Sex Differences in Endothelial Cell Angiogenic Capacity

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Abstract

There is a sex-specific difference in susceptibility to develop obesity-related disorders. Our lab's previous study on high-fat fed mice indicated higher angiogenesis in visceral adipose tissue of female mice, which accompanied preserved metabolic function of adipose tissue and better systemic glucose metabolism. The goal of this study was to determine if there are sex-related differences in endothelial cells (EC) angiogenic capacity that may contribute to higher adipose tissue angiogenesis in females.

To achieve this, visceral adipose tissue explants as well as cultured EC of male and female mice were used. Female EC showed higher sprouting success *ex-vivo*. Furthermore, female EC had higher proliferation *in vitro*. Protein levels of FoxO1 and phosphorylation of p38 MAPK were higher in females. Inhibition of PFKFB3 decreased proliferation only in males.

This project provided evidence for inherent sex-related differences in EC angiogenic capacity that may be related to the higher adipose tissue angiogenesis in females.

Dedication

This thesis is dedicated to my late mother Zohreh Naziri, whose sincere love and support has continually inspired my life.

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AKT	Protein kinase B
ANG-1	Angiopoietin 1
AT	Adipose tissue
CPT1a	Carnitine palmitoyl- transferase 1A
Dll4	Delta like ligand 4
E2	Estradiol
EC	Endothelial cell
ECM	Extra cellular membrane
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitric oxide synthase
ERK1/2	Extracellular signal-regulated kinase1/2
ESR1	Estrogen receptor 1
ET-1	Endothelin 1
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FFA	Free fatty acid
FGF	Fibroblast growth factor
FoxO1	Forkhead box protein O1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GIST-T1	Gastrointestinal stromal tumor cells
GPCR	G protein coupled receptor
GTP	Guanosine triphosphate
HDL	High-density lipoprotein
Hes1	Hairy and enhancer of split-1
Hey1	Hairy/enhancer-of-split related with YRPW motif protein 1
HFD	High-fat diet
HK2	Hexokinase-2
HPMEC	Human pulmonary microvascular endothelial cells
Hprt1	Hypoxanthine-guanine phosphoribosyltransferase
HUVEC	Human umbilical vein endothelial cell
ICAM	Intercellular Adhesion Molecule
IL-1	Interleukin-1
Jag1	Jagged1
JNK	c-Jun N-terminal kinase
Kdr	Kinase insert domain receptor
LDL	Low-density lipoprotein
MAPK	Mitogen-activated protein kinase
MAPKK/MEK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase

MMP	Matrix metallopeptidases
MST1/2	Mammalian sterile 20-like kinases 1 and 2
NCID	Notch intracellular domain
PDGF	Platelet-derived growth factor
PECAM1	Platelet endothelial cell adhesion molecule 1
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6 bisphosphatase 3
PGF	Placental growth factor
PI3K	Phosphoinositide 3-kinase
PLC ɣ	Phospholipase C ɣ
q-RT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RTK	Receptor tyrosine kinase
SHB	Src Homology-2 domain containing protein B
siRNA	Small interfering RNA
SVF	Stromal vascular fraction
TGF-β1	Transforming growth factor beta 1
TNFα	Tumor necrosis factor α
TXA-2	Thromboxane A2
VCAM	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
VPF	Vascular permeability factor
VWF	Von Willebrand factor
WI-38	Fibroblasts derived from lung tissue
YAP-1	Yes-associated protein 1

Chapter 1. Literature Review

1.1 Microvasculature

Survival of mammalian cells is dependent on blood vessels of the circulatory system that provide oxygen and nutrients as well as transferral of metabolic by-products from all tissues in the body. The recognition of a blood circulation system as it is known now dates back to the 16th century and the pioneering experimental methods of William Harvey, which is considered a turning point in the annals of biomedical history (Bylebyl, 1978).

The circulatory system is one of the first functional systems to form during embryogenesis. Blood vessels comprise only 7% of the total body volume, yet they are ubiquitously distributed throughout the body as a result of their highly organized and complex branching pattern and in the adults add up to 90,000 km in total length. (Carmeliet, 2000; Eelen et al., 2017; Gutterman et al., 2016). This system is comprised of the macrovasculature (arteries and veins) responsible for transporting large volumes of blood rapidly toward or away from the organs and microvasculature (arterioles, venules and capillaries) that forms a network that conducts oxygen and nutrient exchange at the cell level in the tissues (Yuan and Rigor, 2011). Arterioles are involved in controlling the local blood flow to the tissue, whereas capillaries and postcapillary venules are the main sites of gas and nutrient exchange (Levick, 1991).

The capillary structure was first described by Theodor Schwann in the nineteenth century. The term endothelium was later used by Wilhelm His to

describe the internal layer of cells lining the capillaries (Bikfalvi, 2017). These quiescent cells form a monolayer called phalanx cells that are connected to each other by junctional molecules such as vascular-endothelial (VE) cadherin, claudins and platelet endothelial cell adhesion molecule (PECAM1) (Carmeliet and Jain, 2011). On the lumenal side, endothelial cells (EC) express surface glycoproteins; while on the basolateral sides these cells express cell surface integrins to attach to the basal membrane. The basement membrane consists of laminin, collagen IV and other extracellular matrix (ECM) molecules depending on the type of microvessel. Capillaries are composed of a layer of EC surrounded by basement membrane and a sparse layer of perivascular (mural) cells such as pericytes. EC produce several factors including PDGF-B, TGF-β1 and VEGF required for pericyte recruitment, differentiation, proliferation and migration (Betsholtz, 2004; Darland and D′Amore, 2001; Kashiwagi et al., 2005; Ribatti et al., 2011). As the vessel structure increases in complexity, pericytes and smooth muscle cells wrap around the EC to help maintain the integrity of the vessel structure (Jain, 2003).

EC are indispensable for proper function of vascular system. Since they are in constant contact with the flowing blood stream, they are perfectly positioned to modulate a number of biological systems in blood. EC are involved in safeguarding transport of blood, controlling vascular permeability and regulation of vascular tone as well as inflammation (Rohlenova et al., 2018; Sturtzel, 2017). The permeability of the blood vessel is mostly controlled by

EC-EC junctions (Claesson-Welsh, 2015; Levick, 1991). The endothelium releases a number of vasoactive factors that are either vasodilatory such as nitric oxide and prostacyclin or vasoconstrictive such as endothelin-1 and thromboxane (Sandoo et al., 2010). In tissue trauma, EC maintain their barrier function in cooperation with platelets which leads to the process of coagulation. The coagulation cascade culminates in the formation of clots that plug the injured blood vessel. EC play an essential role in regulating the coagulation cascade especially by expressing procoagulant molecules such as von Willebrand factor (VWF) (Engelmann and Massberg, 2013; Kazmi et al., 2015; Yau et al., 2015). EC actively mediate immune responses at places of injury or infection (Pober and Sessa, 2007). EC paracrine function is essential in modulation of immune cells as they secrete chemokines, interleukins, interferons and growth factors. Moreover, EC express adhesion molecules such as endothelial-selectin, platelet-selectin, intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 and regulate recruitment of immune cells in inflammation (Vestweber, 2012).

1.2 Vasculogenesis and Angiogenesis

The first blood vessels in body are formed during embryogenesis through in situ differentiation of precursor cells (angioblasts) to EC that assemble into a primitive vascular web (plexus) in a process termed vasculogenesis (Carmeliet, 2003). After formation of the primary vascular plexus, generation of more EC leads to development of new capillaries. In adults, existing blood vessels undergo diameter increase through a process called arteriogenesis.

New capillaries on the other hand, are formed from pre-existing capillaries through angiogenesis (Carmeliet, 2000; Heil et al., 2006). John Hunter, a British surgeon, was the first scientist who used the term angiogenesis in 1787 (Hall, 2005). Carl Thiersch demonstrated the formation of new blood vessels in tumors in 1865. In the early 20th century, scientists started to focus on angiogenesis and vascularization as a potential approach to predict the tumor malignancy as well as studying the delivery of therapeutic agents administrated against these tumors.

In healthy adults, capillary EC are quiescent and are maintained in this state by shear stress as well as by signaling from pathways like NOTCH, angiopoietin-1 (ANG-1) and fibroblast growth factors (FGFs). Production and maintenance of a complex network of vessels requires an intricate progression of events and signaling pathways controlling angiogenesis as well as modulation of EC survival and maintenance of vessel integrity by mural cells such as pericytes (Murakami, 2012; Quaegebeur et al., 2010). Physiological angiogenesis occurs in the female reproductive system, during wound healing, in response to exercise in skeletal muscles and during expansion of adipose tissue whereas pathological angiogenesis causes diabetic retinopathy and also is considered to be one of the hallmarks in tumor growth (Carmeliet and Jain, 2000; Gustafsson, 2011).

1.3 Mechanisms of Angiogenesis

New capillaries are formed through angiogenesis by two different mechanisms. In (abluminal) sprouting angiogenesis, growth of endothelial

sprouts from pre-existing capillaries gives rise to new capillaries (Carmeliet, 2000). In this process, degradation of ECM is followed by proliferation and migration of EC. This rearrangement of EC leads to formation of the new blood vessel lumen and functional maturation of the new capillary (Risau, 1997). Sprouting angiogenesis can be prompted by a number of different angiogenic stimuli including inflammation and hypoxia. Factors such as VEGF, FGF and ANG-1 are released by a hypoxic or inflammatory cell, which activate EC within a quiescent vessel and coordinate the proliferation and migration of EC (Carmeliet, 2000; Ferrara et al., 2003; Phng and Gerhardt, 2009; Risau, 1997). Initiation of sprouting is accompanied by pericytes releasing themselves from the ECM by proteolytic degradation mediated by matrix metalloproteinases (MMPs). Subsequently, EC loosen their junctions and migrate into the surrounding ECM. At the same time, protrusions called filopodia from activated EC start to invade the basement membrane (Arroyo and Iruela-Arispe, 2010). Specialized actin-based structures called podosomes are critically involved in degrading the ECM and promoting invasion and migration of EC and this process is modulated by VEGF and Notch signaling (Murphy and Courtneidge, 2011). To prevent the activation and migration of all EC towards the angiogenic signal, some cells are selected as guiding tip cells and the neighbours assume the stalk cell phenotype (Arroyo and Iruela-Arispe, 2010; Carmeliet, 2003; Carmeliet and Jain, 2011; Risau, 1997). Tip cells are characterized by their migratory behavior that guides the growing sprout towards the angiogenic cues in the

environment whereas stalk cells are highly proliferative and support the stability of the vessel and formation of the lumen (Gerhardt et al., 2003).

Another type of angiogenesis, which is called intussusceptive or non-sprouting angiogenesis, involves splitting of a pre-existing vessel by transcapillary pillars of ECM to create two parallel vessels or to remodel the existing blood vessel (Makanya et al., 2009). This process begins with migration of opposing endothelial walls toward each other forming an intraluminal pillar followed by reorganization of EC-EC junctions. The newly formed pillar is invaded by pericytes and myofibroblasts depositing ECM into the pillar. Splitting of the capillary into two new capillaries is finalized when several pillars fuse with each other (Spiegelaere et al., 2012).

Intussusceptive angiogenesis requires lower rates of EC proliferation compared to sprouting angiogenesis. Moreover, it is accomplished in a shorter time and with minimal tissue degradation (Makanya et al., 2009). Intravascular blood flow is the main factor in initiating and modulating intussusceptive angiogenesis (Mentzer and Konerding, 2014). Non-sprouting angiogenesis was first described by Caduff et al. (1986) in rat pulmonary vessel development during embryogenesis. Their observations indicated that in the phase of rapid alveolarization and capillary growth in rat embryos, new capillary formation is dependent solely on intussusceptive rather than sprouting angiogenesis (Caduff et al., 1986). Non-sprouting angiogenesis has been reported to occur in myocardium and skeletal muscle vascular bed especially in response to exercise training (Caduff et al., 1986; Van

Groningen et al., 1991; Zhou et al., 1998). Moreover, some tumors such as human colon adenocarcinoma are vascularized through intussusceptive angiogenesis (Krishna Priya et al., 2016). Molecular mechanisms involved in modulation of intussusceptive angiogenesis are not as well understood compared to sprouting angiogenesis (Spiegelaere et al., 2012) and thus I will not focus more on it in this thesis.

1.4 Endothelial cell angiogenic pathways

Sprouting is coordinated by multiple cellular and molecular pathways. The following are some of the major endothelial angiogenic pathways involved.

1.4.1 VEGF family

Vascular endothelial growth factor, VEGF-A is the main growth factor in an angiogenic superfamily that also includes VEGF-B, C, D, E and placenta growth factor (PGF) (Kowanetz and Ferrara, 2006). The existence of VEGF was first reported by Senger and Dvorak in 1983. This vascular permeability factor (VPF) was purified from tumor cells and was able to promote accumulation of ascites fluid in the abdominal cavity (Senger et al., 1983). In 1989, VPF was isolated and purified from tumors in the Folkman lab and at the same time it was purified and sequenced by Ferrara and was called VEGF (Ferrara and Henzel, 1989; Rosenthal et al., 1990).

Hypoxia is a major inducer of VEGF production. Under hypoxic conditions, hypoxia inducible factors (HIFs) are stabilized and bind to the promoter region of *Vegfa* enhancing transcription (Pugh and Ratcliffe, 2003; Wang and Semenza, 1993). VEGF gene expression also is upregulated by a number of

growth factors and cytokines including platelet-derived growth factor A (PDGF-A), FGF, epidermal growth factor (EGF), Tumor necrosis factor α (TNF α), transforming growth factor beta 1 (TGF-β) and interleukin-1 (IL-1) (Carmeliet, 2005; Ferrara et al., 2003).

The rapid increase in vascular permeability was the first known function of VEGF (Senger et al., 1983; Thomas, 1996). Early studies also showed the mitogenic effect of VEGF on vascular EC (Leung et al., 1989; Pepper et al., 1992). Subsequently, Alon et al. (1995) proposed the instrumental role of VEGF in maintaining the viability of immature vasculature. Their study showed that the hyperoxia-induced withdrawal of VEGF leads to apoptosis and regression of retinal capillaries in retinopathy of prematurity (Alon et al., 1995). Additionally, the effect of VEGF on mediating chemotaxis and motility of EC was studied by Koch et al. in 1994. Studies with partial and complete genetic ablation of VEGF and its receptors in mice have shown the critical role of VEGF signaling in blood vessel formation and viability of embryos. Studies have shown that ablation of VEGF is lethal in mouse embryo as a result of impairment of angiogenesis and several developmental anomalies (Carmeliet et al., 1996; Ferrara et al., 1996).

In adults, autocrine VEGF has different effects on angiogenic activity of EC compared to paracrine VEGF. A study by Lee et al. (2007) using mice with VEGF ablation in EC showed the importance of autocrine VEGF in maintenance of vascular homeostasis. The systemic EC apoptosis observed in $VEGF^{ECKO}$ mice were not compensated by the effect of paracrine VEGF

produced by other cell types neighbouring EC. Furthermore, this study showed that autocrine VEGF was not involved in modulation of angiogenic response as the mice that lacked autocrine VEGF production had the same vascular density and patterning as the control group. Paracrine VEGF on the other hand is considered the main growth factor involved in angiogenic response (Gerber et al., 1999).

VEGF-A functions mainly through VEGF receptor 2 (VEGFR-2 or FLK1 or KDR) signaling. The activity of VEGFR-2 is enhanced by VEGF co-receptors called Neuropilins (NRP1 and NRP2). In addition to VEGFR2, there are 2 other tyrosine kinase receptors that bind to various VEGF isoforms. VEGF-B and PGF selectively bind to VEGFR-1 (Flt-1) and VEGF-C and D activate VEGFR3 (Alitalo et al., 2005). VEGFR1 and 2 are expressed in a number of different cells including vascular EC, monocytes, macrophages and hematopoietic stem cells. VEGFR3 on the other hand is mostly expressed in lymphatic EC and is involved in lymphangiogenesis (Alitalo et al., 2005). VEGFR3 has also been shown to be expressed in EC tip cells during sprouting (Tammela et al., 2008). Although both VEGFR1 and 2 can be activated by VEGF-A, VEGFR2 is responsible for most of VEGF signaling in vascular EC (Olsson et al., 2006). The activity of VEGFR2 is regulated by the availability of its ligand. Upon binding VEGF, VEGFR-2 dimerizes and several of its tyrosine residues are phosphorylated and depending on the phosphorylated residue, different downstream intracellular signal mediators are activated (Eliceiri et al., 1999; Guo et al., 1995).

Phosphorylation of Y951 leads to activation of Src protein, which in turn activates PI3K/AKT pathway, promoting cell adhesion, vascular permeability and cell survival. Phosphorylation of Y1175 mobilises SHB which subsequently activates FAK and controls cell attachment and migration. Y1175 phosphorylation also leads to recruitment of PLC- γ, which in turn upregulates the expression of MEK/ERK pathway leading to increased cell proliferation and migration. Finally, phosphorylated Y1214 activates p38MAPK, leading to cell migration (Gerber et al., 1998; Peach et al., 2018; Takahashi et al., 1999).

Alternative splicing of VEGF mRNA leads to production of nine VEGF protein isoforms that exert different effects on angiogenic activity of EC (Gerber et al., 1998; Kroll and Waltenberger, 1997, 1999; Waltenberger et al., 1994). These isoforms have different C-terminal sequence and therefore differ in their ability to bind to ECM molecules such as heparan sulfate proteoglycans and co-receptors such as Neuropilin-1. For example, VEGF 121 contains no heparin binding domain and is freely diffusible, whereas VEGF 189 with two heparin binding domains is found tightly associated with ECM and VEGF 165, with one heparin binding domain, has intermediate diffusive properties (Park et al., 1993). The difference in ECM binding and proteolytic release of VEGF isoforms from ECM leads to formation of a gradient of VEGF and this spatial distribution is a key regulator of angiogenesis (Vempati et al., 2014). The role of VEGF gradients in directing the formation of new capillaries has been studied in the vascularization of

mouse retina (Gerhardt et al., 2003). This model showed that VEGF gradients stimulate and guides EC at the tip of new vascular sprouts. Tip cells lead the growing sprout towards the VEGF whereas stalk cells respond to absolute concentrations of VEGF rather than the gradient and proliferate and form the lumen of the blood vessel (Phng and Gerhardt, 2009). Mice expressing only VEGF 188 (VEGF 189 ortholog), which binds strongly to ECM, have shown enhanced vascular branching and increased vascular density whereas mice expressing only VEGF 120 (VEGF 121 ortholog), which is soluble, have poor branching, enlarged vessel diameter and lack of filopodia extension in their EC (Ruhrberg et al., 2002).

1.4.2 The Notch signaling pathway

The Notch pathway controls two main processes involved in angiogenesis, proliferation and migration, by regulating the tip and stalk cell phenotypes of EC. The Notch pathway is activated when a Notch ligand on one cell interacts with a Notch receptor on the adjacent cell, which triggers proteolytic cleavage of the Notch receptor by an intracellular enzyme called γ-secretase. The released Notch intracellular domain (NCID) translocates to the nucleus and binds to its target transcription factor CSL (RBP-Jk) and upregulates the expression of target genes hairy, hairy enhancer of split (Hes-1, 5, 7) and Hes related proteins (Hey 1-3) (Iso Tatsuya et al., 2003). Activation of VEGFR2 signaling on a tip cell induces the expression of Notch ligand Dll4. Consequently, Dll4 binds NOTCH1 receptors on the neighbouring cells and inhibits expression of Hey1 and 2. Inhibition of these transcription factors

leads to decreased expression of VEGFR2 and subsequently reduces these cells' responsiveness to VEGF and elicits a stalk cell phenotype (Blanco and Gerhardt, 2013). The crucial role of Notch signaling in vascular biology has been clearly demonstrated in different rodent studies. In mice, mutations of Notch receptors, ligands and effectors cause abnormalities in the vascular system (Iso Tatsuya et al., 2003). For instance, disruption of Dll-4 or CSL in mice models results in lethality due to defects in vascular remodeling and angiogenesis (Krebs et al., 2004).

Although Dll4 is mainly expressed on tip cells, it has been shown that stalk cells also express Dll4 in lower levels and could potentially activate Notch receptors on tip cells. It is proposed that another Notch ligand called Jag1, which is strongly expressed on stalk cells, antagonizes the activation of Notch by Dll4 and effectively blocks the signaling from stalk cells back to the tip cells as well as inhibiting over activation of Notch on nearby stalk cells (Benedito et al., 2009).

1.4.3 MAPK pathway:

Mitogen-activated protein kinases (MAPK) are a highly conserved family of specialized serine/ threonine kinases (Cohen, 1997). Mammalian cells express three distinct group of MAPKs namely, extracellular signal-related kinases (ERK1/2), Jun amino-terminal kinases (JNK1/2/3) and p38 MAPK proteins (Chang and Karin, 2001). A number of receptor tyrosine kinases (RTKs), G protein-coupled receptors (GPCRs), small GTPases and integrins can activate this pathway (McKay and Morrison, 2007; Shaul and Seger,

2007). Activation of MAPK proteins requires their phosphorylation by an upstream MAPK kinase (MAPKK) such as MAPK/ERK kinase (MEK) which in turn is phosphorylated by a MAPK kinase kinase (MAPKKK) such as members of the Raf family. MAPKs respond to these signals, and through cooperation with other signal transduction pathways, can alter gene expression that ultimately regulate different cell functions such as proliferation, differentiation, development, apoptosis and inflammation (Zhang and Dong, 2007). One of the inducers of MAPK activation is VEGFR2 signaling, which leads to a series of phosphorylation cascades that are essential in regulation of EC motility and proliferation (Pontes-Quero et al., 2019).

ERK1/2:

Extracellular signal-regulated kinase-1 and 2 (ERK1/2), also known as p44/p42 MAPK, are the prototypical kinases of this family in mammalian cells. In response to signaling from VEGF, Ras/Raf/MEK/ERK1/2 are activated and upregulate angiogenic processes including proliferation and migration in EC (Hood et al., 2003; Serban et al., 2008). A study on mice showed that embryos lacking ERK1/2 are not viable due to reduced angiogenesis caused by decreased proliferation and migration in EC isolated from the aorta (Srinivasan et al., 2009). ERK1/2 promotes EC proliferation and survival as well as facilitates anti-inflammatory signals that inhibit the expression of adhesion molecules (Abe Jun-ichi et al., 2000; Tedgui Alain and Mallat Ziad, 2001). ERK1/2 promotes cell proliferation by activating

growth factors such as Elk-1 and Sep-1a in the nucleus, leading to enhanced transcription of growth related proteins such as c-Fos (Marais et al., 1993). Moreover, by regulating the expression of proteins such as Paxillin and focal adhesion kinases that are required for cell migration, ERK1/2 controls EC migration during angiogenesis (Srinivasan et al., 2009).

JNK:

cJun-N-terminal kinases (JNKs) are members of the MAPK family that are capable of phosphorylating a transcription factor called cJun. Unlike ERKs, JNKS are activated more potently in response to cytokines, cellular stress such as hypoxia and glucose deprivation and oxidative stress rather than to mitogens (Chang and Karin, 2001; Hibi et al., 1993). Studies have shown that JNKs can also be activated in response to physiological stimuli such as growth factors and shear stress (Kito et al., 2000; Kyriakis and Avruch, 2001). JNK1 and 2 are ubiquitously expressed whereas JNK3 expression is limited to brain, testis and β cells of pancreas (Solinas and Becattini, 2016). In EC, physiological JNK activation contributes to promotion of proliferation, migration and proteolysis of ECM and angiogenesis (Uchida et al., 2008).

p38:

Stress activated kinase -2 (p38) is a 38-kDa protein of the MAPK family. The p38 kinases are involved in regulation of inflammation, cell growth and differentiation, cell cycle and cell death (Ono and Han, 1999). A study by

Rousseau et al. (1997) indicated p38 MAPK mediates actin organization and cell migration in response to activation of VEGFR2 signaling. p38 is also activated in EC by cytokines such as tumor necrosis factor-a (TNFα) and interleukin-1 (IL-1) and it promotes the expression of proinflammatory molecules such as vascular cell adhesion molecule-1 (VCAM-1) (Surapisitchat et al., 2001). Furthermore, it has been shown that p38 is activated by several growth factors including FGF-2 and negatively regulates endothelial cell differentiation during tubular morphogenesis (Matsumoto et al., 2002). Inhibition of p38 leads to an increase in the formation of blood vessels, although most of these vessels are non-functional (Ono and Han, 1999). A study on chick embryo chorioallantoic membrane (CAM) angiogenesis indicated treating CAMs with p38 MAPK inhibitor in the presence of FGF-2, an angiogenic factor, leads to formation of new blood vessels with partially or entirely closed lumina due to hyperplasia of EC (Matsumoto et al., 2002).

In summary, members of MAPK pathway modulate angiogenic processes in EC in different ways. ERK1/2 is involved in promotion of EC proliferation, migration and survival. JNK is activated in response to cellular stress and hypoxia and promotes angiogenesis by increasing EC proliferation and migration.

1.4.4 FoxO1

Forkhead box O (FoxO) proteins are a group of transcription factors that link cell growth and metabolism (Vander Heiden et al., 2009). They are

downstream effectors of the phosphatidylinositol-3-OH kinase (PI3K)/AKT pathway. FoxO proteins are inhibited by PI3K signaling and the kinase activity of AKT, which leads to their translocation out of nucleus and inhibition of their action (Salih and Brunet, 2008). FoxO1 is a member of FoxO family that is expressed in high levels in EC. It is critically involved in regulation of vascular growth and coordination of metabolic and proliferative activity of EC (Eijkelenboom and Burgering, 2013). EC specific deletion of FoxO1 in mice results in embryonic lethality at day 10 accompanied with cardiovascular defects (Sengupta et al., 2012). FoxO1 keeps EC in quiescence, inhibits glycolysis and mitochondrial respiration through suppression of MYC signaling, which is a driver of anabolic metabolism and proliferation (Wilhelm et al., 2016). Studies on the subcellular distribution of FoxO1 in postnatal retinas in mice have shown higher nuclear localization in the cells forming the blood vessel plexus compared to the areas that are expanding through angiogenesis and require high EC proliferation (Wilhelm et al., 2016).

1.5 EC Metabolism

A growing body of evidence indicates that EC metabolism can regulate vessel sprouting and tip/stalk cell phenotype (De Bock et al., 2013a; Schoors et al., 2015). EC phenotypes (tip, stalk and phalanx cells) determine energy requirements and therefore metabolic states of cells.

1.5.1 Glycolysis

EC rely heavily on glycolysis compared to other cells in the body (Culic et al., 1997; Krützfeldt et al., 1990). Glycolysis is especially important in filopodia of the tip cells as they extend into hypoxic tissues and also for rapid revascularization of ischemic tissue given that at increasing distances from a capillary, interstitial levels of O_2 drop faster than levels of glucose (De Bock et al., 2013b; Gatenby and Gillies, 2004). Glycolysis is mainly regulated by two enzymes, 6-phosphofructo-2-kinase/fructose-2,6 biphosphatase 3 (PFKFB3) and Hexokinase 2 (HK2) (Yu et al., 2017). Regulating the activation of these enzymes is essential in determining the metabolic state of the EC and therefore their phenotype. A study by De Bock et al. (2013) showed that EC-specific deletion of PFKFB3 caused vascular defects, diminished the number of branch points and led to vascular regression in mouse retina. A number of growth factors and signaling pathways are involved in regulating the expression of PFKFB3 and HK2. The biomechanical stimulus of shear stress via expression of the transcription factor Kruppel-like Factor 2 (KLF2) modulates PFKFB3 transcription in EC. In mature EC in resting states, the laminar shear stress generated by the blood flow supresses the expression of PFKB3 through Kruppel-like Factor 2 (KLF2) mediated signaling, preventing the activation of the EC (Doddaballapur Anuradha et al., 2015). As mentioned previously, FoxO1 is also involved in inhibiting glycolysis via suppression of MYC, which results in decreased transcription of *HK2* (Wilhelm et al., 2016). Angiogenic growth

factors such as VEGF and FGF are also involved in regulation of glycolysis. VEGF promotes transcription of *PFKFB3* and FGF is known to induce expression of MYC and its downstream target HK2 (Yu et al., 2017).

1.5.2 Fatty Acid Oxidation

Unlike other cells, EC rely on fatty acid oxidation (FAO) for biosynthesis of precursors to make deoxynucleotides (dNTP) rather than energy production (Schoors et al., 2015). dNTP synthesis is required in proliferating stalk cells for biomass synthesis (Vander Heiden et al., 2009). Schoors et al. (2015) studied the function of FAO in proliferation and migration of EC *in vitro* and *in vivo* by inhibiting a rate controlling enzyme of FAO called Carnitine palmitoyltrasferase 1a (CPT1a). Their study indicated that inhibiting FAO diminished EC sprouting *in vitro* and reduced the number of branch points in vivo by selectively inhibiting EC proliferation without affecting the migratory properties of the EC (Schoors et al., 2015).

In summary, sprouting angiogenesis involves EC proliferation and migration and these activities are coordinated through a large array of cell signaling pathways that alter EC gene transcription and metabolic activity to support the angiogenic process. My main interest in the current project is the regulation of angiogenesis as it relates to adipose tissue expansion in the context of obesity related disorders in males and females.

1.6 Adipose Tissue

Adipose tissue acts as a very efficient energy storage in the body. It is involved in storing lipids under conditions of excess energy intake and the

release of energy dense substrates when needed by other tissues between meals or during physical activity, thus effectively regulating whole-body metabolism. Adipose tissue is capable of responding very rapidly and dynamically to increases in nutrient intake through adipocyte hypertrophy or hyperplasia (Galic et al., 2010; Sun et al., 2011; Zore et al., 2018). The first evidence for the role of adipose tissue in controlling whole-body energy homeostasis was presented by Kennedy and Sterling (1953) when they found the presence of a hunger-inhibiting substance secreted by adipose tissue that controlled energy expenditure and food intake through a negative feedback signal. Years later, studies on genetically obese and diabetic mice confirmed the presence of such a factor and eventually in 1994, the gene and its protein product were discovered and called Leptin (Zhang et al., 1994). Leptin is not the only endocrine factor released by adipose tissue, in fact adipose tissue is considered to have a very crucial secretory function as the body's largest endocrine gland (Trayhurn and Beattie, 2001). Adiponectin is another adipocyte specific hormone involved in systemic insulin sensitizing, which also has anti-inflammatory properties (Fang and Judd, 2018). Studies using mice have demonstrated its role in insulin sensitivity as deficiency in adiponectin leads to insulin resistance while over-expression of adiponectin preserves insulin sensitivity even in obese mice (Scheja and Heeren, 2019). Other adipokines (peptides) and lipokines (lipids) produced by adipocytes have systemic effects on lipid and glucose homeostasis, energy balance, inflammation and tissue repair in liver and skeletal muscles, brain and

pancreatic beta cells (Lynes et al., 2017; Yore et al., 2014). Additionally, adipose tissue has a very important role in modulating inflammation. Adipocytes regulate local and systemic inflammatory responses through expression of factors such as TNF-α, IL-6, 8, 10 and TGF-β in the adipose tissue microenvironment (Berg and Scherer, 2005).

At a cellular level, adipose tissue is divided into two major different types: white adipose tissue (WAT) and brown adipose tissue (BAT). In BAT, adipocytes expend energy via thermogenesis and thus maintain body temperature. In humans, brown adipose tissue is more abundant in newborn babies than adults and is located in intrascapular and supraclavicular regions (Sacks and Symonds, 2013). White adipocytes on the other hand are the most abundant type of adipose tissue and are involved in both energy-storing and secretory functions of adipose tissue (Matsuzawa, 2006). White adipose tissue is further classified based on the location of the depot in the body. Visceral adipose tissue (VAT) includes intra-abdominal (mesenteric), perirenal and pericardial adipose tissue. VAT, specifically mesenteric WAT, has been the subject of interest because its accumulation is closely associated with a number of metabolic abnormalities such as insulin resistance (González-Muniesa et al., 2017). However, more than 80% of total body fat is stored in subcutaneous depots including abdominal and gluteal-femoral regions (Lee et al., 2013). Subcutaneous AT has a protective effect against development of insulin resistance and other metabolic complications during weight gain because of its capacity to store excess energy, which prevents

lipid deposition within muscle, liver and visceral fat depots (Hardy et al., 2012). These protective effects are associated with its greater capacity for absorption of free fatty acids and triglycerides, higher preadipocyte differentiation, smaller adipocyte size and smaller number of inflammatory and immune cells compared to VAT (Bruun et al., 2005; Freedland, 2004; Wajchenberg, 2000).

1.6.1 Adipose Tissue Angiogenesis

Healthy expansion of AT is dependent on a concomitant remodeling and expansion of its vascular supply to maintain adequate nutrient and oxygen levels in the tissue (Cao, 2013; Nishimura et al., 2009). Reduced AT angiogenesis leads to tissue inflammation and fibrosis (Halberg et al., 2009; Lee et al., 2014) and has been considered an important contributor to the pathogenesis of dysfunctional AT in obese subjects (Crewe et al., 2017). AT modulates angiogenesis by secreting several adipokines including Leptin, VEGF, FGF-2, TNF-α and TGF-β, which are released by adipocytes as they grow (Cao, 2010; Sierra-Honigmann et al., 1998).

EC on the other hand contribute to adipogenesis through interactions with adipocytes in the AT microenvironment (Rafii and Carmeliet, 2016). EC are capable of producing paracrine growth factors and cytokines such as PPAR-γ ligands that promote growth of adipocytes (Gogg et al.; Powell, 2007). Furthermore, there is evidence showing that vascular pericytes and EC are capable of differentiating into preadipocytes and adipocytes (Tang et al., 2008; Tran et al., 2012).

1.6.2 Obesity

Obesity is defined by world health organization as excessive fat accumulation that might impair health and is diagnosed as a body mass index of greater or equal to 30 kg/m^2 (Prospective Studies Collaboration, 2009). The primary cause of obesity is long term greater caloric consumption vs expenditure. From an evolutionary point of view, it is proposed that a genotype that favours eating more food and retaining more calories that leads to adipose tissue expansion has been selected so that humans and their predecessors could survive periods of undernutrition (Yanovski, 2018). In recent decades overnutrition, and its consequences, has emerged as a bigger health issue than undernutrition especially in developed countries (Blüher, 2019). Some of the obesity related conditions such as visceral obesity, hypertension and hyperglycemia are considered as features of metabolic syndrome, which is a major risk factor for cardiovascular disease and type 2 diabetes (Alberti et al., 2009; Wyatt et al., 2006). The most recent study providing worldwide trends in BMI indicates that obesity prevalence increased in every country between 1975 and 2016. With the exception of regions in sub-Saharan Africa and parts of Asia, more people are obese than are underweight throughout the world (Abarca-Gómez et al., 2017; Yanovski, 2018).

1.6.3 Obesity and Angiogenesis

A number of studies have shown reduced capillary density in adipose tissue of obese humans and animal models, indicating diminished angiogenesis

during the pathological adipose tissue expansion that occurs in obesity (Gavin et al., 2005; Gealekman et al., 2011; Lash et al., 1989; O′Rourke et al., 2011; Pang et al., 2008; Pasarica et al., 2009; Spencer et al., 2011). The importance of angiogenesis in maintaining the metabolic homeostasis of AT has been explored in studies where overexpression of VEGF-A significantly increased white AT vascularization and prevented undesirable obesity-related metabolic effects such as insulin resistance (Gupta et al., 2012; Park et al., 2017; Zhao et al., 2018). Studies in rodents have shown that, under persistent metabolic challenge of chronic high fat diet (HFD), the capacity to form new blood vessels is poor although the demand for oxygen is great (Lijnen et al., 2006). Chronic HFD leads to decreased levels of VEGF in adipose tissue of obese mice and humans (Pasarica et al., 2009). Furthermore, unresolved inflammation during chronic obesity, marked by adipocyte death due to their increased size beyond physiological limits, leads to pro-inflammatory signaling that leads to oxidative damage to other cells including EC in obese adipose tissue (Furukawa et al., 2004).

Overall, the reduced capillary density observed in adipose tissue in obesity causes adipocyte dysfunction and thus inflammation and diminishes the capacity of the adipose tissue to store excess calories in the form of triglycerides leading to ectopic lipid deposition into muscle and liver. This in turn causes obesity-related complications such as tissue inflammation, insulin resistance and metabolic disorders associated with it (Corvera and

Gealekman, 2014; Hardy et al., 2012; McLaughlin et al., 2011; Virtue and Vidal-Puig, 2010).

1.7 Sex Differences in Obesity and Metabolic Disorders

Nearly all aspects of metabolism such as energy balance, glucose and lipid metabolism are regulated in a sexually dimorphic manner (Fatima et al., 2017). In animal models it has been shown that females have more brown adipose tissue than males which is more metabolically active than white adipose tissue (Rodríguez-Cuenca et al., 2002). This leads to higher contribution of fat mass to resting metabolic rate in females compared to males (Nookaew et al., 2013).

In humans, females are at greater risk of obesity compared to males because of an increased tendency to gain fat. However, females can tolerate higher levels of body fat as they have lower amount of abdominal adipose tissue (Kelly et al., 2008). It is very well established that women store more adipose tissue in subcutaneous fat depots in contrast to the preferential visceral deposition in men (Enzi et al., 1986). Although, females have more adipose tissue mass and more circulating free fatty acid (FFA) compared to males, they are as sensitive to insulin as males and are more resistant to FFA-induced insulin resistance (Frias et al., 2001). Additionally, females have higher insulin sensitivity and greater glucose uptake within skeletal muscle compared to males when they are matched for physical fitness based on their VO_2 max (Nuutila et al., 1995). These sex-based differences manifest

themselves on the pathogenesis of metabolic disorders such as diabetes and obesity (Mauvais-Jarvis, 2015).

A study in our lab showed that female mice have higher vascularity and a more pro-angiogenic environment in their visceral adipose tissue compared to males in response to a high fat diet. This increased vascular density in adipose tissue was associated with preservation of adipose tissue function and insulin sensitivity (Rudnicki et al., 2018). Observed sex differences in obesity and metabolic disorders could be the result of differential effects of sex hormones in males and females. Alternatively, it could be due to genetic differences arising from the male and female sex chromosomes. These possible mechanisms for sex-related metabolic differences will be discussed below.

1.7.1 Sex Hormones in Obesity and Metabolic Disorders

The most widely studied cause of sex-related difference in males and females in the context of metabolic disorders is the influence of gonadal hormones. Studies comparing pre- and post-menopausal women have demonstrated the role of reduced levels of the female sex hormone estradiol (E2) on metabolic changes related to this transition (Carr, 2003). Studies have demonstrated increased risk of cardiovascular disease (CVD) in post-menopausal women compared to pre-menopausal women (Gohlke-Bärwolf, 2000). The loss of estrogen with menopause is also related to increased visceral obesity (Garaulet et al., 2002). Moreover, the incidence of metabolic syndrome and

its related disorders such as insulin resistance, hypertension and diabetes increase with menopause (Everette and Zajacova, 2015; Lima et al., 2012; Lobo, 2008). Estrogens protect against body adiposity through suppression of appetite and increasing energy expenditure (Asarian and Geary, 2002; Clegg et al., 2006). There is evidence for regulation of adipose tissue angiogenesis by activation of estrogen receptor 1 (ESR1) signaling. A study conducted by Deborah Clegg's group indicated that VEGF-A expression is regulated by E2 action and that selective knockdown of ESR1 in adipocytes decreased VEGF expression in adipose tissue of the female mice (Fatima et al., 2017). No study, however, has yet directly assessed vascular density as a function of E2 levels.

Similarly, low serum testosterone and late-onset hypogonadism in males have been associated with abdominal obesity, cardiovascular disease, the metabolic syndrome and type 2 diabetes (Araujo et al., 2011; Blouin et al., 2008). Testosterone is believed to stimulate lipolysis, decrease lipogenesis and enhance β-oxidation (Kelly and Jones, 2015). Reductions in total testosterone levels in overweight and moderately obese men could be a consequence of decreased sex hormone binding globulin (SHBG) caused by obesity-related hyperinsulinemia and not the free testosterone (Bhasin et al., 2010). SHBG is a glycoprotein with high affinity for testosterone and E2 and is involved in transport of these sex hormones in plasma (Selby, 1990). In cases of severe obesity, however, free testosterone levels are decreased. This decrease in free testosterone level is a result of production of aromatase from

inflamed, insulin-resistant adipose tissue, which converts testosterone to estradiol (E2) (Boer et al., 2005). Estradiol acts on the hypothalamo-pituitary axis as a negative feedback signal and subsequently supresses gonadal testosterone release. Therefore, obesity lowers testosterone and further induces adiposity (Cohen, 1997).

Adipose tissue distribution is affected by both androgens and estrogens. Testosterone inhibits the activity of lipoprotein lipase in the subcutaneous depot in men, which gives rise to abdominal adiposity and the apple shape body type in overweight and obese males (Kelly and Jones, 2015). E2 on the other hand, enhances the activity of lipoprotein lipase in subcutaneous fat depots. This helps storing excess energy in subcutaneous depots that leads to the pear shaped body type in overweight and obese females (Arner et al., 1991; Ramirez et al., 1997).

1.7.2 The Genetic Basis for Sex Differences in Obesity and Metabolic Disorders

Any biological difference between males and females results from the presence of male and female sex chromosomes, which in turn determine the types and levels of sex hormones produced (Link and Reue, 2017). Sex chromosomes themselves can be contributors to sex-related differences in metabolic characteristics (Link and Reue, 2017). Studies on individuals with different sex chromosome anomalies have shown the effects of having irregular number of sex chromosomes on some metabolic related disorders. For instance, men with Klinefelter syndrome (XXY) have an almost five-fold higher incidence of developing metabolic syndrome (Bojesen et al., 2006).

However, distinguishing the influence of other side effects of having these conditions on metabolic disorders from the sex chromosome number itself is difficult (Gravholt, 2004; Hagen et al., 2010). One approach to look at the effect of sex chromosomes on different biological processes at a cellular level independent of sex hormones is studying early embryonic growth prior to the development of gonads and their hormones. For instance, in most mammals, early male embryos are larger than female embryos (Bukowski et al., 2007; Burgoyne, 1993; Burgoyne et al., 1995). One model that has been used extensively to study the effect of sex chromosomes separately from the sex hormones is the Four Core Genotypes (FCG). In this model, mice with male gonads are generated which contain XX chromosomes and mice with female gonads have XY chromosomes. Using this approach, traits are compared and the influence of sex chromosomes or gonadal hormones on these traits are evaluated. In C57BL/6 FCG mice that were gonadectomized before the experiment to remove any effects of circulating sex hormones, XX mice consumed more food and gained more weight and accumulated more adipose tissue in subcutaneous depots compared to XY mice (Chen et al., 2015). Moreover, XX mice developed obesity-related conditions such as fatty liver, elevated insulin levels (Chen et al., 2012). Furthermore, studies with the FCG mouse model indicated the effects of X chromosome dosage on cholesterol levels. Basal levels of HDL in XX mice fed a chow diet were higher than in XY mice whereas LDL levels were not affected by chromosome complement. By contrast, triglyceride and free fatty acid levels were higher

in the mice with male gonads compared to females regardless of their sex chromosome complement (Chen et al., 2012). Based on these results, sex chromosome complement has been identified as a factor responsible for observed sex differences in obesity and metabolism (Chen et al., 2012). Although metabolic differences between males and females are well-established, whether regulation of adipose angiogenesis contributes to these differences is not known.

1.7.3 Male and Female Endothelial Cells

In recent years, a number of studies have investigated the inherent sex-related differences in EC independent of the effects of E2. Studies on human umbilical vein EC (HUVEC) have shown higher levels of prostacyclin and prostaglandin E2 synthesis in response to thrombin in males compared to females which could contribute to higher rates of atherosclerosis and thrombosis in males (Batres and Dupont, 1986). Other studies have shown higher migratory capacity, transcription of pro-angiogenic genes in response to shear stress, expression of endothelial nitric oxide synthase (eNOS), cell viability after 20 hours of serum starvation and tube formation capacity in female HUVEC compared to males (Addis et al., 2014; Cattaneo et al., 2017; Lorenz et al., 2015). Vanetti et al. (2017) illustrated some of the metabolic differences of male and female HUVEC in terms of substrate utilization. Using EC spheroids, they observed higher inhibition of sprouting in male

cells compared to female ones when cultured in media stripped of all fatty acids, indicating higher dependence of male EC on free fatty acids for proliferation and *in vitro* angiogenesis compared to females. The underlying mechanisms for these observed differences in male and female EC are yet to be identified and could be possibly explained by differences in key intracellular signaling and metabolic pathways, such as PFKFB3 expression and glycolytic rate, which are known to regulate angiogenic behaviour. Overall, there are little data on differences of male and female signaling pathways and metabolic functions that may cause differences in angiogenic capacity of EC. There are still obvious gaps in knowledge on this topic that should be studied further. A logical hypothesis is that sexual dimorphism observed in angiogenesis of adipose tissue is caused by differences in angiogenic capacity of male and female EC.

1.8 Study Rationale

It is well established that expansion of adipose tissue in males is associated with higher incidence and severity of obesity-related disorders compared to females (Logue et al., 2011; Steinarsson et al., 2018; Tchernof and Després, 2013). In line with this, rodent studies using obese mice have shown the same trend of developing cardiometabolic disorders with lower BMI in males compared to females (Macotela et al., 2009; Medrikova et al., 2012). Diminished adipose tissue vascularization has been indicated as one of the

contributors to pathogenesis of dysfunctional adipose tissue, which causes obesity-related cardiometabolic complications such as insulin resistance and type II diabetes (Crewe et al., 2017). A recent study from our lab examined the differences in visceral AT angiogenesis of high fat fed obese male and female mice. This study provided evidence for higher visceral AT vascularization in females and reduced visceral AT vascularization in males compared to normal chow fed mice, indicating the maintenance of angiogenesis in obese females but diminished angiogenesis in obese males white AT. (Rudnicki et al., 2018). This points to sex-related differences in angiogenesis of AT; however, the mechanisms are not known. It is established that female sex hormone, estradiol (E2), enhances the angiogenic activity of EC (Fatima et al., 2017; Geraldes et al., 2002; Morales et al., 1995; Zhao et al., 2008), however, a number of studies have indicated that EC themselves show sex specific differences independent of E2 (Addis et al., 2014; Cattaneo et al., 2017; Huxley et al., 2018; Lorenz et al., 2015; Vanetti et al., 2016, 2017). Most of these studies were conducted *in vitro* with HUVEC and there is no knowledge about the sex-related differences of visceral AT EC and its association with the observed higher angiogenic response in visceral AT of female mice. My thesis will fill this gap in knowledge by examining sex-specific differences in angiogenic characteristics of visceral AT EC in mice. Studying these differences might contribute to a better knowledge of the role of EC in the sexual dimorphism observed in physiology and pathophysiology of the cardiometabolic system.

1.9 Hypothesis

There are differences in male and female EC angiogenic capacity, and these variations are the results of intrinsic differences in pathways controlling proliferation, migration and metabolism.

1.10 Study Objectives

1- To compare angiogenic capacity of male and female EC, including expression levels of angiogenic pathway proteins as well as proliferation, migration and sprouting capacity.

2- To test the involvement of MAPK, FoxO1 and Notch pathways in controlling proliferation and migration of male and female EC.

Chapter 2 Methods

2.1 Ethical approval

Animal studies were approved by York University Committee on Animal Care and performed in accordance with the Canadian Council for Animal Care Guidelines.

2.2 Cells

Male and female microvascular EC were isolated from VAT of male and female C57BL/6 mice. White VAT was extracted and pooled from each sex and digested with 0.5% Type I collagenase (#17100-017, ThermoFisher Scientific, USA) for 20 minutes at 37 °C with shaking. Adipocytes were separated from the stromal vascular fraction (SVF) by centrifugation (300xg for 5 min) and the SVF was resuspended and passed through a cell strainer (100µm) and then incubated with biotinylated rat anti-mouse CD31 antibody-coated streptavidin (BD IMag, USA) -coupled Dynabeads (ThermoFisher Scientific, USA). Isolated EC were plated on gelatin-coated plates and maintained in high glucose (4.5 g/L) DMEM (#11960069, Gibco, USA), 1% Penicillin-Streptomycin 10,000µg/mL (#15140122, Gibco, USA), 1% GlutaMAX™ L-glutamine 200mM (#35050061, Gibco, USA), 1% Sodium Pyruvate 100mM (#11360070, Gibco, USA) and 20% FBS (#10082-147, Lot# 1913800, Gibco, USA). The FBS lot used contains estradiol (40.5

pg/mL), Progesterone (<0.05 ng/mL) and Testosterone (<0.10 ng/mL). Cells used for experiments were between passages 2-4.

2.3 Adipose tissue Ex-Vivo Sprouting Assay

Small pieces of visceral AT were extracted from 10 weeks old C57BL/6 male and female mice and were cut in ~1mm^2 washed with PBS and kept in DMEM at room temperature. Explants were embedded in 3mg/ml type I rat tail collagen (A1048301, Gibco, USA). 50µL of collagen neutralized with NaOH 5N was added to each well in a 96 well plate and incubated for 1 hour in 37°C to polymerize. Explants were treated with 50ng/ml human recombinant VEGF 121 (R and D systems, MN, USA) and allowed to grow for 1 week. The explants were fixed with 3.7% paraformaldehyde and stained for imaging using alkaline phosphatase substrate SIGMA*FAST*™ BCIP®/ NBT according to the kit instructions (B5655, Sigma, MO, USA). Images of whole explants were taken using a stereo microscope. The sprouting areas were calculated by subtracting the explant area from the total sprouting area in ImageJ software. Integral density of explant was calculated by subtracting the integral density of explants from the total integral density of sprouting front in ImageJ software.

2.4 RNA extraction

10-15 mg of frozen visceral white adipose tissue was homogenized in Qiazol buffer (Qiagen, ON, Canada) using the Retsch MM400 tissue lyser. Total

RNA was purified using RNeasy Mini Kit for Adipose (74104, Qiagen, ON, Canada) as per the manufacturer's instructions.

2.5 q-RT PCR

For each sample, 190 ng of RNA was reversed transcribed to cDNA in a two-step PCR reaction on a Thermal cycler (2720 cycler, Applied Biosystems, CA, USA) using [dNTP (#N0447S, New England Bioabs, ON, Canada), Oligo DT (#100002344 Invitrogen, CA, USA), Ribolock RNase inhibitor (#EO038, Thermo Fisher Scientific, MA, USA), Random hexamers (#100026484, Invitrogen, CA, USA), M-MuLV reverse transcriptase and buffer (New England BioLabs, ON, Canada)]. The cDNA was diluted in 40 µl of RNase free water. 2 µl of cDNA was combined with Taqman® Fast Advanced Master Mix (#4444557, Applied Biosystems, Thermo Fisher Scientific, CA, USA) and TaqMan® FAM-Labelled probe sets for murine *Hprt1* (Mm00446968-m1), *Kdr* (Mm01222421-m1), *Pecam1* (Mm00476712-m1), *Vegfa* (Mm00437306-m1). q-RT PCR was performed using the PCR cycler Rotor-Gene Q system (Qiagen, ON Canada) in the following thermal conditions: 60 °C for 30 minutes, 95 °C for 10 minutes for 40 cycles. Samples were assessed in duplicate. To determine the relative amount of target sample mRNA, the average cycle threshold (CT) was calculated and compared to the average CT of Hprt1 (housekeeping gene) for the same samples with the formula ΔCT= Average CT (gene of interest)-Average CT (housekeeping gene). The amount of target gene amplification relative to the control was calculated using the formula $2^{\Delta CT}$.

2.6 Protein extraction

EC were plated in high glucose DMEM at 70-80% confluency and incubated overnight with or without treatments (FoxO1 inhibitor: AS1842856 (10 µM) and p38 MAPK inhibitor: SB203580 (10 µM)). Protein was extracted the following day using RIPA (50mM Tris base, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing dissolved PhosSTOP™ and cOmplete™ Protease Inhibitor Cocktail (Sigma-Aldrich, USA).

2.7 Protein quantification

Protein extracts were quantified by bicinchoninic acid assay (BCA) (Pierce, Fisher Thermoscientific, ON, Canada). Pure protein lysates were loaded in triplicates on 96 well plates with working reagent and compared to a standard curve using serial dilutions of bovine serum albumin (BSA: 0-2000µg/ml, Pierce, Thermo Fischer scientific, ON, Canada). Sample absorbance at 562nm was measured using the Cytation3 microplate reader (BioTek, Vermont, USA).

2.8 Western Blot

Western blots were done using 15-30 µg of total protein per sample prepared in 4X dithiothreitol (DTT) loading buffer containing 0.72M β-mercaptoethanol, 69.4mM sodium dodecyl sulfate, 32% glycerol, 14.29 µM bromophenol blue, 62.4 mM Tris pH 6.8, and RIPA lysis buffer as a diluent. Samples were heated at 99° C for 5 minutes, centrifuged, and placed on ice. The prepared lysates were loaded (15-30 µg per lane) and separated by

electrophoresis through 8%, 10% or 12% SDS-Polyacrylamide gels under reducing conditions. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon P, EMD Millipore, ON, Canada) using wet transfer at 100V for varying durations, higher molecular weights for around 90 minutes and lower molecular weights for 60 minutes. Membranes were blocked for one hour with 5% milk in a 0.05% Tween Tris-buffered saline solution (TTBS). After blocking, membranes were incubated with primary antibodies Phospho-Akt-ser473 (1:1000; #4058, Cell Signaling, ON, Canada), Akt (1:1000; #9272, Cell Signaling), Phospho-p38 Thr180/Tyr182 (1:1000; #9211, Cell Signaling), p38 (1:1000; #9212, Cell Signaling), αβ-Tubulin (1:1000; #2148, Cell Signaling) and β-Actin (1:1000; #4967, Cell Signaling), Phospho-SAPK/JNK Thr183/Tyr185 (1:1000; #9251, Cell Signaling), SAPK/JNK (1:1000; #9252, Cell Signaling), Phospho-p44/42 MAPK (Erk1/2) Thr202/Tyr204 (1:1000; #4377, Cell Signaling), p44/42 MAPK (Erk1/2) (1:1000; #4695, Cell Signaling), Phospho-FoxO1-ser256 (1:1000; #9461, Cell Signaling), FoxO1 (1:1000; #2880, Cell Signaling), Hexokinase II (1:1000; #2867T, Cell Signaling), PFKFB3 (1:1000; #13123S, Cell Signaling) in 5% BSA (Bioshop, Canada Inc, ON, Canada) in 0.05% TTBS overnight at 4°C. Membranes were washed with TTBS and incubated with secondary antibodies [goat anti-rabbit IgG-horseradish peroxidase (#111-035-003, Jackson ImmunoResearch Laboratories Inc., USA) in 5% BSA for 1 hour at room temperature. For experiments measuring phosphorylated protein and total protein on the same membrane, a membrane

stripping method using 0.7% β-mercaptoethanol at 50 º C for 8 minutes was used and then the membranes were washed for 1 hour in TTBS and blocked and incubated in the antibody as described above. The antibodies were detected by enhanced chemiluminescence (Pierce, Fisher Thermoscientific, ON, Canada) using Microchemi DNA Bio-imaging system (Neve Yamin, Israel). Blots were quantified and analyzed using Image J software.

2.9 Proliferation assay

Male and female EC were plated in 96 well plates at a density of $8x10^3$ per well. To assess the effect of pathway inhibition on cell proliferation, EC were incubated overnight with Notch inhibitor (10μM DAPT #2634, Tocris, Bristol, UK), p38-MAPK inhibitor (10 μM SB203580, #1202, Tocris, Bristol, UK), ESR1 inhibitor (10μM MPP dihydrochloride hydrate, M7068, Sigma, MO, USA), PFKFB3 inhibitor (10 μM 3PO, #525330, EMD Millipore, MA, USA) and FoxO1 inhibitor (1μM AS1842856, #344355, EMD Millipore, MA, USA) were used. 1μL of DMSO (D2650, Sigma, MO, USA) was used as the vehicle control. The following day, EC were incubated with 10μM of EdU provided in Click-iT EdU Imaging Kits (Invitrogen, Carlsbad, CA, USA) for 4 hours. To generate confluent cells, EC were plated in 96 well plate at a density of $8x10^3$ per well and incubated for 3 days without treatment for cells to reach confluency and then underwent treatment as described above. Cells were fixed using provided reagent in the kit and Click reaction was performed according to the manufacturer's instructions for visualizing the proliferating cells with Alexa Fluor488 Azide conjugated with

EdU incorporated into DNA. All cell nuclei were stained with DAPI (D3571, Molecular probes, OR, USA). Imaging and counting were performed using CellInsight CX7 High-Content Screening (HCS) Platform (Thermo Fisher Scientific, USA). The number of EdU-positive and number of DAPI-positive cells were counted separately, and the percentage of proliferating cells was expressed as (EdU/ DAPI) x 100.

2.10 Migration assay

Cells were plated at a density of 10^4 cells/well in a collagen I coated OrisTM 96 well plate with Oris™ Cell Seeding Stoppers (Platypus Technologies, WI, USA) inserted to create a cell-free zone in the middle of each well. After allowing cells to attach, they were incubated overnight with different inhibitors for each well (DAPT, SB203580 and AS184285 as described above). 500ng/ml of Mitomycin C (Tocris, Bristol, UK) was added to each well to stop cell proliferation. The following day the stoppers were removed, and the cells were allowed to migrate into the cell-free zone for 24 hours and then were fixed with 3.7% paraformaldehyde. Cells were then stained with Alexa Fluor™ 488 Phalloidin (P5282, Sigma, MO, USA) and imaged using CellInsight CX7 High-Content Screening (HCS) Platform (Thermo Fisher Scientific, USA). Reference cell-free area was determined from wells that were fixed immediately after removal of stoppers (Fig 2-1). Each well's cell free area was compared to the reference area after 24 hours of incubation with different treatments. Cell migration was measured using Image J

software as the area fraction percentage of reference cell free zone covered by migrating cells.

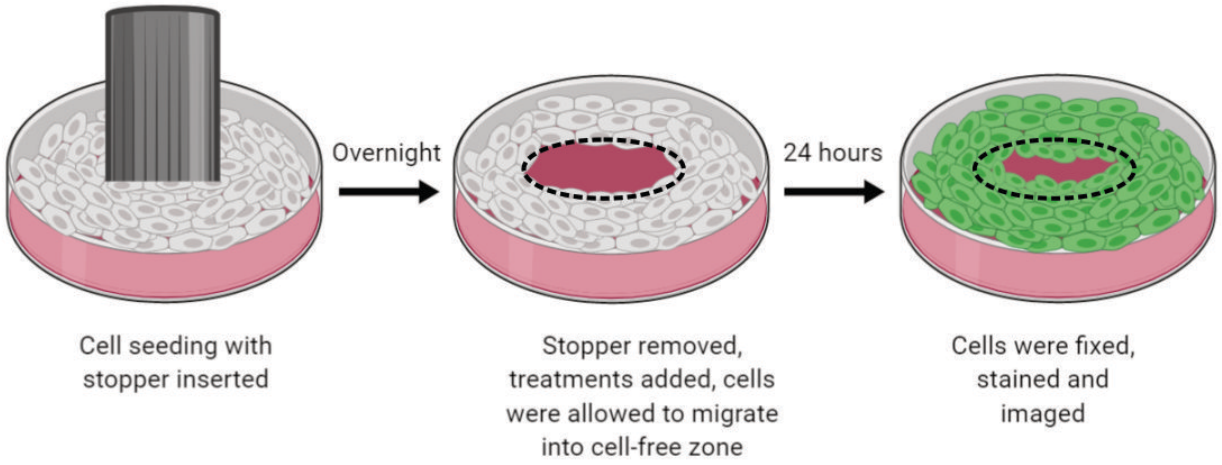


Figure 2-1: **Migration assay**. Schematic depicting the steps for measuring the migration of EC into the cell free zone (Created with BioRender).

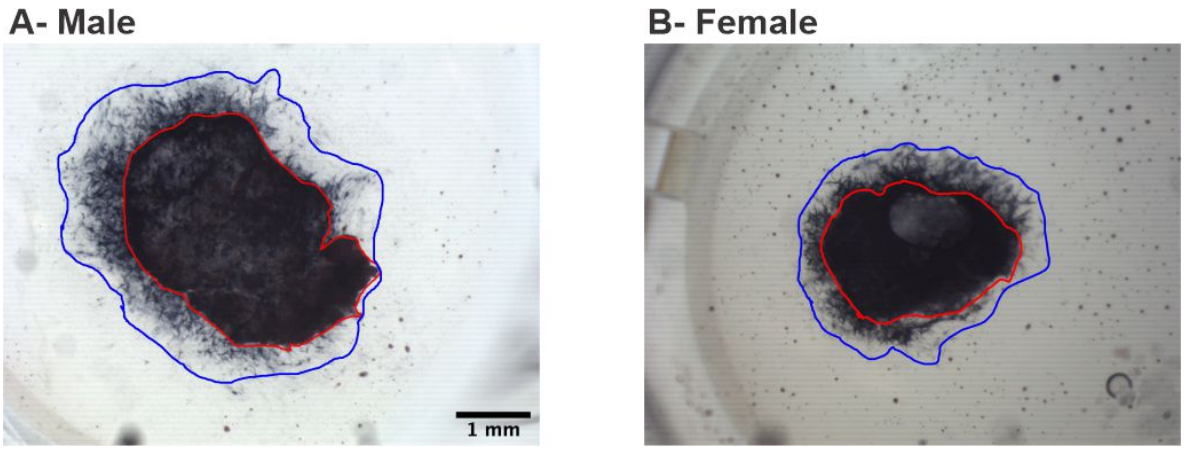
2.11 Statistical Analysis

Results are presented as Mean ± S.E.M. Adipose tissue explants, adipose tissue mRNA expression, baseline EC migration and proliferation and baseline protein levels were analyzed using unpaired student's t-test. Protein level measurements with treatments (VEGF, FoxO1 inhibitor) and proliferation assays with both p38 MAPK and FoxO1 inhibitors were analyzed using two-way ANOVA. Migration and proliferation assays using inhibitors were analyzed by repeated measures ANOVA. Post hoc analyses were performed using Sidak's multiple comparisons test (Prism8; Graphpad software Inc; La Jolla, CA, USA). In all cases, $p < 0.05$ was considered statistically significant.

Chapter 3 Results

3.1 Distinct sprouting capacity of male and female adipose tissue ex-vivo:

To compare the angiogenic capacity of male and female visceral AT, a sprouting assay was performed using adipose tissue explants embedded in type I collagen and treated with VEGF for 7 days (Fig 3-1A-D). In line with our previous study, which indicated higher AT angiogenesis in females (Rudnicki et al., 2018), explants from AT of females were more successful in initiating sprouting compared to males (83.3% vs 50%) (Fig 3-1E). Surprisingly, male explants successful in sprouting had a higher density of cells and a larger area of sprouting (Fig 3-1F, G). The higher density of sprouting may be due to higher EC proliferation and the larger area could be a result of higher proliferation and/or migration of EC.



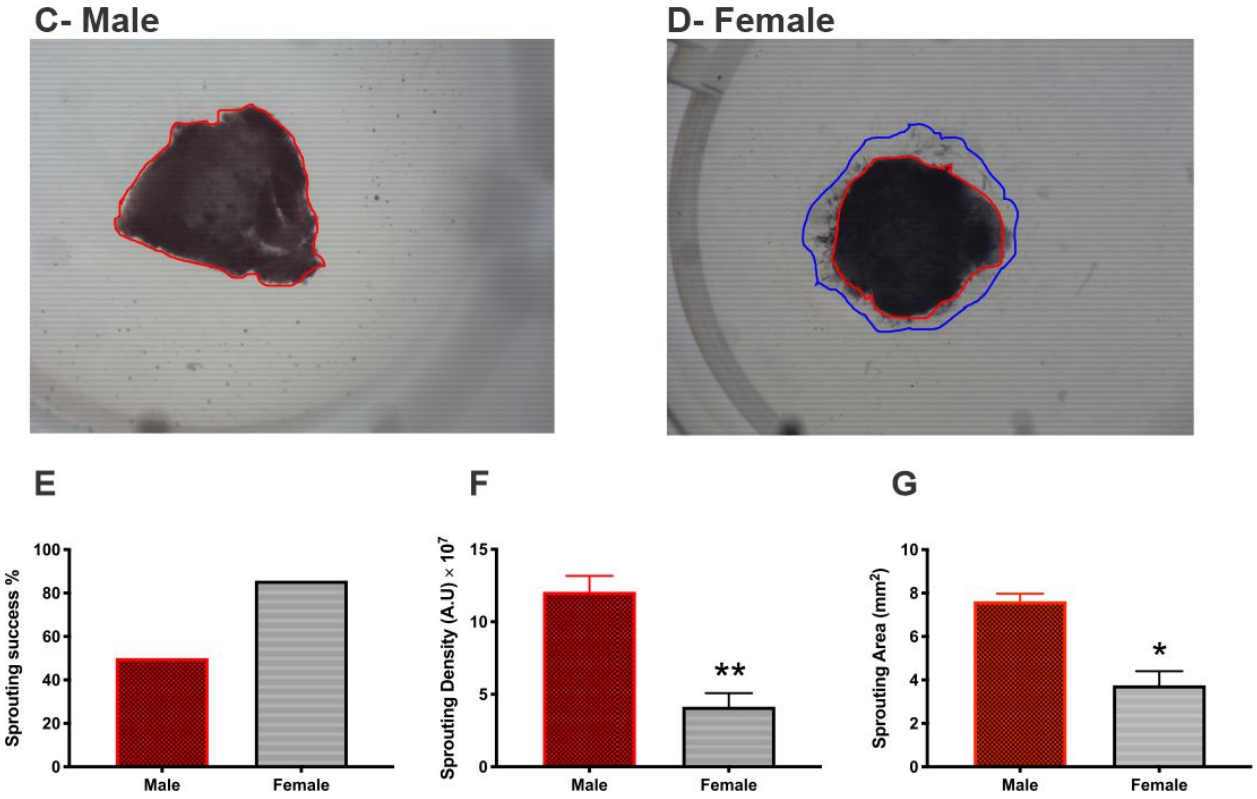


Figure 3-1: **Distinct sprouting patterns in male and female adipose explants.** Representative images of male **(A, C)** and female **(B, D)** explants. The red lines encircle the explant area and the blue lines indicate the sprouting area. **E)** Sprouting success of male and female explants. **F)** Density of cells within the sprouting area of explants **G)** Sprouting area of male and female explants in mm^2. Data analyzed using unpaired student t-test. Mean +/-S.E.M. *p<0.05, **p<0.01 (n=6 for males and 12 for females)**.**

3.2 Similar AT vascularization and angiogenic environment in male and female mice

Baseline differences in EC content and angiogenic environment of male and female AT could have an effect on the rate of sprouting from the explants. To examine this, gene expression analysis was performed on AT of the same 10 weeks old male and female C57/BL6J mice that were used for the explant sprouting assay. mRNA expression for EC markers *Pecam1* and *Vwf* showed

no significant difference between males and females, suggesting no disparity in EC content of visceral AT in these mice (Fig 3-2A and B). In line with this, mRNA expression of the main VEGF receptor, *Kdr*, was not different in AT of males and females (Fig 3-2C). Similarly, mRNA expression of a major angiogenic factor, *Vegfa,* was not significantly different in AT of males and females (Fig 3-2D).

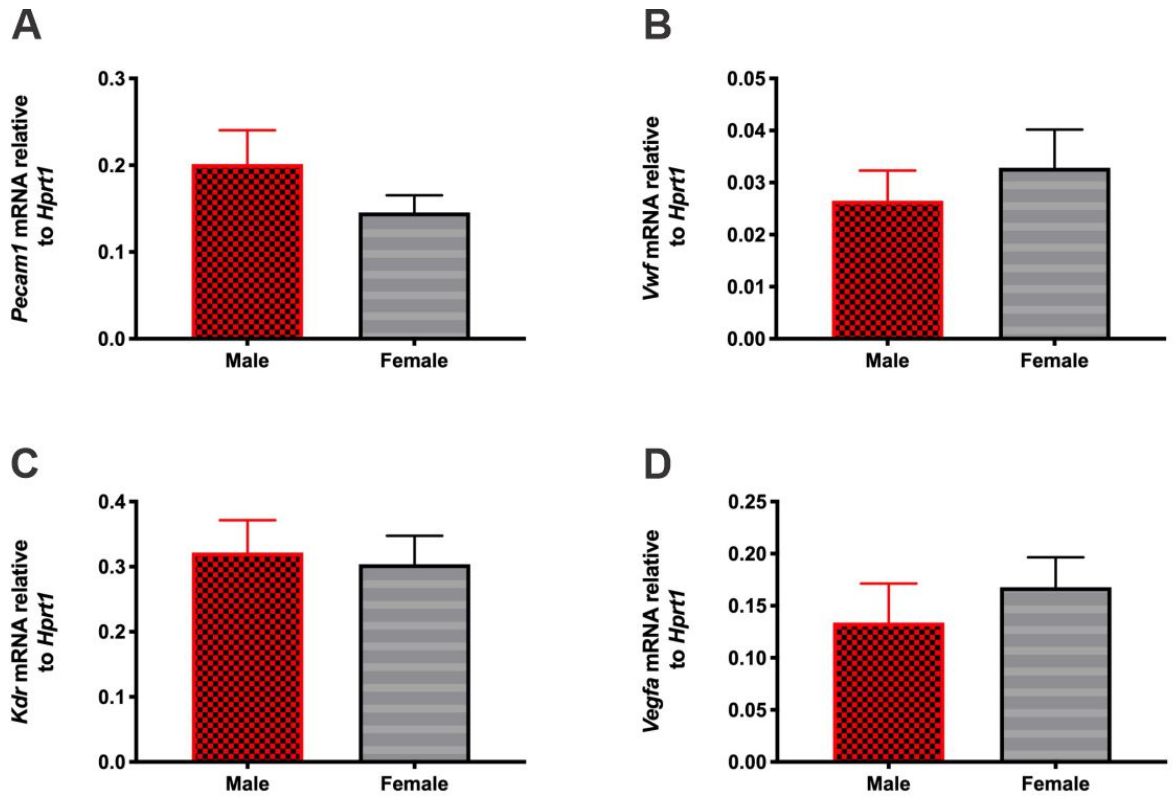


Figure 3-2 : **Similar mRNA expression of endothelial markers and angiogenic related genes in male and female visceral AT. (A-D)** Visceral white AT gene expression analysis by qPCR and presented relative to the housekeeping gene *Hprt1*. Data were analyzed using unpaired student t-tests. Mean +/-S.E.M. (n=7-8).

3.3 Male and female EC migration

Sex-specific differences in EC migration may explain the observed higher sprouting density and area of AT explants from males. To examine if there were any differences in EC migration between males and females, a migration assay was performed. Male and female EC without any treatment showed no significant difference in their migration after 24 hours, with EC from males covering 20% and females covering 24% of the cell-free zone (Fig 3-3A-D). However, the results of this assay were inconclusive because of high variability due to poor cell adhesion to the plate.

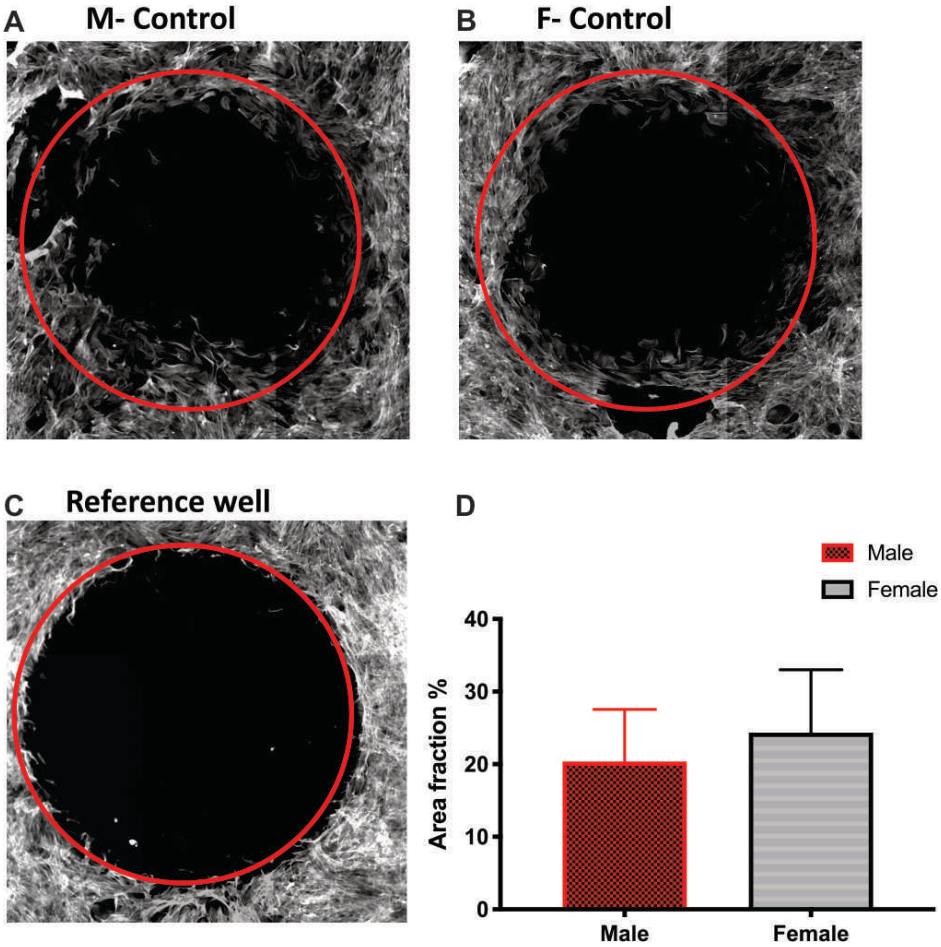


Figure 3-3: Migration of male vs female EC. **(A, B)** Representative images of male and female EC and **(C)** Representative image from the reference well. The cell free area indicated inside the

red circle is used as a reference. (**D**) Area fraction of EC migrated into the cell free zone**.** 500ng/ml of Mitomycin C was added to all wells including control wells to inhibit proliferation of EC. Data analyzed using unpaired student t-test (n= 3).

3.4 Higher *in vitro* female EC proliferation compared to males

We hypothesized that sex-related differences in EC proliferation may be a contributor to the initial successful growth of female explants and higher in vivo angiogenesis in females observed previously. I initially tried measuring EC proliferation using the CyQuant™ assay, which measures the DNA content of cells. The CyQuant assay failed to produce consistent results from one experiment to another. Due to these issues, another proliferation assay measuring the percentage of cells incorporating EdU was used, which allowed me to count the number of proliferating cells as a proportion of the total number of cells (Fig 3-4A, B). My results indicated significantly higher proliferation in female EC compared to males when EC were plated at a low density (21% vs 16% of cells incorporated EdU) (Fig 3-4C). Proliferation of EC in confluent plates showed lower EC proliferation compared to EC in low density in both sexes as expected (Fig 3-4E). Under both conditions, female EC were proliferating at a higher rate compared to males (Fig 3-4D).

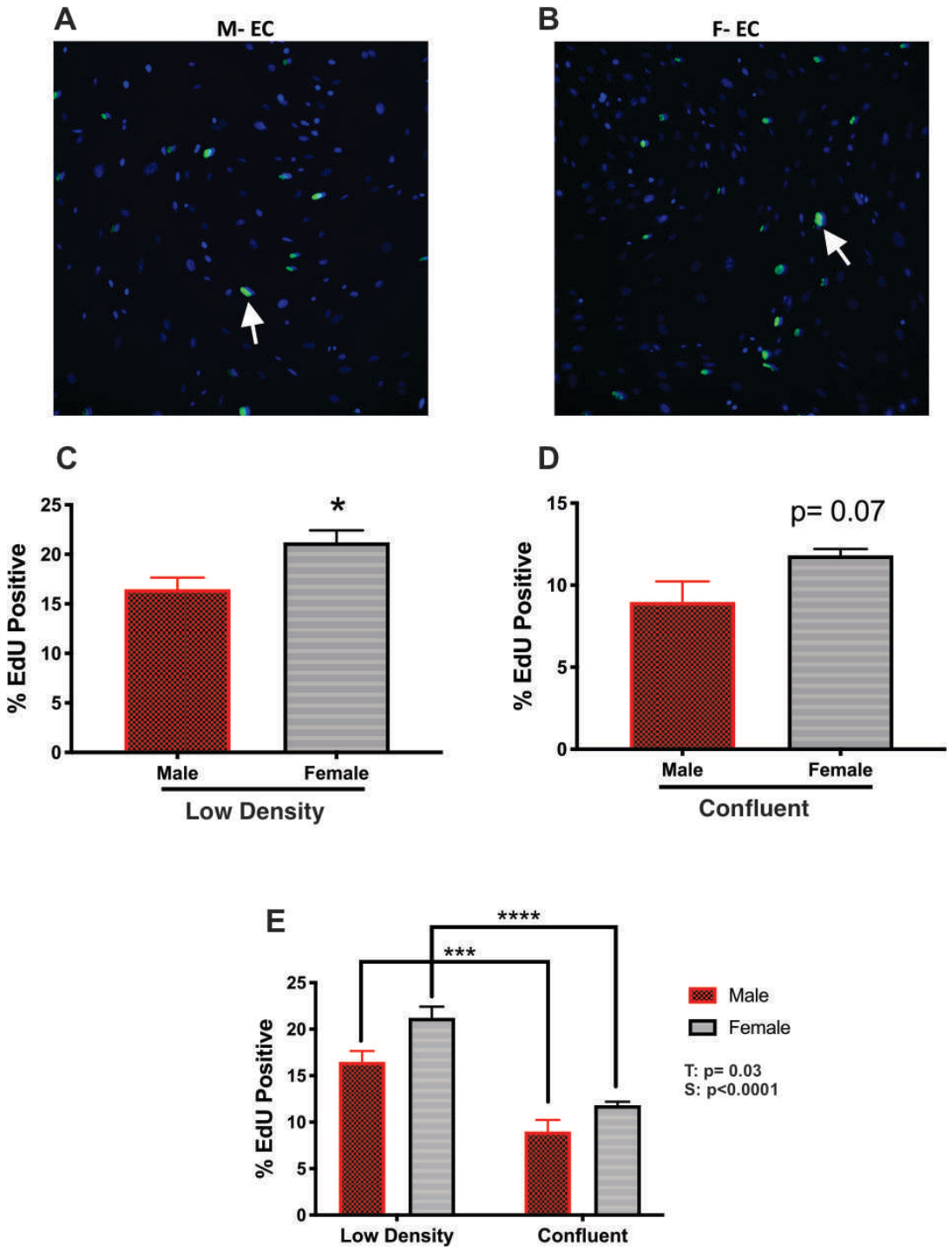


Figure 3-4: **Proliferation of male vs female EC.** Representative images of proliferation assays with EdU-positive cells (green Alexa Fluor 488), indicated with a white arrow) in (**A**) males (MEC) and (**B**) females (FEC). All cells were stained with DAPI (blue). Proliferation of EC after 4 hours of EdU incubation expressed as percent EdU positive in low density (**C**) and confluent (**D**) EC. (**E**) Comparison of % EdU positive cells in male and female confluent and low-density male and female EC. Data analyzed using unpaired student t-test(**C,D)** and two-way ANOVA (**E**). Mean +/-S.E.M. *p<0.05 (n=4-6).

3.5 Higher phosphorylation levels of p38 MAPK in females EC compared to males

Next, I examined the signaling pathways that affect angiogenic capacity of EC to see if they differ in male and female EC when cultured at high density. Since the MAPK pathways are involved in modulation of EC proliferation and migration, I measured the baseline protein phosphorylation of MAPK members in cultured EC extracted from visceral AT. Female EC had significantly higher p38 MAPK phosphorylation levels compared to males (Fig 3-5A). There were no significant differences in basal phosphorylation levels of ERK1/2 or JNK (Fig 3-5B and C).

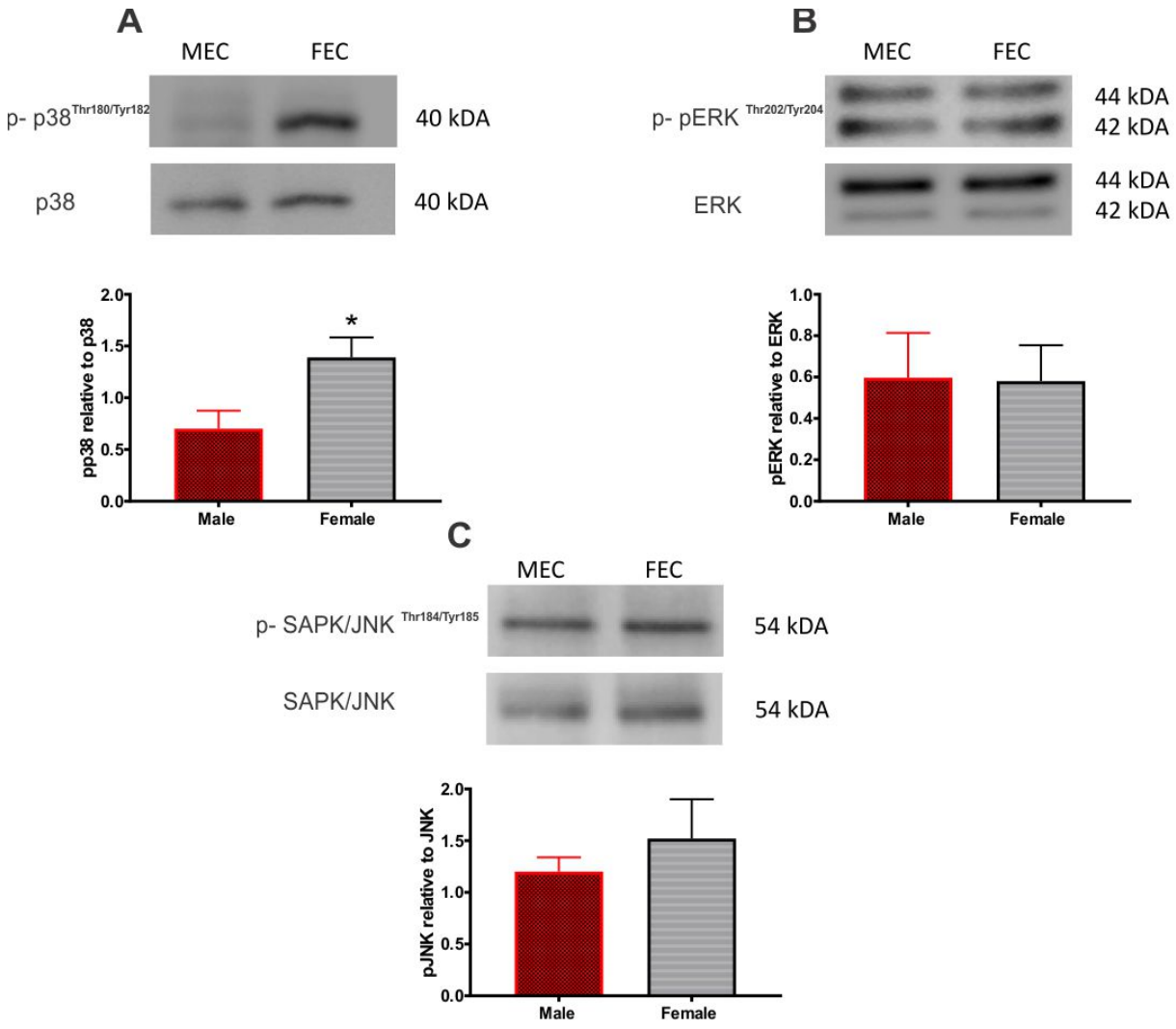


Figure 3-5: **Phosphorylation levels of MAPK pathway in male vs female EC.** Representative western blots and protein quantification of **(A)** p-p38 MAPK-Thr180/Tyr182 and total p38; **(B)** p-ERK-Thr202/Tyr204 and total ERK; and **(C)** p-SAPK/JNK-Thr183/Tyr185 and total JNK in male (MEC) and female (FEC) cells. Results are expressed as phosphorylated protein over total protein. Data analyzed using unpaired student t-test. Mean +/-S.E.M. *p<0.05 (n=3-4).

Since VEGFA activates MAPK pathways as well as Akt in EC, the effect of VEGFA on activation of MAPK members and Akt was examined. Basal levels of phosphorylation of Akt were not different between sexes. Surprisingly, VEGFA did not phosphorylate Akt in EC (Fig 6A). Furthermore, phosphorylation levels of p38 and ERK1/2, both of which are

also downstream of VEGFR2 signaling pathway, were not significantly affected by VEGFA treatment (Fig 3-6B and C).

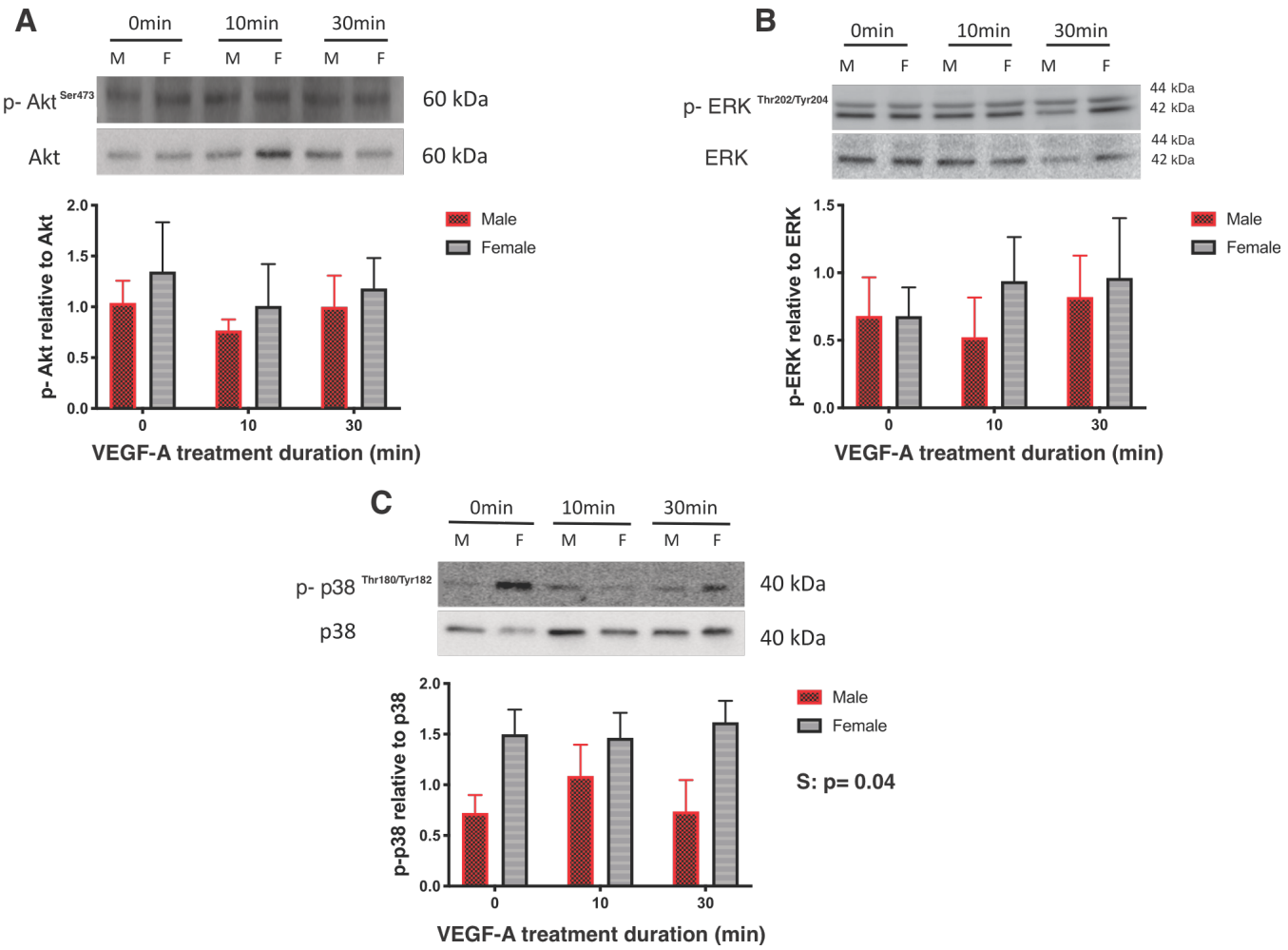


Figure 3-6: **Phosphorylation levels of AKT and MAPK in male vs female EC in response to VEGFA treatment.** Representative western blots and protein quantifications of **(A)** p-AKT-Ser 473 and total AKT; **(B)** p-ERK-Thr202/Tyr204 and total ERK; and **(C)** p-p38 MAPK-Thr180/Tyr182 and total p38 in males (M) and females (F). EC were serum starved for 2 hours and then treated with 50ng/ ml VEGF for 10 and 30 minutes. Results are expressed as phosphorylated protein over total protein. Data analyzed using two-way ANOVA, testing main effects of Sex (S) and Treatment (T). Mean +/-S.E.M. (n=5).

3.6 Higher FoxO1 protein level in female EC compared to males

FoxO1 inhibits proliferation by decreasing metabolic rate and inhibiting cell cycle progression in EC (De Bock et al., 2013b; Wilhelm et al., 2016). Since FoxO1 maintains the quiescent state of EC, based on our *in vivo* and *ex vivo* results, we expected to detect higher FoxO1 levels in males. Surprisingly, protein levels of total FoxO1 were higher in female EC compared to males when cultured at high density (Fig 3-7A). Phosphorylation state of FoxO1 affects its activity and protein level. Phosphorylation of PI3K/ AKT pathway leads to FoxO1 phosphorylation and as a result FoxO1 is deported from nucleus and is targeted for ubiquitination and degradation. Our results indicated that phosphorylation levels of FoxO1 and Akt were not different in male and female EC (Fig 3-7 B, C).

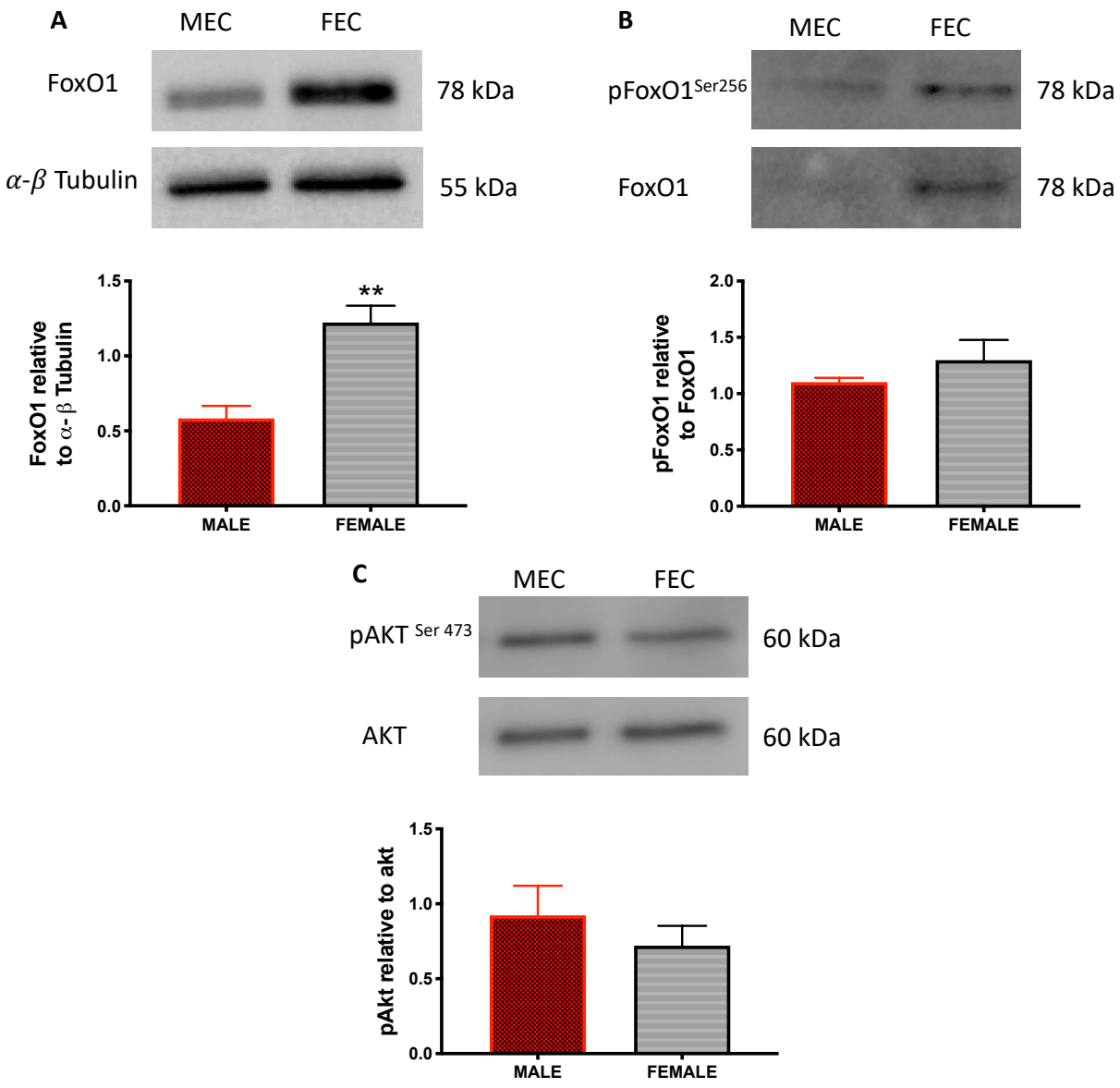


Figure 3-7: **Protein levels of FoxO1, p-FoxO1 and pAKT in male vs female EC.** Representative western blots and protein quantification of **(A)** FoxO1 and αβ-Tubulin (**B)** and p-FoxO1-Ser 256 and total FoxO1 (**C)** p-Akt-Ser 473 and total Akt in males (MEC) and females (FEC). Data analyzed using unpaired student t-test. Mean +/-S.E.M. **p<0.01 (n=4).

3.7 Glycolytic protein levels in male and female EC

Glycolysis is a major modulator of EC proliferation and migration (De Bock et al., 2013b). Baseline protein levels of important glycolytic enzymes,

glyceraldehyde 3-phosphate dehydrogenase (GAPDH), hexokinase 2 (HK2) and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) were measured in EC cultivated at high density. No significant differences in baseline protein levels were observed between male and female EC (Fig 3-8A, B and C). There was, however, a trend for higher levels of PFKFB3 in female EC, which was observed consistently in each experiment.

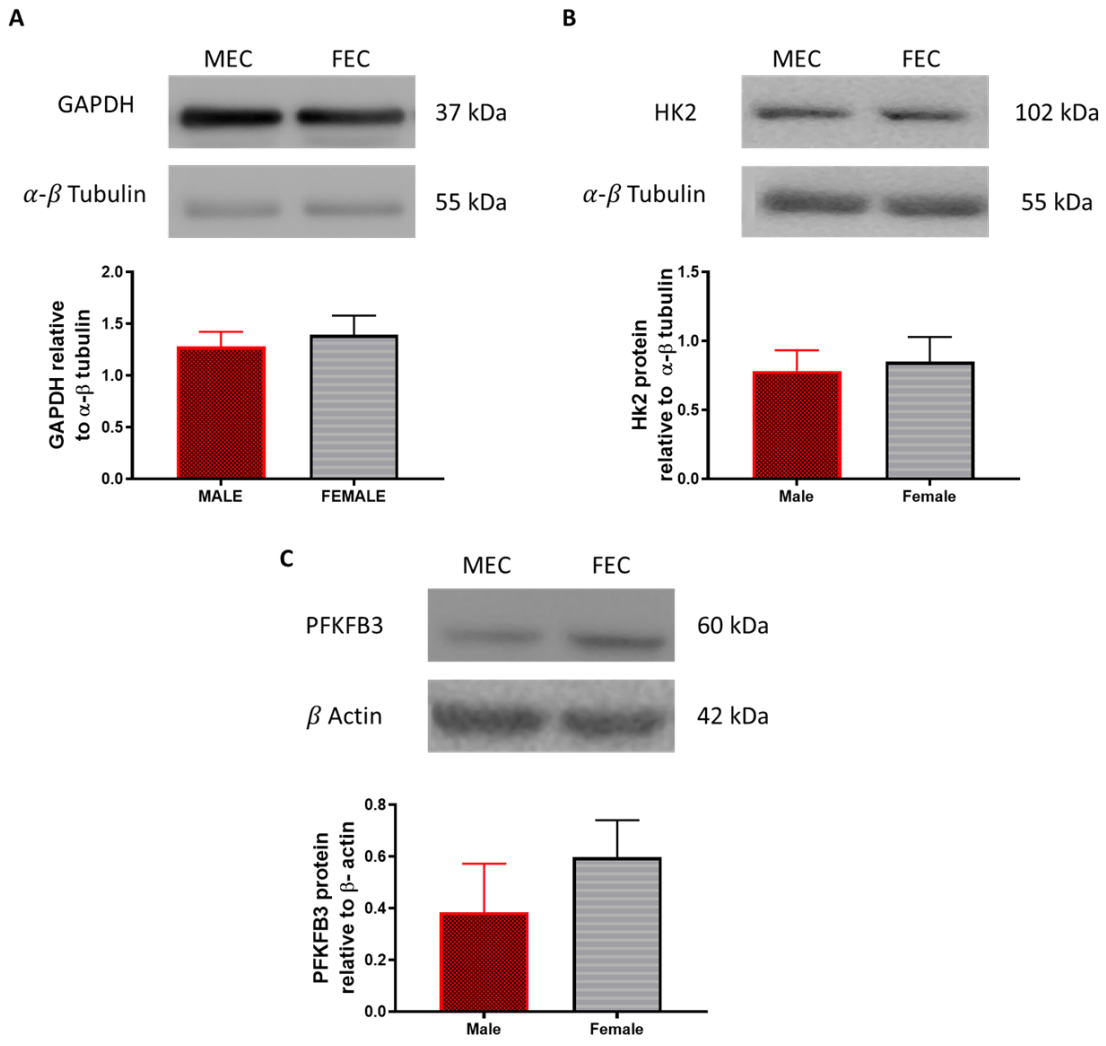


Figure 3-8: **Protein levels of GAPDH, HK2 and PFKFB3 in male vs female EC.** Representative western blots and protein quantification of **(A)** GAPDH and αβ-Tubulin; (**B)** HK2 and αβ-Tubulin; and, (**C)** PFKFB3 and β-actin in males (MEC) and females (FEC). Data analyzed using unpaired student t-test. Mean +/-S.E.M. (n=3-6).

Since the protein level of FoxO1 was significantly higher in female EC compared to males, and FoxO1 is a known modulator of glycolysis in EC (Wilhelm et al., 2016), we analyzed the effect of inhibition of FoxO1 on protein levels of HK2 and PFKFB3. Inhibition of FoxO1 did not significantly change the protein levels of either of these proteins (Fig 9A and B).

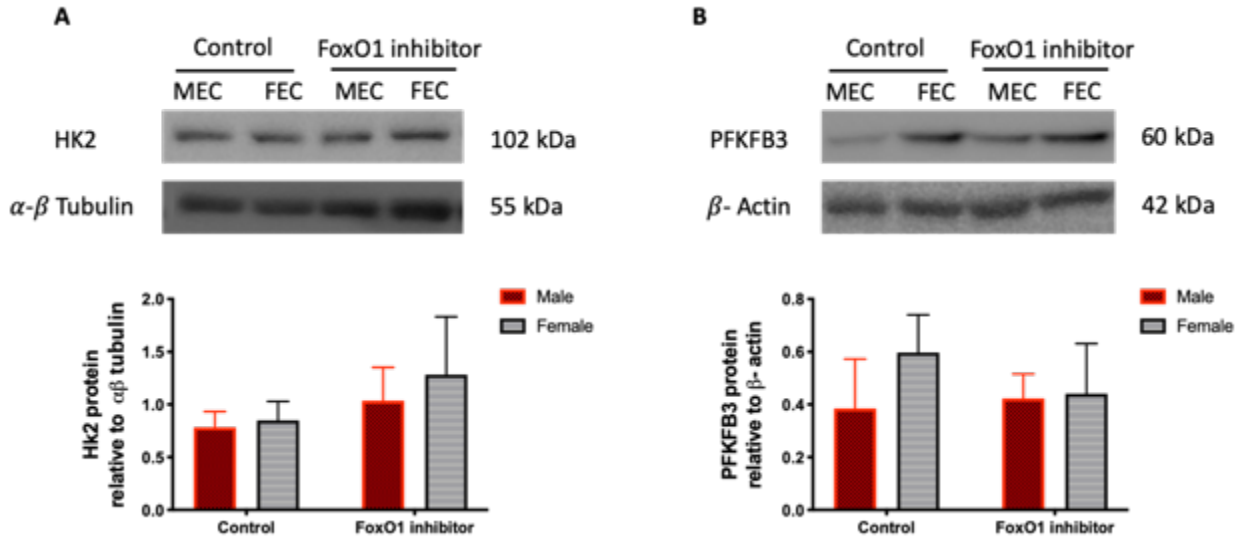


***Figure 3-9*: Effect of FoxO1 inhibition on protein levels of HK2 and PFKFB3 in male vs female EC.** Representative western blots and protein quantification of control and with FoxO1 inhibition (AS1842856-1 μM) **(A)** HK2 and αβ-Tubulin and **(B)** PFKFB3 and β-Actin in males (MEC) and females (FEC). Data analyzed using two-way ANOVA. Mean +/-S.E.M. (n=3).

3.8 Function of angiogenic pathways in male and female EC migration

The effects of p38 MAPK, Notch and FoxO1 inhibition on migration were examined (Fig 3-10D-L). There was a trend for increased migration in male EC with inhibition of Notch (Fig 3-10I) which is consistent with an inhibitory role of Notch signaling on migration of EC (Phng and Gerhardt, 2009). Furthermore, there was a trend for decreased migration with inhibition of

FoxO1 (Fig 3-10L), which is consistent with the reported role of FoxO1 in modulating polarity and the evidence for impaired EC migration and sprouting with inhibition of FoxO1 (Kim et al., 2019).

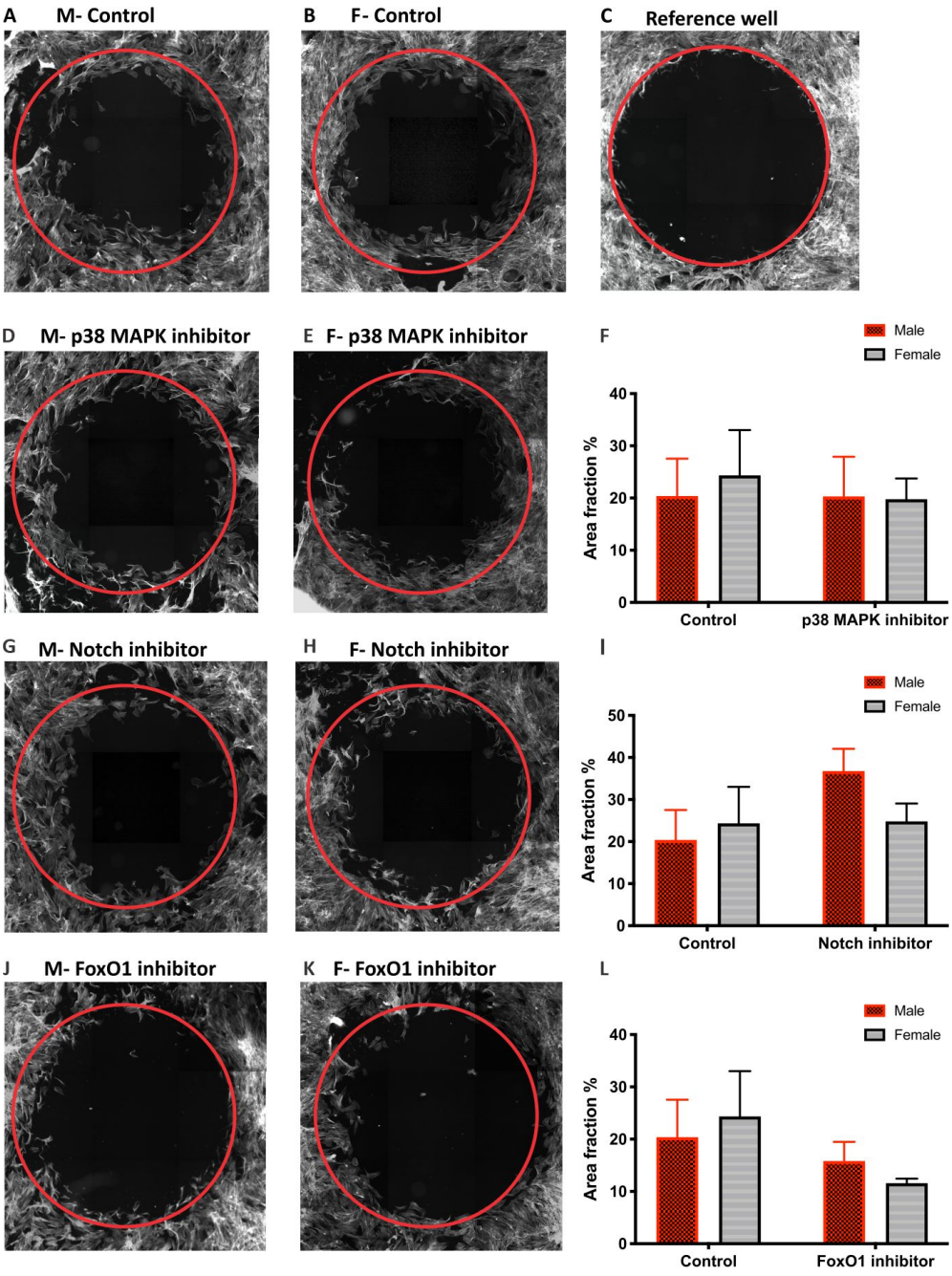


Figure 3-10: **Contribution of cell signaling pathways to migration of male vs female EC.** Representative images of male and female EC in (**A, B**) Control or under treatment with (**D, E**) p38 MAPK inhibitor (SB203580-10µM); (**G, H**) Notch inhibitor (DAPT-10µM); or, (**J, K**) FoxO1 inhibitor (AS1842856-1 µM). (**C**) Representative image from the reference well fixed immediately

after removing the stopper. The cell free area indicated inside the red circle is used as a reference for wells with cell migration. Area fraction percentage of male and female EC migrated into the cell free zone under treatment with (**F**) p38 MAPK inhibitor (SB203580-10µM); (**I**) Notch inhibitor (DAPT-10µM); and (**L**) FoxO1 inhibitor (AS1842856-1 µM). Data analyzed using unpaired student t-test and repeated measures ANOVA (n= 3).

3.9 Function of angiogenic pathways in male and female EC proliferation

Cell density could affect the function of some signaling pathways. For example, cell-cell contacts induce anti-proliferative signals leading to cell quiescence in a mechanism referred to as contact inhibition (Eagle and Leviene, 1967). As an effector of the PI3K/AKT pathway, FoxO1 is involved in inducing endothelial quiescence (Wilhelm et al., 2016). p38 MAPK has also been shown to be involved in the signaling cascade of contact inhibition (Faust et al., 2005). To assess the involvement of signaling pathways at different cell confluency, plates were seeded at high and low cell density and the effects of inhibition of these pathways on EC proliferation was measured.

3.9.1 Low density EC

p38 MAPK inhibition increased proliferation (main effect: p=0.004). Post hoc comparisons indicated a significant effect of the inhibition of MAPK in increasing proliferation only in male EC (Fig 3-11A). Neither FoxO1 nor Notch inhibition altered proliferation in male and female EC (Fig 3-11B, C). Estradiol signaling is known to induce EC proliferation (Zhao et al., 2008) and in line with this, inhibition of ESR1 significantly decreased proliferation in both sexes. There was, however, no significant difference in proliferation between male and female EC with inhibition of ESR1 (Fig 3-11D). Inhibition of the glycolytic enzyme PFKFB3 significantly decreased proliferation in

males but not females and thus PFKFB3 inhibition exaggerated the difference in proliferation between male and female EC (Fig 3-11E). Mitomycin C, a potent inhibitor of proliferation was used as a control in the proliferation assay and significantly inhibited the proliferation of EC (Fig 3-11F).

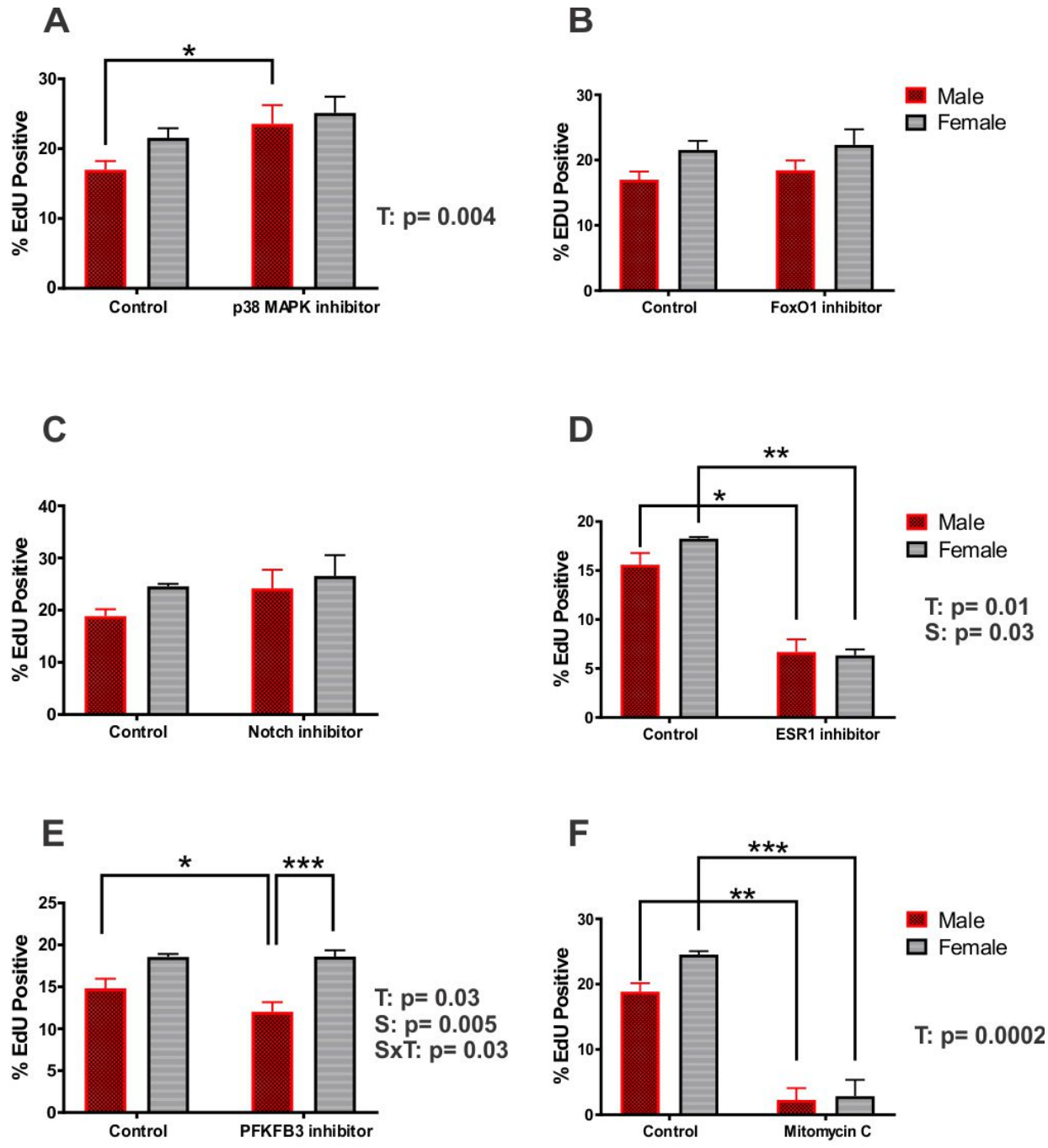


Figure 3-11: **Signaling pathways contribution to the proliferation of sub-confluent male and female EC:** Proliferation assay results in male and female EC with inhibition of **(A)** p38 MAPK (SB203580-10µM); **(B)** FoxO1 (AS1842856-1 µM); **(C)** Notch (DAPT-10µM); **(D)** PFKFB3 (3po-10µM); **(E)** ESR1 (MPP dihydrochloride-10µM); and treatment with Mitomycin C (500ng/ ml) **(F).** Data analyzed using repeated measures ANOVA. Main effects are Sex (S) and Treatment (T). Mean +/-S.E.M. *p<0.05, **p<0.01 ***p<0.001 (n=4-6).

3.9.2 Confluent EC

Male and female confluent EC showed significantly lower proliferation compared to low density cells, consistent with contact inhibition (Fig 3-12A). Proliferation rates at high confluency were higher in female EC compared to males ($p=0.07$) which was consistent with sex-related difference in proliferation observed in low density EC (Fig 3-12B). Inhibition of p38 MAPK increased proliferation independent of EC sex ($p=0.04$). (Fig 3-12C). Inhibition of FoxO1 significantly increased proliferation in both male and female confluent EC (Fig 3-12D). Simultaneous inhibition of p38 MAPK and FoxO1 significantly increased proliferation in both male and female confluent EC (Fig 3-12E). Interestingly, proliferation of EC with inhibition of both p38 MAPK and FoxO1 was significantly higher than proliferation of EC with inhibition of either FoxO1 (Fig 3-12F) or p38 MAPK alone (Fig 3-12G). Inhibition of PFKFB3 had no effect on proliferation of male and female EC and there was only a significant main effect for sex ($p=0.01$) (Fig 3-12H). ESR1 inhibition significantly decreased proliferation in both male and female EC which is consistent with the observed effect of ESR1 inhibition in low density EC (Fig 3-12I).

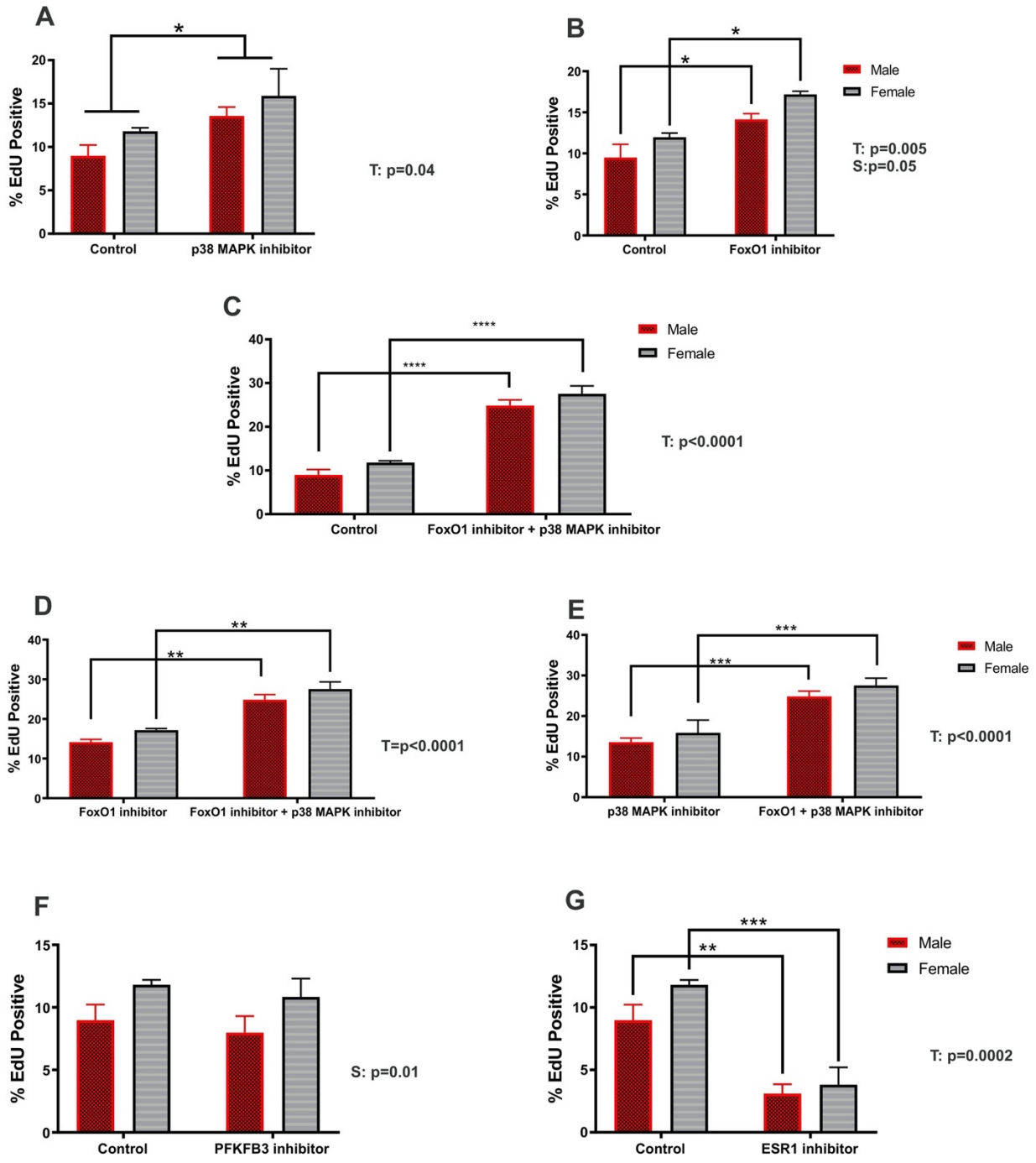


Figure 3-12: Signaling pathway contributions to proliferation of confluent EC. Proliferation assay results in male and female EC following inhibition of **(A)** p38 MAPK (SB203580-10µM); **(B)** FoxO1 (AS1842856-1 µM); **(C)** p38 MAPK and FoxO1 compared to control; **(D)** FoxO1 compared to p38 MAPK and FoxO1; **(E)** p38 MAPK compared to p38 MAPK and FoxO1; **(F)** PFKFB3 (3PO-10µM); **(G)** ESR1 (MPP dihydrochloride-10µM). Data analyzed using repeated measures ANOVA (**A, B, F** and **G),** two-way ANOVA **(C, D** and **E)**. Main effects are Sex (S) and Treatment (T). Mean +/-S.E.M. *p<0.05, **p<0.01 ***p<0.001.

Chapter 4 Discussion

In my thesis, I have established the existence of sex-specific differences in the angiogenic capacity of male and female EC *in vitro*. Female EC from the visceral AT explant were more successful in sprouting *ex-vivo* compared to males. There was, however, no sex-specific difference in gene expression levels of EC markers or VEGF in AT *in vivo*. My experiments showed that female EC have a higher proliferation rate *in vitro* compared to males. Surprisingly, I observed higher protein levels of FoxO1 and phosphorylated p38 MAPK in female EC. Furthermore, I provided evidence showing that FoxO1 is involved in inhibition of proliferation in confluent EC. p38 MAPK on the other hand suppressed proliferation of EC under low confluency in males, whereas in confluent EC, p38 MAPK was involved in inhibition of proliferation independent of the sex. Additionally, these two pathways acted independently in confluent EC since inhibiting both at the same time resulted in a greater decrease in cell proliferation.

4.1 Sex differences in sprouting capacity

Our lab's previous study provided evidence for higher angiogenesis in high fat-fed female mice compared to males (Rudnicki et al., 2018). Studying AT sprouting *ex-vivo* has been shown to reflect angiogenic growth in-vivo (Gealekman et al., 2011; Greenway et al., 2007). Utilizing this method, I was able to study angiogenesis in a controlled environment. The higher sprouting success of female AT explants was in line with the higher female AT

angiogenesis observed previously. It is unlikely that the higher sprouting success of female AT explants could be due to a higher number of EC present in the explant at the start of the assay, since my results indicated no disparity in EC content in AT of males and females. Male AT explants on the other hand, exhibited higher area and density of sprouting compared to females, which contrasts with our previous findings of lower AT angiogenesis in males. An important consideration here is that in the previous study, lower angiogenesis was observed in males compared to females when under a high fat diet. In the current project, explants were from normal chow fed mice. It is possible that a different outcome would have been observed if the *ex-vivo* experiments were repeated using AT from high-fat fed mice.

The higher density and area of sprouting in male explants could be an indicator of higher proliferation and migration of EC *ex-vivo*. The higher sprouting success of female explants, on the other hand, could be indicative of a higher capacity of female EC to initiate proliferation and migration and transform into the tip and stalk cells required for sprouting (Gerhardt et al., 2003). This higher propensity to initiate sprouting could be either a result of higher inherent female EC capacity to proliferate and migrate or the presence of a more pro-angiogenic environment in female visceral AT.

4.2 No difference in VEGFA expression in male and female ex-vivo

Examining the angiogenic microenvironment of a tissue requires measuring the gene expression and protein levels of several of angiogenic factors. There

was no sex difference in the gene expression of VEGFA in AT of 10-week old mice. Based on this, I conclude that the distinct sex-related patterns in AT sprouting *ex-vivo* was not due to differences in VEGFA expression. Although VEGFA is considered to be a major angiogenic factor, there are other pro-angiogenic growth factors and pathways such as angiopoietin (ANGPT), Notch and PDGFB (Hellström et al., 2007; Papetti and Herman, 2002; Yancopoulos et al., 2000). While our lab's previous study on 16-week old mice, indicated higher gene expression of *Angpt2* in females AT compared to males (Rudnicki et al., 2018), the same study did not find any sex-specific differences in Notch pathway ligands, Dll4 and Jag1. In summary, I observed no difference in *Vegfa* mRNA expression in AT but comparing the pro-angiogenic state of male and female AT requires a more extensive examination of additional angiogenic factors.

4.3 EC angiogenic capacity

During sprouting angiogenesis, specialized endothelial tip cells migrate and connect with each other to form new sprouts while stalk cells are proliferating to form the lumen and elongate the new vessel (Gianni-Barrera et al., 2011). The lack of sex difference in migration of EC observed in the present study is in contrast with the higher sprouting success of female AT observed in our *ex-vivo* assay as well as higher AT angiogenesis in female mice documented in our previous study (Rudnicki et al., 2018). Due to limitations with my migration assay that will be discussed later, I am

reluctant to draw any conclusions for the functions of signaling pathways in migration of male and female EC.

The higher proliferation of female EC *in vitro* provides a tissue environment-independent explanation for the observed higher angiogenesis in high-fat fed female mice. Proliferation is a major process in sprouting angiogenesis (Risau, 1997). Several studies have compared the proliferation of EC extracted from various tissues in males and females. Consistent with our results, a study comparing male and female HUVEC provided evidence for higher female EC proliferation with 10% FBS without addition of E2 to the media (Addis et al., 2014). Another study using human pulmonary microvascular endothelial cells (HPMEC) showed higher female proliferation compared to males after 72 hours of *in vitro* cell growth with 5% FBS and no E2 supplementation (Zhang et al., 2018). Conversely, male rat skeletal muscle microvascular EC showed higher proliferation compared to females with 20% FBS and growth factors in a study by Huxley et al. (2018), indicating possible differences in EC proliferation based on the species and/or the tissues from which the EC were extracted and the cell culture conditions.

4.4 Differences in signaling pathways and their contributions to proliferation of male and female EC

Several signaling pathways control proliferation in EC. I examined the differences in protein expression and functions of MAPK, FoxO1, ESR1 pathways as well as PFKFB3, a glycolytic enzyme, in male and female EC to

understand the mechanisms underlying the observed sex-specific difference in EC proliferation.

ERK1/2, JNK and Akt

Members of MAPK pathway, ERK1/2 and JNK are involved in regulation of angiogenesis in EC. ERK1/2 promotes EC proliferation and survival whereas JNK is involved in glucose deprivation and cellular stress response (Abe Jun-ichi et al., 2000; Chang and Karin, 2001). The results of my protein measurements showed no sex-specific difference in phosphorylation levels of these proteins in EC. Therefore, the observed higher proliferation of female EC appears unlikely to be due to differences in activities of ERK1/2 and JNK.

Similarly, the phosphorylation levels of another kinase involved in modulating angiogenesis, Akt, did not show any differences in male and female EC. Akt is involved in promoting EC survival, proliferation and migration (Kureishi et al., 2000; Morales-Ruiz et al., 2000).

VEGFR2 activation leads to phosphorylation of MAPK proteins as well as Akt (Dimmeler et al., 2000; Lamalice et al., 2006). Under my experimental conditions, however, I observed no effect of VEGFA on phosphorylation of ERK1/2, p38 MAPK and Akt. This is in contrast with the known effect of VEGF on these proteins activation in other types of EC (Aiken and Birot, 2016; Ren et al., 2010; Romano et al., 2014; Sack et al., 2016). This may indicate that visceral AT EC are not responsive to VEGFA. Alternatively, it could indicate that other growth factors present in the media can compensate

in the absence of VEGF and therefore other signaling pathways such as epidermal growth factor receptor pathway (EGFR) are active which are capable of activating the same downstream targets as VEGFR2 pathway (Sigismund et al., 2018). Measuring VEGFR2 phosphorylation would give a better understanding of the activity of this pathway in the absence and presence of VEGFA. Although in my experiments I serum-starved the cells for 2 hours, longer serum-starvation may help to control for confounding variables such as serum that contains a host of growth factors including VEGF, FGF and EGF.

FoxO1 and p38 MAPK

The higher protein levels of FoxO1 and p-p38 MAPK in female EC compared to males were unexpected as both p38 MAPK and FoxO1 are involved in controlling proliferation by inhibiting a number of cell cycle related factors such as c-MYC and CyclinD1 (Dang, 2013; Wilhelm et al., 2016; Zhang and Liu, 2002).

In justification of this apparent paradox between FoxO1 level and proliferation, it is important to consider that protein levels of FoxO1 may not directly be associated to its transcriptional activity. FoxO1 is regulated by a wide range of factors such as insulin, cytokines and oxidative stress (Daitoku et al., 2011). These stimuli mainly regulate FoxO1 activity by altering its post-translational modifications such as phosphorylation, acetylation and methylation (Daitoku et al., 2011). These modifications in turn control FoxO1 localization, protein levels and DNA-binding properties (Calnan and

Brunet, 2008). One of the best known post-translational modifications of FoxO1 is Akt-mediated phosphorylation, which leads to FoxO1 translocation from the nucleus and subsequent degradation in cytoplasm (Biggs et al., 1999; Dong et al., 2008; Matsuzaki et al., 2003; Tzivion et al., 2011). However, my experiments did not indicate any sex difference in p-FoxO1 levels. As mentioned before, there are other relevant modifications that I have not assessed in this project. For this reason, measuring nuclear FoxO1 content or its transcriptional activity, may give a better understanding of protein activity. I did attempt to measure the nuclear FoxO1 protein level but my efforts for extracting cytoplasmic and nuclear content of EC and measuring FoxO1 levels were unsuccessful due to presence of cytoplasmic proteins in the nuclear extract, indicating a failure to efficiently separate the nuclear and cytoplasmic fractions.

Higher protein levels of FoxO1 and phosphorylation of p38 MAPK in female EC may be a compensatory response to the higher proliferation rate observed in these cells as part of a negative feedback loop. There is evidence for higher expression of FoxO1 in cells with high proliferative rate. For instance, Wang et al. (2018) compared the protein levels of FoxO1 in gastrointestinal stromal tumor cells (GIST-T1) with the normal human fibroblast (WI-38) and found higher levels of FoxO1 in the faster proliferating GIST-T1 cells. Similarly, there is abundant evidence for upregulation of p-p38 MAPK in a number of tumor cell types including lymphoma, glioma and head and neck squamous cell carcinoma (Demuth et al., 2007; Elenitoba-Johnson et al., 2003; Junttila

et al., 2007), which may be a compensatory response to high rate of proliferation in these cells. It is worth mentioning that higher levels of activation of p38 MAPK in tumor cells may be related to other roles of p38 MAPK including migration, invasion, response to stress and DNA damage (Wagner and Nebreda, 2009). For instance, a few studies have examined the increase in activity of p38 MAPK in response to DNA damage induced by UV irradiation (Wood et al., 2009). Similarly, in female EC p38 MAPK may be upregulated to induce the DNA damage mechanisms repair system that is required when cells are proliferating at higher rates.

The proliferation assay results showed a role for FoxO1 in inhibition of proliferation in confluent cells, indicating that FoxO1 is involved in contact inhibition of proliferation. Contact inhibition is described as the tendency of cultured cells to stop or slow their proliferation as they get close to confluence (Motti et al., 2005). Cell-cell adhesion molecules such as cadherins, particularly VE-cadherin in EC, are considered as the initiators of contact inhibition of proliferation (Niessen et al., 2011). E-cadherin activates the Hippo pathway, mainly through catenins such as β-catenin bound to E-cadherin (Kim et al., 2011). Activation of the Hippo pathway inhibits the action of Yes-associated protein (YAP)-1, which is involved in upregulation of proliferation-promoting genes such as Myc and Cyclin D1 (Schlegelmilch et al., 2011; Silvis et al., 2011). Cyclin D1 is a known target of FoxO1 and it has been shown that inhibition of Cyclin D1 is associated with contact inhibition in confluent cells (Gookin et al., 2017). In summary, I conclude

that in EC under my experimental conditions, FoxO1 is mainly involved in contact inhibition of proliferation.

The greater influence of p38 inhibition in sub-confluent male vs. female EC could indicate a greater role of p38 in suppression of proliferation in male EC. However lower baseline (control groups) proliferation of male EC compared to females could exaggerate the effect of inhibition of p38 MAPK in male EC observed in sub-confluent cells. Baseline proliferation rate of male EC was ≃17% compared to ≃22% in females after 4 hours of incubation with EdU. Inhibition of p38 MAPK increased proliferation to 24% and 25% in males and females respectively. Since females' baseline proliferation was closer to their maximum proliferative capacity (observed by inhibiting both p38 and FoxO1 simultaneously), inhibition of p38 MAPK led to a smaller increase in their proliferation whereas male baseline proliferation was considerably lower and the increase as a result of p38 MAPK inhibition was significant. The results of proliferation assays in confluent cells are in line with this hypothesis. EC of both sexes had a lower proliferation rate in confluent cells; inhibition of p38 MAPK had a significant effect in increasing the proliferation in both and there was no sex related difference in this observed effect.

p38 MAPK was involved in inhibition of proliferation regardless of the confluency of plated EC whereas FoxO1 had a significant effect on proliferation only in confluent EC. Based on this observation, I hypothesize that these two pathways act independently of each other in inhibition of

proliferation. This hypothesis is supported by the effects of inhibition of both p38 MAPK and FoxO1 in EC, which indicated an additive effect of each pathway in proliferation. Overall, my thesis provides evidence for the anti-proliferative function of p38 MAPK in both confluent and sub-confluent EC whereas FoxO1 appears involved in contact inhibition of confluent EC.

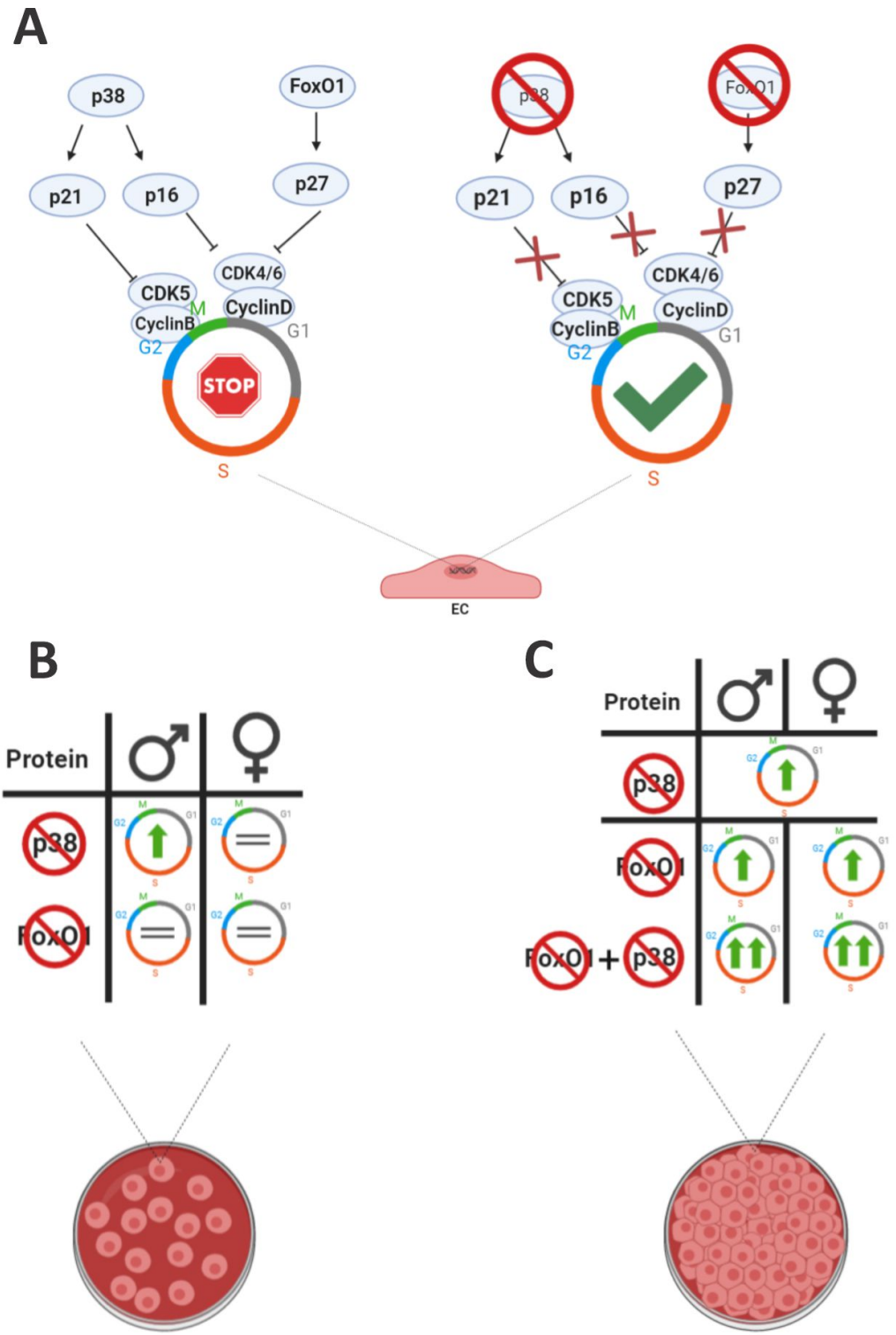


Figure 4-1: **Summary of the effects of p38 and FoxO1 on the proliferation of male and female EC. (A)** Schematic depicting FoxO1 and p38 MAPK function in EC proliferation. Effects of inhibition of p38 MAPK and/or FoxO1 in **(B)** sub-confluent and **(C)** confluent male and female EC (Created with BioRender).

PFKFB3

EC rely on glycolysis for ATP production and PFKFB3 is the most potent stimulator of glycolysis (De Bock et al., 2013b). This enzyme is involved in modulation of EC proliferation and migration. Although there was a trend for higher PFKFB3 protein levels in female EC, the difference was not significant. There is evidence for the importance of fatty acid oxidation in EC metabolism (Bruning et al., 2018; Kalucka et al., 2018; Patella et al., 2015; Schoors et al., 2015; Vanetti et al., 2017). The higher proliferation in female EC may indeed be a result of a higher capacity of these cells to utilize fatty acid as an energy source. Furthermore, the reduction in proliferation in male but not female EC with PFKFB3 inhibition may indicate a higher dependence of male EC on glycolysis for energy production whereas female EC may switch to other energy pathways such as fatty acid oxidation to replenish ATP production. A study by Vanetti et al. (2017) concluded that male HUVEC have higher dependence on fatty acid oxidation for *in vitro* sprouting compared to females which is in contrast to our findings. No effect of PFKFB3 inhibition in confluent EC is consistent with studies indicating that quiescent EC upregulate fatty acid oxidation (Kalucka et al., 2018). Overall, flexibility of female EC in using different energy sources compared to males, which are mostly dependant on glycolysis for proliferation, could be one of the underlying reasons for higher proliferation in female EC. Further experiments measuring glucose uptake and lactate production as well

as measuring protein levels of fatty acid oxidation related protein carnitine palmitoyl- transferase 1A (CPT1a) would provide a better understanding of metabolism in male and female EC.

ESR1

In my experiments, inhibition of the ESR1 pathway eliminated the observed sex difference in EC proliferation. This suggests a role of ESR1 signaling in the higher proliferation of female EC compared to males. ESR1 signaling is mainly activated through ligand (E2) binding. Although I did not add E2 to the cell culture media, there is the possibility for some residual activation of this pathway. The concentration of estrogen in the media was very low (40.5 pg/mL). There is, however, evidence for activation of E2 signaling when media containing FBS is not charcoal-stripped (Cao et al., 2009; Tekkatte et al., 2011). Moreover, phenol red, which is a common component of cell culture media, is known to have estrogenic activity (Liu et al., 2013; Welshons et al., 1988). E2 exerts its angiogenic effect mainly through ESR1 signaling, which leads to increased EC proliferation and migration (Concina et al., 2000; Morales et al., 1995; Simoncini et al., 2006). E2 enhances angiogenesis through ESR1 signaling by upregulating VEGF expression in AT (Fatima et al., 2017). It is worth mentioning that ESR1 signaling could also get activated in a ligand-independent manner and with cross-talk with other pathways including EGF and IGF-1 (Ignar-Trowbridge et al., 1995; Lupien et al., 2010; Straus and Takemoto, 1990).

Higher activation of ESR1 pathway in females could be a result of higher expression of ESR1 protein in female EC. A study on the ESR1/2 protein level of male and female HUVEC by Addis et al. (2014) did not find any difference in their protein level. My western blot experiments to measure protein levels of ESR1 in EC were unsuccessful. E2 has been shown to activate the ERK1/2 pathway, resulting in stimulation of proliferation and migration of EC (Geraldes et al., 2002; Lantin-Hermoso et al., 1997). My results, however, did not indicate any differences in ERK1/2 phosphorylation, therefore E2 activation of ERK1/2 could not explain the observed higher proliferation in female EC. Examining the function of ESR1 signaling in the observed sex-specific difference in EC proliferation requires more experiments that will be discussed later.

Chapter 5 Conclusion

5.1 Significance

My Master's thesis provides evidence for a higher proliferative capacity of female EC compared to males as well as differences in the activation of signaling pathways involved in angiogenesis in culture conditions used in the current project. Our lab's previous work has indicated that female mice have increased AT vascularity in response to a high fat diet whereas male mice have diminished AT vascularization (Rudnicki et al., 2018). My thesis is a step toward determining the mechanisms accounting for this observed sex related difference in AT angiogenesis in vivo. Based on my findings, I

propose that the higher proliferative capacity of EC in females is a novel underlying mechanism for higher angiogenesis in female AT. Furthermore, I have established the existence of sex differences in protein levels of FoxO1 and the phosphorylation of p38 MAPK, which are important signaling pathways involved in the regulation of EC proliferation. Finally, my thesis provides evidence for the lack of effect of VEGFA in the activation of MAPK pathways in EC extracted from visceral AT under the culture conditions used in my experiments. This is in contrast with the observed effects of VEGFA in activating MAPK in EC extracted from tissues other than visceral AT and other species (Aiken and Birot, 2016; Ren et al., 2010; Romano et al., 2014; Sack et al., 2016). This is in line with a number of studies that illustrate differences in characteristics of EC based on the tissue from which they are extracted (Gogg et al.; Huxley et al., 2018). This lack of response of visceral AT EC to VEGFA requires further confirmation. Based on this observed heterogeneity in EC behaviour, it is not appropriate to assume that all EC will respond the same way to the same stimuli.

5.2 Limitations

There were several methodological limitations that may have impacted the results of my experiments. The small pieces of AT used for the *ex-vivo* sprouting assay were isolated from 12 female mice irrespective of the ovarian cycle. E2 is a known regulator of a number of angiogenic factors including VEGF and leptin, and differences in estrous cycle of mice at the time of tissue collection could have introduced variability in the results related to

explant sprouting capacity (Wang et al., 2000; Yamaguchi et al., 2002; Zysow Bernice R. et al., 1997). Another possible source of variability in the *ex-vivo* sprouting assay was the variation in the size of AT explants that were used. Larger explants tend to contain higher amounts of angiogenic growth factors and higher numbers of EC that may lead to higher sprouting capacity.

Most of assays I conducted were dependent on comparing male and female EC *in vitro*. Culturing cells provided the opportunity to perform experiments on cells in similar environments, using the same incubation conditions and the cell culture media used had the same constituents. However, differences in growth rate of male and female EC led to difficulties in maintaining the cells at the same level of confluency. This could affect the EC behaviour, gene expression and protein levels and introduce variability to the results.

Since my proliferation assays were performed *in vitro* in an isolated setting that is not identical to the in-vivo environment, the results may not provide a full explanation for possible sex related differences in EC. Therefore, addressing other factors such as exposure to sex hormones, growth factors and cytokines that are normally present in vivo is necessary to get a clearer picture of sex related differences in EC. The EC used in this project were cultured in 20% FBS media without additional growth factors (VEGF or FGF). We stopped treating EC with VEGF after observing the lack of effect of VEGF on activation of a number of signaling pathways including Akt and MAPK pathways. However, recent unpublished data from our lab has identified increased levels of expression of mesenchymal cell marker genes

which may indicate de-differentiation of EC to mesenchymal cells of EC in the absence of some important growth factors and this could affect outcomes being measured.

Certain assays used to compare EC angiogenic capacity in my project could limit interpretations of the results. In order to measure proliferation, I initially used the CyQuant™ assay, which measures the DNA content of cells. For CyQuant™ assay, control plates frozen at time zero had to have the same starting cell count as the proliferation plates and any small variations could affect the reproducibility of measurements. As a result of this, getting reproducible results was a challenge. Since in the EdU corporation assay, I was able to count the total number of cells plated in each well and count the cells that were proliferating (Fig 4A and B), it was not prone to errors arising from possible variation in starting cell count.

The results of the migration assay were not conclusive. The 96 well collagen coated plate used for this assay had to be reused as stoppers creating the cell free zone were specific to that type of plate and I was analyzing fewer than 96 wells in each experiment. As a result of this, long term use of the same plate for different experiments could have impacted the ECM coating of the plate leading to poor adhesion of cells. Using freshly coated collagen plates for each experiment could eliminate this issue with cell adherence and result in more consistent results. Notch pathway functions in the context of sprout formation and EC-EC interactions during lumen formation where cells are interacting with each other in all directions and not only as a monolayer

(Phng and Gerhardt, 2009; Zhao et al., 2017). The migration assay I used was performed in 2D while assays that allow for EC-EC interactions in 3D would be more useful in determining the effects of Notch in male and female EC. Notch ligands are expressed on tip cells and activate Notch pathway in the surrounding EC in response to VEGF gradient. Without the VEGF gradient, the Notch ligands are not expressed on tip cells and activate the Notch pathway will not happen. For these reasons, testing the Notch pathway *in vitro* using the migration assay that I used may not reflect the in vivo conditions. I tried measuring migration/invasion distance of EC using a monolayer of cells invading into collagen. The results, however, were inconsistent due to difficulties with cutting the collagen in thin sections that were needed for imaging.

I relied on inhibitors to determine the function of different signal pathways in both proliferation and migration assays. One issue with using inhibitors is their off-target effects that may affect the results. As an example, there is evidence for an off target effect of ESR1 inhibitor MPP dihydrochloride which could lead to activation of apoptotic pathways and thus decrease proliferation independent of the effect of E2 (Al-Khyatt et al., 2018). The p38 MAPK inhibitor, SB203580, used in my project is known to have an off target effect on blocking Akt phosphorylation, which is an important upstream kinase of p38 MAPK (Lali et al., 2000). Another inhibitor used in my experiments, 3PO which inhibits the activity of PFKFB3 enzyme, could inhibit other PFKFB isoenzymes, due to similarities of kinase active sites

across all PFKFB proteins (Houddane et al., 2017). An alternative approach that could prevent the off-target effect of inhibitors would be to use small interfering RNA (siRNA) to decrease the expression of the target gene rather than to inhibit the activity of the target protein.

5.3 Future Directions

If I was continuing, I would test culturing cells in a hormone-free media, such as charcoal stripped serum (CSS), which could establish if the observed higher proliferation of female EC is due to their inherent higher capacity to respond to E2 or if there is another inherent sex related difference in EC. Using this approach, measuring FoxO1 and p-p38 in hormone free media with and without addition of E2 helps determine the effect of ESR1 signaling on p38 MAPK activation. It should be noted that one limitation is that charcoal stripping also removes fatty acids that are essential for EC growth, which may impede the normal proliferation, migration and gene expression in EC (Vanetti et al., 2017).

The higher FoxO1 protein levels in females could lead to higher activity of ESR1 in female EC. Using a biotin-DNA pull down assay, Foulds et al. (2013) identified FoxO1 as a co-regulator that binds to E2-liganded ESR1. Furthermore, by using siRNA against FoxO1 and measuring its effect on reporter genes, they indicated that FoxO1 is a co-activator of ESR1 pathway (Foulds et al., 2013). Another study by Schuur et al. (2001) showed FoxO1 and ESR1 interaction using a yeast two-hybrid screen and reported that

FoxO1 enhances ESR1 transactivation. Conversely, their study also showed an inhibitory effect of E2-liganded ESR1 on FoxO1 mediated transactivation.

There is evidence for E2 mediated activation of p38 MAPK by non-genomic ESR1 signaling (Anter et al., 2005; Hsu et al., 2007; Kan et al., 2008). The observed higher levels of phosphorylation of p38 MAPK in female EC in my project may be a result of greater activation of ESR1 signaling in females in response to E2 present in the cell culture media.

In summary, ESR1 signaling is activated by ligand-dependent or ligand-independent mechanisms. To assess which type of ESR1 signaling activation affects proliferation, charcoal-stripped serum media could be used to culture cells and assess cell proliferation. ESR1 signaling could upregulate phosphorylation of p38 MAPK and FoxO1 is a co-activator of ESR-1 signaling.

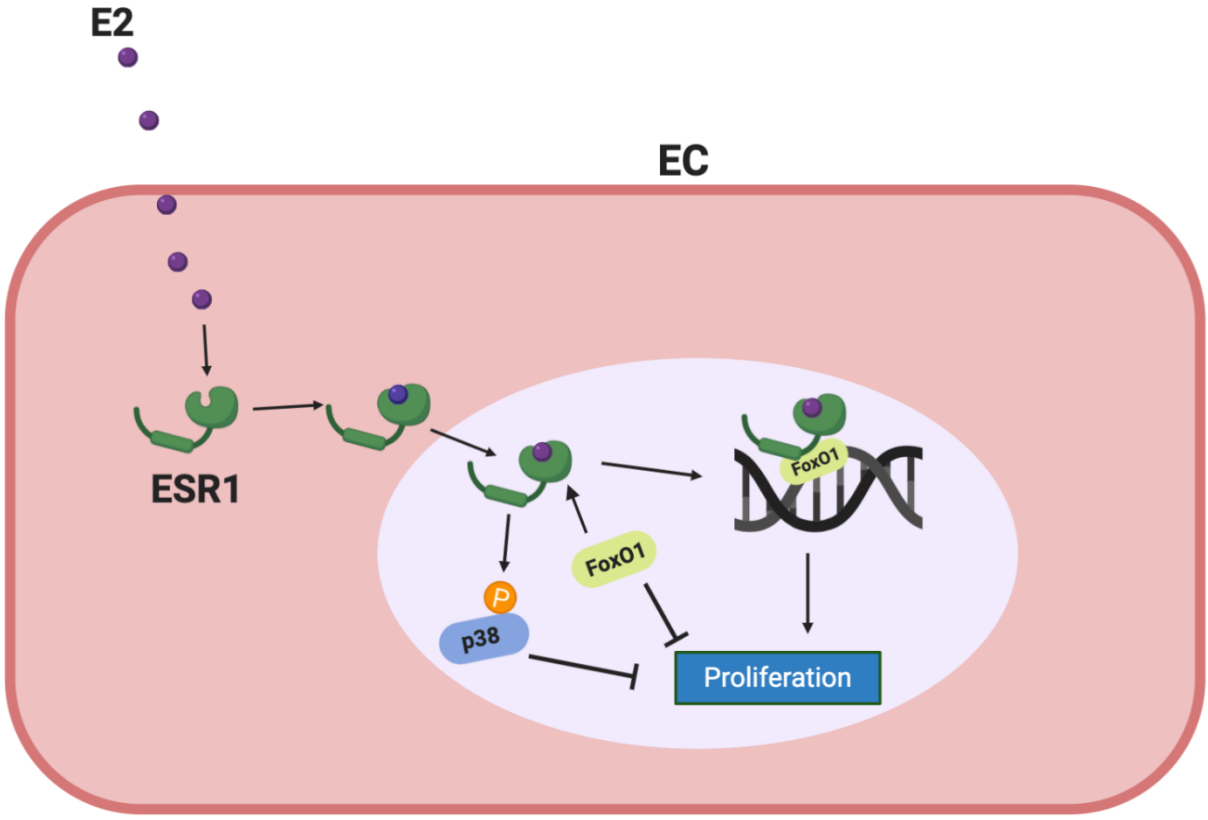


Figure 5-1: **Schematic depicting potential interactions between ESR1 signaling, FoxO1 and p38 MAPK in the regulation of EC proliferation.** ESR1 signaling could activate p38 MAPK, which in turn inhibits proliferation. FoxO1 is a co-activator of E2-liganded ESR1.

In vivo, any sexual dimorphism in the angiogenic behaviour of EC is a result of a combination of inherent differences arising from sex chromosomes and the effect of continuous exposure to sex hormones as well as angiogenic microenvironment of the tissue (Kim-Schulze et al., 1996; Losordo and Isner, 2001; Morales et al., 1995; Venkov et al., 1996). My project focused on inherent sex differences in the angiogenic capacity of EC in a controlled environment in isolation. To get a clearer picture of underlying mechanisms

responsible for sex related differences of EC, *in vivo* models that could characterize the effects of both sex hormones and sex chromosomes in the microenvironment of the tissue may be more informative. To achieve this, surgical removal of the gonads in male and female mice (gonadectomy) could be used to compare the male and female EC angiogenic capacity *in vivo* independent of the effect of the sex hormones. Furthermore, by adding the sex hormones in controlled dosage to each sex, the contribution of these hormones to EC angiogenic capacity could be determined.

These proposed future experiments will be valuable in further clarifying the underlying mechanisms for higher female AT angiogenesis which is associated with lower susceptibility to obesity related disorders in females. Using this approach, the contribution of inherent differences in male and female EC to observed disparity in AT angiogenesis will be clarified. This in turn may lead to better understanding of physio-pathological differences in male and female cardio-metabolic function. A greater appreciation of inherent sex-related differences in EC may suggest new targets for preventative and therapeutic approaches in treating obesity-related disorders such as CVD and type II diabetes.

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