measured after 4h exposure to vehicle (control) or LPS in HUVECs transfected with control mimic or miR-146b-5p mimic. *P<0.05, compared to control. $^{\#}$P<0.05 compared to cells transfected with control mimic. **D-E)** TNFα, IL1β, IL6 and IL8 mRNA expressions in HUVECs measured after 6h exposure to vehicle (control) or LPS in HUVECs transfected with control mimic or miR-146b-5p mimic. *P<0.05, compared to control. $^{\#}$P<0.05 compared to cells transfected with control mimic. **F)** IL6 and IL8 protein levels measured with ELISAs in response to 6h exposure to vehicle (control) or LPS in HUVECs transfected with control mimic or HUVECs pre-treated for 24h with PBS or Ang-1 and then exposed to vehicle (control) or

miR-146b-5p mimic. *P<0.05, compared to control. #P<0.05 compared to cells transfected with control mimic.

Figure 4.6: miR-146b-5p attenuates LPS-induced TLR4 signaling

A-B) Representative immunoblots and means ± SEM of optical densities of phosphorylated/total p38, SAPK/JNK and ERK1/2 measured in response to vehicle (control, 0 time) and *E. coli* LPS in HUVECs transfected with control mimic or miR-146b-5p mimic. *P<0.05, compared to control. #P<0.05 compared to cells transfected with control mimic. **C)** Means ± SEM of NFκB reporter activity (expressed as relative light units) measured after 6h of vehicle (control) or LPS exposure in HUVECs transfected with control mimic or miR-146b-5p mimic. *P<0.05, compared to control. #P<0.05 compared to cells transfected with control mimic.

Figure 4.7: miR-146b-5p inhibits IRAK1 and TRAF6 expressions in HUVECs

A) IRAK1 and TRAF6 mRNA expressions measured in HUVECs transfected with control mimic or miR-146b-5p mimic. Data are means± SEM and is expressed as fold change from values measured in HUVECs transfected with control mimic. **B)** Representative immunoblots of IRAK1 and TRAF6 proteins measured in HUVECs transfected with control mimic or miR-146b-5p mimic. **C)** Means ± SEM of IRAK1 and TRAF6 protein optical densities measured in HUVECs transfected with control mimic or miR-146b-5p mimic. Optical densities are normalized per optical densities of TUBULIN and are then expressed as fold changes from values measured in cells transfected with control mimic. *P<0.05 compared to cells transfected with control mimic. **D-E)** Regulation of luciferase reporters containing the 3'UTRs of IRAK1 or TRAF6 by miR-146-5p HUVECs are

transfected with luciferase reporters expressing wild type 3'UTR of IRAK1 or TRAF6 along with control mimic or miR-146b-5p mimic. After 48h of transfection, luciferase activity was measured and was normalized per Renilla luciferase (to control for transfection efficiency). Shown are mean ± SEM of relative luciferase activity (expressed as fold change from values measured in HUVECs transfected with control mimic). *P<0.05 compared to control mimic.

Figure 4.8: Essential role of miR-146b-5p in the inhibitory effects of Ang-1 on LPS-induced responses

A) HUVECs are transfected with control inhibitor or miR-146b-5p inhibitor. After 36h recovery period, cells are pre-treated for 24h with PBS or Ang-1 and are then exposed for 6h to vehicle (control) or E. coli LPS. Shown are means± SEM of number of adhered U937 cells to HUVECs in response to 6h exposure to vehicle (control) or LPS. *P<0.05, compared to control. #P<0.05 compared to cells pre-treated with PBS. **B)** IL6 and IL8 levels in the culture medium of HUVECs which are transfected with control inhibitor or miR-146b-5p inhibitor and are then pre-treated for 24h with PBS or Ang-1. Shown are means± SEM of IL6 and IL8 protein levels (normalized for total cell protein) measured in response to 6h exposure to vehicle (control) or *E. coli* LPS. *P<0.05, compared to control. #P<0.05 compared to cells pre-treated with PBS.

Figure 4.9: Role of miR-146b-5p in the inhibitory effects of Ang-1 on IRAK1 and TRAF6 expressions

A) Sequence alignment of miR-146b-5p and its target sites in 3' UTRs of IRAK1 and TRAF6. **B)** Relative activity of luciferase reportes expressing wild type (wt) and mutated

(mut) 3'UTRs of IRAK1 and TRAF6 in HUVECs after 24h of pretreatment with PBS or Ang-1. #P<0.05 compared to cells pretreated with PBS.

C) Representative immunoblots and means ±SEM of IRAK1 and TRAF6 protein levels (normalized to TUBULIN) in HUVECs transfected with control inhibitor or miR-146b-5p inhibitor and are pre-treated for 24h with PBS or Ang-1. #P<0.05 compared to cells pretreated with PBS.

Supplementary Figure S4.1 Expression of miR-146b-5p in HUVECs transfected with control or miR-146b-5p mimic (100nM) after 48h. Values expressed as means ± SE. *P<0.05 compared to control. N=3.

Supplementary Figure S4.2 Expression of miR-146b-5p in HUVECs transfected with miScript miRNA control or miR-146b-5p inhibitors (50nM) after 48h. Values expressed as means ± SE. *P<0.05 compared to control. N=3.

4.10 TABLES

Table 4.1: Primers used for real-time PCR experiments

Type	Sequence (5'→3')	Accession Number	Expected Size (bp)
ICAM1	**F:** GGGAACAACCGGAAGGTGTA **R:** TGGCAGCGTAGGGTAAGGTT		191
VCAM1	**F:** CATTGACTTGCAGCACCACA **R:** GATGTGGTCCCCTCATTCGT	NM_080682	202
E-Selectin	**F:** AGAAAATCTGGCACCACACC **R:** GGGGTGTTGAAGGTCTCAAA	NM_000450	128
IRAK1	**F:** CAGGTTTCGTCACCCAAACA **R:** GGGCTGTACCCAGAAGGATG	NM_001025242	142
TRAF6	**F:** TGGTTGCCATGAAAAGATGC **R:** CAAGGCGACCCTCTAACTGG	NM_004620	200
TLR4	**F:** CAGAGTTTCCTGCAATGGATCA **R:** GCTTATCTGAAGGTGTTGCACAT	NM_138557	106
MyD88	**F:** GGCTGCTCTCAACATGCGA **R:** TGTCCGCACGTTCAAGAACA	NM_001172567	177
IRAK4	**F:** CTTGGATGGTACTCCACCACT **R:** CCCTGAGCAATCTTGCATCTC	NM_001114182	298
TAK1	**F:** GGCAGATGGGATAGACACCAG **R:** CGAGCCCAGTGCATTGAGAG	NM_003298	379
β-Actin	**F:** AGAAAATCTGGCACCACACC **R:** GGGGTGTTGAAGGTCTCAAA	NM_001101	123
18S	**F:** GAGGCCATGATTAAGAGGGATG **R:** AAACTCCGACTTTCGTTCTTGG	NC_000024.8	140
hsa-mir-146b-3p	TGCCCTGTGGACTCAGTTCTGG	MIMAT0004766	-
hsa-mir-146b-5p	GTGAGAACTGAATTCCATAGGCT	MIMAT0002809	-
hsa-mir-146a	GTGAGAACTGAATTCCATGGGTT	MIMAT0000449	-
U6 snRNA	ACTAAAATTGGAACGATACAGAGA	NR_004394.1|	-

Chapter 5

Original Contribution to Scientific Knowledge

The research carried out to produce this thesis has generated several original contributions:

Chapter 2: Regulation of angiopoietin-1 signaling in endothelial cells by dual-specificity phosphatases 1, 4 and 5

1- In ECs, Ang-1 induces transient increases in the expressions of DUSP1, DUSP4, and DUSP5 through activation of the ERK1/2, p38, and SAPK/JNK signaling pathways

2- Ang-1 induction of the expressions of DUSP1, DUSP4, and DUSP5 is achieved through enhanced transcription, which is mediated by AP-1 and Egr-1 transcription factors

3- DUSP1 and DUSP5 regulate Ang-1-induced p38 and ERK1/2 phosphorylation, respectively, while DUSP4 regulates Ang-1-induced ERK1/2, p38, and SAPK/JNK phosphorylation

4- DUSP4 plays an important role in Ang-1-induced EC survival and inhibition of apoptosis

5- DUSP1 and DUSP4 play important roles in Ang-1-induced cell migration.

Chapter 3: Estradiol-dependent regulation of angiopoietin expression in breast cancer cells

1- Ang-1 expression inversely correlates with the presence of ERα in breast cancer cell lines

2- Treatment with estradiol inhibits the mRNA expression of Ang-1, Ang-2 and Ang-4 in ERα+ breast cancer cells

3- Ang-1 expression in vivo is higher in ERα− xenografts than in ERα+ xenografts, and correlates with markers of angiogenesis.

4- Reducing the circulating levels of estrogen by ovariectomy in animals with ERα+ xenografts results in increased Ang-1 expression

Chapter 4: Angiopoietin-1 inhibits Toll-Like Receptor 4 signaling in endothelial cells: role of miR-146b-5p

1- Prolonged treatment of ECs with Ang-1 (24h) results in significant attenuation of LPS-induced leukocyte adhesion, adhesion molecule expression, pro-inflammatory cytokine production, p38 and SAPK/JNK phosphorylation, and NFκB activation

2- Prolonged treatment of ECs with Ang-1 (24h) reduces the expression of IRAK1 and TRAF6 protein expression

3- Expression of high levels of miR-146b-5p in ECs using a miRNA mimic results in significant attenuation of LPS-induced leukocyte adhesion, adhesion molecule expression, pro-inflammatory cytokine production; as well as p38, SAPK/JNK and NFκB activation

4- Prolonged Ang-1 treatment reduced the expression of IRAK1 and TRAF6 proteins through the induction of miR-146b-5p in ECs

5- Inhibition of miR-146b-5p with a selective miRNA inhibitor reverses the inhibitory effect of Ang-1 on LPS-induced leukocyte adhesion, cytokine production and IRAK1 and TRAF6.

6- In contrast with Ang-1, Ang-2 significantly reduces the expression of miR-146b-5p in ECs

Chapter 6

Discussion and Opportunities for Future Research

Regulation of angiopoietin-1 signaling in endothelial cells by dual-specificity phosphatases 1, 4 and 5

Ang-1 simultaneously activates the MAPK signaling pathways ERK1/2, p38 and SAPK/JNK to exert its biological functions in the vasculature [157, 159]. Previously, an analysis of the Ang-1 transcriptome performed in our lab suggested that Ang-1 induces the expression of immediate-early genes as a mechanism to coordinate pro-angiogenic and pro-survival signaling in ECs [463]. Our results show that DUSP1, DUSP4 and DUSP5 are transiently induced by Ang-1 as a negative feedback mechanism to effectively inactivate ERK1/2, p38 and SAPK/JNK. Moreover, each DUSP showed distinct substrate specificity and their function was found to be non-redundant. DUSP1 dephosphorylated p38, DUSP4 inactivated all three MAPKs ERK1/2, p38 and SAPK/JNK, and DUSP5 was specific for ERK1/2.

The induction of DUSPs as negative modulators of MAPK signaling pathways activated by angiogenic factors has been described before for VEGF and thrombin [247, 248]. Our observations that Ang-1-induced DUSP1 inactivates p38 and regulates the migratory response of ECs correlate with those described for VEGF and thrombin [247, 248]. Our results also show that Ang-1 induces DUSP5 in a similar manner to VEGF [248]. However, DUSP5 was not involved in the proliferation of ECs, perhaps due to the fact that Ang-1 is a weaker mitogen than VEGF [13]. Interestingly, when Ang-1 and VEGF were given in combination DUSP1 and DUSP5 mRNA induction was potentiated, suggesting that these phosphatases might play a role in the synergistic effect of VEGF and Ang-1 in angiogenesis [464-466].

The induction of DUSP4, a phosphatase known to regulate apoptic processes, by Ang-1 and not by VEGF represents one of the main findings of this study [54, 467][36]. VEGF and Ang1 have both, independent and complementary functions in angiogenesis and

vascular integrity [468]. In contrast with VEGF, Ang-1 has low mitogenic activity but exerts a strong pro-survival effect, which according to our observations is partially mediated by the induction of DUSP4. DUSP4 was the only inducible DUSP able to dephosphorylate SAPK/JNK, a central pathway in the regulation of cellular death in response to cytokines, UV irradiation and cellular stress [469, 470]. Moreover, DUSP4 was the only phosphatase required for the anti-apoptotic effect of Ang-1 under conditions of serum deprivation. In the absence of serum ECs activate the SAPK/JNK pathway and undergo apoptosis. It has been demonstrated that Ang-1 is able to reverse apoptosis caused by serum deprivation through the inhibition of SAPK/JNK activation [159]. Based on this evidence, we speculate that Ang-1-induced DUSP4 could be one of the mechanisms through which Ang-1 attenuates the SAPK/JNK pathway in ECs under conditions of serum deprivation. DUSP4 has been described as an inhibitor of apoptosis in various systems through its ability to limiting SAPK/JNK activation [257, 264, 265]. Adenoviral expression of DUSP4 was able to rescue HUVECs from TNF-α-induced apoptosis when NF-κB signaling was repressed using a DN-IKKβ by selectively dephosphorylating SAPK/JNK [264], and overexpression of DUSP4 in HEK-293 cells treated with UV and cisplatin partially rescued these cells from apoptosis also by reducing SAPK/JNK activation [265]. Additionally, the transcription factor HoxA10 inhibits apoptosis in undifferentiated myeloid cells by activating DUSP4 transcription and thus impairing SAPK/JNK activation [257]. DUSP1 and DUSP4 are also required for Ang-1-induced migration *in vitro*. Both phosphatases are able to inactivate p38, and influence EC migration in a similar way as VEGF signaling [248].

In this study, we did not observe changes in the mRNA stability of Ang-1-induced DUSPs and promoter assays for DUSP1 and DUSP4 revealed that Ang-1 can induce the

transcription of both proteins. Additionally, we found that the transcription factors AP-1 and Egr-1 play a role in DUSP1, DUSP4 and DUSP5 induction by Ang-1. However, additional experimentation is required to elucidate the exact transcriptional mechanisms behind DUSP induction by Ang-1 in detail. Moreover, we do not exclude the possibility that post-traductional mechanisms cooperate with DUSP induction to inactivate MAPKs downstream of Ang-1. DUSPs are very sensitive to post-traductional modifications such as oxidation, phosphorylation and acetylation, which additionally regulate DUSP activity and stability [136, 243, 244, 246, 475]. DUSPs contain a redox-sensitive cysteine motif in their catalytic domain and can undergo oxidation by ROS, which results in the loss of phosphatase activity [240, 245]. Ang-1 signaling in ECs induces the transient production of ROS by a Rac-1-dependent NADPH oxidase [159]. Moreover, inhibition of ROS production by NADPH oxidase increases p38 basal phosphorylation and Ang-1-induced ERK1/2 phosphorylation without affecting AKT or SAPK/JNK activation [159]. The effect of ROS generated by Ang-1 signaling on MAPKs has led us to hypothesize that perhaps oxidation of DUSPs in response to Ang-1-induced ROS production could be potentiating p38 and ERK1/2 activation; and it would be interesting to address this issue in future studies. DUSP1 is a potential candidate for the observed increase in p38 phosphorylation in the absence of ROS since this phosphatase not only inactivates p38 in ECs, but also has been shown to suffer oxidation in various models [245]. For instance, ROS produced by the NADPH oxidase 4 in response to TFG-β increase the amount of thiol modifications in DUSP1 which results in increased phosphorylation of p38 and expression of plasminogen activator inhibitor-1 (PAI-1) in fibroblasts [245].

In summary, our results highlight the importance of phosphatases in the regulation of MAPK signaling pathways activated by Ang-1, and demonstrate that the magnitude and duration of MAPK phosphorylation is largely responsible for the coordinated angiogenic and pro-survival responses that we observe in ECs stimulated with Ang-1.

Estradiol-dependent regulation of angiopoietin expression in breast cancer cells

An important step in tumor progression occurs when small, avascular lesions change the balance between pro- and anti-angiogenic factors, promote angiogenesis and take over the host vasculature [290, 309, 311]. Cells from within the tumors frequently express VEGF, FGF-2, and angiopoietins, which can influence their growth and metastatic potential [310, 311, 313]. In this study, we examined the expression of Ang-1, Ang-2 and Ang-4 in human breast cancer cell lines and the effect of estrogen in their expression. We found that Ang-1 expression inversely correlated with ERα expression in breast cancer cell lines and tissues. These results suggest that Ang-1 expression is higher in ERα- tumors, characterized by an invasive phenotype that often correlates with poor prognosis [298]. The loss of hormonal dependence in breast cancers has been linked with the appearance of mesenchymal features in the tumors and increased metastasis [476, 477]. Epithelial-mesenchymal transition (EMT) describes a process through which cells lose their epithelial characteristics in order to acquire a mesenchymal phenotype that increases their motility and facilitates their invasion into the surrounding microenvironment [477, 478]. The implications of EMT in the progression of breast cancers that are unresponsive to estrogen are beginning to emerge. The zinc-finger transcriptional repressors Snail and Slug have been described as central regulators of EMT in epithelial-derived carcinomas, and various studies have suggested a possible role for

estrogen in inhibiting EMT by regulating their expression [479, 480]. Moreover, there is evidence that hormonal resistance in breast tumors correlates with increased Snail expression [481, 482]. Several growth factors and signal transduction pathways such as Wnt, Notch and integrins are able to induce EMT in epithelial cells [477, 478]. Interestingly, Ang-2 has been found to strongly promote breast cancer invasion and metastasis by inducing EMT through the activation of signaling cascades downstream of ß1 integrin [206, 369]. Ang-2 induces loss of E-cadherin expression, activates the glycogen synthase kinase-3 beta (GSK-3β)/Snail/E-cadherin pathway, and stimulates motility in MCF-7 cells which do not express Tie-2 receptors [206]. Furthermore, blocking β_1 or α_5 integrins reduces the effect of Ang-2 on EMT [206]. Additional studies have demonstrated that Ang-2 can also interact with $\alpha_v\beta_1$ integrins in glioma cells to induce the expression of matrix metalloproteinase-2 and increases their invasiveness [205]. Initially, angiopoietins were considered to signal exclusively on ECs through Tie-2 receptors, but increasing evidence has shown that angiopoietins can affect the survival, migration and adhesion of fibroblasts, cardiac and skeletal myocytes, and cancer cells through integrins [195-198]. Moreover, in ECs both Ang-1 and Ang-2 can engage $\alpha_5\beta_1$ and $\alpha_v\beta_5$ integrins to activate ERK1/2 and FAK; and a monomeric Ang-1 variant containing the fibrinogen-like receptor-binding domain only can bind to $\alpha_5\beta_1$ integrin to stimulate adhesion and ERK1/2 activation [195, 483]. These observations raise the question as to whether the high expression of Ang-1 that we detected in ERα- cell lines, like MDA-MB-231, is capable to signal autocrinally and thus contribute to the mesenchymal phenotype and the invasiveness found in these cells in a similar manner as Ang-2.

The role of Ang-1 in tumor growth is controversial. Our results show that cell growth and tumor volume are inhibited in breast cancer cells transfected with ERα (S30), as compared to ERα− (MDA-MB-231) cells, both *in vitro* and in murine mammary xenografts. Furthermore, the inhibitory effects of ERα on tumor growth are partly reversed when estrogen is depleted, a finding in accordance with other studies [484, 485]. In the case of S30 cells the levels of Ang-1 are lower than in their counterparts MDA-MB-231 cells, which could suggest that lower levels of Ang-1 coincide with reduced growth. However, studies in mice have demonstrated that ectopic expression of Ang-1 reduces xenograft tumor growth of MCF-7 breast cancer cells, A431 squamous cell carcinoma cells and HT-29 colon cancer cells as a result of increased pericyte coverage and reduced angiogenesis [332-334]. In our *in vivo* model, we observed that ER+ xenografts had more CD31 positive vessels a finding that correlated with increased VEGF and Ang-1 expression. In accordance with these results are studies in which Ang-1 overexpression also increased the growth of human cervical cancers in mice and gliomas in rats by promoting angiogenesis [335, 336]. It is important to mention that the effect of Ang-1 on tumor progression has been centered on its effect in angiogenesis and vessel stabilization, while the effect of Ang-1 directly on tumor cells has been largely ignored [332-336]. For this reason, broader studies in which the effects of Ang-1 on both the tumor and the tumor microenvironment are taken into account are urgently needed.

In this study, we focused mainly on the effect of ERα on tumor growth *in vitro* and in mammary xenografts. However, the fact that Ang-1 expression seems to correlate with the aggressive breast cancer cell line MDA-MB-231 suggests that Ang-1 could potentially influence breast cancer metastasis. Although the role of Ang-2 in metastasis has been

extensively documented, few studies have addressed the contribution of Ang-1 to this process [206, 337, 345, 353, 368, 369]. A study in which Ang-1 was administered systemically into mice via adenoviral vectors showed that Ang-1 increased metastasis when LNM35/Luc lung cancer cells were implanted subcutaneously [337]. Moreover, Ang-1 caused vessel enlargement which facilitated the dissemination of tumor cells in these mice [337]. The effect of Ang-1 on the vasculature is highly exaggerated in this model, possibly generating physiological changes that are probably not present in a real scenario of tumor dissemination and metastasis. For this reason, it would be interesting to explore animal models of metastasis in which the tumor cells themselves expressed varying levels of Ang-1 in order to understand how Ang-1 expression affects their metastatic potential. Additionally, Ang-1 is a strong inhibitor of vascular permeability and inflammation, both processes relevant for tumor progression and metastasis, and it is unknown how Ang-1 affects them in breast cancer [13, 168, 171].

In summary, our results strongly suggest that Ang-1 expression in breast cancer cells is hormonally regulated by the ERα. In the search for novel therapeutic interventions that could translate into better clinical outcomes, the angiopoietins are attractive targets to be combined with chemotherapeutic agents and/or other anti-angiogenic therapies such as VEGF blockade [10, 11]. Therefore, dissecting the individual functions of Ang-1 and Ang-2 in cancer could lead to more effective therapies in the treatment of breast cancer. Moreover, a peptide-Fc fusion protein which potently and selectively inhibits Ang-1 and Ang-2 by binding to Tie-2 receptors called AMG 386 is already being tested in clinical trials and represents a promising therapy for the treatment of solid tumors [486, 487].

Angiopoietin-1 inhibits Toll-Like Receptor 4 signaling in endothelial cells: role of miR-146b-5p

The angiopoietins not only regulate angiogenesis and vascular homeostasis, but they can also influence inflammation. Ang-1 has been described as an anti-inflammatory agent, whereas Ang-2 is known to prime the endothelium to potentiate pro-inflammatory responses [13, 169, 488]. The effect of Ang-1 on endothelial inflammation has been mostly studied at short-term, often when administered simultaneously with inflammatory stimuli, and observations of long-term Ang-1 signaling have been largely overlooked [115, 175, 178]. Moreover, sustained expression of Ang-1 in murine models of sepsis has been shown to reduce lung injury and improve survival [408, 410, 411, 489]. Here, we demonstrate that prolonged Ang-1 treatment can limit the inflammatory responses initiated by the innate immune system and we propose a mechanism through which Ang-1 signaling directly affects the outcome of TLR activation through miRNA regulation.

There is increasing evidence implicating miRNAs in the control of angiogenesis and inflammation [414, 490]. In this study, we were interested in finding miRNAs whose expression is modulated by Ang-1 signaling in ECs. Data obtained from miRNA affymetrix arrays performed in our lab showed that Ang-1 not only induced the expression of miR-146b-5p, but also was able to induce and/or downregulate several other miRNAs at 12, 24 and 48h; stressing the idea that angiogenic factors regulate the expression levels of miRNAs to carry out their functions. Some of the miRNAs induced by Ang-1, like miR-424 and miR-126, have been described before as regulators of vascular processes [491-493]. Expression of miR-424 has been found to be upregulated in ECs by VEGF and FGF-2, and modulates these signaling pathways by directly targeting the receptors [491]. Similarly, miR-126 regulates Ang-1 signaling and vessel stabilization by targeting the PI-3 kinase regulatory subunit 2

(p85β) [493]. However, the large majority of the miRNAs modulated by Ang-1 have unknown functions and targets, opening new and exciting possibilities for the study of miRNAs as downstream effectors of Ang-1 signaling in the vasculature. It would be interesting to elucidate how the migration, survival and differentiation of ECs, all biological processes strongly modulated by Ang-1 signaling, are influenced by miRNAs.

The miR-146b-5p belongs to the miR-146 family, whose roles in innate immune responses have been largely studied [380, 385, 414, 494]. Although others have linked miR-146b-5p to NF-κB signaling, the function of this miRNA in inflammation has not been studied in detail [385]. Our current knowledge on the miR-146b gene, located on the long arm of human chromosome 10 and transcribed independently from miR-146a, is very limited [380, 449]. There is only a report showing that miR-146b upregulation by IL-1β depends on the ERK1/2 and SAPK/JNK signaling pathways [454]. In our study, we did not elucidate the signaling pathways activated downstream of Ang-1 that are responsible for miR-146b transcription, nor the transcription factors implicated in this process. For future studies it would be important to examine how miR-146b-5p is transcribed in ECs in response to Ang-1. Furthermore, it would be relevant to determine if the induction of miR-146b-5p is dependent on the activation of Tie-2 receptors, as well as to asses the role of integrin signaling in the induction of its expression by Ang-1.

In this study, we describe a new function for miR-146b-5p in the endothelium distinct from miR-146a, which was not induced by Ang-1 in our model. Interestingly, we observed that treatment with Ang-2 dramatically decreased miR-146b-5p expression, suggesting that Ang-1 and Ang-2 have opposing roles in the expression of this miRNA. Moreover, Ang-2 treatment also decreased miR-146a expression. Ang-2 is an antagonist of

Tie-2 receptors that can also behave as a context-dependent agonist [79, 88, 95, 96]. In contrast with Ang-1, Ang-2 is a destabilizer of the vasculature that potentiates angiogenic and inflammatory responses [186, 187]. In Ang-2 deficient mice, TNF-α is unable to elicit a rapid pro-inflammatory response and fails to induce adhesion molecule expression [488]. Our results are consistent with with studies that demonstrate Ang-2 clearly opposes the anti-inflammatory effects of Ang-1 [169, 488].

In this study, we found that Ang-1 induces the expression of miR-146b-5p as a mechanism through which Ang-1 signaling modulates the activation of TLR4 signaling, and thus influences innate immune responses. Ang-1 reduces the levels of IRAK1 and TRAF6 proteins via miR-146b-5p expression which results in a decrease in MyD88-dependent signaling activation, cytokine expression and leukocyte adhesion upon stimulation with LPS. Our results are consistent with studies that highlight the importance of IRAK1 and TRAF6 in TLR signaling activation. For instance, in IRAK1 knockout mice the activation of NF-κB, p38 and SAPK/JNK by TLR4 and IL-1R is impaired, as are the expression of IL-6 and TNF-α [495-497]. Similarly, ECs deficient in TRAF6 exhibit defective expression of pro-inflammatory cytokines and adhesion molecules after stimulation with oxLDL, a modified lipid able to initiate TLR signaling in a model of atherosclerosis [498]. Additionally, mice deficient in IRAK1 are less susceptible to the lethal effects of LPS when compared to wild-type mice, and an intronic SNP in the TRAF6 gene that increases its mRNA expression has been linked to a higher risk of sepsis-induced acute lung injury [496, 499]. miRNAs have many possible targets with divergent functions, and one of their many functions is to mediate crosstalk between signaling pathways [500, 501]. For these reasons, we do not exclude that the induction of miR-146b-5p by Ang-1 could possibly affect not only

proteins involved in TLR signaling, but also other signaling cascades. Evidence from cancer studies implicates miR-146b-5p in the regulation of the TGF-β signaling pathway by targeting *SMAD4;* and in the modulation of cell migration by targeting epidermal growth factor receptor (EGFR) and matrix metalloproteinase 16 (MMP16) [456, 502]. These studies suggest that Ang-1-induced miR-146b-5p could potentially affect the TGF-β and EGFR signaling pathways and thus impact the angiogenic and migratory effect of Ang-1 in ECs. Moreover, Ang-1-induced miR-146b-5p could have additional mRNA targets that have not yet been described and it would be interesting to address this issue in future studies.

Our study also raises the question as to whether Ang-1-induced miR-146b-5p affects other signaling pathways that depend on IRAK1 and/or TRAF6. A couple of studies have highlighted the potential role of TRAF6 in the control of angiogenesis. In ECs IL-33 signaling through ST2 receptors promote angiogenesis and vascular permeability through TRAF6-mediated activation of PI-3K, AKT and eNOS [503]. Furthermore, IL-1β is able to assemble a pro-angiogenic signaling complex comprised of caveolin-1, TRAF6, p38 and MK2 [504].

Finally, several studies have suggested Ang-1, or Tie-2 agonists, could have a potential therapeutic use in the treatment of septic shock [408, 410, 411]. The pathophysiology of severe sepsis is associated with high mortality rates and it is the host rather than the nature of the pathogen what largely determines the patient outcome [505]. Our findings suggest a widespread role of Ang-1 in limiting inflammatory responses of the innate immune system initiated not only by LPS but by other microbial components recognized by TLRs, and support a potential role for miR-146b-5p as a novel approach in the treatment of sepsis.

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