

Examining sex differences of adipose endothelial cells and the effects of FoxO1 deletion

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Abstract

During obesity, adipose tissue undergoes significant remodeling to store excess energy. A key aspect of healthy expansion is adequate vascular growth, which ensures proper oxygen and nutrient supply and waste removal. Without sufficient vascularization, adipose tissue can become hypoxic and dysfunctional, promoting insulin resistance and metabolic disease. Thus, coordinated angiogenesis is critical for metabolic health during obesity. Our lab previously found that female mice exhibit greater adipose tissue angiogenesis than males during high-fat feeding. This dissertation aimed to uncover the factors behind this sex-based difference. Transcriptomic analysis of adipose endothelial cells (ECs) from 7-week high-fat fed mice revealed that female ECs displayed a more proliferative and chromatin-remodeled profile, while male ECs were enriched in inflammatory and senescence-associated signatures. These findings were confirmed in vivo and in vitro where female adipose tissue had more proliferating ECs, while male ECs were more responsive to inflammatory stimuli. Similar sex differences were observed in aging ECs. To assess the influence of sex hormones, I used a gonadectomy model combined with high-fat feeding. Surprisingly, the loss of gonadal hormones increased capillary density despite reduced levels of vascular endothelial growth factor-A (VEGFA) in the adipose microenvironment. However, gonadectomy had no long-term effect on EC metabolism in culture, though direct estradiol stimulation did increase mitochondrial ATP production. These results suggest that the enhanced angiogenesis in females is largely independent of sex hormones, pointing instead to intrinsic properties of female ECs. One candidate regulator is the endothelial quiescence-inducing transcription factor forkhead box O1 (FoxO1), which is more highly expressed in female ECs despite higher angiogenic activity. FoxO1 knockdown or inhibition in females led to a dysfunctional EC phenotype which was marked by inflammation, oxidative stress, impaired

mitochondrial function, and DNA damage. These endothelial disturbances caused by FoxO1 loss were accompanied by lower capillary content in the females, a response not mirrored in males. Overall, these studies demonstrate that female ECs exhibit a healthier phenotype under obesogenic conditions, a trait that appears to be largely independent of sex hormones. Instead, FoxO1 emerges as a major contributor to this protective and resilient female endothelial profile.

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List of Abbreviations

ADK – adenosine kinase

AGO1 – argonaute 1

Ang2 – angiopoietin 2

AR – androgen receptor

ATGL – adipose triglyceride lipase protein

ATP – adenosine triphosphate

BH4 – tetrahydrobiopterin

BMP – bone morphogenic protein

BP – biological processes

Cav1 – caveolin-1

CDK – cyclin dependent kinases

CPT1A – carnitine palmitoyltransferase

CREB – cAMP response element-binding

CVD – cardiovascular disease

DAVID – Database for Annotation, Visualization and Integrated Discovery

DEG – differentially expressed gene

DHT – dihydrotestosterone

DLL4 – notch ligand Delta-like 4

E2 – estradiol

EC – endothelial cell

ECM – extracellular matrix

ER – estrogen receptor

ETC – electron transport chain

eNOS – endothelial nitric oxide synthase

FABP – fatty acid binding proteins

FA – fatty acid

FATP – fatty acid transport proteins

FC – fold change

FDR – false discovery rate
FFA – free fatty acid
FGF – Fibroblast growth factors
FOXO – forkhead box O
FVB – Friend Virus B
GABBR2 – gamma-aminobutyric acid type B receptors subunit 2
GADD45 – DNA Damage-inducible protein 45
GLUT1 – glucose transporter 1
GO – gene ontology
GPx – glutathione peroxidase
GSEA – gene set enrichment analysis
GTT – glucose tolerance test
H3 – histone 3
HDL – high-density lipoprotein
HF – high fat
HIF – hypoxia inducible factor
HUVECs – Human umbilical vein endothelial cells
ICAM1 – intracellular adhesion molecule 1
IRS1 – insulin response substrate 1
ITT – insulin tolerance test
KD – knockdown
KO – knockout
LPL – lipoprotein lipase
MAPK – mitogen activated protein kinase
MFN – mitofusin
MMP – matrix metalloproteinase
mTORC1 – mammalian target of rapamycin complex 1
NADPH – nicotinamide adenine dinucleotide phosphate
NC – normal chow
NES – normalized enrichment score

NF- κ B – Nuclear Factor kappa-light-chain-enhancer of activated B cells
 NO – nitric oxide
 OPA1 – optic atrophy protein 1
 p70S6K – p70 ribosomal protein S6 kinase
 PAF – platelet-activating factor
 PCA – principal component analysis
 PDGF – platelet-derived growth factor
 PDK – 3-phosphoinositide-dependent protein kinase 1
 PECAM1 – Platelet endothelial cell adhesion molecule 1
 PFKFB3 – glycolytic activator 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
 PI3K – phosphoinositide 3-kinase
 PIP3 – phosphatidylinositol (3,4,5)-trisphosphate
 PPAR γ – peroxisome proliferator activated receptor γ
 PP2A – Protein Phosphatase 2
 PR – progesterone receptor
 PRMT1 – arginine methyltransferase-1
 PTPMT1 – protein tyrosine phosphatase mitochondrial 1
 ROCK1 – rho-associated coiled-coil containing protein kinase 1
 ROS – reactive oxygen species
 SASP – senescence associated secretory phenotype
 SEM – standard error of the mean
 SGK – serum-and glucocorticoid-inducible kinase
 SIRT – sirtuin
 SOD – superoxide dismutase
 TGF β – transforming growth factor beta
 TNF α – tumour necrosis factor alpha
 TSP1 – thrombospondin-1
 UCP1- uncoupling protein 1
 VCAM1 – vascular cell adhesion molecule 1
 VE-cadherin – vascular endothelial cadherin

VEGFA – vascular endothelial growth factor-A
 VEGFR2 – vascular endothelial growth factor receptor 2
 VWF - von Willebrand Factor

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Chapter 1: Literature Review

1.1 The Microvasculature

The circulatory system is a complex network responsible for transporting blood, nutrients, oxygen, carbon dioxide, and hormones throughout the body. It consists primarily of the heart and blood vessels working together to maintain homeostasis and support the body's vital functions (Pittman, 2011). The heart acts as a pump, sending oxygen-rich blood through arteries to tissues and organs, while veins carry oxygen-depleted blood back to the heart and lungs for reoxygenation. This continuous circulation is essential for sustaining life, enabling tissues to receive what they need and dispose of waste efficiently (Tucker et al., 2025). Capillaries are the most numerous and smallest blood vessel in the body, made up of one layer of endothelial cells (EC) and facilitate the exchange of nutrients, oxygen, and waste between the blood and the surrounding tissues (Eelen et al., 2018). Apart from capillaries having the role of providing organs with necessary oxygen and nutrients, they also aid in organ morphogenesis by releasing angiocrine signals that stimulate surrounding tissues to grow and maintain their proper function (Carmeliet, 2005).

1.2 Endothelial Function

The endothelium is a highly dynamic and heterogeneous tissue whose function varies significantly depending on the type of blood vessel and the physiological or pathological context (Aird, 2007). From regulating vascular tone in arteries to mediating immune cell trafficking in veins and facilitating nutrient exchange in capillaries, ECs are finely tuned to meet the unique demands of their local environment.

1.2.1 Vascular Tone

One of the main roles of arteriole ECs is to regulate vascular tone and release vasodilatory and vasoconstrictive molecules that modulate the diameter of the vessel and allow for more blood flow during periods of exercise. The ECs use the endothelial nitric oxide synthase (eNOS/NOS3) enzyme to generate nitric oxide (NO), which stimulates production of cGMP in vascular smooth muscle cells that leads to vasodilation and relaxation (Oliveira-Paula et al., 2016). This response is activated by increased shear stress on the ECs caused by increase in blood flow which upregulates Ca^{2+} , which in turn activates eNOS. Once activated, eNOS uses L-arginine and oxygen and cofactor tetrahydrobiopterin (BH_4) to form NO and L-citrulline (Oliveira-Paula et al., 2016). Studies involving eNOS knockout models show a significantly higher incidence of ischemic strokes and atherosclerotic cardiovascular disease (Le Gouill et al., 2007; F.-F. Liao et al., 2021), highlighting the critical role of EC-derived nitric oxide in maintaining vascular tone, preventing inflammation, and protecting against vascular injury.

1.2.2 EC function in capillaries

Capillaries mediate the transfer of oxygen and nutrients to the parenchymal tissues and remove waste and are characterized into three different categories: continuous non-fenestrated, continuous fenestrated and discontinuous (Aird, 2007; Poole et al., 2020). Capillaries have differing levels of permeability depending on the organ they are found in and on the physiological context. Tight junctions and adherence junctions determine the permeability of the endothelium and vary among vascular beds and are required for migration of immune cells during infiltration (Komarova et al., 2017). During inflammation, the ECs can be activated by cytokines such as IL6, IL-1 β and tumour necrosis factor (TNF) and increase expression of adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM1), intracellular adhesion molecule 1 (ICAM1) and

selectins (Amersfoort et al., 2022; C. Zhang, 2008). This process allows the attachment of immune cells and recruits them into the tissue. ECs can also release cytokines themselves, further contributing to inflammatory state of the tissue (Kulovic-Sissawo et al., 2024; Teijaro et al., 2011).

Aside from capillary permeability, gap junctions in ECs allow for direct communication between cells and allow for transport for small molecules such as Ca^{2+} (Komarova et al., 2017). Adhesion proteins such as platelet endothelial cell adhesion molecule 1 (PECAM1) and claudins are involved in gap junctions, while proteins such as vascular endothelial (VE)-Cadherin are involved in adherence junctions. The endothelium also helps with injury by releasing pro-thrombotic factors such as Von Willebrand Factor (VWF) that induce blood clotting when needed. In sum, the vascular endothelium is highly dynamic and helps maintain whole body homeostasis by maintaining vascular tone, immune responses, maintaining the barrier between the tissue and blood, and contributing to the coagulation process.

1.2.3 Angiogenesis

Angiogenesis is the process of expansion of existing capillaries by sprouting new branches generally during tissue remodelling such as expansion, wound healing, or increasing physiological metabolic tissue demands (Blanco & Gerhardt, 2013). ECs are able to quickly switch from a resting quiescent state to a highly migratory and proliferative state during vessel sprouting and this is often activated by various growth factors (Eelen et al., 2015). Angiogenic expansion may be driven by signals from the surrounding tissue as it grows, or it could be triggered by metabolic cues that occur alongside tissue enlargement, however it is likely that it is a combination of both (Herold & Kalucka, 2021). The growing tissue's increased demand for oxygen and nutrients stimulates non-vascular cells to produce growth factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), transforming growth factor (TGF)- β , angiopoietin-1 and

2, and other pro-angiogenic signals that promote blood vessel formation (Eelen et al., 2018; Matsumoto & Ema, 2014). These growth factors bind to receptors on the ECs, such as VEGF binding to VEGF receptor 2 (VEGFR2) and this triggers a series of events including untightening of EC cell-cell contacts, pericyte detachment, and basement membrane breakdown, which allow a new vessel to sprout and angiogenesis to occur (Eelen et al., 2018).

Angiogenesis involves different EC subtypes: tip cells that are highly migratory but non-proliferative that guide the sprouting in the correct direction, and stalk cells that are highly proliferative and elongate the vessel (Potente & Carmeliet, 2017). The more mature ECs are called phalanx cells, a quiescent subtype of ECs that line the inner surface of blood vessels, particularly in stable vasculature. Unlike tip and stalk cells involved in active angiogenesis, phalanx cells maintain vascular stability and barrier function, playing a crucial role in sustaining vascular homeostasis and tissue oxygenation. Tip ECs respond to VEGFA by binding it to their surface receptor VEGFR2, which activates intracellular signaling pathways that guide their migration toward the VEGFA source, and instruct surrounding cells to adopt a stalk phenotype through DLL4 production that binds to Notch1 receptors in the surrounding cells (Potente & Carmeliet, 2017). The tip cells lead and guide new blood vessel sprouts by extending filopodia toward angiogenic signals and connecting the growing sprout to nearby vessels, and the stalk cells establish tight junctions to promote sprout stability, in turn forming the lumen in which blood flows through. When the new capillaries are formed in the tissue and sufficient oxygenation is achieved, the tissue reduces the release of proangiogenic factors, and the vessel undergoes maturation and stabilization by recruiting mural cells (Geudens & Gerhardt, 2011).

1.3 Endothelial Cell Metabolism

ECs are required for a plethora of functions among the tissues and need to be specialized to meet the individual tissue's needs. Differences in signals from the surrounding tissue environment influence EC behavior, leading to heterogeneity of endothelial and capillary structure and function (Aird, 2005). ECs adapt by changing their function and even structure depending on the vascular bed (Aird, 2007). Endothelial metabolism refers to the method ECs generate and manage energy to support their functions, relying primarily on glycolysis rather than oxidative phosphorylation to fuel processes such as barrier maintenance, angiogenesis, and vascular homeostasis. It has been shown that ECs from different vascular beds have differing metabolic profiles, as liver sinusoidal ECs stray from common glycolytic phenotype of ECs, and have high levels of mitochondrial respiration and constitutive low-levels of glycolysis (Dudek et al., 2022). EC can adapt their metabolic pathways in response to signals from the surrounding tissue, allowing them to meet local demands and support tissue-specific vascular functions.

1.3.1 Quiescent EC metabolism

As the majority of ECs remain quiescent through adulthood, these cells have reduced levels of glucose uptake and mitochondrial content to maintain a quiescent phenotype (Eelen et al., 2015). However, relative to other non-active quiescent cell types, such as immune cells, ECs have a high baseline glycolytic level to maintain critical EC functions such as maintaining vascular integrity and tight barrier functions, facilitating metabolite transport and inhibiting inflammation (De Bock, Georgiadou, & Carmeliet, 2013; Du et al., 2021; Eelen et al., 2015). Quiescent ECs also use fatty acid (FA) oxidation to sustain the tricarboxylic acid cycle for redox homeostasis. Quiescent ECs that have impaired FA oxidation have a dysfunctional phenotype with higher immune cell

infiltration, by increasing oxidative stress (Kalucka et al., 2018). FA oxidation also maintains EC phenotype by preventing endothelial-to-mesenchymal transition (Xiong et al., 2018).

1.3.2 Proliferative EC metabolism: glycolysis and mitochondrial function

Although ECs are highly exposed to oxygen, stalk ECs that are required to proliferate have a high reliance on glycolysis and generate around 85% of the total adenosine triphosphate (ATP) through glycolysis (De Bock, Georgiadou, Schoors, et al., 2013; Sabaratnam & Svenningsen, 2021). It is suggested that using less oxygen allows higher transfer of oxygen to surrounding organs and is also beneficial for ECs to keep their reactive oxygen species (ROS) production low (Eelen et al., 2015; Zecchin et al., 2017). When ECs are stimulated by VEGFA, the ECs double their glycolytic rate to meet the energy demands required during vascular remodelling, and have higher expression of the glycolytic activator 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) (De Bock, Georgiadou, Schoors, et al., 2013).

Proliferating ECs are mainly glycolytic, but the mitochondria are used to a lower extent during proliferation. It was reported that proliferating human umbilical vein ECs (HUVECs) are also dependent on FA β -oxidation for de novo nucleotide synthesis required for DNA replication (Schoors et al., 2015). FAs are metabolized using mitochondrial FA oxidation, yielding high levels of ATP (Kulovic-Sissawo et al., 2024). However, mitochondrial content in the ECs is low compared to other cell types, only occupying 2-6% of the cytoplasmic volume in rat ECs, compared to 32% in cardiac myocytes (Kluge et al., 2013).

1.3.3 Mitochondrial networks in the ECs

Mitochondrial networks are essential for EC function, contributing to energy balance, redox signaling, and metabolic adaptation necessary for vascular homeostasis and angiogenesis.

These networks undergo dynamic cycles of fission and fusion to maintain mitochondrial integrity and function. Mitochondrial fission, primarily mediated by Drp1 and Fis1, involves the division of a single mitochondrion into two, a process important for isolating and removing damaged components, especially under cellular stress. In contrast, fusion, regulated by genes such as optic atrophy protein 1 (Opa1) and mitofusin (Mfn)1/2, joins adjacent mitochondria, promoting ATP production by preserving electrical and biochemical connectivity (Kluge et al., 2013). Disruption in this balance, such as excessive mitochondrial fragmentation, has been linked to metabolic disease (Kluge et al., 2013; Y. J. Liu et al., 2020). Studies show that impairing mitochondrial fusion reduces oxidative phosphorylation and diminishes the angiogenic response to VEGFA (Lugus et al., 2011). Additionally, aging HUVECs exhibit decreased mitochondrial content and membrane potential (Kulovic-Sissawo et al., 2024). While glycolysis remains the primary energy source in ECs, mitochondrial function is indispensable for maintaining a healthy endothelial phenotype.

1.3.4 Transporters and other proteins involved in EC metabolism

ECs depend on specialized glucose and FA transporters to support their distinct metabolic needs, enabling energy production, biosynthesis, and functional responses such as angiogenesis and vascular homeostasis. Although ECs are insulin-responsive, glucose is taken up by ECs through the glucose transporter 1 (GLUT1) which works independently of insulin signalling (Moessinger et al., 2020). ECs with a high proliferation rate have upregulated expression of GLUT1, suggesting its expression is coupled with glycolysis-driven angiogenesis (Veys et al., 2020). Lipoprotein lipases (LPLs) on the EC membrane break down triglycerides which transporters, such as FA transport proteins (FATP)1/3/4 and CD36, transport into the EC. Once in the EC, the FAs bind to FA binding proteins such as FABP4/5, where they can then either be stored, utilized for FA oxidation, or passed to the surrounding tissue (Rudnicki et al., 2022a). FABP4 has

been shown to have a pro-angiogenic effect in ECs, as FABP4 deficient HUVECs had higher apoptosis, decreased migration and impaired capillary network formation (Elmasri et al., 2009). In the heart, ECs have a regulatory role in FA transfer mediated by Meox2/Tcf15 regulation on endothelial CD36 expression (Coppiello Giulia et al., 2015). Mice with an EC-specific deletion of Cd36 had reduced uptake of FAs into the heart, skeletal muscle and brown adipose tissue and reduced lipid droplet accumulation, and after high-fat feeding, these Cd36 deficient mice had improved glucose tolerance and insulin sensitivity (Son et al., 2018). EC Notch signalling is one possible regulator of FA transport genes, as inhibition of Notch impaired expression of CD36 and Fabp4 (Jabs et al., 2018).

EC metabolism plays a crucial role in angiogenesis as the ECs rely on tightly regulated metabolic pathways to fuel the energy demands of proliferation, migration, and sprouting during vessel formation. Indeed, many groups have demonstrated that the downregulation of glycolysis through the inhibition of PFKFB3 leads to reduced vessel sprouting due to the inhibition of EC proliferation (Abdali et al., 2021; De Bock, Georgiadou, Schoors, et al., 2013; Schoors et al., 2014). It was further demonstrated that the inhibition of glycolysis led to reduced expression of inflammatory genes in inflamed ECs (Abdali et al., 2021). Downregulation of glycolysis through other mechanisms such as knockdown (KD) of gamma-aminobutyric acid type B receptors subunit 2 (GABBR2) also leads to impaired angiogenesis (H. Zhang et al., 2021). Furthermore, inhibiting carnitine palmitoyltransferase (CPT1A), a rate limiting enzyme of FA oxidation causes impaired angiogenesis due to impaired proliferation (Schoors et al., 2015). Inhibition of mitochondrial respiration causes EC metabolic adaptation by increasing glycolysis and vice versa, however both glycolysis and mitochondrial respiration were shown to be required for sprouting angiogenesis (Yetkin-Arik et al., 2019). In addition to proliferation, glycolysis was shown to have an essential

role in tip cell differentiation and that PFKFB3 KD diminishes tip cell behaviour (De Bock, Georgiadou, Schoors, et al., 2013; Yetkin-Arik et al., 2019). In sum, disrupted endothelial metabolism impairs the energy and substrate availability required for angiogenic processes, ultimately leading to dysfunctional angiogenesis.

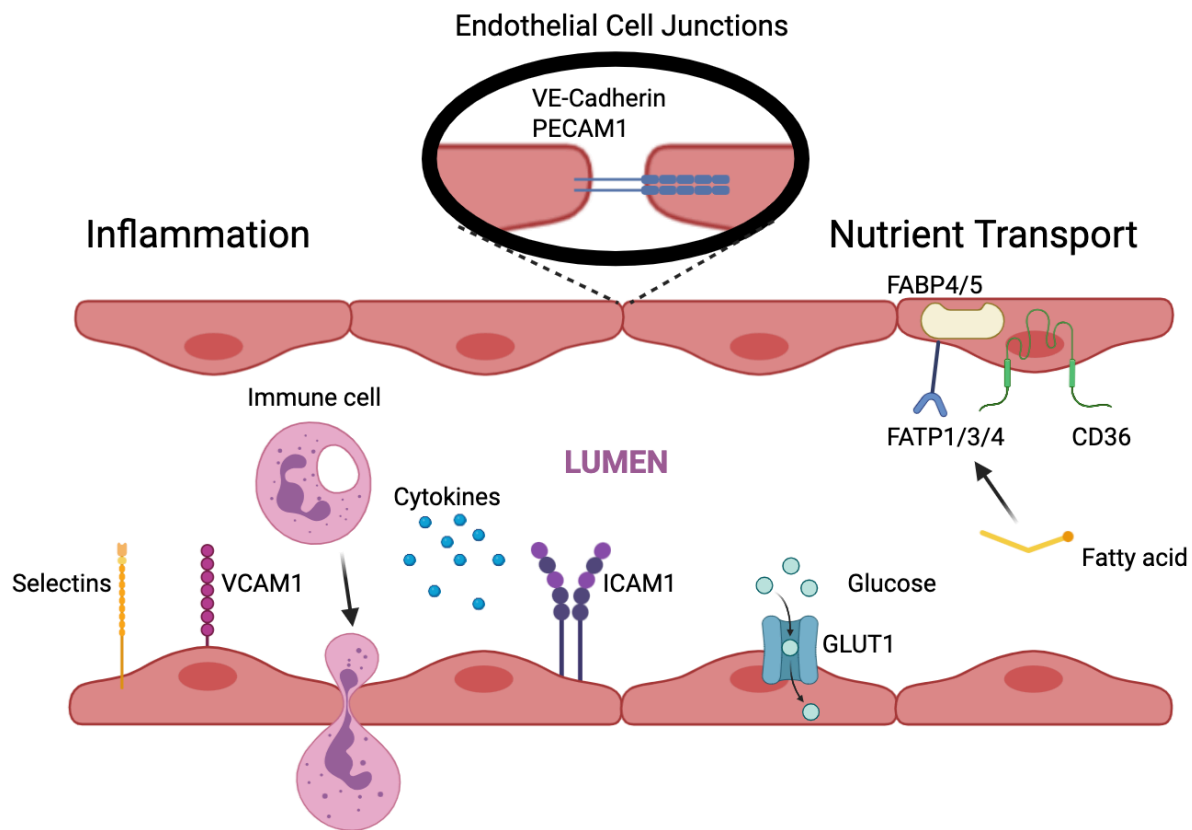


Figure 1.1 Schematic illustrating the roles of endothelial cells in inflammation and nutrient transport. Cytokine stimulation activates receptors on endothelial cells, triggering intracellular signaling pathways that upregulate the expression of adhesion molecules such as ICAM-1, VCAM-1, and E-selectin. These molecules facilitate the binding and transmigration of circulating immune cells across the endothelial barrier. Cytokines can also increase vascular permeability, making it easier for immune cells to infiltrate the affected tissue. Endothelial cell junctions are specialized structures that connect adjacent endothelial cells and regulate vascular permeability and integrity and proteins such as VE-Cadherin and PECAM1 help maintain endothelial junction integrity. Endothelial cells also play a key role in nutrient transport, including the uptake and delivery of fatty acids and glucose to surrounding tissues. Fatty acid transport across the endothelium involves proteins such as FABP4/5, which facilitate intracellular fatty acid uptake. For glucose transport, endothelial cells rely primarily on GLUT1. These nutrient transport mechanisms are used to supply energy sources for both the endothelial cells, and to the surrounding tissue. Figure was created in Biorender.

1.4 Endothelial Cell Dysfunction

EC dysfunction refers to the impaired functions of the endothelium that leads to a cascade of morbidities in the body and can lead to cardiovascular disease. Factors that contribute to EC dysfunction include obesity, low physical activity, diabetes, smoking, aging and more. EC dysfunction is characterized by a reduction in of vasodilators such as NO or an increase of endothelial-derived vasoconstrictors (Hadi et al., 2005). Additionally, this dysfunctional EC phenotype is also described by a proinflammatory and procoagulant phenotype that further contributes to the dysfunctional phenotype (Hadi et al., 2005; C. Zhang, 2008).

1.4.1 Oxidative stress

One key player in EC dysfunction is oxidative stress that is driven by ROS. ROS are produced from oxygen as free radicals and are defined as having at least one unpaired electron that causes their high reactivity (Caminiti et al., 2024). The ECs have an antioxidant response mechanism including superoxide dismutase (SOD)s, glutathione peroxidase (GPx), and catalases, that can counteract oxidative stress. Oxidative stress occurs during the imbalance of antioxidant defence mechanism and excess ROS which is known to contribute to EC dysfunction (Caminiti et al., 2024; Higashi, 2022). ROS can contribute to EC dysfunction and disease progression through multiple pathways, such as impacting the mitochondria or causing DNA damage in the ECs and causing genomic instability (Srinivas et al., 2018).

The mitochondria is the source of around 90% of ROS generation, and when kept at physiological levels, it can be beneficial for the mitochondria (Kulovic-Sissawo et al., 2024). The electron transport chain (ETC) in the mitochondria have multiple constituents that contribute to ROS. During oxidative phosphorylation, electrons are transferred through the mitochondrial ETC complexes, however a small percentage of electrons leak through the ETC and reduce oxygen to

the superoxide anion, O_2^- , which is a primary ROS (R.-Z. Zhao et al., 2019). ROS can also react and damage parts of the mitochondria, such as lipids, proteins and DNA and unrepaired mitochondrial DNA damage can lead to defective ETC complex function that can further result in an upregulation of superoxide anion production, and subsequently lead to mitochondrial dysfunction (Guo et al., 2013). Mitochondrial dysfunction is defined as a decrease in the efficiency of the ETC that results in reduced synthesis of ATP, and can further contribute to ROS generation (Aldosari et al., 2018; Kulovic-Sissawo et al., 2024). It has been shown that in rat aortic ECs, high glucose and FA treatment leads to mitochondrial structure changes, that is accompanied by elevated ROS levels. This was associated with increased mitochondrial fission and increased EC apoptosis (N. Liu et al., 2017). Another study also using rat aortic ECs show that $TNF\alpha$ is sufficient to induce mitochondrial fission and ROS production, which consequentially triggers EC inflammation and immune cell signalling (Forrester et al., 2020). Thus, the relationship between mitochondrial dysfunction and endothelial dysfunction is highly related.

Another source of ROS could be through the uncoupling of eNOS. This occurs when there is a reduction or absence of l-arginine or the cofactor BH₄. In this situation, oxygen becomes the terminal electron acceptor, thus forming O_2^- instead of NO. Moreover, O_2^- can react with NO to form ONOO⁻ that leads to subsequent oxidation of eNOS (Łuczak et al., 2020; D. Zheng et al., 2022). This oxidative and nitrosative stress can promote inflammation in the EC through leukocyte recruitment and stimulating cytokine production.

Furthermore, obesity itself can lead to EC dysfunction through various pathways, such as adipocyte dysfunction and increased cardiovascular risk factors, which cause an upregulation in inflammation and oxidative stress (Kajikawa & Higashi, 2022). Hyperglycemia, a consequence of obesity, causes an increase of EC glucose concentration, that can induce oxidative stress and EC

dysfunction (Eelen et al., 2018). Mice that are fed a high fat diet also have a reduction in insulin receptor substrate in the ECs and this can reduce glucose uptake in surrounding tissue (Sabaratnam & Senningsen, 2021). Long term HF diet feeding was shown to increase mitochondrial ROS production and contribute to mitochondrial dysfunction that can subsequently lead to EC dysfunction (Vial et al., 2011; Zalewska et al., 2019). In sum, EC dysfunction, a precursor to cardiovascular disease, can result from mitochondrial dysfunction, uncoupled eNOS, and obesity, all of which impair nitric oxide signaling and promote vascular inflammation and oxidative stress.

1.5 Endothelial Senescence

Cellular senescence is described by permanent cell arrest in response to stressors and is mediated primarily by the p53/p21 or p16/RB pathways (Hwang et al., 2022). EC senescence is triggered by stressors such as oxidative stress, DNA damage, and mitochondrial dysfunction and is associated with the development of cardiovascular disease (CVD) (Han & Kim, 2023). Oxidative stress can induce EC senescence by Sirtuin (SIRT)6 downregulation, which in turn increases endothelial proinflammatory cytokines and increases NF- κ B transcriptional activity, which promotes immune cell infiltration (R. Liu et al., 2014). It was demonstrated that eNOS and NO activity was lower in senescent human ECs in comparison to young ECs (Matsushita et al., 2001). SIRT1 can protect against EC dysfunction and downstream senescence by increasing EC NO bioavailability (Man et al., 2019). The senescent ECs can be described by having loss of vasodilation control, inflammation and immune cell infiltration, altered glucose metabolism with increased oxidative stress. Senescent ECs have an enlarged and flattened morphology and have been shown to have an upregulated production of fibronectin, ICAM1 and reduced NO bioavailability (Hwang et al., 2022).

Senescent cells are characterized by a pro-inflammatory, pro-oxidative senescence associated secretory phenotype (SASP) which is activated within a few days of the induction of senescence and is irreversible (Coppé et al., 2010; Donato et al., 2015). Despite the permanent growth arrest, senescent ECs still show high metabolic activity, which can contribute to SASP. A group showed that compared to young and healthy ECs, senescent ECs had a lower glycolytic rate but an increase in FA oxidation associated with higher intracellular ATP (Giuliani et al., 2023). When FA oxidation was inhibited in these ECs, it ameliorated the SASP in the senescent cells. Interestingly, it was reported that senescent HUVECs were resistant against mitochondrial damage and had extended and interconnected mitochondria and accompanied by the reduction of mitochondrial fission proteins (Mai et al., 2010). Through the secretory phenotype, an increase in the abundance of EC senescence could cause inflammation and oxidative stress that can impair EC function (Bloom et al., 2023). It has also been shown that endothelial SASP can lead to CVD progression, as it can impair the surrounding tissue function and lead to insulin resistance (Barinda et al., 2020). Endothelial senescence and the associated SASP create a pro-inflammatory, pro-oxidative vascular environment that impairs nitric oxide signaling, and promotes immune cell infiltration, ultimately accelerating the development of cardiovascular disease.

1.6 Insulin signalling

Insulin is a pancreatic peptide hormone secreted in response to increase blood glucose levels to promote uptake into tissues such as skeletal muscle and adipose tissue for usage and storage (Ghaben & Scherer, 2019). Its signalling is mediated by the insulin receptor, located on the cell membrane, which phosphorylates the insulin response substrates (IRS1) downstream, which then activates phosphatidylinositol 3-kinase (PI3K). PI3K adds a phosphate group to the phosphatidylinositol (3,4,5)-trisphosphate (PIP3) that then binds to Akt that leads to the activation

of cAMP response element-binding (CREB), mTOR and forkhead box O (FOXO) proteins (Ghaben & Scherer, 2019). The glucose transporter GLUT4 is downstream of Akt and is recruited to the cell membrane to aid in glucose uptake from the blood. In dysfunctional adipose tissue, chronic low-grade inflammation was demonstrated to interfere with insulin signalling, as studies have shown that the cytokine TNF α downregulates insulin response in the adipocytes, thus reducing levels of GLUT4 and resulting in high blood glucose (Cawthorn & Sethi, 2008; Hafidi et al., 2019). Insulin signalling also promotes lipid synthesis and inhibits their degradation, and in the adipocytes it can activate lipid synthetic enzymes such as pyruvate dehydrogenase, FA synthase, and acetyl-coA carboxylase (Saltiel & Kahn, 2001). Insulin resistance and adipocyte hypertrophy are known to be correlated, and metabolic dysfunctions have been shown to occur when lipid storage capacity of the adipose is exceeded leading to ectopic deposition in the liver and the muscle, which further accelerates insulin resistance (Lemoine et al., 2013). Furthermore, apart from glucose and lipid metabolism regulation, insulin has been shown to act in ECs to promote NO synthesis via eNOS (Zocchi et al., 2022). Insulin is crucial for regulating glucose uptake and storage, as well as facilitating lipid synthesis and storage in adipocytes, thereby maintaining energy balance and metabolic homeostasis.

1.7 Forkhead Box O1

FoxO1 is part of the Forkhead-type O transcription family, characterized by a highly conserved DNA binding domain (Forkhead box) and is downstream of the insulin signaling pathway involving PI3K and Akt (Furuyama et al., 2004). The four domains of the FoxO1 proteins include the Forkhead domain which is a winged-helix type of DNA-binding domain, the nuclear localization signal, and nucleus export sequence, and a transactivation domain (van der Horst & Burgering, 2007). Four FoxO isoforms FoxO1/3/4/6 have been identified in mammals, however

FoxO6 is localized only in the brain (Santos et al., 2023). FoxO1 is generally expressed in insulin-responsive tissues and organs such as the liver, pancreas, skeletal muscle, and adipose tissue (J. Chen et al., 2019). Both global and EC-specific FoxO1 deletions cause an embryonic lethal phenotype (Furuyama et al., 2004; A. Sengupta et al., 2012) while KD of FoxO3 and FoxO4 result in viable offspring (Oellerich & Potente, 2012), showing FoxO1's importance in development. Although FoxO3 and FoxO4 are dispensable in embryonic vascular development, inactivation of Fox3/4 along with FoxO1 show exaggerated effects than when compared to FoxO1 KD alone (J.-H. Paik et al., 2007). The genes that the FoxO isoforms regulate are overlapping but not fully redundant, displayed by FoxO1 and FoxO3 inhibiting EC sprouting and migration, but FoxO4 having no effect on these (Potente et al., 2005). Moreover, FoxO3 overexpression in ECs cannot rescue the effects of FoxO1 KD (Matsukawa et al., 2009), highlighting the potent regulatory mechanism of FoxO1.

Genes regulated by FoxO proteins are grouped into 4 different categories: cell cycle arrest and DNA repair, apoptotic genes, oxidative stress genes, and genes involved in energy metabolism (Furuyama et al., 2004). FoxO1 activates or represses transcription of target genes by binding to the conserved recognition motif TTGTTTAC. Furthermore, FoxO1 can directly bind to histone 3 (H3) via basic regions in the C-terminal of the DNA binding domain, enabling it to function as a “pioneer factor” (J. Chen et al., 2019). While FoxO4 is abundant in the skeletal muscle, FoxO1 mRNA is abundantly found in the adipose tissue and plays a role in adipocyte differentiation (Furuyama et al., 2004). In the adipocytes, FoxO1 is known to act as an antagonist for peroxisome proliferator activated receptor γ (PPAR γ), which could inhibit lipogenesis and adipocyte differentiation (J. Chen et al., 2019). Systemic haploinsufficiency of FoxO1 was shown to increase the number and size of adipocytes in mice fed a high-fat diet (J. Chen et al., 2019).

1.7.1 Regulation and localization of FoxO1

Both transcriptional and post-translational regulation have an impact on FoxO1 expression and activity. It contains three highly conserved phosphorylation sites that are targeted by Akt, which its phosphorylation inhibits the transcriptional activity mediated by FoxO1, due to its nuclear exclusion (J. Chen et al., 2019). Phosphorylation of FoxO1 at S256 by AKT decreases the binding affinity of FoxO1 to the target DNA and increases phosphorylation at the other sites by AKT and other kinases, and thus its activity can be regulated by their cellular localization (J. Chen et al., 2019). Protein phosphatase 2 (PP2A) dephosphorylates FoxO1 and leads to nuclear relocation. In contrast phosphorylation by stress-activated JNK and energy-sensor AMPK facilitates FoxO1 activation in the nucleus (Oellerich & Potente, 2012). Another post-translational regulation that causes nuclear exclusion is acetylation of FoxO1, which can promote Ser256 phosphorylation and it has been shown that deacetylation of FoxO1 by SIRT1/2 may retain FoxO1 in the nucleus (X.-J. Yang & Seto, 2008). FoxO1 can also be methylated by arginine methyltransferase-1 (PRMT1) which interferes with the ability of AKT to phosphorylate FoxO1 (J. Chen et al., 2019).

FoxO1 can also localize to the mitochondria, where its phosphorylated form binds to the mitochondrial D-loop under basal nutrient conditions (S. Chen et al., 2024; Lettieri-Barbato et al., 2019). Localization of FoxO1 in the mitochondria was shown to cause inhibition of mitochondrial respiration and also induce oxidative stress (S. Chen et al., 2024). However, in response to nutrient stress, protein tyrosine phosphatase mitochondrial 1 (PTPMT1) dephosphorylated FoxO1 and it shuttles out of the mitochondria towards the nucleus (Lettieri-Barbato et al., 2019).

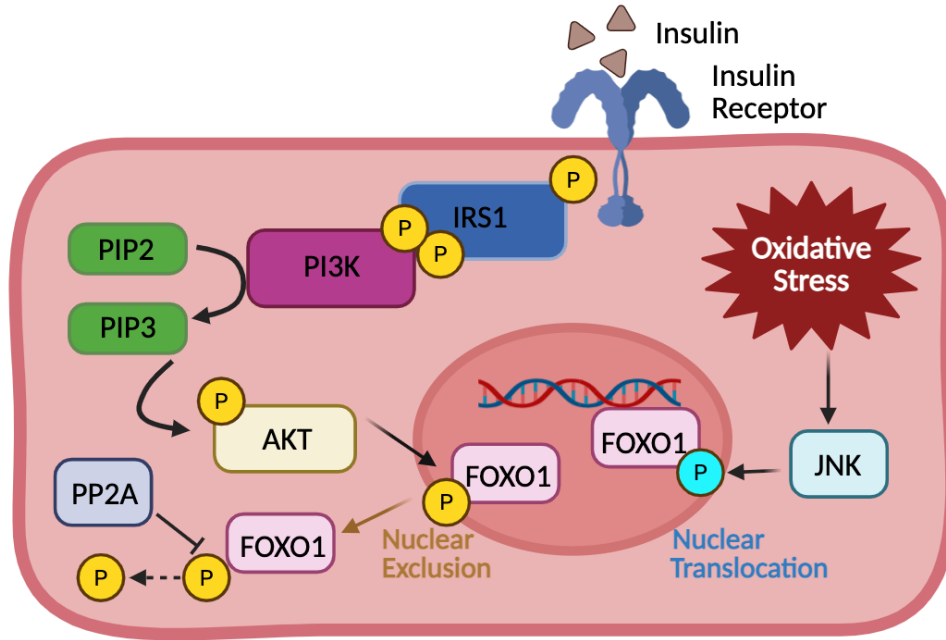


Figure 1.2 Schematic illustrating FoxO1 regulation and localization. Insulin signaling activates the PI3K-Akt pathway, leading to the phosphorylation of FoxO1 by Akt. This phosphorylation causes FoxO1 to be excluded from the nucleus, preventing it from binding DNA and regulating gene expression. In contrast, oxidative stress activates the JNK pathway, which phosphorylates FoxO1 at different sites, promoting its nuclear localization and transcriptional activity. Additionally, the phosphatase PP2A can dephosphorylate FoxO1, reversing the effect of Akt and allowing FoxO1 to relocate to the nucleus. Figure was created in Biorender.

1.7.2 Endothelial-specific FoxO1

EC-FoxO1 is shown to be important in development as EC expression of FoxO1 partially rescues the lethality of global FoxO1 KO (Dharaneeswaran et al., 2014). FoxO1 acts as a gatekeeper of EC quiescence by reducing glycolysis and mitochondrial respiration, as shown by EC-specific deletion causing EC hyperplasia and vessel enlargement (Rudnicki, et al., 2018a; Wilhelm et al., 2016). Consistent with this, FoxO1 overexpression inhibits angiogenesis through a downregulation in migration, sprouting and tube formation (Oellerich & Potente, 2012; Potente et al., 2005). One way FoxO1 restricts angiogenesis is by regulating EC homeostasis, with its inactivation causing an increase in EC proliferation (J.-H. Paik et al., 2007; Wilhelm et al., 2016). Potente's group showed that FoxO1 reduces glycolysis and mitochondrial respiration through the

suppression of c-MYC, which is a potent driver of metabolism and growth, and that restoration of MYC signalling in FoxO1-overexpressing ECs normalizes metabolic and angiogenic activity (Wilhelm et al., 2016). VEGFA was shown to signal the PI3K/FoxO1 pathway and promote EC survival, proliferation and cell-cycle progression, causing FoxO1 nuclear exclusion and preventing cell cycle suppressor p27 (Abid et al., 2004). FoxO1 is important in quiescent phalanx cells by reducing both glycolysis and ox-phos activity through the suppression of MYC (Potente & Carmeliet, 2017). FoxO1 plays a critical role in maintaining vascular homeostasis by suppressing EC proliferation, acting as molecular brake, ensuring controlled endothelial growth and preventing excessive vascular expansion. This function underscores FoxO1 as a key negative regulator of EC proliferation.

Although downregulation of FoxO1 leads to upregulation of angiogenesis, studies have shown that a balance of FoxO1 signalling is required for tissue vascularization. A deletion of EC-FoxO1 leads to the loss of tip cell polarity during sprouting angiogenesis (Y. H. Kim et al., 2019; Miyamura et al., 2024). FoxO1 may also have an effect on chromatin organization within ECs that regulate gene expression. It was reported that FoxO1 can bind to condensed chromatin near tip-enriched genes as a pioneer factor (Miyamura et al., 2024). Furthermore, FoxO1-deficient ECs showed changes in Notch signalling and genes involved in vascular health (Dharaneeswaran et al., 2014). Dharaneeswaran *et al.* also showed that constitutively active FoxO1 leads to an increase in EC size mediated by the Akt-mammalian target of rapamycin complex 1 (mTORC1) pathway, corresponding with growth arrest and vascular occlusion. Consistent with this, overexpression of FoxO1 showed enhanced p70 ribosomal protein S6 kinase (p70S6K) phosphorylation in ECs (Cao et al., 2006), which is a substrate of mTOR and contributes to increases in cell size. Moreover, mice expressing an inactive form of PI3K-C2b β , upstream of FoxO1 signalling, displayed large

blood vessels, corresponding to larger ECs caused by mTORC1 activation (Kobialka et al., 2023). Interestingly, PI3K-Akt inhibitors and mTORC1 inhibitors induce EC elongation and this was specific to EC, as it did not have the same effect in other cell types. Furthermore, FoxO1 KD in these ECs failed to elongate in response of the inhibitors, indicating that elongation of ECs is FoxO1 dependent (Tsuji-Tamura & Ogawa, 2016). While over and under expression of FoxO1 appear to have opposite roles in the various contexts such as vascularization, transcriptomic analysis comparing downregulated genes of FoxO1 KD and upregulated genes by FoxO1 overexpression reveals that the intersection of these genesets is relatively small (Miyamura et al., 2024). The same phenomenon was found in the opposite scenario (upregulated genes by FoxO1 KD vs genes enriched by FoxO1 overexpression), underscoring its broad effects and suggesting that it must be tightly regulated at physiological levels to maintain endothelial homeostasis and prevent pathological outcome. The seemingly paradoxical phenotypes stress the importance of the coordination of FoxO1 signalling within ECs during angiogenesis to enable a balanced vascular response to angiogenic signals.

The role of FoxO1 in the ECs is multifaceted and helps with the regulation of EC functions and therefore helps mitigate whole body metabolic dysfunctions. It has been suggested that retention of FoxO1 in senescent adipose ECs is negatively correlated with nuclear PPAR γ transcriptional activity and leads to decreased lipid handling by the ECs. In contrast, FoxO1 KD in HUVECs caused a decrease in FABP4, suggesting that FoxO1 is necessary in lipid handling (Hwangbo et al., 2017). It was reported that FoxO1 inhibits mitochondrial enzyme 2-oxoglutarate dehydrogenase and causes quiescence in ECs, suppressing proliferation and causing vascular rarefaction (Andrade et al., 2021). FoxO1 also regulates expression of angiopoietin 2 (Ang2), and represses eNOS expression, suggesting it plays a role in vascular tone (Potente et al., 2005).

Inflammation has been shown to be mediated by FoxO1 through metabolic pathways, as it was shown that inflammatory stimuli increase cellular metabolism by enhancing glycolysis and ox-phos in ECs. Enhanced ox-phos was attributed to the suppression of FoxO1, however the restoration of FoxO1-PDK4 signalling decreased inflammatory-induced ox-phos but worsened inflammation (Xiao et al., 2021). Interestingly, inflammation was upregulated by the increase of Pfkfb3-regulated glycolysis (Xiao et al., 2021), which FoxO1 is known to suppress (Rudnicki, et al., 2018a), therefore showing the regulation of FoxO1 has to be tightly controlled, as even slight deviations can disrupt EC functions and dynamics.

1.7.3 Protective effect of FoxO1

Outside of angiogenesis, FoxO1 is known to have a protective role in cells, and has a defensive effect against high glucose-induced ROS accumulation and mitochondrial dysfunction (Jerome et al., 2022). When there is excessive ROS, FoxO1 is phosphorylated by JNK and p38 mitogen activated protein kinase (MAPK) which induces translocation of it from the cytosol to the nucleus where it becomes active and help reduce oxidative stress (Ju et al., 2014; Klotz et al., 2015; van der Horst & Burgering, 2007). FoxO1 upregulates several antioxidant enzymes such as catalase and MnSOD and helps to scavenge oxidative stress produced in peroxisomes and mitochondria (Ponugoti et al., 2013; van der Horst & Burgering, 2007). FoxO1 KD was reported to have increased ROS levels that led to mitochondrial dysfunction, while overexpression of FoxO1 showed reduce ROS levels (Jerome et al., 2022). In ECs, FoxO1 induced mitochondrial fission through activation of Rho-associated coiled-coil containing protein kinase 1 (ROCK1), which phosphorylates Drp1 (Shi et al., 2018). In contrast, FoxO1 inhibition reduced expression of the fission protein Drp1, but also in the fusion proteins OPA1 and MFN1/2 in epithelial cells (D. Wang et al., 2020). Moreover, FoxO1 plays a pivotal role in cell cycle arrest, apoptosis and DNA

damage repair by upregulating p27, Bim and Growth Arrest and DNA Damage-inducible protein 45 (GADD45) (Dijkers et al., 2000; Furukawa-Hibi et al., 2002; Ju et al., 2014). It was shown that DNA damage causes both an increase in FoxO1 protein levels, as well as its nuclear import. In ischemic muscles, FoxO1 expression was increased in the capillaries compared to the control and in nutrient deprived ECs, and this was related to upregulation of p27, suggesting that nutrient stress is enough to elevate FoxO1 levels (Milkiewicz et al., 2011).

Because of its role in cell cycle arrest, cell death, and cellular oxidative stress followed by DNA repair or cellular damage, FoxO1 signalling is similar to p53 in many ways. Both genes regulate cell cycle arrest through cell cycle inhibitors, and activate the BH3 proteins during apoptosis (van der Horst & Burgering, 2007). There is evidence that p53 regulates FoxO1 function by upregulating the expression of serum-and glucocorticoid-inducible kinase (SGK), a negative regulator of PI3K. However, FoxO1 and p53 work together, and FoxO1 regulates expression of certain proteins in a p53-dependent manner (van der Horst & Burgering, 2007).

Although FoxO1 is known to have a protective role in the ECs, its suppression of angiogenesis makes it a promising candidate of pharmacological targeting in low vascularization contexts. Within ischemic muscles, a EC-directed deletion of FoxO1 improved both blood flow recovery and angiogenesis (Roudier et al., 2013). FoxO1 deletion had improved metabolic outcomes, increased angiogenesis and led to healthier adipose tissue compared to control male mice (Nwadozi et al., 2016; Rudnicki, et al., 2018a). In the skeletal muscle, downregulation of FoxO1/3 increases exercise-induced angiogenesis (Slopack et al., 2014). Pharmacological antagonism and biological silencing of FoxO1 was also shown to restore eNOS activation and improve insulin resistance in the adipose and ECs of obese subjects (Karki et al., 2015). Contrary to FoxO1 being involved in DNA damage repair, it was also shown that FoxO1 KD avoided

persistent DNA damage and cellular senescence under high glucose in ECs by promoting DNA repair in response to oxidative stress injury (Sun et al., 2022), suggesting that DNA repair is glycolysis-dependent. Despite the established role of FoxO1 in EC regulation, the extent to which its signaling pathways differ between sexes remains largely unexplored. Understanding whether sex-specific factors influence FoxO1 activity could provide important insights into vascular biology and disease susceptibility, warranting further investigation.

1.8 The Adipose Tissue Under Homeostasis and Expansion

The adipose tissue is supplied by a dense network of capillaries lined with specialized ECs and plays a vital role not only in energy storage, but also in regulating metabolism and vascular function. The adipose tissue can be classified into three types according to the kind of adipocytes present in each. The white adipose tissue is primarily responsible for fat storage and contains adipocytes which have a large lipid droplet occupying most of the cell's volume, giving it the white colour (Fitzgerald et al., 2018; Richard et al., 2000). The brown adipose tissue has adipocytes with a large number of mitochondria and the colour is provided by the cytochromes in the mitochondria. Its primary function is thermoregulation but also has autocrine, paracrine and endocrine roles (Fitzgerald et al., 2018). To help with thermoregulation, these adipocytes have high levels of uncoupling protein 1 (UCP1) (Hafidi et al., 2019). The beige adipose tissue is a mix of both white and brown AT.

The white adipose tissue is a highly dynamic metabolic organ that can expand in periods of high calorie consumption (Fitzgerald et al., 2018). It contains a variety of cell types, such as adipocytes, which are the main cells, as well as ECs, immune cells and fibroblasts. The role of the adipose tissue is to store fat and properly sequester lipids to prevent lipotoxicity in other tissues such as the liver and heart (Hafidi et al., 2019). Apart from lipid storage, it can also maintain

glucose homeostasis, inflammatory and immune response (Fitzgerald et al., 2018). However, during obesity, the adipose tissue can become dysfunctional and is characterized by factors such as impaired metabolism and production of pro-inflammatory cytokines (AlZaim et al., 2023; Goossens & Blaak, 2015). When the adipose tissue is dysfunctional and cannot store lipids or releases excessive levels of free FAs, the risk of lipotoxicity heightens and this contributes to insulin resistance, inflammation and cell death (Hafidi et al., 2019; Richard et al., 2000; Xu et al., 2024). These morbidities lead to the development of metabolic syndrome, characterized by visceral obesity, low high-density lipoprotein (HDL)-cholesterol, high triglycerides, high hypertension and high blood sugar.

1.8.1 Adipose tissue expansion

When the tissue expands in response to nutrient overload, it can do so through two different mechanisms: hyperplasia (increasing adipocyte number) and hypertrophy (increasing the size of the adipocytes). During hyperplasia, new adipocytes form through the differentiation of precursor cells known as preadipocytes. These cells restrict their fate to the adipogenic lineage and become incapable of forming other mesenchymal cells such as myoblasts (Choe et al., 2016; Ghaben & Scherer, 2019). Bone morphogenic protein (BMP)2 and BMP4 are sufficient to drive adipocyte commitment in cultured fibroblasts and the mature adipocytes start to accumulate triglycerides (Hafidi et al., 2019). One of the most important proteins of adipogenesis is the master regulator PPAR γ , as it is indispensable for adipocyte differentiation. This nuclear hormone receptor regulates FA storage and stimulates lipid uptake (Corvera & Gealekman, 2014). Adipose remodeling through hyperplasia is generally considered healthy because it preserves adequate blood vessel growth and maintains adiponectin signaling, a hormone that helps regulate insulin sensitivity (Ghaben & Scherer, 2019). Adiponectin also has anti-inflammatory effects by inhibiting TNF α signalling,

reducing Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF- κ B) activation, and decreasing monocyte adhesion to ECs (Shah et al., 2008).

While healthy adipose expansion involves the increase in adipocyte number, hypertrophy is associated with insulin resistance and increased inflammation, fibrosis, and an altered adipokine secretion profile (Ghaben & Scherer, 2019; Sabaratnam & Senningsen, 2021). The relationship between adipocyte size and insulin resistance is widely studied showing that smaller adipocytes have higher responsiveness to insulin (Abranches et al., 2015; Arner et al., 2010; Cotillard et al., 2014; Hammarstedt et al., 2012; Weyer et al., 2000; J. Yang et al., 2012). Additionally, a decrease in adiponectin, which promotes glucose and FA metabolism is also associated with hypertrophy and obesity (Brakenhielm et al., 2004; Sabaratnam & Senningsen, 2021). Adipocytes uptake free FAs which are stored in the form of triglycerides, and can undergo lipogenesis or lipolysis depending on the metabolic need of the body (Hafidi et al., 2019). It has been shown that it takes as little as 1 week of high-fat feeding in rodents for the adipocytes to grow in size, but also can decrease in size after only 24 hours of fasting (Crewe et al., 2018). When there is an energy deficiency, triglycerides are hydrolyzed to free FAs and glycerol and then released into the circulation using the capillaries (Hafidi et al., 2019).

1.8.2 Healthy adipose tissue angiogenesis

Sufficient vascularization is important in expanding adipose tissue during adipogenesis to deliver oxygen and nutrients to the expanding tissue and allow hyperplasia to occur (AlZaim et al., 2023). Each adipocyte in the white adipose tissue is in direct contact with the capillary network in a circular manner and this helps regulate the transport of oxygen, nutrients and hormones (Z.-Z. Liao et al., 2022). During healthy adipose expansion, there is a parallel increase in the capillary network as this allows crosstalk between vascular cells and adipocytes to promote recruitment,

commitment and differentiation of new preadipocytes (Hammarstedt et al., 2018). The angiogenic expansion may be triggered by proliferating or expanding adipocytes, or may be triggered by metabolic signals in parallel to the enlargement of adipocytes (Corvera & Gealekman, 2014). The adipose releases proangiogenic factors and this triggers the adipose EC to start sprouting and proliferating in the direction of adipose growth, and is considered to have one of the highest angiogenic capacities among all tissues (Lemoine et al., 2013).

As adipocytes grow in size, they reach the diffusional limit of oxygen, causing local mild hypoxia and activating the transcriptional activity of hypoxia inducible factor (HIF) α to induce production of VEGFA in the adipocytes (Crewe et al., 2017). A study had shown that inhibiting VEGFA signalling in the adipose tissue not only restricted angiogenesis but also adipogenesis, indicating that these processes are coupled and mediated by VEGFA (Nishimura et al., 2007). Moreover, the adipose tissue also releases proangiogenic factors such as angiopoietin-2, adipokines, leptin and adiponectin that also stimulate angiogenesis and allow for sufficient vascularization (Cao, 2013; Herold & Kalucka, 2021).

1.8.3 Endothelial Cells of the Adipose Tissue

The ECs form networks of capillaries with a total length of almost one meter in each cubic millimeter of adipose tissue (Z.-Z. Liao et al., 2022). The capillary ECs communicate with adipocytes via paracrine signalling pathways, extracellular components, and direct cell-cell interactions, and express factors that guide preadipocyte migration towards developing capillary networks to ensure coordination of both adipogenesis and angiogenesis (Cao, 2007). ECs are not only heterogeneous within different vascular beds, but also within the adipose tissue itself (Emont et al., 2022). Different depots also show different clusters of ECs, with ECs from the visceral adipose exhibiting markers of angiogenesis and inflammation, along with a downregulation of

metabolism related genes. The ECs from the visceral depot also had an enriched senescent profile compared to those of the subcutaneous adipose tissue, and this was further validated by exposure to visceral adipocytes causing more EC senescence-associated β -galactosidase activity than when exposed to subcutaneous adipocytes (Villaret et al., 2010).

Single-cell sequencing of the adipose tissue identified distinct populations of ECs that had angiogenic and adipogenic expression profiles (Massier et al., 2023; D. T. Paik et al., 2020). Adipose ECs have upregulated genes relating to lipid metabolism such as FABP4, CD36 and PPAR γ , compared to ECs of other tissues, supporting that the adipose endothelium mediates lipid storage and release of the adipose tissue (AlZaim et al., 2023). Similarly, a study using microvascular ECs from human adipose tissue identified that the adipose ECs had lipid handling genes such as FATP1/3/4 and Cd36 as well as FABP4, the expression of which is regulated by PPAR γ . Furthermore, this group demonstrated that senescent ECs had a downregulation of FA transport and an upregulation in inflammation (Briot et al., 2018).

1.8.4 Crosstalk of the endothelium and adipose tissue

The interactions and crosstalk between ECs of the adipose capillaries and other cells of the adipose tissue are necessary for the coordination of both normal capillary and adipose function. The capillaries in the adipose tissue express high levels of LPL, an enzyme produced in the adipocytes and mobilized to the luminal surface of ECs (Scalia, 2013). LPLs have a major role in hydrolyzing triglycerides, resulting in the release of free FAs (FFA) from the adipose tissue to other organs, based on metabolic need (Goldberg et al., 2009). Conversely, FFA uptake and release by the capillary ECs into the adipose tissue is required in order for FFA to be available to the adipocytes, where they are stored (Scalia, 2013). Preadipocytes and adipocytes coordinate angiogenesis by releasing a number of non-specific and adipose-specific factors that stimulate EC

growth and motility, such as VEGF, adiponectin and leptin, as previously described (Scalia, 2013). Another example of an adipose-angiogenic molecule is 1-butyryl-glycerol, which is upregulated around 200-fold during adipocyte differentiation and stimulates angiogenesis *in vivo* and capillary EC *in vitro* (Dobson et al., 1990). It has been reported that senescent ECs impair mature adipocyte functions through SASP, with these adipocytes also then showing evidence of senescence accompanied by impaired insulin signalling (Barinda et al., 2020). These adipocytes showed higher oxidative stress and the senescent ECs consequently impaired systemic metabolic health.

Bidirectional signalling between the capillary ECs and adipocytes is found within the adipose, showing that ECs release a number of angiocrine signals that increase preadipocyte proliferation (Hutley et al., 2001). Communication of ECs to the adipocytes is also evident in a study exploring extracellular vesicle (EV)-mediated signalling (Crewe et al., 2018). Levels of caveolin-1 (Cav1) remain abundant in the adipocytes with an ablation of Cav1, owing to ECs transfer of EVs containing Cav1 (Crewe et al., 2018). Adipocytes release EVs back to ECs which contain proteins and lipids that modulate downstream signalling pathways (Crewe et al., 2018; Zocchi et al., 2022). Another group reported that ECs in the adipose tissue produce polyamines through mTORC1 signalling that stimulate adipocyte lipolysis and regulate adipose homeostasis (Monelli et al., 2022).

Moreover, FAs increased PPAR γ activation in human adipose ECs, which in turn increased FA transport and the expression of the lipid transporters FABP4 and CD36 (Gogg et al., 2019). In this study FAs were bidirectionally exchanged between the ECs and adipose cells, and the EC PPAR γ activation was dependent on the adipose triglyceride lipase protein (ATGL), while the ECs also release lipids that activate PPAR γ signalling in adipocytes (Gogg et al., 2019). Similarly, another study demonstrated that PPAR γ signalling in ECs facilitates FA uptake through the

upregulation of FABP4 and CD36 (Goto et al., 2013). Furthermore, studies have shown that EC can release 3-phosphoinositide-dependent protein kinase 1 (PDK) and adenosine kinase (ADK) that can impair adipose insulin sensitivity and glucose levels by inhibiting AKT activity (Z.-Z. Liao et al., 2022). These examples of crosstalk between capillary ECs and adipose tissue cells demonstrate that the adipose plays a role in modulating capillary formation and function, as well as ECs of the capillaries proliferating and providing adipose-specific signals required in the expansion of the adipose tissue.

1.8.5 Changes of adipose endothelial cells in response to obesity

Single-cell profiling of ECs from high-fat-fed mice revealed that sustained obesity lowered the proportion of angiogenic and proliferating adipose ECs, consistent with defective angiogenesis during clinical obesity (Bondareva et al., 2022). These ECs also had higher levels of FA transporter proteins and Hif α . Furthermore, the inflammatory phenotype of visceral adipose tissue in obese subjects has been shown to be linked to EC senescence (Lemoine et al., 2013). However, proliferating and angiogenic ECs were also identified in the adipose of these tissues and they were associated with increase of integrin and focal adhesion protein expression. Moreover, it was recently reported that adipose ECs have a higher level of mitochondrial ox-phos proteins compared to ECs of the mesentery during normal chow diet. However, HF diet intervention reduced the heterogeneity of ECs in the adipose tissue and made them more transcriptomically similar to ECs of different subtypes (Dunaway et al., 2023). Obesogenic conditions can alter the phenotype and function of adipose ECs, potentially impairing their ability to support angiogenesis and contributing to adipose tissue dysfunction.

1.8.6 Dysfunctional adipose tissue angiogenesis

During obesity, the rapid expansion of the adipose tissue can outpace the formation of new blood vessels. Limited adipose angiogenesis during obesity can lead to pathological adipose tissue expansion and obesity progression accompanied by hypoxia, macrophage infiltration and low-grade inflammation (Goossens, 2017; Lemoine et al., 2013). An acute effect of HIF α signalling is normal in healthy adipose expansion, however if the adipose depots grow beyond the capacity of sufficient angiogenesis, the adipose becomes chronically hypoxic and fibrosis, senescence and adipocyte death will occur (Crewe et al., 2017). HIF α is elevated in the adipose of obese patients, and is reduced with weight loss (Lemoine et al., 2013). Immune cells migrate to areas of hypoxia where they differentiate to pro-inflammatory macrophages, and macrophage infiltration can occur within a day of high-fat feeding. This immune cell infiltration, along with the proinflammatory factors that the adipocytes release, can trigger angiogenesis, however when there is unresolved inflammation caused by chronic hypoxia, this can contribute to the impaired angiogenesis and fibrosis within the adipose tissue (Crewe et al., 2017). This is because persistent stress signaling caused by long-term and severe hypoxia fails to induce VEGFA and results in limited angiogenesis (Crewe et al., 2017), thus a tight regulation of hypoxia signalling is needed for healthy adipose expansion.

1.8.7 Angiogenic signalling in the adipose tissue

The VEGFA/VEGFR2 signalling network is considered one of the most potent activators of the adipose tissue. Overexpression of VEGFA in the adipose tissue led to increase vessel number and size in the white adipose tissue and was associated with improved whole-body insulin sensitivity and glucose tolerance, showing the importance of adipose vascularization for overall metabolic health (Chakraborty et al., 2019; Elias et al., 2012; Sung et al., 2013). Similarly, adipose

specific deletion of VEGFA led to reduced adipose vascular density accompanied by increased adipose hypoxia, apoptosis and inflammation and metabolic defects when fed a HF diet (Sung et al., 2013). Argonaute 1 (Ago1) is an essential regulator in the EC's response to hypoxia and VEGFA signalling, with its suppression resulting in VEGFA-induced angiogenesis (Z. Chen et al., 2013). A mouse model with an EC-specific deletion of Ago1, showed increased vascularity in the adipose which was associated with lower body fat and improved whole-body metabolism (Tang et al., 2020). Moreover, it was reported that hypoxic-induction of VEGFA is impaired in obese mice and this causes a decrease in vascular density (Y. S. Lee et al., 2014), showcasing the impact of obesity on impaired angiogenic signaling.

However, it has been suggested that human adipose tissue does not show increased levels of hypoxia during obesity, yet it still exhibits poor capillary growth, insulin resistance, and signs of inflammation (Goossens et al., 2011). Hypertrophic adipocytes have an altered secretory adipokine profile that can lead to impaired angiogenic signalling, independent of hypoxia signalling (Ghaben & Scherer, 2019). Large adipocytes in obese individuals produce lower levels of adiponectin, but induced overexpression of adiponectin leads to the increase of adipose vasculature during HF-feeding, and ablates the immune cell infiltration in the adipose tissue (Aprahamian, 2013). Additionally, the adipose tissue secretes lower amounts of adiponectin than in healthy individuals which has been linked to impairment in endothelial-dependent vasodilation (Zocchi et al., 2022). Similarly, leptin is a potent angiogenic factor that binds its receptor (OB-Rb) on the ECs and promotes cell growth and proliferation (Cao, 2007), however its signalling is shown to be disrupted during obesity, which can affect angiogenesis (Picó et al., 2022). When ECs had a leptin receptor KO, the phenotype of the adipose tissue presented as more dysfunctional with increased inflammation (Gogiraju et al., 2023).

PPAR γ is another example of an adipose angiogenesis regulator, where its overexpression leads to increased capillary density and sprouting capacity of the adipose tissue. Known for its role in adipogenesis, its inhibition in pre-adipocytes not only prevents differentiation of adipocytes but also prevents formation of vasculature (Corvera & Gealekman, 2014). Without sufficient angiogenesis, the adipose expansion becomes dysfunctional and not only leads to inflammation and metabolic disturbances, but also causes ectopic lipid accumulation in peripheral tissues such as liver and skeletal muscle (Sabaratnam & Svenningsen, 2021). These studies underline the critical role of angiogenesis in adipose tissue remodeling during obesity, and when sufficient vascularization accompanies this remodeling, it enables healthy adipose tissue expansion, helping to maintain metabolic homeostasis and reduce inflammation-associated dysfunction.

1.9 Sex Differences in the Adipose and Endothelium and its Contributors

Many aspects of cardiovascular health are sex-specific and are driven by various factors (Fitzgerald et al., 2018; Gavin & Bessesen, 2020; Spiering et al., 2025). Sex differences in the body are due to a combination of genetic, hormonal and environmental factors (Szadvári et al., 2023). Differential epigenetic regulations, such as methylation, acetylation and ubiquitination, can also lead to sex differences without affecting the DNA sequence. The main epigenetic mechanisms contributing to sex differences include X chromosome inactivation in the females and genomic imprinting (Szadvári et al., 2023). Thus, a wide range of biological factors contribute to the complex and multifaceted nature of sex differences.

1.9.1 Sex hormones

Estradiol, an estrogen hormone, also known as E2, is a female sex hormone primarily synthesized in the granulosa cells of the ovaries in the females and Sertoli cells in the males (Boese et al., 2017). Progesterone is another female sex hormone and is mainly produced by the corpus

luteum cells that form after ovulation (Cable & Grider, 2023). Androgens are a group of hormones that are important in the expression of the male phenotype and are synthesized in testicular Leydig cells and ovarian theca cells (McEwan & Brinkmann, 2000; Stanhewicz et al., 2018). Although estrogen is considered the female hormone and androgens the male hormones, they both have important signalling functions in both males and females (Boese et al., 2017). Sex hormones are not only produced in the gonads but are also found to be produced in the adrenal glands (Jackson et al., 2020). The main androgen in males is testosterone, which around 10% a day is converted into dihydrotestosterone (DHT) by the enzyme 5-alpha-reductase (Kinter & Anekar, 2021; McEwan & Brinkmann, 2000). A major source of E2 in men comes from testosterone, which is converted to E2 using the enzyme aromatase (Brooks et al., 2020; Carreau et al., 2010). DHT is more potent than testosterone, with around double the binding affinity to the androgen receptor and one fifth of the disassociation rate, and cannot be converted to E2 (Kinter & Anekar, 2021). Aromatase is found to be expressed in ECs of male mice and humans, and it was found that there is an increased risk of cardiovascular events associated with aromatase inhibitors (Brooks et al., 2020; Carreau et al., 2010; Khosrow-Khavar et al., 2020).

Both E2 and androgens have receptors that they need to bind to in order to activate downstream events. The estrogen receptor (ER) has two subtypes, ER α and ER β , and is sensitive to circulating plasma estrogen levels cells (Mendelsohn, 2002; Stanhewicz et al., 2018). Expression of the ER is dependent of the tissue type with ER β being more widely distributed in the body than ER α , however ER α is a stronger transactivator at low receptor concentrations in response to estradiol (Hodges et al., 2000; Orshal & Khalil, 2004). When E2 binds to the ER, the complex undergoes homodimerization or heterodimerization and this is then able to bind specific DNA sequences to upregulate transcription (Mendelsohn, 2002). There are many targets of which

the ER can affect their expression, such as eNOS, matrix metalloproteinase (MMP), VCAM-1, elastin and more (Mendelsohn, 2002). Progesterone is another type of female sex hormone that have shown to have two main progesterone receptors (PR) and when it is bound to the receptor, it has role in regulation of gene expression, similar to E2 (Orshal & Khalil, 2004; Vázquez et al., 1999). Similarly, androgens regulate gene transcription when they bind to the androgen receptors (AR) and undergo conformational changes (Davey & Grossmann, 2016). Although androgen effects are primary regulated by AR, some effects are mediated by the ER, after aromatase converts testosterone into E2 (Stanhewicz et al., 2018). All the mentioned receptor types were found to be expressed in ECs and can regulate their gene expression and phenotypes (Stanhewicz et al., 2018; Vázquez et al., 1999).

1.9.2 Cardiovascular and adipose sex differences

CVD is a leading cause of mortality worldwide, and sex differences within the cardiovascular context have been widely studied and are highly documented within literature. Vascular tone, defined by the degree of constriction of a blood vessel relative to its max diameter when dilated, shows sex differences in various vascular beds, both in humans, and in animals, such as rats (Orshal & Khalil, 2004). Additionally, CVD incidences are more prevalent in men and post-menopausal women compared to premenopausal women (Khalil, 2005). Chronic heart disease is known to have a higher prevalence in males compared to females, however menopause diminishes this sex difference (Betai et al., 2024). It has been reported that men develop CVD at earlier ages compared to women and this sex difference is seen until women reach menopause (Reckelhoff & Fortepiani, 2004; Stanhewicz et al., 2018). The incidence of new onset CVD increases rapidly when women reach menopause, coinciding with the decline of the female sex hormones

(Stanhewicz et al., 2018). The ERs have a significant effect on EC function, as a homozygous knockout of the ER in mouse aorta ECs heightens the risk for CVD (Rubio-Gayosso et al., 2002).

One possible explanation for the worsened cardiovascular outcomes in males could be due to sex differences in adipose distribution. Normalizing for BMI, women normally have higher levels of body fat compared to men throughout their entire life span and have a higher risk to become obese (Fitzgerald et al., 2018; Gavin & Bessesen, 2020; Karastergiou et al., 2012). However, the way the fat is stored in the body also differs by sex. Females have a higher proportion of subcutaneous adipose tissue with greater accumulation in the gluteofemoral region, whereas males have increased proportion of intra-abdominal visceral fat depots (Gavin & Bessesen, 2020). This fat distribution in females may be beneficial to provide an additional buffer for fat storage during periods of nutrient excess, in place of ectopic fat deposition. A study on ovariectomized female mice showed around a 52% higher weight gain on a HF diet and higher fat mass (Ludgero-Correia et al., 2012), suggesting that sex hormones also have a positive effect on adiposity. Furthermore, visceral adiposity is associated with poor metabolic health and worse health outcomes overall. The visceral adipose tissue has larger adipocytes than those of the subcutaneous adipose tissue and has a greater influence on the development of metabolic disease, such as type 2 diabetes much more than overall body mass (Dhokte & Czaja, 2024). Additionally, females are known to have higher proportions of brown adipose tissue, increased levels of leptin and adiponectin, and direct free FA uptake, all which lead to an improved cardiometabolic health outcome compared to males (Karastergiou et al., 2012).

The female sex hormones are found to be beneficial in terms of cardiovascular disease development, as when females reach menopause, they often lose the protective effect that was observed in the presence of the sex hormones. Mice with an ER knockout had increased insulin

resistance and an increase in adiposity (Kuryłowicz, 2023). Lower estrogen led to metabolically unhealthy hypertrophic visceral adipose expansion in females (Steiner & Berry, 2022). However, the influence of sex hormones goes beyond adipose distribution and can directly affect cell metabolism. Lipid storage is dependent on FA uptake by the adipocytes and their conversion into triglycerides. In contrast, triglycerides are hydrolyzed and released into the circulation depending on the metabolic demand of the body (Steiner & Berry, 2022). Female sex hormones can directly inhibit lipid storage genes and instead activate lipolytic pathways. Estradiol was shown to directly inhibit PPAR γ -induced adipogenesis and reducing adiposity through the PPAR γ pathway (Jeong & Yoon, 2011). In sum, estrogen plays a vital role in promoting healthy adipose tissue metabolism by regulating fat distribution, enhancing insulin sensitivity, and reducing inflammation, ultimately supporting overall metabolic health.

1.9.3 Sex hormone impact on ECs and angiogenesis

Many separate studies have demonstrated that sex hormones upregulate EC proliferation from various vascular beds and ultimately, angiogenesis through various pathways. One study demonstrated that treating porcine pulmonary arterial EC with E2 induced their sprouting and increased angiogenesis (Seo et al., 2004). Estrogen was shown to cause the activation of NF- κ B, inducing angiogenesis using a mechanism involving platelet-activating factor (PAF)-mediated NF- κ B activation (Seo et al., 2004). E2 treatment on HUVECs enhanced their adhesion to matrix proteins, promoting migration in a wounded monolayer of cells as well as increasing proliferation and capillary-like networks (Morales David et al., 1995). A separate group also reported that E2 leads to proliferation in HUVECs by the gene thrombospondin-1 (TSP-1), a known negative regulator of angiogenesis. The downregulation is mediated through nongenomic ER/mitogen-activated protein kinase (MAPK)/extracellular-regulated kinase (ERK) 1/2 and c-Jun NH(2)-

terminal kinase (JNK)/stress-activated protein kinase signaling pathways (K. Sengupta et al., 2004).

Considering MAPK is involved in EC proliferation, E2 was reported to induce phosphorylation and activation of MAPK (Khalil, 2005). One group of researchers demonstrated that E2 promotes phosphorylation of p42/44 and p38 MAPK in porcine aortic ECs, allowing the cells to enter mitosis and increasing the migration and proliferation (Gerald et al., 2002). Further, E2 can activate the PI3K pathway also involved in EC proliferation and migration (X. Zhao et al., 2008). Another group showed that E2 treatment led to an upregulation of HUVEC proliferation and migration due to an increased gene expression of Rho GEF-1 and RhoA (Oviedo et al., 2011). The same group showed that p27 levels decreased and upregulated the expression of cyclins and cyclin dependent kinases (CDK), which are important for the cell cycle progression during mitosis (Oviedo et al., 2011). Furthermore, stimulation of cardiac microvascular ECs with E2 led to increased expression of the ER α , accompanied by increased proliferation, migration, and tube formation (H. Liu et al., 2018). Another group investigated the effects of this female sex hormone on VEGFA signalling and reported that E2, but not progesterone, increased the expression of VEGFR in human myometrial microvascular ECs (Gargett et al., 2002).

Estradiol can upregulate EC proliferation by modulating cellular metabolism, enhancing the energy production and biosynthetic pathways necessary for cell growth and division. It was reported that E2 administration to both female and male mice resulted in elevated capillarization of the bones, as well as increase of the total number of ECs (Rodrigues et al., 2022). The group showed that estradiol had a direct effect on EC usage of FAs, by upregulating the expression of the FA transported FABP4 and increasing the amount of FA found in the ECs, associated with increased EC proliferation and reduced number of adipocytes (Rodrigues et al., 2022). Moreover,

treatment on brain ECs with estradiol increased mitochondrial cytochrome C, as well as reduced mitochondrial ROS levels (Razmara et al., 2008). Estradiol is associated with mitochondrial function, and this was observed as female mice EC had enhanced mitochondrial membrane potential as well as a higher mitochondrial content. The ECs of these female mice had enhanced Ca^{2+} uptake, attributable to the presence of more mitochondria (Damacena de Angelis et al., 2022). Estradiol also plays a role in EC glycolysis, with studies showing that E2 increases the glycolytic protein PFKFB3, as well as the glucose transporter GLUT1 (Boscaro, Carotti, et al., 2020; Trenti et al., 2017). The binding of E2 to the cytosolic ER leads to the translocation of the ER bound to the E2 into the nucleus (Duckles & Miller, 2010). In the nucleus the ER binds to co-regulators and DNA response elements on the eNOS gene that increases the transcription and, ultimately, the production of the eNOS gene (Duckles & Miller, 2010; Stirone et al., 2003). Ovariectomized female rats were treated with estrogen and were found that the levels of eNOS protein increased at a 17.4 fold change in the microvessels compared to rats that did not receive estrogen (McNeill Anne et al., 1999). Females that were not ovariectomized also expressed eNOS protein at 9.2 fold higher than males (McNeill Anne et al., 1999). Estradiol supports EC metabolism by enhancing mitochondrial function, promoting nitric oxide production, and improving glucose and lipid utilization, all of which contribute to vascular health and cellular energy balance.

While the roles of estradiol on ECs have been widely reported, there is little information on the other female sex hormone, progesterone, and its effect on ECs. In contrast to estradiol, progesterone was shown to inhibit EC proliferation and have a proapoptotic effect on the ECs, the opposite effect of E2 (Vázquez et al., 1999). Studies of human dermal ECs and human coronary ECs demonstrated that progesterone treatment sent cells into cell arrest during the G_1 phase,

attenuating proliferation (Vázquez et al., 1999). Another group demonstrated that the inhibition of EC proliferation by progesterone is mediated by the p53 pathway (Hsu et al., 2008). In contrast, progesterone was shown to increase HUVEC migration by activating focal adhesion kinase (S. Zheng et al., 2012). Although both estrogen and progesterone are female sex hormones, it is evident that the effects of these hormones differ due to the pathways through which they activate downstream genes.

1.9.4 Sex chromosomes

Apart from sex hormones, sex chromosomes also contribute to sex differences in the body (X. Chen et al., 2013; Fuente-Martín et al., 2013; Spiering et al., 2025). Females are genetically defined by XX and males are defined by XY (V. Miller, 2010). Numerous differentially expressed genes were reported between male and female adipose tissue, with some being located on sex chromosomes (Fuente-Martín et al., 2013). The most studied gene on the Y chromosome is the *Sry* gene which is associated with severity of hypertension (V. Miller, 2010). Polymorphisms of genes on the X chromosome are expressed in the males but will show a mosaic expression on females due to X inactivation (Fuente-Martín et al., 2013). Thus, sex chromosomes can independently contribute to sex-specific differences in ECs, highlighting the combined impact of genetic and hormonal factors on vascular health.

To independently determine the effect of sex chromosomes and hormones on sex differences, the four core genotype model is used to separate the effects of the two factors. The four core model involves moving the testis-determining gene, *Sry*, to an autosome, creating four distinct groups: XX and XY gonadal males, and XX and XY gonadal females (Regitz-Zagrosek & Kararigas, 2017). Using this system allows comparison of the difference of sex chromosomes while controlling for the sexual phenotype, gonad type, and sex hormone level.

1.9.5 Endothelial Sex Differences

As a result of sex hormones and sex chromosomes, male and female ECs display transcriptomic and functional differences. Primary cultured rat ECs from skeletal muscle retained intrinsic sex differences in a gonadal-free-environment, suggesting that these sex differences are due to sex chromosomes (J. Wang et al., 2010). Hartman *et al.* reported that 14-25% of the EC transcriptome is sex biased, and that female adult ECs are enriched in estrogen responses, while oxidative phosphorylation was enriched in male ECs (Hartman et al., 2020). Consistent with the increased vasodilation observed in females, female ECs express higher eNOS mRNA and protein levels (Cattaneo et al., 2017). Transcriptomic analysis of the EC from five major organs (including the adipose) in young mice and old mice found 590 differentially expressed genes (DEGs) between females and males in the younger group and 252 DEGs in the older group. In the younger group, the female adipose DEGs were enriched in pathways relating to innate immunity, while the male DEGs were involved in EC organization such as cell junction assembly and angiogenesis (X. Huang et al., 2021). However, in the older group, the males had high levels of inflammation and immune cell phenotypes, but the female ECs were enriched in markers of proliferation.

1.9.6 Sex differences in EC proliferation and angiogenesis

In the absence of sex hormones, female HUVECs were enriched in cell proliferation and migration, showing an intrinsic capacity for females to have higher proliferation (Addis et al., 2014). The males from this study also appeared to be more autophagic than female HUVECs which could be a reason of why females have higher angiogenesis. HUVECs treated with synovial fluids from arthritis patients showed that there was enhanced migration and tube formation in females (Baggio et al., 2022). Another group reported that female HUVECs had higher viability after serum starvation and increased tube formation capacity compared to those of males (Lorenz et al., 2015).

HUVECs from female donors display elevated viability and growth rate, as well as angiogenic potential (Boscaro, Trenti, et al., 2020). When these HUVECs were treated with estradiol, the glycolytic gene PFKFB3 was upregulated at a higher potency in female HUVECs compared to the males, suggesting that hormone signalling is sex-dependent. These reports suggest that female ECs are more proliferative than male ECs, a difference that persists even in the absence of circulating sex hormones, indicating that intrinsic genetic or epigenetic factors contribute to this heightened proliferative capacity. This sex-based disparity may underlie observed differences in vascular repair, angiogenesis, and susceptibility to cardiovascular diseases, highlighting the importance of incorporating sex as a biological variable in both basic and translational vascular research.

Indeed, studies have shown that the higher proliferative potential of female ECs is reflected in the increased angiogenesis females display. Our lab reported that when on a high fat (HF) diet, female adipose tissue had higher capillary number and EC markers, which was associated with an improved adipose phenotype (Rudnicki, et al., 2018b). Female ECs also are shown to have a better migratory phenotype, that is needed for angiogenesis (Cattaneo et al., 2017). The higher female angiogenesis is in part attributed to sex hormones, as not only is estrogen a potent proliferative inducer of ECs but was also shown to regulate the expression and activity of the pro-angiogenic adenosine A_{2A} receptors (Troncoso et al., 2020). Moreover, male and female pulmonary ECs exhibit different secretomes, and while both secrete factors that increase length of sprouts in the female, male sprouts were unaffected (Hayward-Piatkovskyi et al., 2023). This study and others (Sieveking et al., 2010) explain that both sex hormones and sex-specific secreted factors influence EC phenotypes in a sex specific manner. While higher VEGFA levels have been identified in the females (Lindholm et al., 2014; Malamitsi-Puchner et al., 2000), this does not explain the higher female proliferative capacity in the absence of exogenous pro-angiogenic signals.

1.9.7 Sex differences in EC oxidative stress response and dysfunction

There is evidence that female ECs exhibit greater resilience to oxidative stress compared to male cells, likely due to enhanced antioxidant defenses and more efficient mitochondrial function. Sex disparities were evident in ECs exposed to hyperoxia, with female HUVECs having higher viability, less oxidative stress and higher angiogenic potential (Y. Zhang & Lingappan, 2017). Estrogen is well documented to reduce ROS in the endothelium, with its suppression increasing O₂ production in HUVECs (A. A. Miller et al., 2007), coinciding with lower free radical levels in female ECs compared to males. A HF diet that induces vascular oxidative stress had worse outcomes for male ECs, indicating that ECs from female vessels are more resilient to diet-induced oxidative stress (Norton et al., 2020). The same group showed that H₂O₂ caused less apoptosis in female ECs compared to those of males (Norton et al., 2019). In the carotid arteries, females were seen to have higher levels of SOD1 compared to males, and it coincided with higher levels of SOD in vascular smooth muscle cells, showing a greater antioxidant stress mechanism (Morales et al., 2015). Furthermore, a group showed that nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase subunits were higher in males than in females. These subunits played a role in the arteries of males but not females, indicative of more oxidative stress in the male vasculature (Kander et al., 2017; Wong et al., 2015). These NADPH oxidases are a major source of ROS, and estrogen can reduce these oxidases while at the same time upregulating antioxidant enzymes, showing its beneficial role in the female oxidative stress regulation (A. A. Miller et al., 2007).

In contrast to literature showing females having a healthier EC phenotype consisting of higher proliferation and antioxidant activity, analysis on the mouse and human aortic endothelium in atherosclerosis models shows that female ECs have higher rates of apoptosis and higher levels of ROS (Shin et al., 2024). The aortic ECs of females also had lower mitochondrial membrane

potential, accompanied by decreased angiogenic potential, such as proliferation, compared to those of males. As expected, the lower angiogenic potential in the females was related to increase lipid accumulation in the aorta, as well as impaired whole-body metabolism (Shin et al., 2024). Similarly, male ECs from mouse lungs showed higher mitochondrial function, and higher rates of proliferation (Zemskova et al., 2020). Sex differences in EC inflammatory response were also reported, with female HUVECs appearing to have more deleterious effects when induced with an inflammatory stimulus (Doro et al., 2024).

It is possible that the effect EC sex differences such as ROS handling and oxidative stress resistance are context specific, as an inflammatory insult increased the antioxidant SOD2 gene in males more than in females in HAECs, but the opposite effect was observed in HUVECs with higher SOD2 expression in females (Najjar et al., 2023). Similarly, male HAECs had enriched expression of the inflammatory pNF- κ B compared to females, but these sex differences were not detected in other EC beds (Najjar et al., 2023). Sex differences relating to EC dysfunction were also shown to be context specific, with higher dysfunction in female cerebral arteries but higher in the male mesenteric arteries (Cole et al., 2021). A comprehensive study examining sex differences from different vascular beds, showed that female ECs are bigger in the aorta compared to those of males, but the opposite effect were seen in the ECs of the skeletal muscle (Huxley et al., 2018). Cell growth in the macrovascular ECs was independent of sex in this study, whereas microvascular ECs grew faster in the male compared to those of females, yet healing of scratch-wounded EC was slower in the males (Huxley et al., 2018). These data underscore the importance of considering the context and tissue-specific environment when studying sex differences in EC, as their phenotype and function can vary significantly across vascular beds and are influenced by local signals and disease states.

1.10 Scope of dissertation

Endothelial sex differences have been widely reported in literature, with many groups showing that females have higher oxidative stress resistance and higher proliferation (Addis et al., 2014; Lorenz et al., 2015; Y. Zhang & Lingappan, 2017). Our lab showed higher adipose tissue vascularization during HF feeding (Rudnicki, et al., 2018b), however the mechanism of the higher angiogenesis is unknown. Thus, my primary aim for this dissertation was to assess sex differences of the adipose ECs. To achieve this, the transcriptome of male and female was compared, as well as functional sex differences were studied in culture. Next, I considered the known positive role of the female sex hormones on angiogenesis (Seo et al., 2004), and sought to investigate if the loss of gonadal sex hormones has an impact on adipose vascularization and EC metabolism. A gonadectomized cohort of mice and two diet types were used for this aim and investigated the effect on sex hormone loss in the mice. Lastly, due to the protective effects of FoxO1 (Jerome et al., 2022) and the higher level RNA and protein in female ECs our lab has reported, I hypothesized that FoxO1 contributes to the EC sex differences observed in the adipose tissue. To address this, I compared the endothelial transcriptome between EC-specific FoxO1 KD and control mice and investigated the changes of the ECs following FoxO1 signalling disruption. These investigations allow for better understanding of the sexual dimorphism in the adipose tissue vascularization and also apply to contexts outside the adipose, which can be used to explain sex differences in CVD. This may be used to identify potential therapies to improve adipose endothelial health during obesity which can lead to better metabolic and cardiovascular outcomes.

1.11 Hypothesis and Objectives

Hypothesis

Female and male adipose EC are transcriptomically distinct, in part due to different functional effects of FoxO1, leading to differences in their phenotype, that can in turn affect adipose health.

Objectives

1. Identify differentially regulated pathways between ECs of males and females that may explain the vascular phenotypic differences observed in the adipose during development of obesity.
2. Investigate if sex hormones impact the observed EC sex differences, by using intact & gonadectomized cohorts of mice and analyzing the changes of adipose vasculature and EC phenotype after sex hormone loss.
3. Study the sex differences of FoxO1 depletion EC gene expression and function, and how the adipose tissue and whole-body metabolism is subsequently affected.

Chapter 2: Transcriptomic profiling reveals sex-specific molecular signatures of adipose endothelial cells under obesogenic conditions

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2.1 Contributions

I was involved in the conceptualization of the study and development of the experimental framework along with TH, MR and ERO. Preparation of the samples for RNA sequencing was done by MR and OR. ERu and TG performed the RNA-seq raw analysis (read mapping, normalization, differential expression analysis). I performed the RNA-seq analysis using the data received from ERu and TG. I generated the second proliferative cohort used for the study and administered the diet and EdU injection. I extracted the adipose tissue and performed the quantitative analysis on it. I isolated endothelial cells and performed the cultured inflammation experiments. I compared our transcriptomic data to the Tabula Muris Senis transcriptomic data. I generated Figures 2.1C, 2.2, 2.3 A-D, 2.4, 2.5, 2.6, S2.4, S2.6 and helped with 2.7. MR generated Figures 2.1A, B, created the summary schematic in 2.7, S2.1, S2.2. MR and EN collected data for Tables 2.1 and 2.3 and I created all other tables. OR performed the *in vitro* endothelial proliferation assay and generated Figure 2.3E. AF helped generate figures S2.2, S2.3, and S2.5 by running western blots for metabolism-related proteins and isolating RNA from whole adipose and running qPCR. I wrote the manuscript along with MR and TH.

2.2 Introduction

Endothelial cells (EC) form the contiguous inner lining of the entire circulatory system. Within this privileged position as the interface between the bloodstream and all parenchymal cells, EC act as gatekeepers of energy balance and tissue homeostasis by controlling nutrient and oxygen supply, coagulation and inflammation (Augustin & Koh, 2017; Pober & Sessa, 2007). EC provide additional support for tissue growth and metabolism by coordinating the growth of new capillaries through angiogenesis (Potente & Carmeliet, 2017). During this process, they exit their usual state of quiescence and adopt specialized transient phenotypes associated with proliferation and motility (Geudens & Gerhardt, 2011; Potente & Carmeliet, 2017). Moreover, EC exhibit organ-specific molecular signatures and functional specializations based on bidirectional paracrine interactions with the tissue parenchyma and adjacent stromal cells.

Extensive evidence from animal and human studies concur that EC dysfunction and impaired angiogenesis contribute to the development of obesity and its related complications (Aoqui et al., 2014; García-Prieto et al., 2015; Molnar et al., 2005). In white adipose tissue of males, insufficient capillary growth compromises healthy tissue expansion to accommodate calorie excess, which ultimately reflects in metabolic disturbances at the systemic level. Our group and others have reported that promoting angiogenesis to increase adipose vascularization can prevent/reverse metabolic disorders associated with excess adiposity (Robciuc et al., 2016; Rudnicki, et al., 2018a; Sung et al., 2013). Although this reinforces the paramount role of adipose EC in the pathogenesis of obesity-related disease, the molecular profiles of adipose EC under conditions of obesity, and the extent to which biological variables may regulate their vulnerability to dysfunction have not been investigated.

Sex is a biological variable that accounts for differences in adipose tissue pathophysiology

and clinical manifestations of obesity (Goossens et al., 2021). However, mechanisms underlying these differences are poorly defined particularly because sex is frequently overlooked in pre-clinical studies (Yoon et al., 2014; Zucker & Beery, 2010). We recently provided the first evidence that female mice have greater adipose angiogenesis than males in response to high-fat diet (HFD) feeding, indicating that sex is a modifier of vascular remodeling. Yet, the underlying mechanisms for this difference remain unknown. Given that several recent studies have reported intrinsic sex differences in gene expression and behavior of human umbilical vein endothelial cells (HUVEC) (Addis et al., 2014; Cattaneo et al., 2017; Hartman et al., 2020; Lorenz et al., 2015, 2019), we hypothesize that EC sex-differences play a substantial role in establishing divergent angiogenic responses in the expanding adipose tissue.

Thus, the present study aimed to analyze the transcriptome of adipose tissue EC of male and female mice to identify molecular signatures that may determine the sex differences in vascular remodeling during HFD-induced expansion of white adipose tissue.

2.3 Methods

2.3.1 Mice

Mouse protocols were approved by the York University Animal Care Committee and all experiments conformed to the standards of the Canadian Council on Animal Care. All mice were maintained in a 12:12 light:dark cycled, temperature-controlled environment with water and diet ad libitum. The first two cohorts of mice utilized genetically matched (FVB;B6) littermates that were bred in-house at York University and fed a high-fat diet (Surwit Diet, 58% kcal from fat, Research Diets) for a total of 7 weeks, starting at 7 weeks of age. In the first cohort (n=4 female and n=4 male), subcutaneous and perigonadal white adipose tissues were harvested for whole tissue RNA analysis and EC isolation for RNA sequencing (RNA-Seq). In the second cohort, (n=4

female, n=3 male), mice were injected with EdU (*i.p.* 1.5 mg in 100 μ L sterile PBS) on two sequential days prior to sacrifice and the perigonadal adipose tissue was used for whole-mount imaging analysis to detect proliferating cells. A third validation cohort of age-matched male and female C57Bl6/J mice (purchased from Jackson Laboratories) were fed a high fat diet for 7 weeks starting at 6 weeks of age (n=6 males; 12 females). Perigonadal white adipose from this cohort of mice was used to isolate EC for qPCR validation of selected genes from the RNA-Seq dataset. C57Bl6/J mice were used to extend the validity of the RNA-seq molecular signatures by testing in a difference mouse strain. To increase sample yield from the C57Bl6/J cohort, perigonadal adipose tissue from 2 females was pooled together for each EC isolation, yielding 6 independent RNA isolations for both females and males. Despite this strategy, the small amount of RNA yielded limited sample sizes for some gene expression analyses may show different sample sizes due to the small amount of starting material for RNA.

2.3.2 Primary murine endothelial cell cultures

EC were isolated from perigonadal white adipose tissue of male or female mice and purified a Streptavidin-magnetic beads coupled to biotinylated rat anti-mouse CD31 antibody (Rudnicki, et al., 2018a). Cells were used in experiments between passages 2 and 5.

2.3.3 Adipose EC isolation for RNA Seq

Perigonadal and inguinal subcutaneous white adipose depots from each mouse were harvested, minced and digested with Type I collagenase (0.5% m/v) in DMEM for 30 minutes at 37°C (or until a single cell slurry was obtained). The resulting stromal vascular fraction was passed through a cell strainer (100 μ m) and ECs were selected by incubation with Dynabeads conjugated to rat anti-mouse VE-cadherin antibody and Streptavidin-magnetic beads coupled to biotinylated rat anti-mouse CD31 antibody (Rudnicki, et al., 2018a).

2.3.4 Bulk RNA Seq

RNA was extracted from freshly EC isolated from adipose tissues (pooled perigonadal and inguinal pads) using RNeasy Mini Kit and its quality was assessed using the RNA 6000 Nano chip on the 2100 Bioanalyzer automated electrophoresis system (Agilent Technologies Inc.). Samples with an RNA Integrity Number (RIN) > 6 (n=4 / group) were processed for RNA sequencing by The Centre for Applied Genomics, The Hospital of Sick Children, Toronto. PolyA-enriched cDNA-libraries were generated and sequenced using a Novaseq SP flowcell (paired end 2x50 bp), generating ~60 million reads per sample. After mapping reads to the mouse genome from Ensembl Mus_musculus.GRCm38.cdna.all.fa, expression levels were normalized as Transcripts Per Kilobase Per Million (TPKM) by dividing the read count of each transcript model with its length and scaling the total per sample to one million using Kalisto V0.4.4. Quality control of raw and mapped reads was done using FastQC version 0.11.8. Transcripts with <20 raw counts per library were deemed unexpressed and were discarded from analysis. Differential expression analysis was performed using the R package EdgeR version 3.34 (Robinson et al., 2010), with the formula $\log_2\text{CPM} = 0 + \text{Sex}$, correcting for common, trended and tagwise disparity. The analysis rendered two-base logarithmic fold change ($\log_2\text{FC}$) differences and false discovery rate (FDR) values, where FDR of <1% was considered significant.

2.3.5 Gene ontology and set enrichment analyses

Gene ontology analysis was performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (D. W. Huang et al., 2009a, 2009b) to identify enriched Biological Processes (BPs) within the genes significantly upregulated in male and female EC. Ontologies with a Benjamini P value < 0.05 were considered significant. Enrichplot (R) was used to generate the dotplot based on the GO output. Gene set enrichment analysis (GSEA)

(Subramanian et al., 2005) was used to test our RNA-Seq dataset for the enrichment of custom gene sets generated from literature. Customized gene sets included: endothelial tip cell markers (Kalluri et al., 2019; Goveia et al., 2020); proliferating EC markers (Li et al., 2019; Goveia et al., 2020; Kalucka et al., 2020; Niethamer et al., 2020; Orsenigo et al., 2020; Tombor et al., 2021); oxidative phosphorylation (Diebold et al., 2019); inflammasome complex/NLRP3 inflammasome complex assembly (GO:0061702 and GO:0044546) and EC senescence associated secretory phenotype (SASP) (Coppé et al., 2008; Basisty et al., 2020; Kiss et al., 2020). A .gmt file was created including all generated gene sets as mouse gene symbols, requiring no collapse or remapping in GSEA. The 14501 genes in our dataset were pre-ranked based on log fold change (positive values for genes upregulated in female, negative for those upregulated in males; zero cross at 7445). Gene sets with an FDR q-value<0.05 were considered significantly enriched.

2.3.6 Bioinformatic inference of transcriptional regulators

iRegulon, accessed via Cytoscape (Shannon et al., 2003), was used to predict transcriptional regulators of the DEGs represented in the male-biased inflammatory ontologies (Immune System Process GO: 0002376; Positive Regulation of Phagocytosis GO: 0050766; Inflammatory Response GO: 0006954; Neutrophil Chemotaxis GO: 0030593; Antigen Processing and Presentation of Exogenous Peptide Antigen via MHC Class II GO: 0019886) and in the female-biased transcription and nucleosome assembly ontologies (Positive Regulation of Transcription GO:0045893; Nucleosome Assembly GO: 0006334). Transcription factor binding motifs were identified 10kB around the transcription start site.

2.4.7 Comparison with publicly available EC data

We also compared our RNA-Seq dataset with the sex-biased EC transcriptional profile of young and aged EC, compiled from multi-organ single cell sequencing analysis of 3- and 18-

month-old mice from the Tabula Muris Senis dataset (Schaum et al., 2020), as compiled by Huang and colleagues (X. Huang et al., 2021). To do this, we conducted GSEA of annotated Gene Ontology Biological Processes (GO BP) of each dataset, pre-ranking each dataset by log fold change. Gene sets were considered significantly enriched using FDR q values of < 0.05 , 0.1 and 0.15 for our dataset and the Huang datasets for young and aged-mice, respectively. The less stringent q value was utilized for the Huang dataset because this single cell sequencing dataset consisted of a much smaller number of genes (6,656 total) compared to our RNA-seq dataset (14,501 total). A clustering strategy was used to aid in describing similar ontologies comprised within each dataset. Biological networks were annotated using the “AutoAnnotate” function in Enrichment Map (Merico et al., 2010) (accessed via Cytoscape) and clustered according to the GLayer clustering algorithm (Su et al., 2010) based on ontology name. Only clusters containing five or more ontologies are reported. Lastly, we identified specific gene ontologies that were enriched in both datasets. The logFC and adjusted p values of selected genes comprising the leading edges of these shared gene sets were compared and displayed by chord plot (Gu et al., 2014).

2.3.8 Whole-mount imaging of adipose tissue

Small pieces of formaldehyde-fixed perigonadal adipose tissue were stained to assess EC proliferation (Rudnicki et al., 2022b). Proliferating endothelial cells (EdU+) were detected by pre-incubation in a solution containing 0.1 mM Azide-fluor 545; 0.1 M Tris, 1 mM CuSO₄, 0.1 M ascorbic acid prior to immunostaining. To visualize EC nuclei, samples were blocked and permeabilized with 0.1% Triton X-100, 5% goat serum in PBS followed by overnight incubation with rabbit anti-ERG (A7L1G) antibody (1:200), and 1 hour incubation with AlexaFluor647 goat anti-rabbit (1:200). Samples were counterstained with DAPI (1:1000) to detect all nuclei and Griffonia simplicifolia lectin I (GSL)-FITC (1:100) to detect capillaries. Single plane images were

captured with a Zeiss LSM700 inverted confocal microscope equipped with a 40×/1.30 Oil EC PLAN-Neofluar objective (York University YSciCore). Microvascular content, EC nuclei and EdU positive nuclei were quantified from 4-5 fields of view per animal using ImageJ Analysis Software.

2.3.9 In vitro proliferation assays

Male and female adipose EC were plated in 96 well plates (8×10^3 cells per well). The following day, ECs were incubated for 4 hours with 10 μ M EdU (Click-iT EdU Imaging Kit) then fixed and Click reaction using Alexa Fluor488 Azide was performed according to the manufacturer's instructions. Cell nuclei were counter-stained with DAPI (1:1000). Imaging and counting were performed using CellInsight CX7 High-Content Screening Platform (Thermo Fisher Scientific, USA) to quantify the number of EdU-positive and number of DAPI-positive cells, and the percentage of proliferating ECs was expressed as $(\#EdU^+ / \#DAPI^+) \times 100$.

2.3.10 In vitro TNF α stimulation

Male and female adipose EC were plated at 4×10^4 cells per well in a 12-well plate. After 3 days, the media was replaced with 10% FBS high-glucose DMEM and then cells remained untreated or stimulated with 5 ng/mL recombinant human TNF α , for 20 hours before RNA extraction and analysis by qPCR.

2.3.11 RNA analysis by quantitative PCR

RNA extracted from EC or whole adipose tissue was reverse transcribed using M-MLV reverse transcriptase and the cDNA was analyzed by real-time PCR on the Rotor-Gene Q platform (Qiagen Inc.) using Fast TaqMan Master Mix and TaqMan primers. Transcript levels of each target gene were calculated relative to an appropriate housekeeping gene (*Hprt1*, *Actb* or *Tbp*) and

expressed as $2^{-\Delta Ct}$.

2.3.12 Western Blot analysis

Total protein extraction from cultured adipose EC was performed using lysis buffer containing 50 mM Tris (pH 8.0), 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate with phosphatase inhibitor (PhosSTOP, Roche # 4906837001) and protease inhibitor (Complete; Roche #11836153001). Following protein separation through a polyacrylamide gel under reducing conditions, western blots were performed using the following primary antibodies: hexokinase 2 (HK2, 1:1,000), 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3, 1:1,000), and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH, 1:1,000), Beta-actin (1:5,000) and alpha/beta Tubulin (1:1,000). Goat anti-rabbit or anti-mouse IgG-horseradish peroxidase secondary antibodies were utilized. Membranes were developed using enhanced chemiluminescence and densitometry analysis was performed with ImageJ Analysis Software (NIH).

2.3.13 Quantification and Statistical analyses

All data reported are from independent biological replicates with each mouse considered as one biological replicate. Averaged values were used when technical replicates (analysis of the same sample in duplicates) were performed. For *in vitro* assays, experiments were repeated at least six times with independent biological samples. No statistical methods were used to predetermine sample size, the experiments were not randomized and no outliers were excluded in our analyses. For 2-group comparisons, datasets with sample sizes greater than 6 were analyzed by unpaired Student's t test and those with samples sizes less than 6 were assessed using the nonparametric Mann-Whitney U test. Gene expression data from TNF α treatments of cultured cells were analyzed

using 2-way repeated measures ANOVA (Sex x Treatment), followed by Sidak's multiple comparison post hoc tests when a significant main effect or interaction was identified. A *P* value of < 0.05 was considered significant for all analyses, except for the DEG analysis for which a *P* value of < 0.01 was the cut-off for significance. Statistical tests were performed using Prism ver. 5 or 7 (GraphPad Software Inc.). Data are shown as means $\pm$ standard errors of the mean (SEM) and *P* values are indicated in each Figure legend.

2.4 Results

2.4.1 Male and female adipose EC exhibit distinct molecular profiles under obesogenic conditions

We recently reported that the expansion of the visceral adipose tissue of female mice is accompanied by a concomitant growth of its vascular network that contrasts with the compromised adipose angiogenesis of male mice following 16 weeks of HFD feeding (Rudnicki, et al., 2018b). Here, we sought to uncover predictive molecular mechanisms that may explain the disparate angiogenic capacity in adipose of female compared to male mice, by focusing on EC gene expression during an earlier phase of adipose tissue remodeling. Thus, we treated male and female Friend Virus B (FVB);B6 mice with a 7-week HFD intervention. Mouse body weight gain and plasma glucose levels of these mice are provided in Table S1. The transcriptome of EC isolated from white adipose tissue of these mice was analyzed by RNA-Sequencing (RNA-Seq). First, we examined the pattern of EC hallmark genes (**Figure S2.1**). As expected, transcripts for EC marker genes *Cdh5*, *Pecam1* and *Ptpnb* were abundant in both male and female EC. Capillary marker genes also were prominent whereas transcripts of arterial and venous EC markers were present at lower levels.

Differential gene expression analysis displayed substantial sex-dependent transcriptional differences in adipose EC, with 1,281 differentially expressed genes (DEGs) between the sexes

when applying an adjusted *P* value of 0.01 as a cut-off (**Figure 2.1A**). The majority of the DEGs (1,225 genes) were distributed among autosomal chromosomes while 56 DEGs were detected on the X chromosome (**Figure 2.1B**). Genes localized to the Y chromosome were not included in the analysis.

Gene Ontology (GO) enrichment analysis of the RNA-Seq dataset was used to identify biological processes (BP) over-represented within the sets of DEGs for each sex. Genes associated with chromatin organization, epigenetic modifications and transcriptional regulation were significantly enriched in the female EC DEGs (**Figure 2.1C**), and suggestive of elevated cellular proliferative activity. Female EC also exhibited an enrichment of the GO BP term ‘ossification’, which largely included genes that have known EC expression and can influence mesenchymal cell behavior (i.e. *Ptn* and *Bmp3*). Conversely, the DEGs that were higher in male EC were dominantly associated with GO BP terms related to inflammation. Interestingly, male EC were enriched in genes belonging to the contrasting ontologies of ‘negative’ and ‘positive regulation of angiogenesis’, which may imply a restriction on angiogenic signaling in these cells. Differing sets of genes encoding extracellular matrix (ECM) and adhesion proteins were enriched in female and male EC, respectively. Complete lists of the significantly enriched GO BPs for each sex are provided in **Table S2.2**. In sum, these data demonstrate that male and female EC display substantially distinct transcriptional profiles under high-fat feeding.

To verify that sex differences in gene expression detected in the RNA-Seq dataset were not limited only to this mouse cohort or strain, we conducted qPCR analysis of selected genes utilizing adipose EC isolated from an independent cohort of 7-week HFD-fed male and female C57Bl6/J. Mouse body weight gain, fasting glucose and glucose tolerance tests of this mouse cohort were reported in **Table S2.3**. Overall, a high level of consistency was observed between gene expression

in this independent cohort compared with the RNA-Seq dataset, with 12 of the 14 genes tested displaying the same patterns of gene expression (**Table S2.4**).

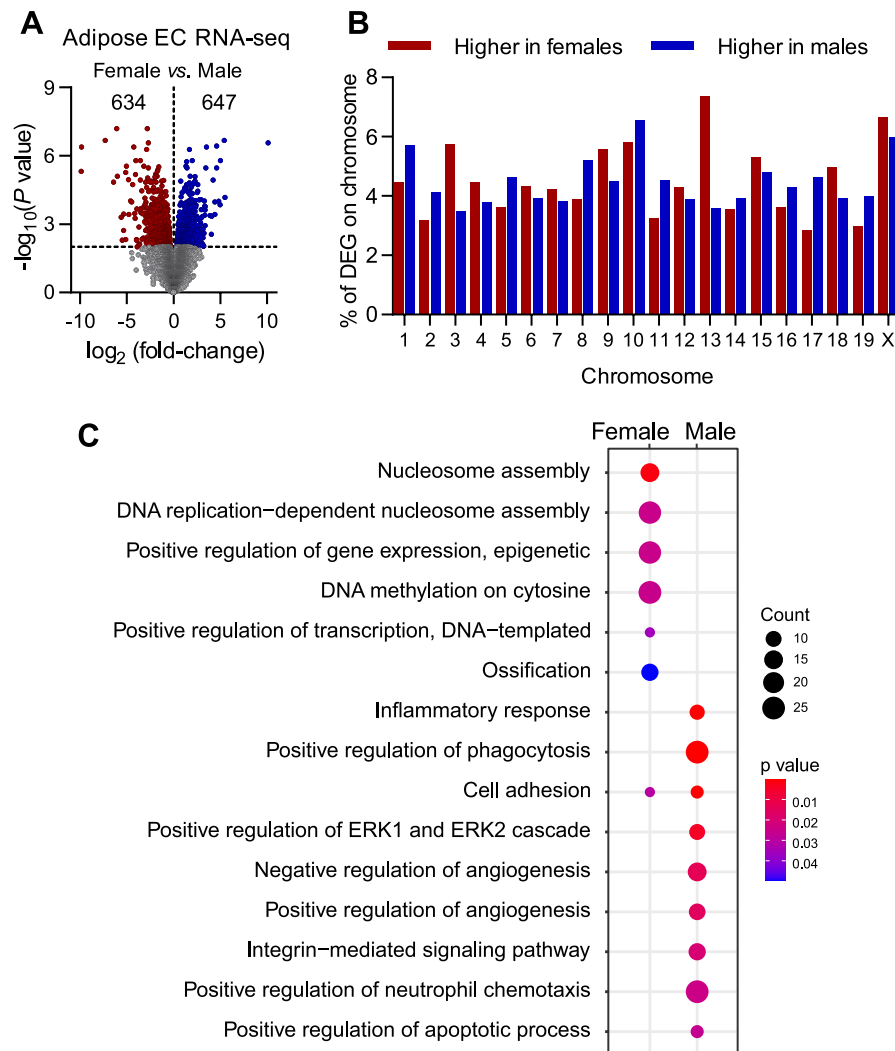


Figure 2.1 Transcriptome analysis of male and female adipose EC from HF-fed mice. **A)** Volcano plot illustrating the expression profile of genes in female and male adipose EC, as determined by RNA-seq of n=4 mice per sex. Negative logFC (red) = up in females; positive logFC (blue) = up in males. A total of 1,281 DEGs were identified, with an adjusted *P* value <0.01 (indicated by the dashed horizontal line). See also Figure S1. **B)** Chromosome distribution of DEGs in female and male adipose EC. The proportions of female and male-biased genes are expressed as a % of the total number of genes on that chromosome. **C)** Dot plot illustrating selected “Biological Process” gene ontologies that were enriched in the DEG upregulated in female and male adipose EC, respectively. The count represents the number of inputted DEGs as a percentage of the total number of genes in that ontology. The Benjamini *P* value for each ontology is shown. All significant ontologies are listed Table S3.

2.4.2 Female adipose EC show a highly proliferative phenotype compared to male counterparts

Angiogenesis requires the transient acquisition of the specialized phenotypes of proliferating stalk and migratory tip cells (Geudens & Gerhardt, 2011; Potente & Carmeliet, 2017), which is supported by metabolic reprogramming (De Bock, Georgiadou, Schoors, et al., 2013; Potente & Carmeliet, 2017). Thus, we assessed within our RNA-Seq dataset the molecular profiles involved in supporting distinct cellular phenotypes and metabolic pathways. First, we conducted GSEA using gene sets comprising markers for proliferative and tip endothelial phenotypes (**Table S2.5**), hypothesizing that both would be enriched in female EC. Indeed, female EC displayed enriched expression of genes associated with proliferation (**Figure 2.2 A,B**). However, enrichment on tip cell genes was detected in male EC (**Figure 2.2 A,B**). Despite the phenotype differences, the transcript levels of genes involved in glycolysis, pentose phosphate pathway, tricarboxylic acid cycle, or glutamine metabolism did not differ between male and female EC (Figure S2A). *In vitro* analysis of cultured male and female EC also did not display sex-differences in protein levels of glycolytic markers hexokinase 2 (HK2), 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (**Figure S2.2 B-D**). In contrast, we observed a substantial enrichment of genes associated with oxidative phosphorylation in female EC (**Figure 2.2 A,B**). This is notable because mitochondrial respiration was reported to be critical to sustain EC proliferation.

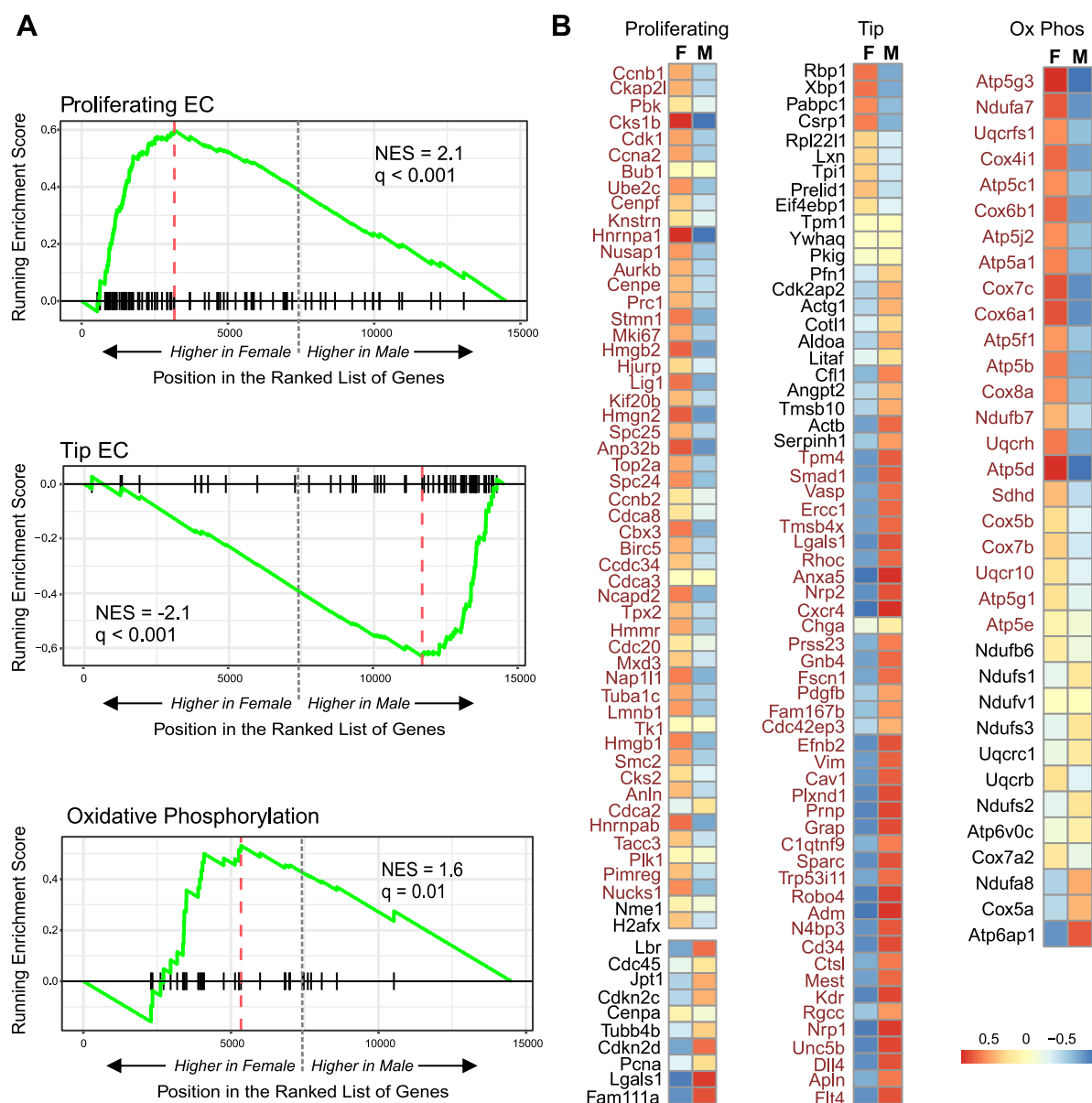


Figure 2.2 Female adipose EC exhibit elevated markers of proliferation and oxidative phosphorylation compared to male EC. A-B) GSEA of endothelial cell proliferating, tip and oxidative phosphorylation genes with corresponding heatmaps. A) GSEA plots of genes representing proliferating EC, mouse tip cell markers; and oxidative phosphorylation machinery. B) Heatmaps corresponding to the genes found in the GSEA plots in (A), displaying average z-scores for each sex, calculated from the gene TPM for each sample. Leading edge genes are indicated in red font. Full list in Table S5. See also Figure S2.2.

To substantiate the greater proliferative potential of female EC under HFD conditions, we used a third cohort of mice that underwent a similar protocol of HF feeding for 7 weeks. Employing the nucleotide analogue EdU to mark cells in S-phase during the last week of the dietary intervention, coupled with ERG staining to identify EC nuclei, we detected proliferating EC in the visceral adipose of both sexes (**Figure 2.3A**). However, a significantly higher proportion of EC were EdU-positive in the white adipose of female mice, corroborating the gene expression analyses (**Figure 2.3B**). Of note, we did not detect differences in mRNA levels of key angiogenic factors *Vegfa* or *Vegfb* (Robciuc et al., 2016; Sung et al., 2013) between male and female white adipose tissue either in the cohort of mice used for assessment of EdU incorporation (**Figure 2.3 C,D**) or in those used for EC RNA-Seq analysis (**Figure S2.3A**). Transcript levels of leptin, which also exerts pro-angiogenic effects (Sierra-Honigmann et al., 1998), were higher in the adipose of males compared to females (**Figure S2.3B**). This suggests that the augmented proliferation of female EC may be independent of differential levels of angiogenic factors present in the microenvironment. *In vitro* studies employing primary cultures of EC (isolated from the adipose tissue of matched sets of young, healthy male and female mice) also demonstrated a greater proliferative capacity of female EC as detected by EdU incorporation (**Figure 2.3E**). Altogether, these experiments reinforce the idea that sex differences in EC proliferation may reflect cell-autonomous differences rather than distinctions in the adipose microenvironment.

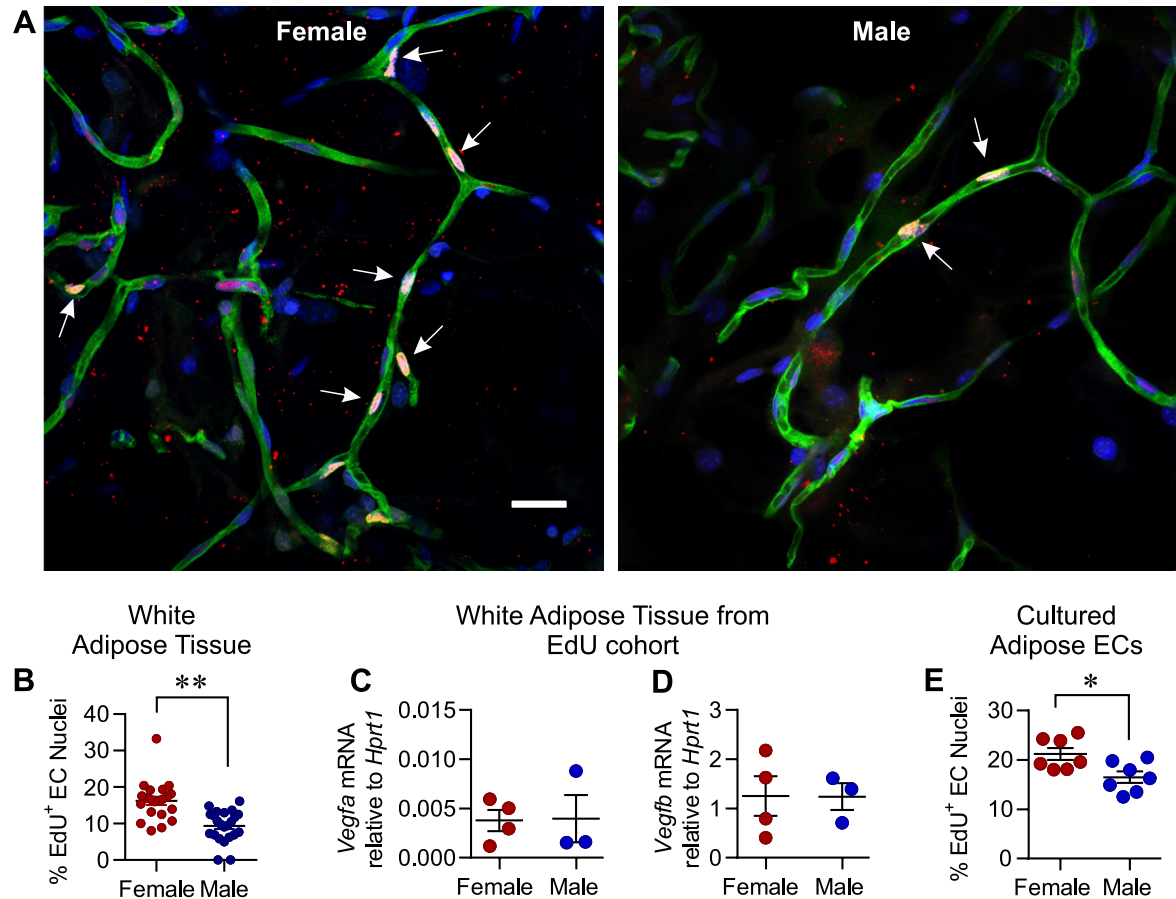


Figure 2.3 Female adipose EC exhibit elevated proliferation rates compared to male EC. A-B) Female and male mice were fed a HFD for 7 weeks. EdU was injected two sequential days prior to tissue extraction. **A)** Whole-mount confocal imaging of white visceral adipose tissue to detect proliferating EC. Green (Griffonia simplicifolia-FITC), capillaries; Blue (DAPI), nuclei; Red (ERG), Endothelial cell nuclei; Yellow (EdU), proliferating cells. Arrows point to EdU-positive endothelial cell nuclei. Scale bar=25 μ m. **B)** Quantification of EdU-positive EC (ERG+) relative to total number of EC per field of view. Data points represent individual fields of view from 4 female and 3 male mice. Bars represent mean $\pm$ SEM. $**P=0.0037$, two-tailed unpaired Student's t-test. **C-D)** RNA was extracted from visceral white adipose tissue of the EdU cohort of female and male mice (n=4 and 3 respectively). Expression levels of *Vegfa* (**C**) and *Vegfb* (**D**) relative to *Hprt1* were quantified by qPCR. Also refer to Figure S2.3. **E)** Adipose EC from young, healthy female and male mice were isolated and cultured under growth conditions. Proliferation was assessed by EdU incorporation. EdU-positive cells were quantified as a percentage of total EC number. N=7 independent experiments/sex; $*P=0.015$, two-tailed unpaired Student's t-test.

2.4.3 Sex-biased inflammatory state under obesogenic conditions may reflect distinct sensitivity of male and female adipose EC to an inflammatory stimulus

Further characterization was also conducted on the predominant transcriptional phenotype detected in adipose male EC under HFD conditions. In association with their pro-inflammatory profile, male EC displayed significant enrichment of genes belonging to inflammasome and inflammasome assembly gene ontologies (**Figure 2.4A and Figure S2.4**). Considering that the pronounced pro-inflammatory profile of adipose EC from male HF-fed mice can be an indicator of cellular senescence (Spinelli et al., 2020; Tchkonina et al., 2010), we assessed if hallmark genes associated with senescent cellular behavior were enriched in male versus female EC. Notably, we found that senescence associated secretory phenotype (SASP)-related genes were enriched in the male adipose EC (**Figure 2.4A and Table S2.5**).

We considered that the greater pro-inflammatory state of the male EC could result from differences in the local adipose microenvironment. However, we did not detect sex differences in the mRNA levels of key inflammatory cytokines *Tnfa* and *Il1b* within the white adipose samples that were used for the EC RNA-Seq analysis (**Figure S2.5**). This suggests that the greater pro-inflammatory state of male EC may not be triggered by the differential levels of these cytokines in the white adipose microenvironment. We sought further insight by assessing if sex differences in genes associated with inflammatory responsiveness remained detectable in primary cultures of adipose EC following TNF α stimulation. We examined the transcripts of multiple inflammation or immune-related genes detected as DEGs in our dataset that have also been previously reported to be expressed in mouse microvascular ECs from multiple organs (Jambusaria et al., 2020). Multiple acute-response inflammatory genes displayed a sex bias in responsiveness to TNF α . *Icam1* mRNA increased in both sexes with TNF α exposure, but this effect was significantly higher

in male versus female EC (interaction between sex and treatment) (**Figure 2.4B**). This concurs with a report that ICAM1 expression and leukocyte adherence were lower in mesenteric microvessels of females compared to male mice (Moss et al., 2019). Transcript levels of the cytokines *Ccl6* and *Tnf* also displayed an interaction between sex and treatment, increasing in male but not female EC in response to TNF α (**Figure 2.4C**; **Figure S2.6**). Multiple immune-related genes displayed a significantly greater expression in the male compared to female EC, but no responsiveness to TNF α treatment. This pattern was observed for *Tyrobp*, an immune-modulatory adaptor protein, the transcriptional regulator *Spi1* (**Figure 2.4 B,C**) as well as inflammatory markers *Cd68*, *Ptpnc* and *Itgax* (**Figure S2.6**). Together, these data indicate that male adipose ECs display a slightly pro-inflammatory profile under basal conditions and they have greater sensitivity to an inflammatory stimulus.

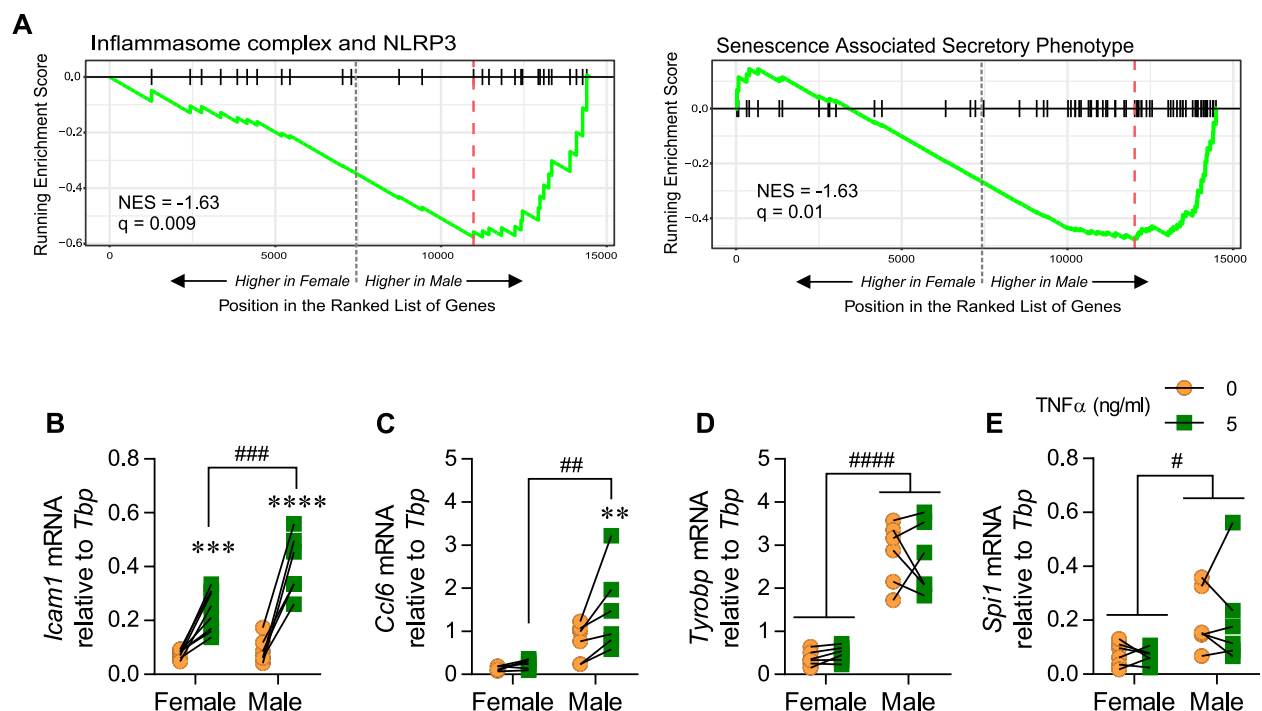


Figure 2.4 Male adipose EC display an inflammatory profile *in vivo* and *in vitro*. **A)** GSEA plots of genes represented in the Inflammasome Complex (GO:0061702) and NLRP3 Inflammasome Complex Assembly (GO:0044546) gene ontologies and SASP-related genes. Corresponding heatmaps in Figure S2.4. See also Figure S2.5. **B-E)** TNF α treatment (5 ng/mL; 20 hours) of cultured adipose EC from female and male young, healthy mice. mRNA levels of target genes were assessed by qPCR and expressed relative to housekeeping gene *Tbp*. Data were assessed by 2-way paired ANOVA (Sex x Treatment) with Sidak's post-hoc comparisons; n=6 independent experiments for female and male EC. **(B)** *Icam1* mRNA *** $P=0.0006$ vs. 0 ng/mL; **** $P<0.0001$ vs. 0 ng/mL; ### Interaction Male vs. Female, 5 ng/mL, $P=0.0003$. **(C)** *Ccl6* mRNA ** $P=0.0071$ vs. 0 ng/mL; ## Interaction Male vs. Female, 5 ng/mL, $P=0.0014$. **(D)** *Tyrob* mRNA ##### $P<0.0001$ Male vs. Female, for both 0 and 5 ng/mL. **(E)** *Spi1* mRNA #Main effect of sex, $P=0.045$ Male vs. Female. Analyses of additional genes in Figure S2.6.

2.4.4 Transcriptional regulators involved in the proliferative and pro-inflammatory profiles of EC

Having established the distinct cellular phenotypes of male and female adipose EC, we considered putative transcription factors that might predict the sex-dimorphic endothelial profiles under HFD feeding. The analysis identified several enriched motifs (NES>3) associated with transcription factors and revealed *Zbtb14*; *E2f6/E2f1*; *Sox4* and *Foxo1/3* as the top predicted regulators of the DEGs associated with ontologies ‘chromatin/transcription’ in female EC. Of these transcription factors, gene expression of *Sox4*, *Foxo1* and *Foxo3* mRNA were significantly higher in EC from female HF-fed mice (-1.83, -0.63 and -0.77 logFC, respectively; **Figure 2.5A**). Interestingly, estrogen receptor binding sites were not identified amongst the top enriched transcription factor motifs for these transcripts. This corroborates a previous report that estrogen signaling accounts for only a portion of sex differences in gene expression (Lopes-Ramos et al., 2020).

Conversely, putative transcriptional regulators that may regulate the male DEGs belonging to “immune response/inflammation” related gene ontologies included *Spi1*; *Gata2*; *Fli1*; *Nfkb1* (**Figure 2.5B**). Of these transcription factors, *Spi1* and *Gata2* mRNA were significantly higher in the EC from male HF-fed mice (1.58 and 0.9 logFC, respectively, vs. female EC). *Spi1* and *Gata2* were recently described as putative regulators of an immune response program in EC (Krausgruber et al., 2020). Notably, *Spi1*, which has been associated with cellular senescence (Delestré et al.,

2017), was elevated in male compared to female EC from the validation cohort of mice (**Table S2.4**) and in cultured cells (**Figure 2.4E**).

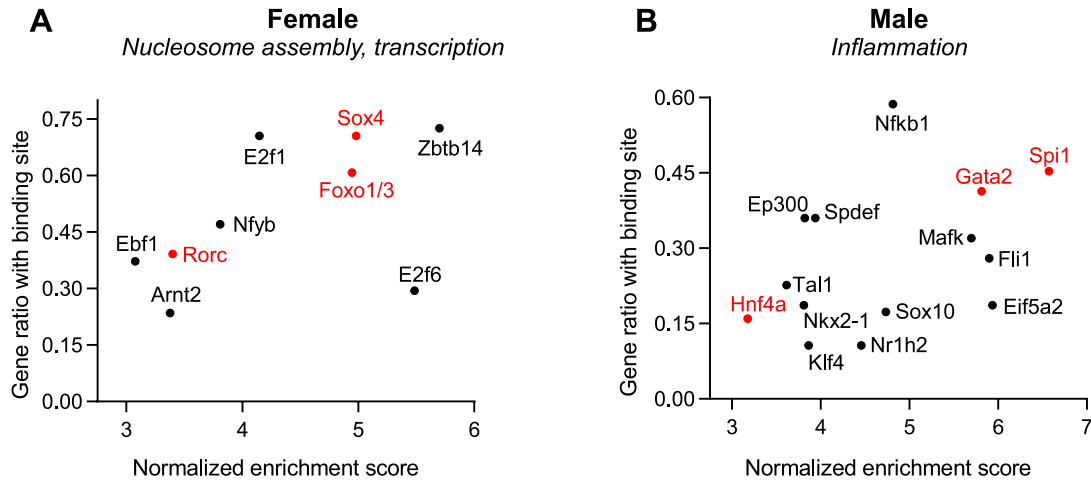


Figure 2.5 Predicted transcription factors associated with dominant female and male enriched ontologies. iRegulon was used to predict putative transcriptional regulators of female DEGs related to nucleosome assembly and positive regulation of transcription and male DEGs related to immune system process, inflammatory response, phagocytosis, neutrophil chemotaxis, and antigen processing and presentation. Scatter plots of resultant analyses are shown for female (**A**) and male (**B**), in which the X-axis indicates the normalized enrichment score (NES) for a given transcription factor and the Y-axis shows the ratio of the genes associated with that factor relative to all inputted genes. Red font indicates transcription factors that were also significantly upregulated in that sex.

2.4.5 The sex differences observed in adipose EC under HFD conditions are also detectable in aged EC

We sought to corroborate and extend the sex-biased transcriptional profiles revealed in our analysis of EC from HF-fed mice by comparing to an independent transcriptional profile of EC in a different pathophysiological context. Considering the SASP phenotype observed in the male EC, we chose to compare our findings to the transcriptomic signature of EC from several organs (including fat) from aged male and female mice, which was generated utilizing Tabula Muris senis single cell sequencing data from 18-month-old mice. Overall, males and females exhibit differential patterns of aging (Hägg & Jylhävä, 2021). Although it is well-documented that

microvascular disease and EC dysfunction increase substantially with aging (Donato et al., 2018), sex differences in aging EC and the impact on capillary content remain poorly characterized. We used GSEA to first identify GO BPs associated with the sex-biased gene sets in each dataset independently. The male-biased BPs (220 and 141 in the HFD and aged EC datasets, respectively) were dominantly clustered in ontologies related to ‘Immune regulation,’ ‘migration/chemotaxis’ and ‘Interleukin production’ (**Table S2.6**). There were 35 identical ontologies displaying male-biased expression in both datasets. Selected ontologies (and their core enrichment genes) that were common between the HFD and aged EC datasets are displayed in **Figure 2.6A**. Conversely, 73 and 40 BPs were female-biased in HFD and aged EC datasets, respectively. Of these, the two major clusters shared between both datasets were associated with DNA organization/ conformation and chromatin remodeling (**Figure 2.6B**).

We further tested if the transcriptomes of young healthy EC from 3 month-old mice (X. Huang et al., 2021; Schaum et al., 2020) display similar sex-biases as seen in the EC from HFD-fed mice. However, biological pathways enriched in DEG higher in young male (vs female) EC clustered substantially with ‘vasculogenesis’ rather than inflammation (**Table S2.6**), consistent with a healthy EC phenotype. Notably, EC from young female mice were enriched in ontologies related to ATP synthesis (oxidative phosphorylation) and ribosomal processes, which more closely paralleled the gene sets enriched in EC of HFD-fed female mice. In sum, these comparisons suggest that HFD and aging evoke similar detrimental effects on male EC whereas EC from female mice appear to resist these deleterious alterations.

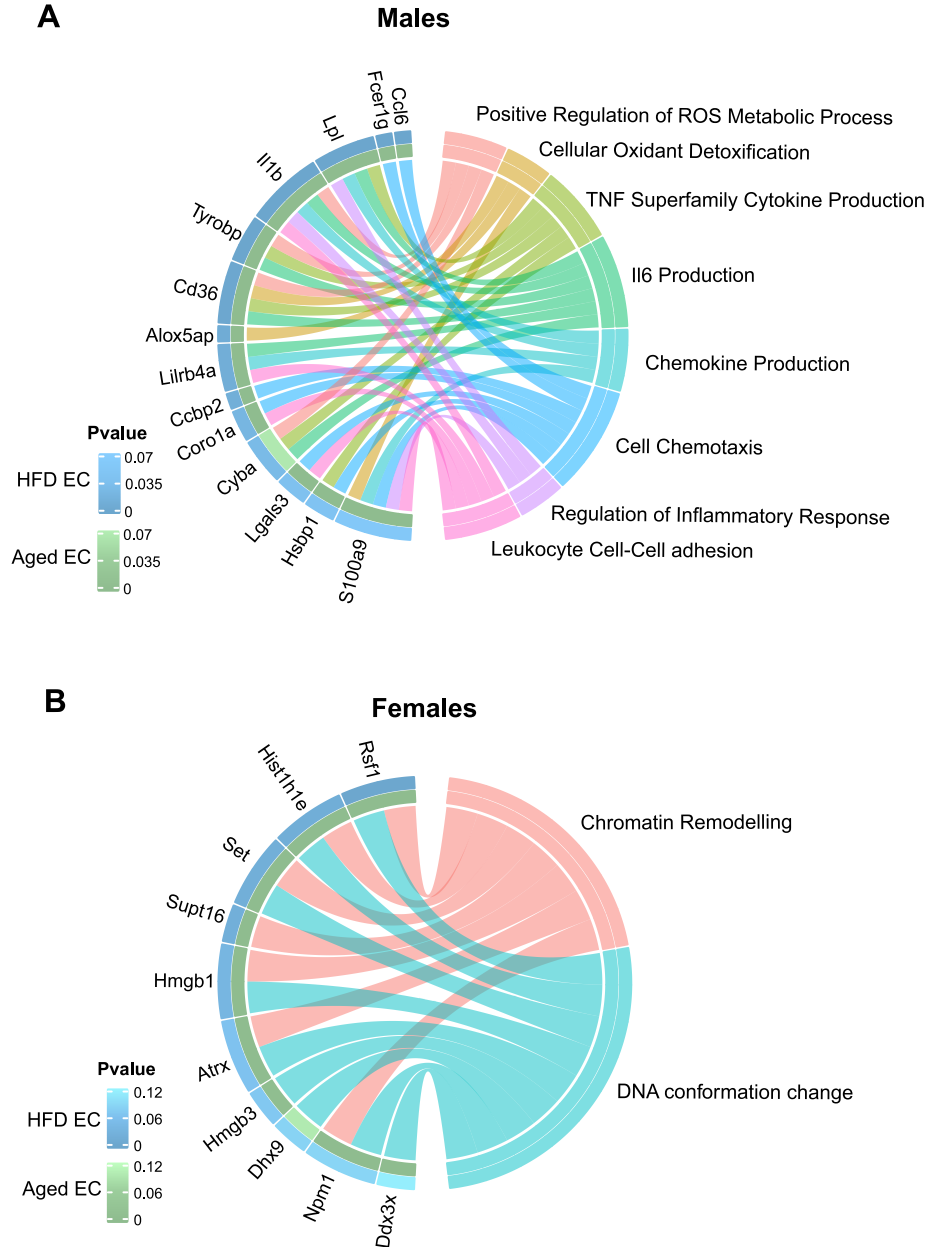


Figure 2.6 Sex-biased Biological Process ontologies and constituent genes in common between EC from HFD-fed and aged mice. GSEA was used to identify sex-biased GO BPs enriched in EC from both HFD-fed and aged mice. The full analysis is in Table S6. Selected genes belonging to the core enrichment of BPs higher in male EC (**A**) and in female EC (**B**) are illustrated by chord plot. For individual genes, the outer ring (blue) represents the adjusted P value of that gene in the HFD RNA-seq dataset and inner ring (green) represents log FC of the gene in the aged mouse dataset (Huang et al, 2021).

2.5 Discussion

Herein, we revealed distinct molecular signatures in male and female EC from white adipose tissue of mice upon 7 weeks of HFD feeding (**Figure 2.7**). Female adipose EC exhibit a transcriptomic signature associated with a predominantly proliferative state, which manifests as a greater proliferative capacity when compared to male EC. Conversely, EC from male counterparts show enrichment in gene sets involved in a pro-inflammatory phenotype, which seems to be triggered in response to the diet but also affected by differential sensitivity to inflammatory stimuli when compared to female EC. These findings suggest that molecular disparities in EC account, at least in part, for the sex-biased adipose tissue angiogenesis under obesogenic conditions.

The enrichment in proliferation-related genes and elevated EdU incorporation in female EC compared to male counterparts demonstrate that female EC retain the capacity to undergo angiogenesis and expand the local vasculature to meet physiological tissue demands even after several weeks of HFD. This is coherent with the prior observation that adipose vascularization is greater in female compared to male mice after 16 weeks of HFD (Rudnicki, et al., 2018b). In the current study, the similar adipose tissue levels of growth factors (i.e. VEGFA, VEGFB) in both sexes and the proliferative advantage detected in cultured female EC imply that this is a cell-autonomous phenotype. Enrichment of transcripts associated with chromatin modifiers was observed in female adipose EC, suggesting that epigenetic regulatory mechanisms maintain the increased proliferation rate in female EC. These findings fit with a growing body of data documenting a higher proliferation of HUVEC and pulmonary artery EC isolated from female compared to male donors (Addis et al., 2014; Qin et al., 2020). The contrasting ontologies representing both positive and negative regulation of angiogenesis enriched in the male adipose EC suggests that inhibition of angiogenic signaling in males may lower the proliferative potential

of male EC under HFD conditions. The elevated levels of typical ‘tip cell’ marker genes in the male EC appear to concur with this outcome, as proliferation is repressed in tip cells (Gerhardt et al., 2003). Interestingly, elevated proliferative activity EC was reported in male mice fed a HFD for just two weeks (but not after five or fifteen weeks) (Voros et al., 2005), indicating that HFD may elicit an initial angiogenic response in adipose male EC but that sustained HFD suppresses their proliferative capacity.

Previous studies have established the paradigm that sustained HFD dysregulates critical EC cellular signaling pathways, inducing oxidative stress, inflammation and senescence (Aoqui et al., 2014; García-Prieto et al., 2015; Molnar et al., 2005; Sárvári et al., 2021; C.-Y. Wang et al., 2009). The overt inflammatory signature prevalent in the male adipose EC in our study provides evidence of EC dysfunction (Pober & Sessa, 2007), agreeing with this established paradigm. The development of inflammation may underlie the enrichment of negative regulators of angiogenesis and the senescence-associated signature (SASP) displayed by the male adipose EC. In turn, these molecular signatures likely contribute to the poor angiogenic response observed in the adipose of male mice during long-term HFD. Of note, SASP is a hallmark of aged or damaged cells that can exert deleterious effects on surrounding cells (Rodier et al., 2009) and may contribute to the sex-biased development of adipose tissue dysfunction with obesity. Considering that higher levels of inflammatory cytokines similar to ones observed in our work were recently reported in male EC from obese compared to lean mice following 18 weeks of HFD, it is probable that the inflammatory profile of male EC in our study is largely induced by the diet (Sárvári et al., 2021). The pro-inflammatory signature in the transcriptome of adipose EC from male HFD-fed mice also was in common with those detected in EC from aged male mice, which concurs with reports that EC

dysfunction and inflammation generally manifests at an earlier age in males than females (Celermajer et al., 1994; Cole et al., 2021).

Our findings that female EC did not develop the same extent of inflammation as seen in the male adipose EC, and instead retained proliferative capacity, suggests a higher resistance of female EC to the stress associated with prolonged HFD. This corroborates several reports showing that cultured female EC exhibited higher viability than male EC when challenged with serum starvation, hyperoxia, shear stress, or oxidative stress (Lorenz et al., 2015, 2019). One plausible contributor to their stress tolerance is the enrichment in genes involved in the electron transport chain in female compared to male adipose EC. This sex-biased pattern of gene expression, also detected in the EC from healthy young mice, has been reported across numerous cell types and species (Chella Krishnan et al., 2021; Lopes-Ramos et al., 2020; Norheim et al., 2019). Indeed, higher oxidative phosphorylation gene content is linked with greater stress resistance and longevity (Kristensen et al., 2019; Magalhaes-Novais et al., 2022) whereas the lower mitochondrial content in the adipose of males has been associated with a greater risk of developing metabolic dysfunction (Norheim et al., 2019).

There is growing awareness that EC express immune-related transcripts and can play tissue-specific immune-modulatory roles (Amersfoort et al., 2022). In addition to the diet effect on inflammatory genes, we found that male adipose EC in culture exhibited higher basal levels of some immune response-related genes than female EC and greater responsiveness to TNF α -stimulated conditions. This suggests a sustained distinction between male and female EC that is not dependent on immediate cues from the environment, which is in line with recent work demonstrating that epigenetic encoding of chromatin accessibility plays a critical role in establishing immune response potential (Krausgruber et al., 2020). Therefore, it is likely that the

EC dysfunction of male adipose ECs results not only from lower resistance to stress but also because of a greater inherent responsiveness to an inflammatory stimulus, which may divert these cells from an angiogenic response under obesogenic conditions. We propose that epigenetic mechanisms support the underlying sex differences in EC phenotype of both male and female EC and that these sex differences are amplified by the obesogenic conditions triggered by HFD.

Overall, the distinctive molecular signatures of male and female EC under HFD conditions provide strong support for the concept that sex differences in EC behavior and angiogenesis contribute to the well-described sex-disparities in the development of obesity and its related complications. This indicates that the male overrepresentation in prior studies has generated an incomplete understanding of the factors impacting vascular remodeling during adipose tissue expansion. Our results suggest that female EC have a better capacity to cope with the stress imposed by HFD. These phenotypic differences may also apply to other conditions such as aging. Thus, this study opens new perspectives on the influence of sex in the pathophysiology of EC in obesity and emphasizes the importance of considering sex as a significant variable in the regulation of vascular remodeling and the contribution of EC in disease states.

2.6 Limitations of the Study

This study was designed to understand molecular mechanisms underlying the greater adipose angiogenesis observed in HFD-fed female versus male mice rather than to study how HFD-feeding alters the transcriptome of male and female adipose EC. Comparison with publicly available datasets from standard diet-fed mice combined with the gene expression analysis of cultured EC allowed us to infer that the extent of inflammation in the male EC was in part induced by HFD. However, future studies should extend this observation by directly assessing the impact of diet on the EC molecular signatures in both sexes. The relative contributions of the gonadal

hormones and genes localized to the Y chromosome to the observed sex-biased EC phenotypes remain undefined, requiring further investigation. Our study relied on tissue dissociation and isolation of EC using a magnetic bead-based enrichment protocol. While *in vitro* approaches employed in our study alongside the comparison of other publicly available EC databases provide support that the major molecular signatures presented in this paper reflect those of EC, we cannot completely exclude the possibility of low-level contamination of the RNA by other cell types as well as the loss of EC during isolation. Future studies combining spatially resolved transcriptional profiling with single-cell RNA sequencing can provide greater insight into distinct EC subpopulations that might be altered by sex or by diet. Exploration of these EC sex differences within human adipose will be important in advancing the health-related implications of our study.

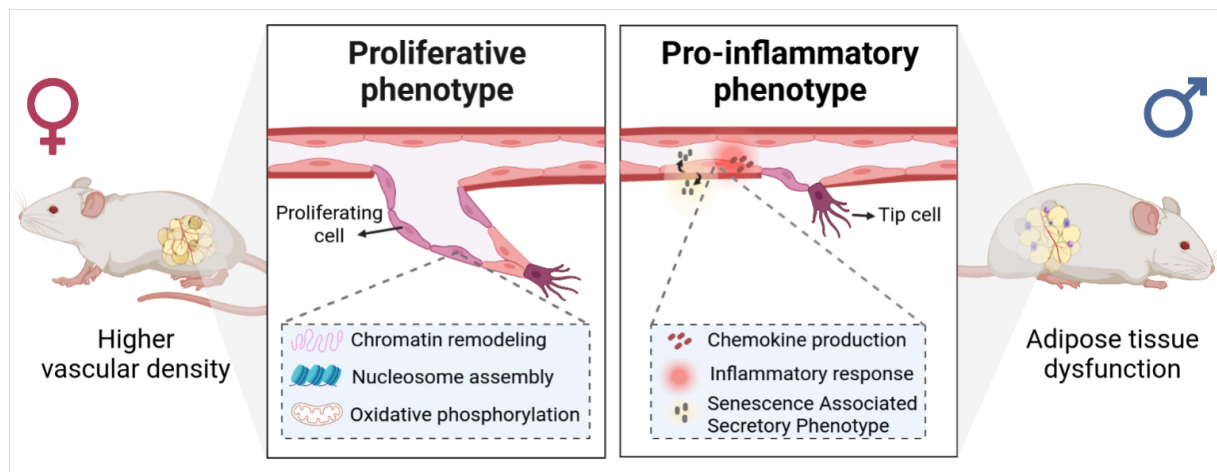


Figure 2.7 Summary Schematic Visual representation of key sex-biased BPs identified by the analysis of genes that were differentially expressed in female versus male adipose ECs under obesogenic conditions. These transcriptional signatures may underlie the divergent angiogenic capacities previously reported within white adipose tissue of male and female high fat diet-fed mice and sex disparities in pathophysiological responses to obesity. Figure was created in Biorender.

2.7 Supplementary

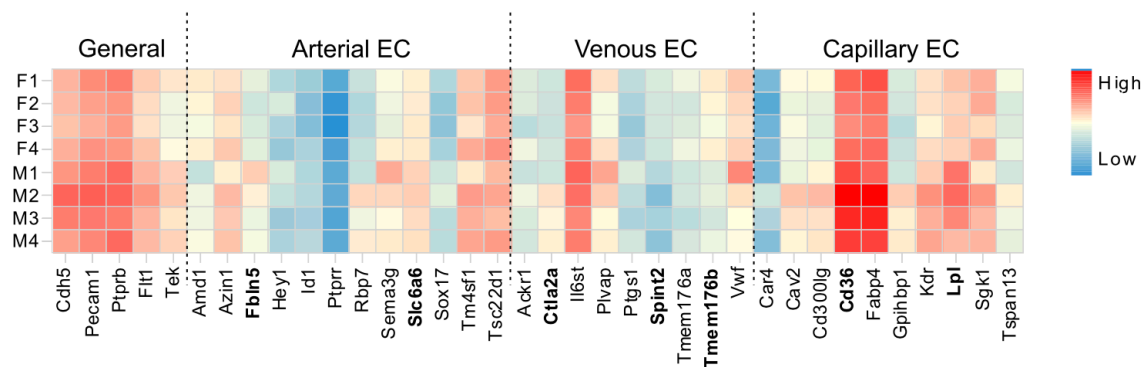


Figure S2.1 Relative levels of endothelial cell hallmark genes in female and male EC. Relative expression of general EC markers, as well as hallmark arterial, venous and capillary EC genes (logCPM of transcripts) within male and female adipose EC isolated from HF-fed mice (n=4/sex), as determined by RNA-seq.

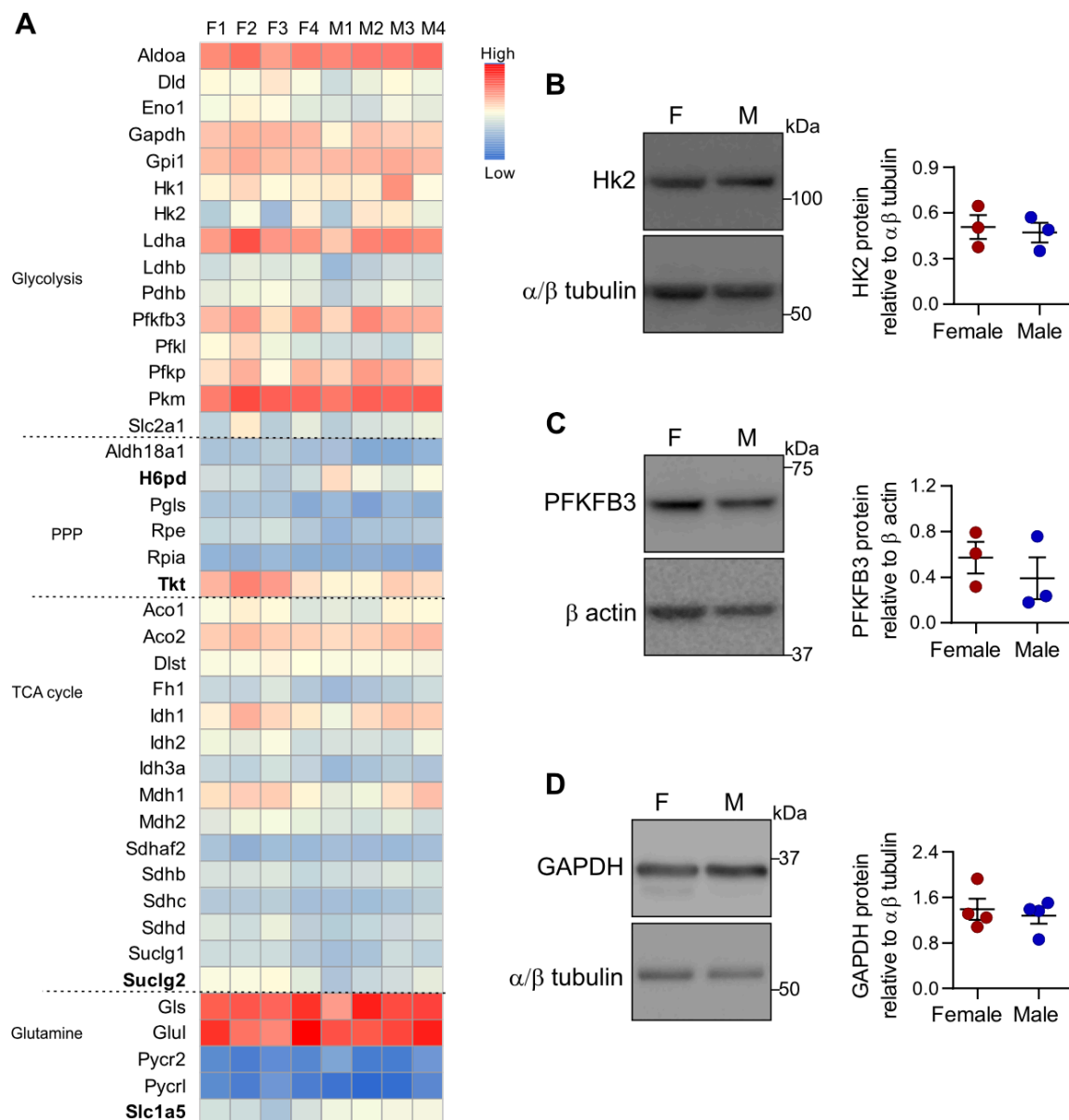


Figure S2.2 Metabolic pathway markers are similar in female and male EC. **A)** Heatmap of RNA-seq transcript levels (logCM) showing glycolysis, pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle and glutamine metabolism-related genes in male and female EC ($n = 4/\text{sex}$). **B-D)** Adipose EC from female and male young standard laboratory diet-fed mice were isolated and maintained in culture for up to 5 passages. Expression levels of glycolytic proteins were assessed by Western blotting. $n=3-4/\text{sex}$. **B)** Hexokinase 2 (Hk2) protein relative to α/β tubulin. **C)** 6-phosphoructo-2-kinase/ fructose-2,6-bisphosphatase 3 (PFKFB3), protein relative to β actin. **D)** Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) protein relative to α/β tubulin.

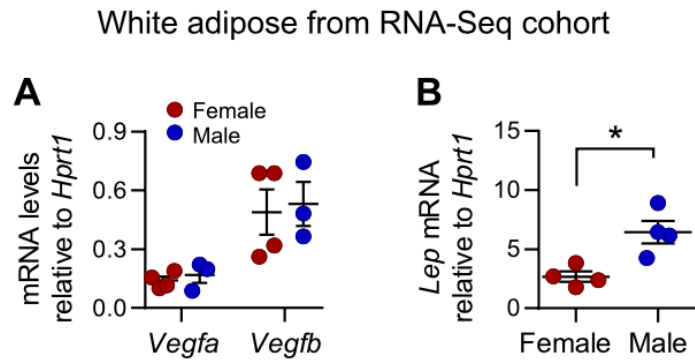


Figure S2.3 RNA levels of angiogenic factors in white adipose tissue. RNA was extracted from visceral white adipose tissue of the RNA-Seq cohort of female and male mice fed a HFD for 7 weeks. mRNA levels of **A)** *Vegfa* and *Vegfb* and **B)** *Leptin* were analyzed by qPCR and expressed relative to *Hprt1*. * $P < 0.05$, Mann Whitney U test; $n = 4$ per sex.

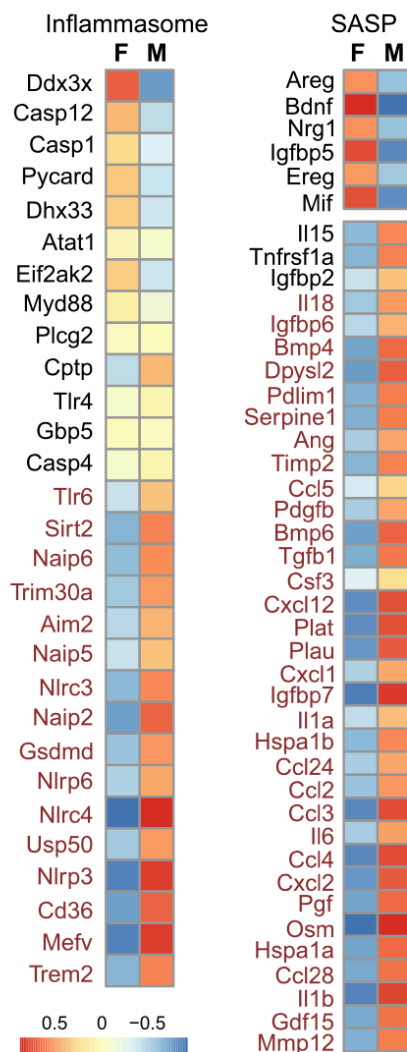


Figure S2.4. Inflammatory profile in male adipose EC from HFD-fed mice. Heatmaps that correspond with GSEA of inflammation-related ontologies shown in Fig. 2.4A, displaying average z-scores (calculated from the gene TPM for each sample) for genes represented in the Inflammasome Complex (GO:0061702) and NLRP3 Inflammasome Complex Assembly GO:0044546) gene ontologies and SASP-related genes. Leading edge genes are indicated in red font. Complete GSEA analysis in Suppl. Table 2.5.

White adipose from RNA-Seq cohort

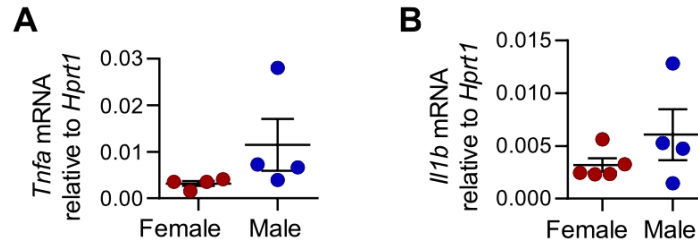


Figure S2.5 RNA levels of cytokines in white adipose tissue. RNA was extracted from visceral white adipose tissue of the RNA-Seq cohort of female and male mice fed a HFD for 7 weeks. mRNA levels of A) *Tnfa* and B) *Il1b* were analyzed by qPCR and expressed relative to *Hprt1*. n=4/sex.

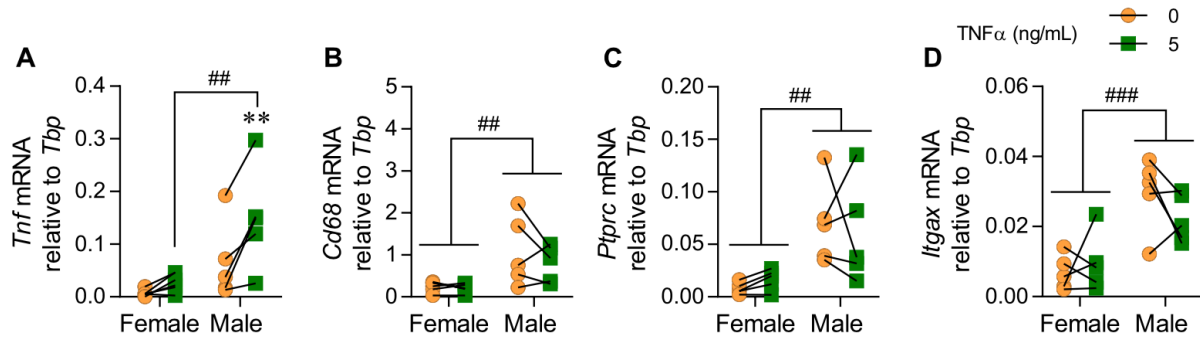


Figure S2.6 Male adipose EC display a greater immune-related profile *in vitro*. Cultured adipose EC from young healthy male and female mice were treated with $TNF\alpha$ (5 ng/mL) for 20 hours. mRNA levels of target genes were assessed by qPCR and expressed relative to housekeeping gene *Tbp*. Data were assessed by 2-way paired ANOVA (Sex x Treatment) with Sidak's post-hoc comparisons. A) *Tnf* mRNA. ** $P=0.0012$ vs. 0 ng/mL; ## $P=0.006$; Interaction Male vs. Female, 5 ng/mL, n=5-6/group. B) *Cd68* mRNA. C) *Ptprc* mRNA. D) *Itgax* mRNA. Main effect of sex, ## $P<0.01$; ### $P<0.001$; n=5-6 per group.

Table S2.1 Phenotypic features of high fat-fed mice from the RNA seq cohort, related Figures 2.1, 2.2

Parameter	Male (mean $\pm$ SEM) n=4	Female (mean $\pm$ SEM) n=4	P value (Mann Whitney U test)
Initial body weight (g)	33.8 $\pm$ 1.6	26.7 $\pm$ 2.5	0.06
Final body weight (g)	39.7 $\pm$ 2.3	29.7 $\pm$ 2.0	0.06
Weight gain (g)	5.9 $\pm$ 1.3	3.0 $\pm$ 0.9	0.2
4 hr fasting blood glucose (mM)	8.0 $\pm$ 0.6	7.3 $\pm$ 0.4	0.4

Table S2.2 GO BP analysis of differentially expressed genes in EC from HF-fed male and female mice, related to Figure 1

A downloadable excel file at the link below:

[Download: Download spreadsheet \(15KB\)](#)

Table S2.3 Phenotypic features of high fat-fed mice from the C57Bl6/J validation cohort, related to Table S2.4

Parameter	Male (mean $\pm$ SEM) n=6	Female (mean $\pm$ SEM) n=12	P value (Mann Whitney U test)
Initial body weight (g)	20.6 $\pm$ 0.41	18.1 $\pm$ 0.25	<0.0001
Final body weight (g)	31.5 $\pm$ 1.0	23.6 $\pm$ 0.5	0.0001
Weight gain (g)	10.8 $\pm$ 1.1	5.4 $\pm$ 0.47	0.0004
Blood glucose, 16 hr fasting (mM)	6.9 $\pm$ 0.41	5.3 $\pm$ 0.29	0.04
GTT (AUC at 180 min)	1346.2 $\pm$ 50.3	1077.0 $\pm$ 27.3	0.0001

GTT; glucose tolerance test

Table S2.4 Validation of gene expression profiles, related to Results, Figure 2.1

Gene	qPCR of EC from C57Bl6/J (target gene RNA relative to <i>Actb</i>)			RNA Seq	
	Female (x E-3 mean $\pm$ SEM)	Male (x E-3 mean $\pm$ SEM)	P-val (Mann Whitney)	Log FC (+ up in male)	Adj. P-val
<i>Ccl6</i>	1.59 $\pm$ 0.41; n=4	4.91 $\pm$ 0.75; n=6	<i>0.010</i>	1.704	<i>5.20E-07</i>
<i>Cd36</i>	4.59 $\pm$ 2.09; n=4	67.9 $\pm$ 21.4; n=6	<i>0.019</i>	1.367	<i>0.002</i>
<i>Cdh5</i>	18.7 $\pm$ 6.17; n=4	27.3 $\pm$ 13.0; n=5	<i>0.56</i>	0.988	<i>0.012</i>
<i>Foxo1</i>	4.77 $\pm$ 0.51; n=5	2.01 $\pm$ 0.26; n=6	<i>0.001</i>	-0.631	<i>0.005</i>
<i>Il1b</i>	4.11 $\pm$ 1.99; n=4	5.49 $\pm$ 3.33; n=6	<i>0.76</i>	2.087	<i>0.0004</i>
<i>Irs1</i>	9.26 $\pm$ 1.30; n=6	2.70 $\pm$ 0.51; n=5	<i>0.002</i>	-1.326	<i>0.0001</i>
<i>Kdr</i>	3.96 $\pm$ 0.88; n=5	5.47 $\pm$ 1.31; n=5	<i>0.36</i>	1.163	<i>0.029</i>
<i>Rbp1</i>	7.64 $\pm$ 1.62; n=4	9.62 $\pm$ 3.55; n=6	<i>0.61</i>	-1.804	<i>0.003</i>
<i>Robo2</i>	1.49 $\pm$ 0.41; n=4	0.32 $\pm$ 0.01; n=5	<i>0.032</i>	-2.669	<i>0.0002</i>
<i>Slc2a1</i>	4.03 $\pm$ 0.84; n=6	4.62 $\pm$ 0.57; n=6	<i>0.67</i>	-0.100	<i>0.91</i>
<i>Spi1</i>	0.15 $\pm$ 0.06; n=4	1.83 $\pm$ 0.42; n=6	<i>0.010</i>	1.585	<i>0.0003</i>
<i>Tgfb1</i>	3.34 $\pm$ 0.99; n=4	7.76 $\pm$ 0.80; n=5	<i>0.032</i>	0.824	<i>0.010</i>
<i>Tyrobp</i>	32.4 $\pm$ 11.3; n=5	138 $\pm$ 23.0; n=6	<i>0.004</i>	2.141	<i>0.0005</i>
<i>Vegfa</i>	2.66 $\pm$ 0.56; n=5	2.61 $\pm$ 0.46; n=5	<i>0.95</i>	-0.299	<i>0.37</i>

Bold font indicates genes with similar expression profile in each dataset

Table S2.5 Genes included in the custom GSEA lists, related to Figures 2, 4, Figure S2.4

Proliferating ECs			Tip EC		Ox-Phos	Inflammasome	SASP	
Ccnb1	Tuba1c	Ran	Rbp1	Fam167b	Atp5g3	Ddx3x	Areg	Igfbp2
Ckap2l	Lmnbl	Ube2s	Xbp1	Cdc42ep3	Ndufa7	Casp12	Bdnf	Il18
Pbk	Tkl	Slbp	Pabpc1	Efnb2	Uqcrfsl	Casp1	Nrg1	Igfbp6
Cks1b	Hmgb1	Usp1	Csrp1	Vim	Cox4i1	Pycard	Igfbp5	Bmp4
Cdk1	Smc2	Lbr	Rpl22l1	Cav1	Atp5c1	Dhx33	Ereg	Dpysl2
Ccna2	Cks2	Cdc45	Lxn	Plxnd1	Cox6b1	Atat1	Mif	Pdlim1
Bub1	Anln	Jpt1	Tpi1	Prnp	Atp5j2	Eif2ak2	Gsto1	Serp1
Ube2c	Cdca2	Cdkn2c	Preli1	Grap	Atp5a1	Myd88	Hmgb1	Ang
Cenpf	Hnrnpab	Cenpa	Eif4ebp1	C1qtnf9	Cox7c	Plcg2	Gapdh	Timp2
Knstrn	Tacc3	Tubb4b	Tpm1	Sparc	Cox6a1	Ctp	Hgf	Ccl5
Hnrnpa1	Plk1	Cdkn2d	Ywhaq	Trp53i11	Atp5f1	Tlr4	Il7	Pdgfb
Nusap1	Pimreg	Pcna	Pkig	Robo4	Atp5b	Gbp5	Tpd52l2	Bmp6
Aurkb	Nucks1	Lgals1	Pfn1	Adm	Cox8a	Casp4	Egfr	Tgfb1
Cenpe	Nme1	Fam111a	Cdk2ap2	N4bp3	Ndufb7	Tlr6	Pkm	Csf3
Prc1	H2afx		Actg1	Cd34	Uqcrh	Sirt2	Kitl	Cxcl12
Stmn1	Dlgap5		Cotl1	Ctsl	Atp5d	Naip6	Fgf2	Plat
Mki67	Rpa2		Aldoa	Mest	Sdhb	Trim30a	Serp2	Plau
Hmgb2	Ranbp1		Litaf	Kdr	Cox5b	Aim2	Iqgap1	Cxcl1
Hjurf	Rrm1		Cfl1	Rgcc	Cox7b	Naip5	Clic1	Igfbp7
Lig1	Lsm2		Angpt2	Nrp1	Uqcr10	Nlrc3	Cxcl16	Il1a
Kif20b	H2afz		Tmsb10	Unc5b	Atp5g1	Naip2	Ctnnb1	Hspa1b
Hmgn2	Nuf2		Actb	Dll4	Atp5e	Gsdmd	Axl	Ccl24
Spc25	Tuba1b		Serpinh1	Apln	Ndufb6	Nlrp6	Mmp14	Ccl2
Anp32b	Tyms		Tpm4	Flt4	Ndufs1	Nlrc4	Tnfrsf18	Ccl3
Top2a	Tubb5		Smad1		Ndufv1	Usp50	Angpt2	Il6
Spc24	Racgap1		Vasp		Ndufs3	Nlrp3	Adipoq	Ccl4
Ccnb2	Dek		Erccl		Uqerc1	Cd36	Icam1	Cxcl2
Cdca8	Gmnn		Tmsb4x		Uqerb	Mefv	Igfbp3	Pgf
Cbx3	Kif23		Lgals1		Ndufs2	Trem2	Plec	Osm
Birc5	Smc4		Rhoc		Atp6v0c		Myl12b	Hspa1a
Ccdc34	Dnajc9		Anxa5		Cox7a2		Cxcl10	Ccl28
Cdca3	Tmpo		Nrp2		Ndufa8		Il1rl1	Il1b
Ncapd2	Rfc2		Cxcr4		Cox5a		Actb	Gdf15
Tpx2	Hint1		Chga		Atp6ap1		Tln1	Mmp12
Hmmr	Cenpw		Prss23				Igfbp4	
Cdc20	Rrm2		Gnb4				Csf1	
Mxd3	Tipin		Fscn1				Il15	
Nap1l1	Tubb6		Pdgfb				Tnfrsf1a	

Table S2.6 Gene set enrichment analysis comparing HF-fed male and female EC with aged male and female EC, related to Figure 2.6

A downloadable excel file at the link below:

[Download: Download spreadsheet \(318KB\)](#)

Table S2.7 TaqMan® primer sets, related to Figures 2.3, 2.4, S2.3, S2.5, S2.6

Gene	Probe ID (ThermoFisher Scientific)
<i>Actb</i>	Mm04394036_g1
<i>Ccl6</i>	Mm00436446_g1
<i>Cd36</i>	Mm00432398_m1
<i>Cd68</i>	Mm00839636_g1
<i>Cdh5</i>	Mm00486938_m1
<i>Foxo1</i>	Mm00490671_m1
<i>Hprt1</i>	Mm00446968_m1
<i>Icam1</i>	Mm00516023_m1
<i>Il1b</i>	Mm00434228_m1
<i>Itgax</i>	Mm00498701_m1
<i>Irs1</i>	Mm01278327_m1
<i>Kdr</i>	Mm01222421_m1
<i>Leptin</i>	Mm00434759_m1
<i>Ptprc</i>	Mm01293577_m1
<i>Rbp1</i>	Mm00441119_m1
<i>Robo2</i>	Mm00620713_m1
<i>Slc2a1</i>	Mm00441480_m1
<i>Spil</i>	Mm01270605_g1
<i>Tbp</i>	Mm00446973_m1
<i>Tnfa</i>	Mm00443258_m1
<i>Tgfb</i>	Mm00450205_m1
<i>Tyrbp</i>	Mm00449152_m1
<i>Vegfa</i>	Mm00437306_m1
<i>Vegfb</i>	Mm00442102_m1

2.8 Acknowledgments

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Chapter 3: The influences of gonadectomy and high fat diet on adipose vascularization and endothelial cell metabolism

Alexandra Pislaru, Christian Martone, Elizabeth Garland, Gisang Lee, Mikayla Hatton, Mark Danesh, Tara L. Haas.

3.1 Contributions

I was involved in the conceptualization of the study and coordinated the administration of diets and weighing of mice weekly. I also participated in the *in vivo* metabolic testing (glucose and insulin tolerance tests). I performed the tissue extraction and endothelial cell purifications. The initial study involved 32 mice on a 15-week diet regiment. CM and EG helped with the feeding, weighing and metabolic assessments, and isolated muscle endothelial cells for analysis not shown here. MD assisted with the metabolic assessments (and collected muscle for parallel analyses). I immunostained and imaged the adipose tissue of the mice and quantified the vascular area. GL assisted with the EC proliferation analysis by quantification of the whole-mount images taken by me. GL also immunostained and imaged adipose paraffin-sections for quantification of capillary to adipocyte ratios. MH quantified the adipocyte size and performed VEGFA isoform measurements using cDNA isolated by me. I extracted RNA from the adipose tissue and performed the qPCR analysis. I performed all the *in vitro* metabolic assessments (Seahorse) and drafted the manuscript. TH conceptualized the study and assisted with *in vivo* metabolic assessments and tissue extraction and edited the manuscript.

3.2 Introduction

Obesity, defined by an excess of adipose accumulation, is a contributor to cardiovascular disease (CVD) (Fruh, 2017). The adipose tissue has a paramount role in fat storage to prevent lipotoxicity in other tissues and whole-body metabolic disturbances, and healthy expansion can be achieved even in the context of obesity (Ghaben & Scherer, 2019; Goossens & Blaak, 2015). Adipose tissue vascularization is necessary for healthy adipose expansion, allowing for hyperplastic adipose growth and prevention of CVD (AlZaim et al., 2023; Corvera & Gealekman, 2014). During this adipose remodelling, the increase of the capillary network is achieved through the crosstalk of the adipocytes with endothelial cells (ECs), which line capillaries and facilitate nutrient and oxygen transport to the adipose tissue (Andrade et al., 2021; Potente & Carmeliet, 2017). Insufficient angiogenesis during development of obesity can lead to pathological adipose expansion and obesity progression that is accompanied by immune cell infiltration and hypoxia (Goossens, 2017; Lemoine et al., 2013). Thus, adipose vascularization serves as a reliable indicator of adipose tissue health, reflecting its overall capacity to maintain systemic energy balance.

Although females have lower probability of developing morbidities associated with obesity, they have a higher proportion of adipose tissue in the body and a higher risk of obesity (Fitzgerald et al., 2018; Gavin & Bessesen, 2020). The underlying causes of these sex differences are multifaceted and affected by various factors. Females have a higher distribution of subcutaneous adipose relative to visceral adipose compared to males, which is associated with improved metabolic health due to smaller adipocytes, healthier adipose environment and higher insulin sensitivity (Chang et al., 2018; Dhokte & Czaja, 2024; Villaret et al., 2010). Within the same depot, females also have healthier expansion of adipose tissue, demonstrated by higher angiogenesis in parallel to adipose remodelling during HF-feeding accompanied by preserved

metabolic function (Rudnicki, et al., 2018b). To understand underlying mechanisms of the higher female adipose angiogenesis, our recent paper investigated sex differences of adipose ECs. Indeed, our recent report describes sexual dimorphism in adipose ECs, where female ECs have a healthier phenotype with higher proliferative capacity and reduced inflammation compared to those of males (Rudnicki et al., 2023). Although these sex differences were described in culture in the absence of sex hormones, we postulated that sex hormones may have a lasting impact on endothelial phenotype that contribute to sex differences.

Sex chromosomes and sex hormones contribute to sex differences in CVD (Spiering et al., 2025) and adipose distribution and health (X. Chen et al., 2013), however, far more research has been done on the effect positive effect of estrogen on effect on adipose function and metabolism. Multiple groups have shown that ovariectomized rodent models show an increase in fat mass and exhibit a more dysfunctional adipose expansion compared to controls, accompanied by higher serum triglyceride levels and larger adipocytes (Amengual-Cladera et al., 2012; Babaei et al., 2010; Ludgero-Correia et al., 2012; Nishio et al., 2019). Moreover, estrogen upregulates angiogenesis and EC proliferation (H. Liu et al., 2018; Seo et al., 2004; Sobrino et al., 2009) and has been shown to increase both EC mitochondrial function (Amengual-Cladera et al., 2012; Damacena de Angelis et al., 2022; Nadal-Casellas et al., 2011) and glycolytic activity (Boscaro, Carotti, et al., 2020; Trenti et al., 2017). Apart from this direct metabolic regulation, it can have a long-lasting impact through epigenetic regulation and miRNA changes (Pérez-Cremades et al., 2021; Y.-M. Yang et al., 2018). In contrast, far less research has been done on the effect of androgens on ECs and there is conflicting literature on the role of androgens in EC proliferation and health showing both negative effects on the vasculature (Costa et al., 2022) and positive effects (Cai et al., 2011).

Considering the protective role of estrogen on ECs and given our reported EC sex differences, we hypothesized that sex hormones may cause sustained changes in ECs that contribute to the sex differences in their phenotype and adipose angiogenesis. In this study we used a gonadectomy model to investigate if sex hormone loss impacts adipose angiogenesis and EC metabolism during both normal and high-fat diets, which ultimately contribute to the sex differences observed in the adipose tissue.

3.3 Methods

3.3.1 Mice

The mouse cohort consisted of 32 C57bl/6J mice (n= 16 per sex) (purchased from Jackson Labs). 8 females (F) and 8 males (M) underwent ovariectomy (Ovx) or castration (Cas), respectively, at 5 weeks of age (conducted by Jackson Labs). At seven weeks of age, mice were divided into normal chow (NC) or high fat diet (HFD) diet conditions, consisting of 10% kcal from fat for NC diet (D12450H Research diet) or 45% kcal fat HFD (D12451 Research diet), and remained on these diets for 15 weeks. This resulted in 8 condition groups (n=4/group): M NC, M HF, F NC, F HF, M NC Cas, M HF Cas, F NC Ovx, F HF Ovx. Body weights and food intake of mice were recorded weekly. Mice received 2 sequential EdU injections (i.p. 1.5 mg in 100 mL sterile PBS) 3 days and 1 day prior to tissue extraction. ITT and GTT (weeks 13, 14 respectively) were performed on the mice (**Figure. 3.1**). Tissue collections occurred at week 15. Animal studies were conducted in accordance with the American Physiological Society's guiding principles in the Care and Use of Animals, following protocols approved by the York University Committee on Animal Care.

3.4.2 *Insulin and glucose tolerance tests (ITT and GTT).*

For ITT, mice were fasted for 2 hr and then received an i.p. injection of insulin (0.5 U/kg BW). For GTT, mice were fasted for 6 hr and then received an i.p. injection of glucose (2 g/kg BW). Blood glucose was measured by glucometer at post-injection time-points 0, 15, 30, 45 and 60 min (ITT) or 0, 15, 30, 60 and 120 min (GTT). Area under the curve (AUC) was calculated for the GTT from the 0 to 120-minute timepoint. Area over the curve (AOC) was calculated for ITT from the 0 to 60-minute time point for the male mice and to the 45-minute end point for the female mice.

3.3.3 *Whole-mount and tissue section imaging of adipose tissue*

The whole-mount imaging of the adipose was performed as described in Chapter 2 (Rudnicki et al., 2023) and analyzed on ImageJ [as described in Chapter 2 and in (Rudnicki et al., 2022b), found in Appendix 1]. Paraffin embedding, sectioning and hematoxylin and eosin staining were carried out by The Centre for Phenogenomics (Toronto, Canada). Adipocyte area was analyzed in four randomly selected fields of view (10x objective) using ImageJ Analysis Software (NIH). For quantification of capillaries, de-paraffinized sections were stained with FITC-conjugated *Griffonia Simplicifolia* Lectin-1 (1:100) and Rhodamine Wheat Germ Agglutinin (1:200).

3.3.4 *Adipose EC isolation and metabolic assays.*

Adipose ECs were isolated and cultured as described in Chapter 2 (Rudnicki et al., 2023). Cells were plated at a density of 15,000 cells/well in Agilent Seahorse 96-well plates and were either treated with FoxO1 inhibitor AS1842856 as previously described or left untreated overnight. The Agilent Seahorse XF Real-Time ATP Rate Assay (103592-100) was conducted according to manufacturer's instructions using the Seahorse XFe96 analyzer. Metabolic assessments were

normalized to cell content per well using CyQUANT (ThermoFisher #C7026) and the Agilent Seahorse Wave software. Some cells were stimulated with 100 μ M estradiol overnight before the Seahorse analysis.

3.4.5 Statistical Analysis

All results presented are mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using Prism 10 (GraphPad Software Inc.). Significance was established at $P < 0.05$, by two-way ANOVA, with factors of gonadectomy and feeding conditions and separated by sex. Each n-value represents one mouse from the cohort. 3 technical replicates were used to get the average for each n for the Seahorse Assay. P-values are provided in each figure legend and indicated in figures as * $P < 0.05$, ** $P < 0.05$, *** $P < 0.005$, **** $P < 0.001$.

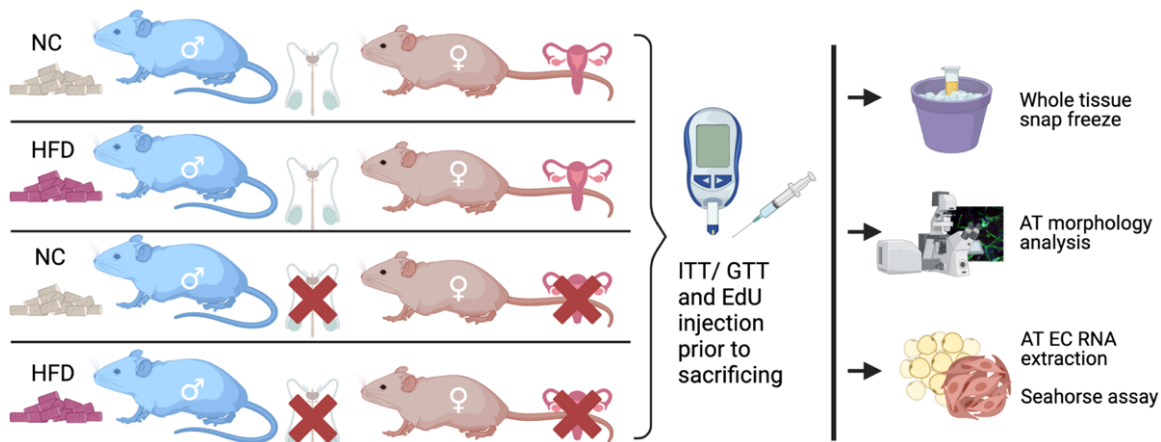


Figure 3.1 Summary schematic for the gonadectomized cohort. The 8 condition groups each included 4 mice. They were fed either normal chow (NC) or a high-fat diet (HFD). Insulin and glucose tolerance tests (ITT, GTT) were performed prior to sacrificing. After 15 weeks of diet intervention, adipose tissues were frozen, fixed for morphology analysis, or used for EC isolation. The isolated ECs were then either lysed for RNA or cultured and underwent Seahorse XF Real-Time ATP Rate Assay. Created by BioRender.

3.4 Results

3.4.1 Ovariectomy, but not castration, causes changes in whole-body metabolism

To understand the metabolic consequences of sex hormone loss in different diet conditions, we monitored body weights and performed whole-body glucose metabolism assessments. HF diet increased the weight in both intact and Ovx group (**Figure. 3.2 A,B**). Unsurprisingly, ovariectomy caused an overall increase in weight gain across both diet groups, corroborating previously published literature (Babaei et al., 2010; Nishio et al., 2019). In the males, castration led to lower body weight of HF-fed mice, suggesting that loss of androgens leads to reduced weight gain or that testes contribute to the body mass (**Figure. 3.2 A,B**).

Six-hour fasting blood glucose level was elevated by HF diet in the females, but not in males (**Figure. 3.2 C,D**). Ovariectomy led to an increase in fasting blood glucose levels in NC-fed female mice, but not in those fed a HF diet (**Figure. 3.2 C,D**). Castration did not have a significant effect on fasting blood glucose levels. Despite weight gain, we found that insulin sensitivity (based on ITT) was greater in the female Ovx groups compared to the intact females, regardless of diet (**Figure. 3.2E**). The ITT was halted after 45 minutes, as many Ovx mice became hypoglycemic. Castration in the male mice did not impact insulin sensitivity in either diet group (**Figure. 3.2 E,F**). Glucose sensitivity was lower in the ovariectomy group compared to the intact female group (**Figure 3.2G**), suggesting that estrogen helps maintain the body's response to glucose. In comparison, the HF diet led to reduced glucose sensitivity compared to the NC diet only in the male Cas group (**Figure. 3.2H**). These findings demonstrate that female sex hormones maintain glucose sensitivity, but androgen loss exerted minimal influence on whole-body metabolism.

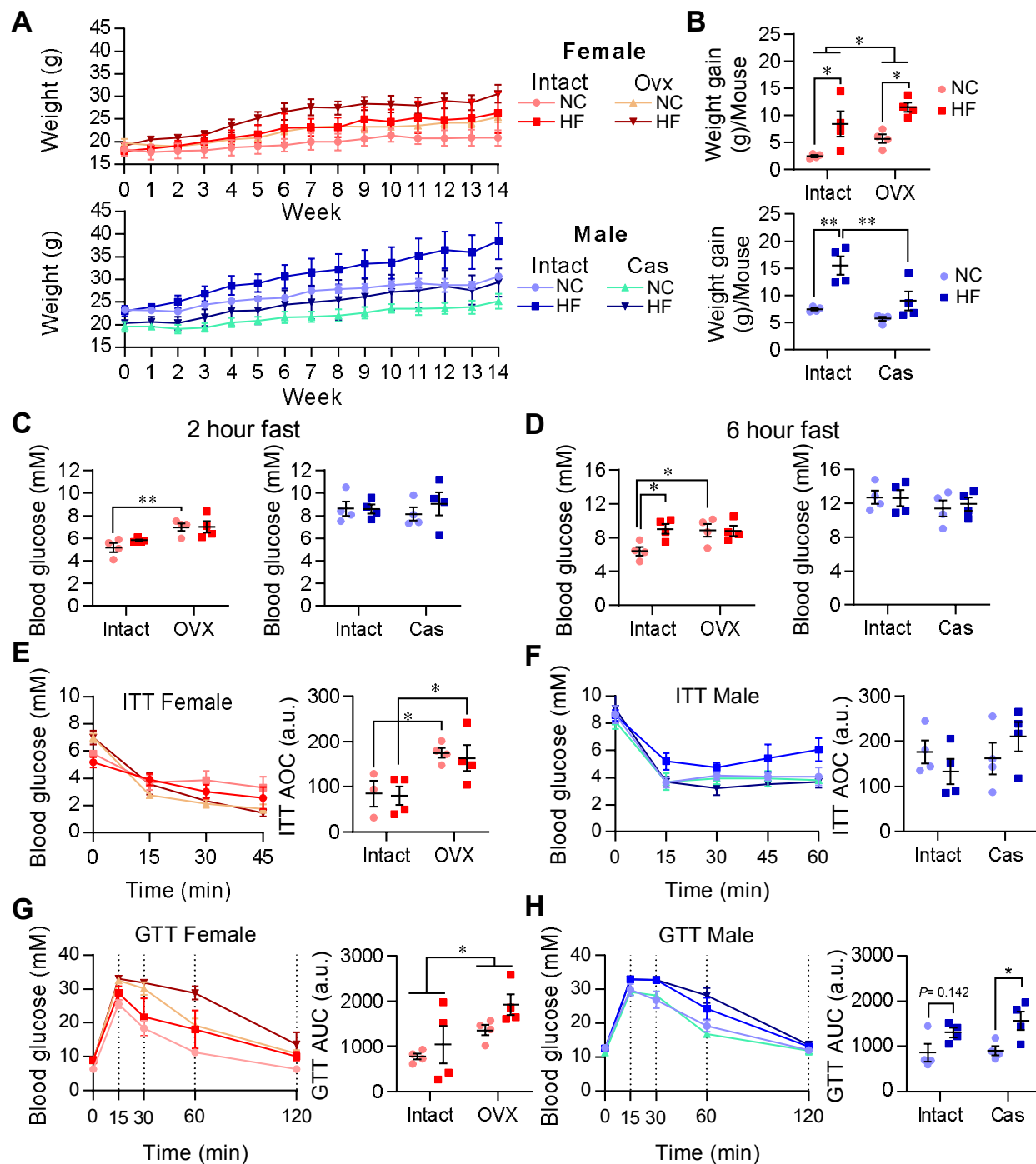


Figure 3.2 Whole-body metabolism of male and female mice either intact or gonadectomized after diet intervention. Mice were fed either a NC or HF diet for 14 weeks. Red and blue data points represent females and males, respectively ($n=4$ /group). **A**) Body weights during 14 weeks of diet. **B**) Summarized weight gain over the course of 0-14 weeks ($n=4$ /group). **C**) Fasting blood glucose levels after 2-hour and **D**) 6-hour fast. Insulin sensitivity of **E**) female or **F**) male mice either intact or gonadectomized after 13 weeks of NC or HF diet and 2-hour fasting. Represented as area over the curve (AOC). Glucose sensitivity of **G**) female or **H**) male mice either intact or gonadectomized after 14 weeks of NC or HF diet and 6-hour fasting. Represented as area under the curve (AUC) Data expressed as $\pm$ SEM. * $P<0.05$; ** $P<0.005$.

3.4.2 Effects of gonadectomy on the adipose tissue morphometry

We extended the metabolic analyses by investigating the morphology of the adipose tissue. Visceral and subcutaneous adiposity increased with ovariectomy in the females; however, HF diet increased subcutaneous adiposity only in the ovariectomized group (**Figure. 3.3 A,B**). Ovariectomy did not affect the weight of the brown adipose tissue (**Figure. 3.3C**) or the weight of the heart, relative to body weight (**Figure. 3.3D**). In the males, HF diet increased the visceral and subcutaneous adipose weight in the intact mice, but the diet did not cause an increase of white adiposity in the castrated group (**Figure. 3.3 A,B**). Notably, castration caused a decrease in the visceral weight in both diet groups. (**Figure. 3.3B**). In both diet groups, castration caused a decrease in relative brown adipose weight (**Figure. 3.3C**), as well as a decrease in the heart weight proportion of the NC diet group (**Figure. 3.3D**).

We next assessed adipocyte morphology following the gonadectomy. Adipocyte size was not significantly altered by diet in the female intact group, but was larger in the male HF-fed mice compared to the NC diet intact mice, similar to our previously reported data (Rudnicki, et al., 2018b). Similar to previous published reports (do Valle Gomes-Gatto et al., 2015; Oliveira & Gonçalves, 2024), ovariectomy led to adipocyte hypertrophy in the HF-fed mice, which tended to increase in the NC-fed mice as well (**Fig 3.3E**). The intact males on a HF diet had larger adipocytes than those on a NC diet. Castration caused a decrease in adipocyte size in the male mice following a HF diet (**Fig 3.3F**). These data indicate opposing effects of ovariectomy and castration on adiposity and adipocyte hypertrophy.

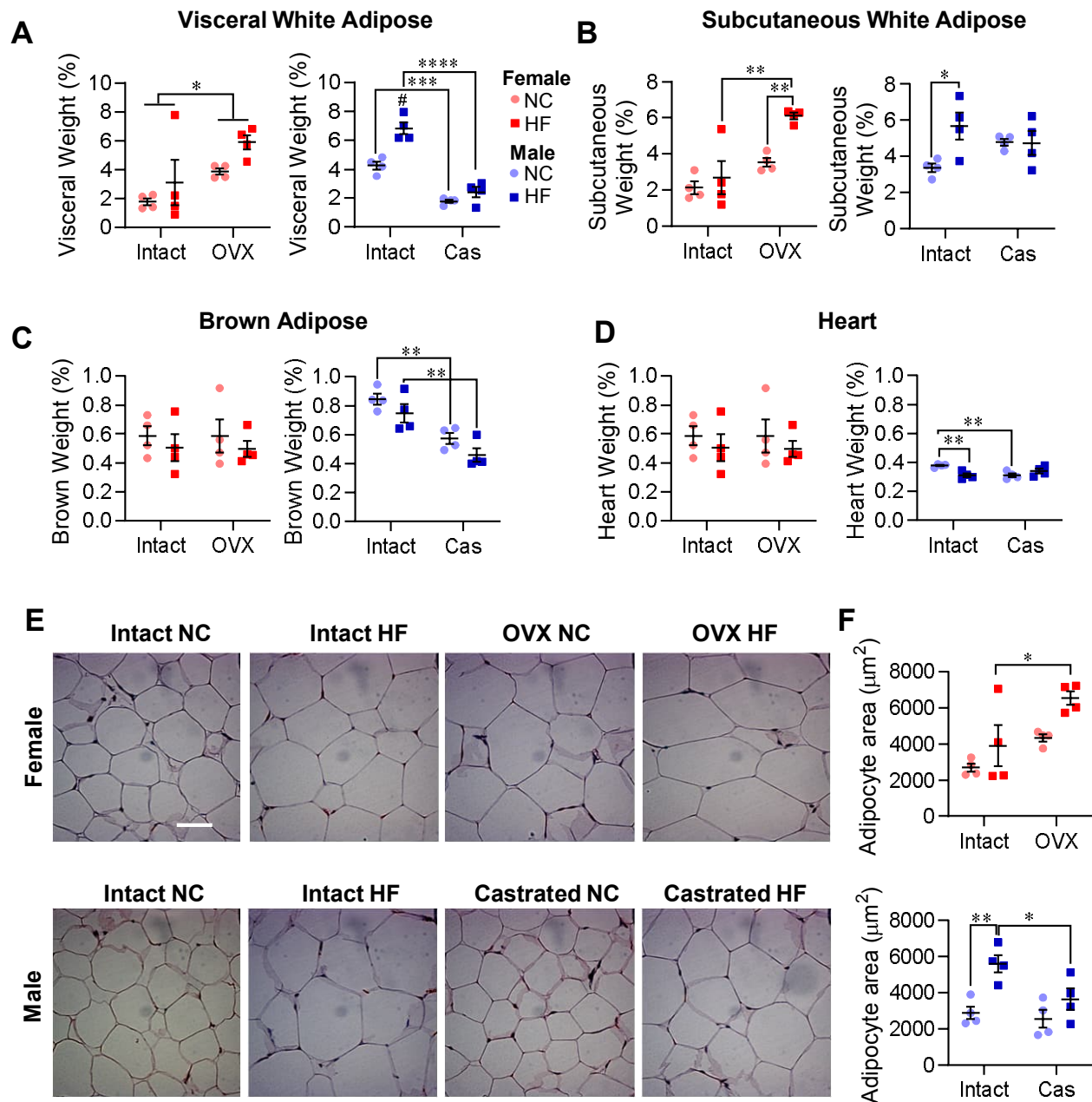


Figure 3.3 Effects of gonadectomy on adipose tissue morphology after diet intervention. Red and blue data points represent females and males, respectively (n=4/group). **A)** Visceral white adipose **B)** Subcutaneous white adipose **C)** Brown adipose and **D)** Heart weight relative to total body weight. **E)** Representative hematoxylin and eosin-stained images from epididymal adipose tissue of female mice and male mice. Scale bar = 50 μm . **F)** Mean adipocyte cross-sectional area of female and male mice. Data expressed as $\pm$ SEM. * P <0.05; ** P <0.005; *** P <0.0005; **** P <0.0001; # P <0.05 compared to intact NC for male visceral weight.

3.4.3 Effects of gonadectomy on vessel density and endothelial content in visceral white adipose

We next investigated adipose tissue capillarization to determine whether vascular content would be affected by the gonadectomy-induced loss of sex hormones. Contrary to published literature showing the positive impact of female sex hormones on vascularization (Baruscotti et al., 2010; H. Liu et al., 2018), ovariectomy caused an increase in vascular density within the female HF group (**Figure. 3.4 A,B**). In males, the HF-diet led to higher vascular density compared to the NC-fed mice, however castration did not impact vascular density (**Figure. 3.4 A,B**). This is surprising considering previous literature showed that a HF stimulus leads to lower adipose vascularization in the males (Gealekman et al., 2011; Rudnicki, et al., 2018b).

Vessel density measurements are influenced by adipocyte size, where enlargement of adipocytes creates an apparent decrease in vascular density (Rudnicki et al., 2022b). Thus, changes in vascular density may not accurately reflect changes in capillary number. Therefore, we quantified the capillary-to-adipocyte (C:A) ratio, which is not affected by adipocyte size. Diet had no effect on C:A in the females, but the HF diet caused a decrease in capillary content in the males, similar to what was reported by Rudnicki *et al.* 2018. Consistent with the increased vessel density, we observed that the Ovx group had higher C:A compared to the intact group fed a HF diet (**Figure. 3.4 C,D**). Similarly, castration led to a higher C:A in the HF-fed group (**Figure. 3.4 C,D**). These data suggest that loss of male and female sex hormones leads to increased adipose vascularization in the presence of HF stimulus.

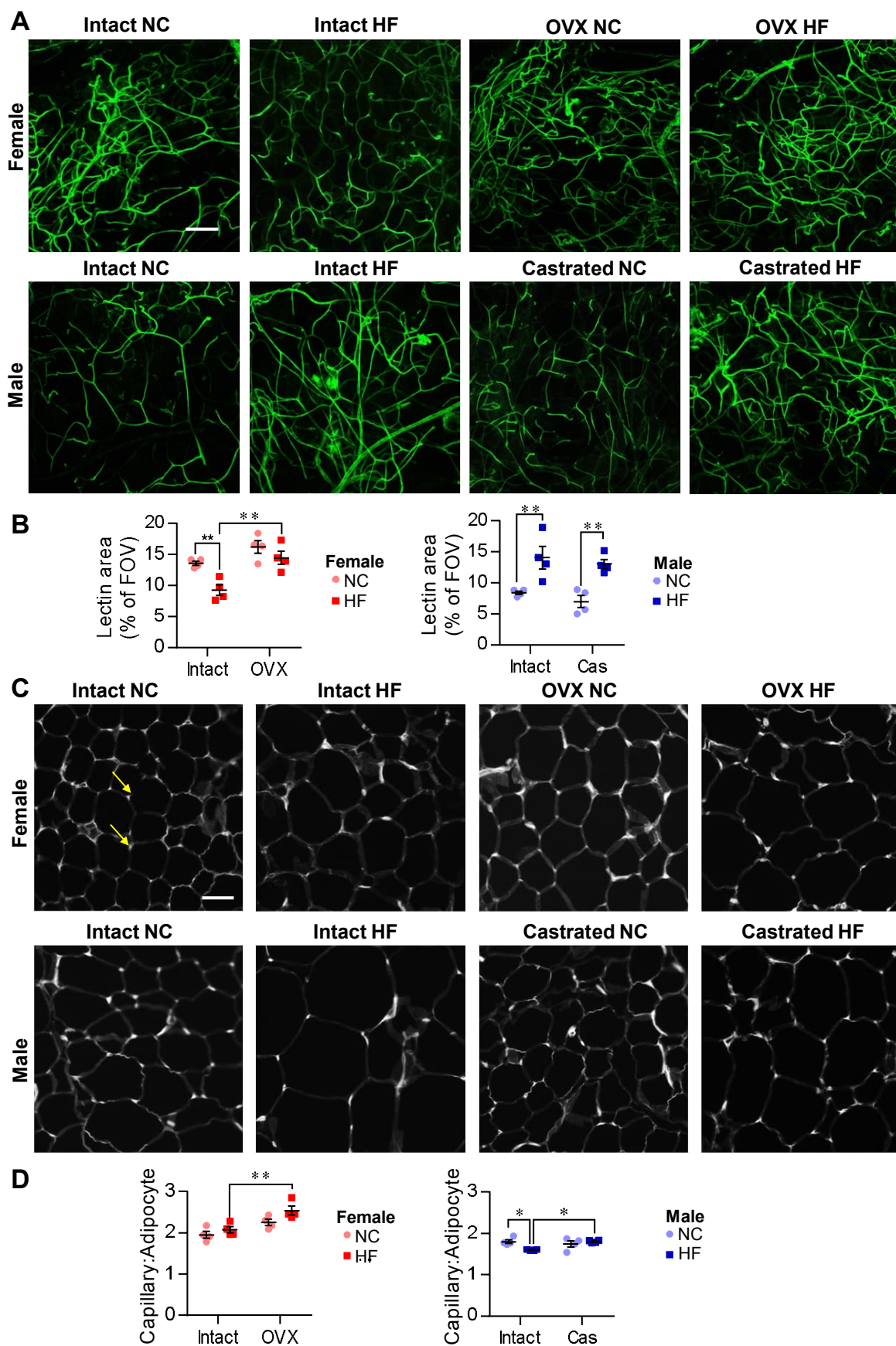


Figure 3.4 Effect of gonadectomy on vessel content after 15 weeks of diet conditioning. Red and blue data points represent females and males, respectively (n=4/group). **A)** Representative confocal images of adipose tissue whole-mount staining with *G. simplicifolia* lectin (green) of female mice and male mice. Scale bar = 100 μ m. **B)** Quantification of female and male vessel density of these images. **C)** Representative images of paraffin-sectioned adipose tissue staining with *G. simplicifolia* lectin of female mice and male mice. Scale bar = 50 μ m. **D)** Quantification of female and male capillary to adipocyte ratio of these images. Data expressed as $\pm$ SEM. * P <0.05; ** P <0.005.

Considering that higher vascularization may be due to an increase in EC proliferation, we next determined the effect of gonadectomy on EC proliferation following 15 weeks of diet intervention. HF diet caused higher EC proliferation in the Ovx group but not the intact group (**Figure. 3.5 A,B**). In the male adipose, neither diet nor castration had a significant effect on the EC proliferation rate (**Figure. 3.5 A,B**). It is noteworthy that the proliferation rate was very low in the adipose ECs of all mice, which may be due to adipose remodelling and angiogenesis occurring prior to the proliferation analysis that occurred at the end of the diet intervention. We also assessed EC nuclei per vessel area, as this could reflect EC proliferation at an earlier timepoint. Strikingly, loss of sex hormones led to an increase of EC number per vessel area in both sexes; however, the HF diet led to a significantly lower EC number compared to the NC diet in the male Cas group (**Figure. 3.5C**).

To investigate the increased capillary content in the Ovx group, we measured the pro-angiogenic cytokine VEGFA levels to assess its potential role in the enhanced adipose vascularization. Surprisingly, there was a significant reduction of *Vegfa* mRNA in the perigonadal adipose tissue of each sex following the loss of sex hormones (**Figure. 3.5D**), implying that the higher vessel density and EC content following the gonadectomy is likely not due to increased VEGFA signalling. Higher capillary content yet lower levels of *Vegfa* were found in the gonadectomy group, thus we considered that the adipose may have higher proportions of VEGF165 isoform, which is matrix bound and elicits prolonged activation of VEGFR2 considered

to be the most pro-angiogenic (T. T. Chen et al., 2010). No differences of VEGFA isoform proportions were found between groups, and *Vegfa165* was unsurprisingly the most abundant in each group, as this is considered to be the dominant form in the body (**Figure. 3.5 E,F**) (Guzmán-Hernández et al., 2014).

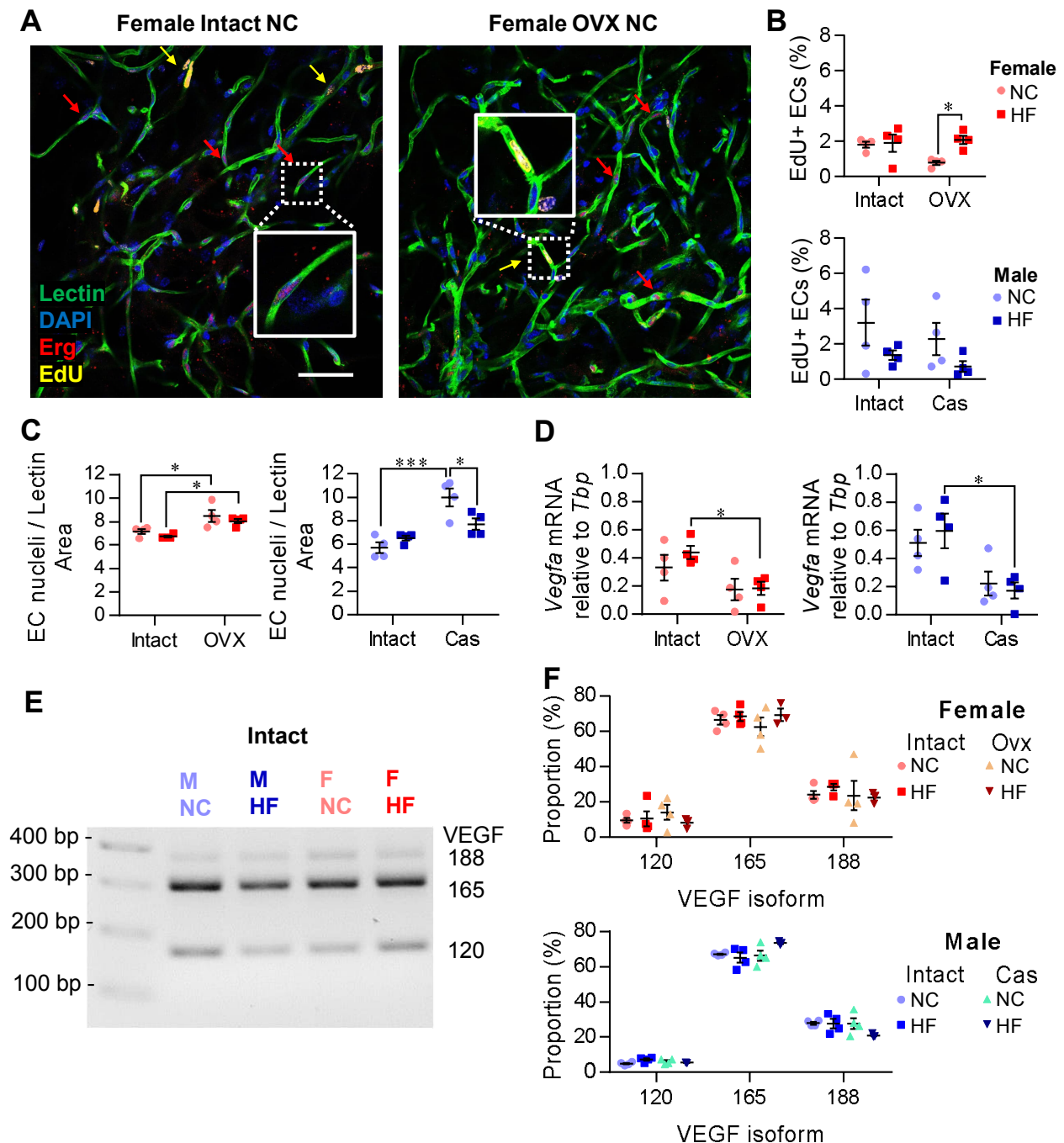


Figure 3.5 Loss of sex hormones leads to increase of EC content per vessel area, but reduction of *Vegfa*. Red and blue data points represent females and males, respectively (n=4/group). **A)** Representative confocal images of adipose tissue whole-mount staining with *G. simplicifolia* lectin (green), EC-specific Erg (red), DAPI (blue) and EdU to assess proliferating cells (yellow). Yellow arrows point to EdU+ ECs, red arrows point to Erg+ cells. Scale bar = 50 μ m. **B)** Quantification of EC proliferation assessed using EdU incorporation and click reaction **C)** Endothelial cell number relative to total lectin area. **D)** *Vegfa* mRNA levels from the visceral adipose tissue normalized to *Tbp* ($2^{-\Delta CT}$). **E)** PCR of *Vegfa* isoforms from cDNA of the whole visceral adipose tissue **F)** Quantification of *Vegfa* isoforms. Data expressed as $\pm$ SEM. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

3.4.4 Sex hormone loss has no long-term impact on EC metabolic profile

Estradiol is known to increase both EC glycolysis and mitochondrial function (Boscaro, Carotti, et al., 2020; Damacena de Angelis et al., 2022; Rodrigues et al., 2022; Trenti et al., 2017), and we previously reported that female adipose ECs have higher levels of ox-phos-related genes (Rudnicki et al., 2023). We confirmed higher mitochondrial ATP production in the female ECs at baseline levels, using metabolic data pooled from various separate cohorts (**Figure. 3.6**). Thus, we assessed adipose EC metabolism to determine if sex hormone loss or diet cause lasting metabolism that can be observed after 1 week *in vitro*. The male Cas ECs had a contamination and were therefore unusable. The isolated ECs were also treated with 100 μ M estradiol to determine if this direct stimulation would impact the EC metabolic profile. We found that ovariectomy had no long-term impact on the total ATP produced (**Figure. 3.7A**). However, we found that total ATP produced increased in both sexes with direct estradiol stimulation (**Figure. 3.7A**).

To further characterize EC metabolism, we identified if the ATP was produced through either glycolysis or mitochondrial oxidative phosphorylation. Ovariectomy did not impact the glycolytic production rate (**Figure. 3.7B**). Likewise, estradiol treatment did not affect the glycolytic ATP production rate in either sex. Interestingly, female ECs originating from HF-fed mice had higher glycolytic ATP production compared to those from NC mice, despite being in

culture for a week (**Figure. 3.7B**), showing the long-term impact on a HF diet on EC glycolysis in females. In contrast, the glycolytic rate of the male ECs did not change based on the diet.

Notably, the mitochondrial ATP rate was not affected by ovariectomy or by diet. However, consistent with previous studies (Razmara et al., 2008), estradiol treatment substantially increased mitochondrial ATP production in both sexes and in both diets (**Figure. 3.7C**). In the males, mitochondrial ATP rate was elevated by the HF diet (**Figure. 3.7C**). Despite no significant differences in total ATP production (**Figure. 3.7A**), the female ECs originating from mice fed a HF-diet had more produced more mitochondrial ATP than glycolytic ATP compared to the NC-fed mice (**Fig 3.7D**). In contrast, there was a trend for the male ECs originating from the HF group to have increased mitochondrial ATP to glycolytic ATP production ratio. In sum, gonadectomy did not have a significant long-term impact on the EC metabolism, and only direct treatment with estradiol caused an upregulation of mitochondrial ATP production. The data suggest that diet also causes long-term metabolic shift in the ECs in a sex-specific manner.

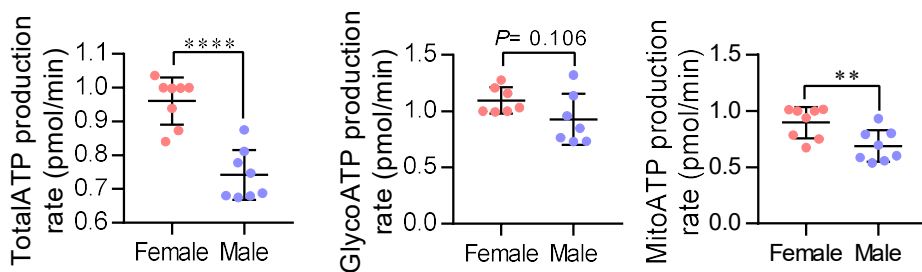


Figure 3.6 Female endothelial cells have higher levels of ATP production. Endothelial cells were isolated from FVB mice fed a NC. TotalATP production rate, glycolytic ATP production rate and mitochondrial ATP production rate (pmol/min) from EC of previous separate experiments showing baseline levels. (n=7-8) Data is collected from independent ATP Rate Seahorse assays. **P<0.005; ****P< 0.0001.

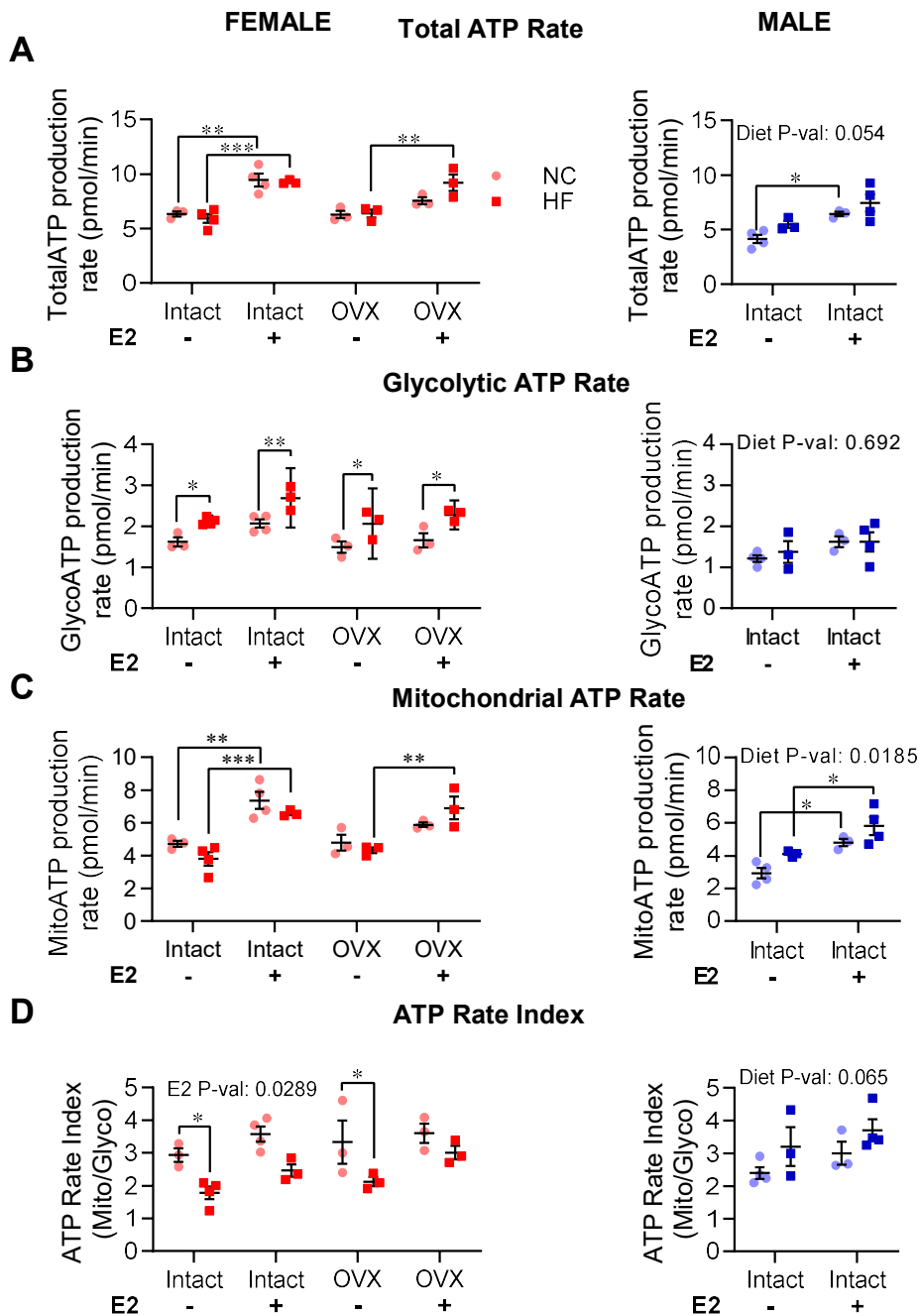


Figure 3.7 Effect of diet and sex hormones on female and male endothelial energy production. Red and blue points represent females and males, respectively. **A-D)** Passage 2 subcutaneous adipose endothelial cells were used (n=3-4 animals/group). A subgroup of ECs was treated with 100 μ M estradiol overnight. Seahorse analysis of **A)** Total ATP production rate. **B)** Glycolytic ATP production rate (pmol/min) **C)** Mitochondrial ATP production rate (pmol/min) and **D)** ATP Rate Index (MitoATP/GlycoATP rate). Data were normalized to DNA content after the Seahorse Assay and expressed as mean $\pm$ SEM (each data point represents cells from one mouse, as the average of 5-6 technical replicates). Diet main effect is written in the graphs. Diet main effect is shown in the males. * P <0.05; ** P <0.005; *** P <0.0005.

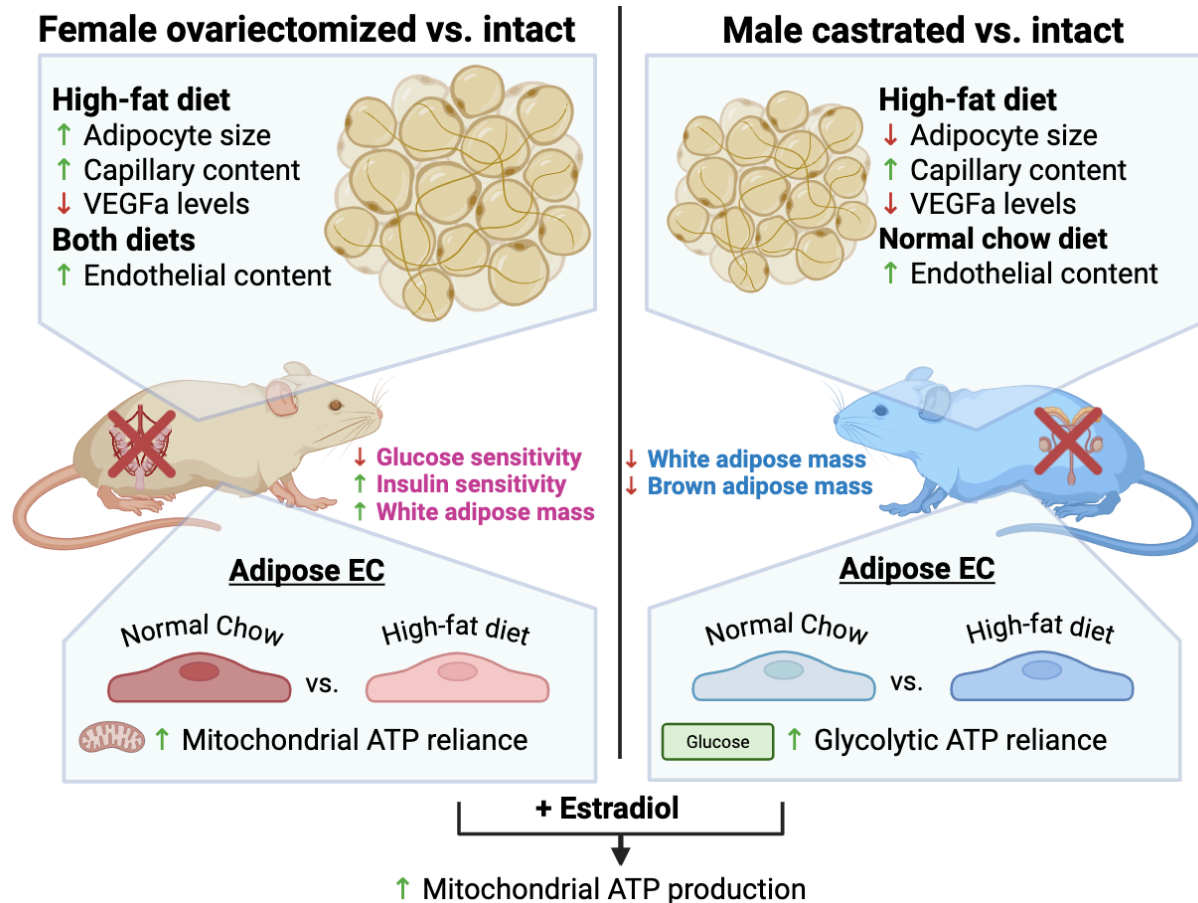


Figure 3.7 Schematic showing the effect of gonadectomy on adipose vascularization. Female mice with an ovariectomy showed adipocyte hypertrophy, higher adipose capillary and endothelial content, and a reduction of a proangiogenic marker in the adipose environment. Castration in the male mice led to the same results, with the exception of lower adipocyte size. The gonadectomy had no lasting impacts on endothelial metabolism, however the high-fat diet caused lower mitochondrial reliance in the females, but opposite results in the males. Treatment with estradiol caused all ECs to increase their mitochondrial ATP production. Figure was created in Biorender.

3.5 Discussion

In the current study, we provide novel evidence that loss of sex hormones leads to an increase of adipose capillarization in mice challenged with a HF diet. Although there is mounting evidence that estrogen promotes angiogenesis and whole-body metabolism (H. Liu et al., 2018; Seo et al., 2004; Trenti et al., 2017), herein we demonstrate that loss of gonadal sex hormones led to higher adipose capillary density, corroborated by increased vascular content (as indicated by the C:A and the EC nuclei per vessel area) when mice were fed a HF diet. Indeed, we do show that

short-term stimulation with estradiol increases metabolic activity of ECs. These findings are summarized in **Figure 3.7**.

However in the context of this study, long-term estrogen presence may actually impede angiogenesis, suggesting a potential inhibitory role, similar to findings from other groups (Ali et al., 2000; Basini et al., 2008). One of the groups showed that overexpression of the ER in tumours led to lower VEGFA levels and inhibition of vascularization (Ali et al., 2000). Similarly, Basini *et al.* also showed reduced levels of VEGFA and that hydroxyestrogens, the main estrogen metabolites, led to inhibited angiogenesis in ovarian follicles (Basini et al., 2008). In HUVECs, androgen receptor (AR) agonists reduce cell migration in a dose-dependent manner, and this effect is reversed by either AR antagonists or AR KD (Huo et al., 2024). However, these effects have not been reported in the adipose until now suggesting that, contrary to expectations, sex hormones restrict adipose vascularization rather than enhancing it when challenged with a HF diet.

Endothelial proliferative differences were not observed following gonadectomy, however the overall proliferation rate among all the groups was low compared to our previous study with only a 7 week diet intervention (Rudnicki et al., 2023). A possible reason for the lower proliferation compared to our previous study is that the current had 15% lower fat content compared to the previously used Surwit Diet. Although the diet in this study had lower fat content and may not have triggered as much adipose tissue remodeling (Poret et al., 2018), the level of adipocyte hypertrophy was similar to previous findings, suggesting similar adipose expansion. Considering we show that the diet altered endothelial metabolism, it is possible that it directly affected EC proliferation. However, it is most probable that the low endothelial proliferative rate is due to the longer duration of the diet intervention compared to our previous study, which likely resulted in the formation of mature, stabilized blood vessels whose ECs are quiescent and non-proliferative

(Eelen et al., 2018; Ricard et al., 2021). Indeed, angiogenesis is an early event that occurs rapidly during tissue remodelling to support expansion and metabolic demand, but this vascular growth tapers off once remodeling is complete and the tissue returns to homeostasis (Gealekman et al., 2008). It is possible had we examined the adipose EC proliferation at an earlier timepoint, we may have captured a more dynamic state of the adipose where EC proliferation rate matched the vascular changes we observed and that provides a better understanding of the EC response to the lack of hormones.

The loss of hormonal signals may alter the adipose tissue microenvironment, leading to cumulative changes that ultimately suppress capillary growth. VEGFA is a potent pro-angiogenic factor and its adipose-specific deletion reduces vessel density, while its induction promotes angiogenesis (Sung et al., 2013). However, *Vegfa* mRNA levels were surprisingly lower in the adipose of the gonadectomized compared to the intact mice of both sexes. This outcome is consistent with previous studies indicating that estradiol promotes the transcription of *Vegfa* in adipocytes and other cell types (Fatima et al., 2017; Kazi et al., 2009). The current accepted relationship is that low VEGFA levels in adipose tissue lead to low capillary numbers and can cause local hypoxia, which not only limits oxygen and nutrient delivery but also triggers inflammatory responses and fibrosis (Crewe et al., 2017). Over time, these changes can disrupt adipocyte function, hinder proper tissue remodeling, and contribute to systemic metabolic dysfunction, including insulin resistance and impaired lipid metabolism. However, the concurrence of both low VEGFA levels and higher capillary content suggests that another factor may be driving the observed increase in angiogenesis. The adipose environment contains many other pro-angiogenic factors (e.g., VEGFB, FGF, angiopoietins, platelet-derived growth factor (PDGF), adiponectin, leptin) that can promote vascularization (Hagberg et al., 2010; Lemoine et

al., 2013; Zocchi et al., 2022) and can be upregulated when VEGFA signaling is impaired as a compensatory mechanism (Casanovas et al., 2005; Lopes-Coelho et al., 2021). To better understand the vascular differences, levels of other angiogenic molecules such as FGF and PDGF should be quantified in the adipose tissue of gonadectomized mice. Examining changes in the local adipose environment may help to determine whether the loss of hormones acts directly on endothelial signaling pathways or indirectly alters EC behavior by reshaping their surrounding microenvironment.

Given the capacity of estrogen to induce epigenetic changes in ECs (Y.-M. Yang et al., 2018), we considered that gonadectomy-induced metabolic changes in ECs might persist in culture, even in the absence of circulating sex hormones. Estrogen has been shown to regulate metabolic proteins like GLUT4 and LPL (Campello et al., 2017; D. Liu et al., 2008), and enhance antioxidant gene expression, while its loss increases vascular free radicals (Strehlow et al., 2003), potentially impairing mitochondrial function. Despite previous studies showing that estrogen can increase both EC glycolysis and mitochondrial function (Boscaro, Carotti, et al., 2020; Damacena de Angelis et al., 2022; Rodrigues et al., 2022; Trenti et al., 2017), we found that the loss of sex hormones did not have long-term effects on either glycolytic or mitochondrial rate. In contrast, treatment of the ECs with estradiol elevated mitochondrial function, based on increased mitochondrial ATP production, suggesting that the mechanism by which estrogen upregulates EC metabolism is by direct signalling, and sex hormone loss is not sufficient to cause a sustained reduction in cellular metabolism.

Our findings demonstrate striking sex differences in terms of the effect of HF diet on metabolic reprogramming of EC. Although we previously showed that female ECs have enhanced oxidative phosphorylation compared to those of males on a HF diet (Rudnicki et al., 2023), here

we demonstrate that the HF diet causes a higher dependency on glycolytic ATP production compared to mitochondrial ATP production in the female ECs. This effect was observed in both intact and ovariectomized groups, despite the diet having no significant differences in total ATP production. The opposite effect can be seen in males, with a trend for the ECs to become more reliant on mitochondrial respiration than glycolysis when on a HF diet. Previous literature suggests that a HF diet stimulus can increase mitochondrial capacity to oxidize fatty acids and increase mitochondrial content (Leduc-Gaudet et al., 2018; Rennison et al., 2007). However, long-term HF-diet intervention can lead to increased mitochondrial ROS production, contributing to mitochondrial dysfunction and various metabolic disorders (Vial et al., 2011; Zalewska et al., 2019). Thus, the adaptive characteristic of female ECs to be more reliant on glycolysis when challenged with a HF diet may give them a mechanism to reduce oxidative stress caused by HF diet and could contribute to the resilient phenotype, however it would be beneficial to also analyze the transcript levels of genes related to these two metabolic pathways.

Although not the focus of the current investigation, our results confirm the previous observation that adipose vascular content is higher in female compared to male mice following long term HF diet (Rudnicki, et al., 2018b). Greater female vascularization may stem from higher EC mitochondrial function and proliferation. While partly intrinsic, our findings show that estrogen enhances mitochondrial ATP production, potentially contributing to increased adipose angiogenesis in females during high-fat feeding. However, the increased EC ATP production by estrogen was not reflected by lower vascularization in the absence of female sex hormones.

In summary, this study was used to understand the impact of sex hormone loss on adipose EC phenotype, and to determine if sex hormones contribute to the previously reported EC sexual dimorphism. Our findings demonstrate that sex hormones are not responsible for the elevated

adipose tissue vascularization observed in females and instead support the idea that these EC sex differences are intrinsic to the cells themselves. We further present novel evidence that loss of sex hormones results in increased vascular content within adipose tissue and that ovariectomy, unexpectedly, enhances insulin sensitivity. Finally, we illustrate that loss of sex hormones has no lasting impact on the adipose EC metabolic profile. In contrast, we show that a HF diet induces a sustained metabolic shift, where female ECs adapt toward a more glycolytic phenotype; a response that may serve a protective role under metabolic stress. These results help provide valuable insight into clinical applications involving reduced hormone levels, such as those observed during aging, suggesting that sex hormone level manipulations may help prevent dysfunctional tissue expansion and metabolic decline. Moreover, the sex differences in the metabolic switch in response to a HF diet may explain why female mice are more resistant to obesity and its accompanying metabolic disturbances. These results collectively advance our understanding of sex-specific endothelial function in adipose tissue and underscore the complexity of hormonal and intrinsic regulation in metabolic adaptation.

3.6 Limitations and Next Steps

Contrary to our hypothesis, loss of the female sex hormone did not cause a decrease in EC content, with higher adipose vascularization in the ovariectomized mice. However, analysis of pro- and anti-angiogenic factors levels in the adipose microenvironment should be done, as well as subsequent tests to determine if they contribute to the higher vascularization observed. In this study, we reported low *in vivo* EC proliferation which may not be reflective of the effect of sex hormone loss on proliferation, thus future studies should aim to assess proliferation at an earlier time point after the gonadectomy and also assess *in vitro* EC proliferation of the cells isolated from these mice. We have isolated RNA from the adipose ECs from all groups, and future transcriptomic

analysis should be performed to determine if pathways relating to angiogenesis and estrogen receptors are different between the intact and gonadectomized groups. This may be useful to understand if the vascularization differences are due to direct downregulation of sex hormone signalling pathways, or if the loss of hormones is acting indirectly by and pathways activated by angiogenic molecules in the microenvironment are changed.

Our study relied on the assumption that the gonadectomy resulted in the absence of sex hormones, and we indeed report changes that indicate there is decreased estrogen, such as reduced VEGFA levels and whole-body adipose and metabolic changes. However, we cannot exclude the possibility of an upregulation of the production of sex hormones from other sources. Sex hormones can also be produced by adrenal glands and from the conversion of testosterone to estrogen by aromatase in the adipose tissue (Robert, 2023). In rodents, ovariectomy can lead to the increased function of the adrenal glands suggesting a compensatory function following loss of hormones made by the sex organs, but it generally does not fully compensate (Jopek et al., 2017). However, it would be of value to measure the levels of sex hormones after the gonadectomy to potentially uncover any compensatory production from the adrenal glands. The gonadectomy may have also had an effect on adipose tissue distribution and mass, considering that the absence of testes in the males caused an almost full reduction in epididymal fat. Therefore, it would be beneficial to explore alternative methods for reducing sex hormone signalling, as gonadectomy is invasive and may introduce confounding variables that could impact the interpretation of experimental outcomes. These methods consist of pharmacological interventions such as estrogen and androgen antagonists and aromatase inhibitors, or genetic knockout (KO) models such as androgen receptor KO or estrogen receptor KO that can be specific to the endothelium. The Four Core Genotypes

model is also a valuable tool for studying the effects of sex hormones as it would allow the separation of the influence of chromosomes from that of gonadal hormones on sex differences.

Chapter 4: FoxO1 protects endothelial cell phenotype in the female adipose tissue

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4.1 Contributions

I was involved in the conceptualization of the study and development of the experimental framework along with TH, MR and ERo. MR generated the original cohorts of FoxO1 KD mice and MR and EN performed the metabolic testing of the mice (Figures 4.2 and S4.3). Preparation of the samples for RNA sequencing was done by MR. ERu and TG performed the RNA-seq raw analysis (read mapping, normalization, differential expression analysis). EY measured the adipose tissue capillary to adipocyte ratio (Figure 4.1D) for the 16-week timepoint and performed quantification of FoxO1 levels in culture (Figure 4.4 A,B). AF measured mRNA levels of genes in both white and brown adipose tissue (Figures 4.1 F,I and S4.1 C,F). I performed the RNA-seq analysis using the data received from ERu and TG. I generated the proliferative cohort used for the study and administered the diet and EdU injection and performed the imaging and analysis of the adipose tissue (Figure 4.3D). I performed all *in vitro* validation experiments using endothelial cells I isolated. I generated the mTmG validation cohort and analyzed it. I also generated one of the 16-week NC cohort, imaged the vasculature in the adipose tissue, and measured the vessel diameters. I generated Figures: 4.1B, 4.1K, 4.3, 4.4 D-G, 4.5, 4.6, 4.7, partially S4.1H, S4.2 B,C, S4.4, S4.5, S4.6. I wrote the manuscript along with MR and TH.

4.2 Introduction

White adipose tissue is a highly dynamic organ with a remarkable capacity for expansion (Choe et al., 2016). Healthy enlargement of this tissue requires coordinated extension of the capillary network (angiogenesis), which maintains tissue homeostasis by ensuring adequate oxygen and nutrient supply (Corvera et al., 2022). However, under conditions of sustained nutrient excess, such as high fat diet (HFD) feeding, endothelial cells (ECs) that comprise capillaries become dysfunctional (Bondareva et al., 2022). This impairs angiogenesis and contributes to adipose tissue malfunction and the development of systemic metabolic disorders (AlZaim et al., 2023; Cao, 2007; Corvera & Gealekman, 2014; Crewe et al., 2017; Eelen et al., 2018; Gogg et al., 2019; Sabaratnam & Svenningsen, 2021). Elucidating the molecular mechanisms that support endothelial resilience, the capacity of ECs to maintain their functional integrity despite metabolic stress, is critical for developing strategies to preserve vascular health in obesity.

Emerging studies have highlighted sex-specific differences in EC function, particularly under metabolic stress (Addis et al., 2014; Lorenz et al., 2015, 2022; Rudnicki et al., 2023). Compared to males, female mice exhibited higher angiogenesis within visceral white adipose tissue in response to HFD, which correlated with greater resistance to develop metabolic dysfunction (Rudnicki, et al., 2018b). The molecular drivers that dictate the differential angiogenic capacity of male and female ECs remain largely unresolved. Transcriptomic analyses have revealed that female ECs possess distinct gene expression profiles compared to male ECs, including an enhanced proliferative signature (Rudnicki et al., 2023).

The transcription factor Forkhead Box O1 (FoxO1) orchestrates a broad range of cellular processes, including metabolism, oxidative stress responses, cell cycle control, and DNA repair. In ECs, FoxO1 is indispensable for vascular development, as its deletion results in embryonic

lethality (Dharaneeswaran et al., 2014; Furuyama et al., 2004). In the postnatal and adult vasculatures, FoxO1 maintains endothelial quiescence and plays an important role in the suppression of angiogenesis (Andrade et al., 2021; Wilhelm et al., 2016). This influence is achieved through upregulation of cell cycle inhibitors such as Cdkn1a (p21^{cip}) and repression of *c-myc*. Beyond this dominant effect, additional cytoplasmic roles for FoxO1 have been reported, including regulation of EC shape (Ghanbarpour Houshang et al., 2025; Matsukawa et al., 2009). Notably, obesogenic conditions increase FoxO1 expression in ECs (Nwadozi et al., 2016) and its endothelial-specific depletion in adult male mice was shown to fuel angiogenesis in adipose tissue depots and skeletal muscle, predominantly when mice were exposed to HFD conditions (Rudnicki, et al., 2018a). This adaptive angiogenic remodeling was associated with a healthier adipose tissue expansion and protection against obesity-associated metabolic dysfunction (Rudnicki, et al., 2018a). Intriguingly, female ECs have significantly higher FoxO1 levels relative to their male counterparts (Rudnicki et al., 2023), despite the greater angiogenic capacity of HFD-fed female versus male mice. This suggests a sex-specific regulation and potential functional divergence of this transcription factor.

In this study, we hypothesized that FoxO1 plays a unique, protective role in female ECs during metabolic stress. To test this, we investigated the effects of EC-specific FoxO1 depletion in female mice, and compared the transcriptomic landscape associated with FoxO1 depletion in female and male ECs. Using genetic models and pharmacological inhibition of FoxO1, we uncovered a critical requirement for FoxO1 in maintaining EC phenotype and promoting metabolic adaptations to obesogenic conditions in females.

4.3 Methods

4.3.1 Animals

All animal procedures were approved by the York University Animal Care Committee. Mice were housed under specific-pathogen-free conditions in ventilated cages and received water and standard chow diet *ad libitum* until use in experiments. For adipose EC isolation, young adult wild-type mice (FVB/N background) fed a standard chow were used. Conditional *Foxo1* mice were maintained on an FVB/N background. To generate mice with EC-specific and deletion of *Foxo1*, *Foxo1^{ff}* were crossed with transgenic mice expressing tamoxifen-inducible Cre recombinase (CreERT2) under the control of the *Pdgfb* promoter (*Pdgfb-iCreER^{T2}*) as described previously (Rudnicki, Abdifarkosh, Nwadozi, et al. 2018). Endothelial specificity of *Pdgfb*-driven Cre expression was validated by crossing *Pdgfb-iCreER^{T2};Foxo1^{ff}* with the double-fluorescent Cre reporter mouse *Rosa^{mT/mG}* (JAX stock # 007676). To induce Cre-mediated recombination mice (4-6 weeks of age) received intraperitoneal injections of tamoxifen (200 μ L *i.p.*; 15 mg/mL in corn oil, Sigma-Aldrich,.) for five consecutive days, as described previously. Age-matched tamoxifen-injected *Foxo1^{ff}* mice lacking Cre were used as controls. One week following the final tamoxifen injection, female endothelial-specific FoxO1 knockdown mice (EC-FoxO1 KD) and control littermates were placed on either a normal chow diet (NCD; 11 kcal% fat w/sucrose, Research Diets D12329) or HFD (58 kcal% fat w/sucrose Surwit Diet, Research Diets D12331) for 4, 7 or 16 weeks. A cohort of male littermates received only the HFD for equivalent durations. For assessment of EC proliferation and transcriptomic analyses, a separate cohort of EC-FoxO1 KD mice (n = 5 females, n = 6 males) was fed the HFD for 7 weeks and injected with EdU (1.5 mg in 100 μ L sterile PBS; *i.p.*) on two consecutive days prior to tissue collection.

4.3.2 Intra-peritoneal insulin and glucose tolerance tests (ITT and GTT)

Insulin and glucose tolerance tests were conducted on female mice fed 16 week-NC and HF diets, at weeks 14 and 15, respectively. For ITT, mice were fasted for 4-h prior to receiving a bolus injection of insulin (1.0 U/kg BW, *i.p.*). For GTT, mice were fasted for 16-h prior to receiving a bolus injection of glucose (2 g/kg BW, *i.p.*). Blood glucose was measured by glucometer at post-injection time-points 0, 20 and 40 min (ITT) or 0, 30, 60, 90, 120 and 180 min (GTT).

4.3.3 Adipose tissue histochemistry

Small pieces of perigonadal white adipose tissue (pgWAT) were fixed (4% paraformaldehyde) and processed for either whole-mount staining or paraffin embedding. For EC proliferation assessment, EdU+ cells were detected using a click reaction (0.1 nM Azide-fluor 545; 0.1 M Tris, 1 mM CuSO₄, 0.1M ascorbic acid) prior to immunostaining, as described previously, following by blocking and permeabilization with 0.5% Triton X-100 (PBS-T) and 5% goat serum at RT. Samples were incubated overnight at 4 °C with primary anti-ERG antibody (1:100) in blocking buffer. Following three 5-min washes with PBS, samples were incubated with fluorescein-labeled *G. simplicifolia* lectin (1:50, GSL I–BSL I; FL-1101, Vector Labs) and the goat anti-rabbit Alexa 647 secondary antibody (1:200) in blocking buffer for two hours. Samples were counter stained with DAPI (1:1000) for 5 min and then washed with PBS (three x 5 min) before mounting. Single plane images were captured with a Zeiss LSM700 inverted confocal microscope equipped with a 40x/1.30 Oil EC PLAN Neofluar objective (York University YSciCore). Microvascular content, vessel diameter, EC nuclei and EdU positive nuclei were quantified from 4-5 fields of view per animal using ImageJ, as previously described. For morphometric analysis of adipocytes, fixed WAT was paraffin embedded, sectioned and stained with hematoxylin and eosin by The Centre for Phenogenomics (Toronto, Canada). Adipocyte areas were analyzed in 4-6 randomly selected fields

of view per mouse (10x objective) using ImageJ. For quantification of capillaries, de-paraffinized sections were stained with *G. simplicifolia* lectin-FITC (1:50) and Wheat Germ Agglutinin-Rhodamine (1:200; RL-1022, Vector Labs) and images captured using a Zeiss 200M inverted microscope, cooled CCD camera and Metamorph software. Capillary and adipocyte counts were obtained from 3-4 independent fields of view per mouse (10X objective).

4.3.4 Skeletal muscle histochemistry

Extensor digitorum longus (EDL) muscles were embedded in optimal cutting temperature (OTC) compound and frozen in liquid nitrogen-cooled isopentane. Transverse 10 μ m cryosections of EDL muscles were co-stained with *G. simplicifolia* lectin FITC (1:100) and wheat germ agglutinin-Rhodamine (1:400) to visualize myofiber membranes. A Zeiss inverted microscope with a cooled CCD camera and Metamorph software was used to capture three to four representative images of each muscle section using a 20X objective. Capillary to fiber ratio (C:F) was measured by counting the number of capillaries relative to the number of myofibers in each field of view.

4.3.5 RNA-Sequencing

Perigonadal and inguinal subcutaneous white adipose depots from each mouse were harvested, pooled and digested with type I collagenase for 30 min at 37 °C as described previously. EC were purified from the filtered stromal vascular fraction by magnetic sorting following incubation with Dynabeads conjugated to rat anti-mouse VE-cadherin antibody and streptavidin-magnetic beads coupled to biotinylated rat anti-mouse CD31 antibody. RNA was extracted from freshly isolated EC using RNeasy Mini Kit and quality-checked using a Bioanalyzer automated electrophoresis system (Agilent Technologies Inc.). Samples with an RNA Integrity Number (RIN) > 6 (n = 4/group) were processed for RNA sequencing by The Centre for Applied Genomics, The

Hospital of Sick Children, Toronto. PolyA-enriched cDNA-libraries were generated and sequenced using a Novaseq SP flowcell (paired end 2x50 bp), generating ~60 million reads per sample. After mapping reads to the mouse genome from Ensembl Mus_musculus.GRCm38.cdna.all.fa, expression levels were normalized as Transcripts Per Kilobase Per Million (TPKM) by dividing the read count of each transcript model with its length and scaling the total per sample to one million using Kalisto V0.4.4. Quality control of raw and mapped reads was done using FastQC version 0.11.8. Transcripts with <20 raw counts per library were deemed unexpressed and were discarded from analysis. Differential expression analysis was performed using the R package EdgeR version 3.34.52 with the formula $\log_2\text{CPM} = 0 + \text{genotype}$ between control and EC-FoxO1 KD for each sex, correcting for common, trended and tagwise disparity. The analysis rendered two-base logarithmic fold change ($\log_2\text{FC}$) differences and false discovery rate (FDR) values, where FDR of <5% was considered significant.

4.3.6 Bioinformatics analyses

Principal components were calculated to reduce dimensionality and capture the major sources of variance within the dataset. The first two principal components (PC1 and PC2), which accounted for the greatest variance, were plotted to visualize the clustering and separation of samples based on gene expression profiles. Gene set enrichment analysis (GSEA) (Mootha et al., 2003; Subramanian et al., 2005) was used to test for enrichment of ontologies from publicly available databases Gene Ontology Biological Process, Molecular Function and Cellular Compartment, Wiki Pathways, Reactome, PID pathways and Kegg, comparing EC-FoxO1 KD to Control for each sex. A custom 'EC proliferation' geneset was formulated based on literature, as described previously. PCA was performed using only the DEGs identified in female samples. Gene expression values were normalized and scaled prior to analysis.

4.3.7 Endothelial Cell Culture

Pooled pgWAT and sWAT depots of wild-type male and female FVB/N mice kept on a standard diet were digested for 30 min at 37 °C with type I collagenase and cells from the stromal vascular fraction were seeded in 1.5% gelatin-coated 25 cm² flasks in high-glucose (25 mmol/L) DMEM supplemented with 20% FBS. After reaching confluence, cells were purified with streptavidin-magnetic beads coupled to biotinylated rat anti-mouse CD31 antibody, as described previously (Rudnicki, et al., 2018a; Rudnicki et al., 2023). Cells were cultured until passage 5 and were plated in multi-well plates for RNA or protein, or 8-well chamber slides coated with rat tail collagen for immunostaining. To inhibit FoxO1 function, cells were treated with the FoxO1 inhibitor AS1842856 (1 µM) for 1 or 6 days. In some experiments, cells were concurrently treated with mitoTEMPO (20 µM; #SML0737, Sigma-Aldrich). Media was replenished every three days with the specified reagents. In some experiments, cells were treated with EdU (10 µM for 4 hours) prior to fixation with 4% paraformaldehyde. In some experiments, cells were incubated with MitoBrilliant™ (100 nM) for 1 hour prior to fixation. To validate the antioxidant effect of MitoTempo, cells were pretreated with MitoTempo or vehicle for 24 hours, then treated with menadione (100 µM) for one hour. These cells were incubated with CellROX™ Green (10 µM; #C10444, Invitrogen) for 30 minutes prior to fixation with 4% paraformaldehyde. For siRNA treatments, cells were plated overnight, then transfected with SMARTpool non-targeting or FoxO1 siRNA (5 nM) using DharmaFECT transfection reagent; Horizon Discovery) then cultured for 24-72 hours. Cells were serum-starved for 2 hours prior to lysis, where specified.

4.3.8 Cell immunostaining

Following fixation with 4% paraformaldehyde, non-specific binding was blocked with 0.1% Triton and 5% goat serum in PBS for one hour, then cells were incubated overnight with

primary antibodies: after washing, cells were incubated for one hour with secondary antibodies diluted in blocking solution. See Supplementary Table 1 for antibody details. Cells were incubated with DAPI (1:1000) to detect the nuclei. Images were captured using a Zeiss LSM700 or a Zeiss Observer Z1 SD confocal (40x objective), maintaining identical gain settings within each experiment. The average pixel intensity per nucleus (CellROX Green, p21, p53, pSmad2) or per cell area (4-HNE) was measured from 5 to 8 fields of view per experiment using ImageJ. DNA damage was assessed by counting the individual γ H2AX foci within each nucleus. Mitochondrial network quantification was performed using Image J (Fiji). Average mitochondria lengths were calculated from thresholded 2D images using the Mitochondria Analyzer plugin (Chaudhry et al., 2020).

4.3.9 Metabolic assessments

Cells were plated at a density of 15,000 cells/well in Agilent Seahorse 96-well plates and were either treated with FoxO1 inhibitor AS1842856 as previously described or left untreated overnight. The Seahorse XF Real-Time ATP Rate Assay (103592-100, Agilent) was conducted according to manufacturer's instructions using the Seahorse XFe96 analyzer, measuring glycoATP and mitoATP production rates, expressed as pmol ATP/min. Metabolic assessments were normalized to cell content per well using CyQUANT (ThermoFisher #C7026) and the Agilent Seahorse Wave software.

4.3.10 RNA isolation and qPCR

RNA extracted from freshly isolated ECs, cultured ECs, pgWAT and brown adipose tissue (BAT) using the RNeasy Mini Kit (Qiagen Inc.) and was reverse transcribed using M-MuLV reverse transcriptase. cDNA was analyzed by real-time PCR on the Rotor-Gene Q platform using TaqMan primer/probe sets (see Supplementary Table 2 for details) and TaqMan Master Mix

(#4444963, Invitrogen or #ABMG890, Diamed). Transcript levels of each target gene were calculated relative to the housekeeping gene and expressed as $2^{-\Delta C_t}$.

4.3.11 Western Blots

Total protein extraction from cultured adipose EC was performed as described previously using lysis buffer containing 50 mM Tris (pH 8.0), 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate with phosphatase inhibitor and protease inhibitor. Following protein separation through a polyacrylamide gel under reducing conditions, western blots were performed using primary and secondary antibodies. Membranes were developed using enhanced chemiluminescence and densitometry analysis was performed with ImageJ Analysis Software (NIH).

4.3.12 Statistical Analyses

For *in vivo* assessments, all data reported are from independent biological replicates with each mouse considered as one biological replicate. Averaged values were used when technical replicates (analysis of the same sample in duplicate) were performed. For *in vitro* assays, experiments were repeated at least six times with independent biological samples. For 2-group comparisons, datasets were analyzed by paired Student's t test. Gene expression data from cultured cells were analyzed using 2-way repeated measures ANOVA (Sex x Treatment), followed by Sidak's multiple comparison post hoc tests when a significant main effect or interaction was identified. A p value of <0.05 was considered significant for all analyses. Statistical tests were performed using Prism ver. 5 or 7 (GraphPad Software Inc.). Individual data points are shown on each graph, together with means $\pm$ standard errors of the mean (SEM) and p values are indicated in each Figure legend.

4.4 Results

4.4.1 Depletion of endothelial FoxO1 causes lower tissue vascularization in female mice.

Female mice display greater adipose angiogenesis than their male counterparts. This occurs despite having higher levels of the anti-angiogenic factor FoxO1 (Rudnicki, et al., 2018b; Rudnicki et al., 2023), as was reported in adipose EC from high fat diet fed mice. We found that FoxO1 mRNA levels were robustly higher in female versus male adipose EC extracted from normal chow fed mice (**Figure 4.1A**), indicating that this sex dimorphism is not restricted to a high fat diet condition. To assess the functional role of FoxO1 in adult female mice, we utilized the *Pdgfb-iCreER^{T2}:FoxO1^{fl/fl}* mouse model to generate an endothelial-specific inducible depletion of FoxO1, as reported previously in male mice. Endothelial cell specificity of the *Pdgfb-iCreER^{T2}* driver was confirmed in mice expressing the mTmG reporter, wherein tamoxifen induced the expression of GFP expression exclusively in endothelial cells (**Figure 4.1 A,B**). Gene expression analysis of adipose ECs extracted from female mice 8 weeks after the administration of tamoxifen showed that *Foxo1* transcript levels in EC-FoxO1 KD were 58% lower than in Control littermates (**Figure 4.1C**), similar to the 50% reduction previously reported in male mice. Since endothelial deletion of FoxO1 in high-fat fed male mice caused greater vascularization in visceral and brown adipose tissue and skeletal muscle (Rudnicki, et al., 2018a), we hypothesized that FoxO1 depletion in females would promote a similar or even greater angiogenic response. Unexpectedly, EC-FoxO1 KD caused the opposite effect on capillary content in high-fat fed females, which displayed a decrease in capillary number within visceral adipose tissue (**Figure 4.1D**). This effect was time-dependent and significant after 7 and 16 weeks of high-fat feeding (**Figure 4.1E**). This decrease in capillary content was not accompanied by lower VEGFA levels in the adipose tissue, as *Vegfa* transcript level did not change following the EC-FoxO1 KD (**Figure 4.1F**). Notably, the reduction

in capillary content was not confined to white adipose tissue. Morphological analysis of skeletal muscle indicated lower capillary to muscle fiber ratio in EC-FoxO1 KD females (**Figure 4.1 G,H**). Furthermore, gene expression analysis showed lower levels of the endothelial cell markers *Pecam1*, *Cdh5* and *Tie1* in the brown adipose tissue of EC-FoxO1 KD females compared to Control littermates after 16 weeks of high-fat diet (**Figure 4.1I**). Lower vascular content also was observed within the same tissues in normal chow-fed EC-FoxO1 KD females (**Figure S4.1 A-F**), indicating that this effect occurred independent of diet. We confirmed the discrepant effects of EC-FoxO1 KD on vascularization in females versus males, using a matched cohort of male mice. Consistent with previous finding, adipose capillary number increased in male EC-FoxO1 KD compared to Control mice after 7 and 16 weeks of high-fat diet (**Figure S4.1G**). Thus, EC-FoxO1 KD broadly impaired capillary content in the females, in stark contrast to the pro-angiogenic effects observed in these tissues in the male mice.

4.4.2 EC-FoxO1 KD promotes capillary enlargement and morphological anomalies.

Capillary diameters in the adipose of EC FoxO1 KD female mice were 3-fold larger than in Control mice after 7 and 16 weeks of high fat (**Figure 4.1 J,K**). We observed that adipose vessels in female EC-FoxO1 KD mice frequently displayed atypical morphologies, including some malformations that appeared monolayer-like and others that displayed a glomerular-like appearance (**Figure S4.2A**). Capillary diameters also increased in male mice with EC FoxO1 KD, as previously reported, but to a lesser extent than in female mice (**Figure S4.1H**). The increased vessel size following EC-FoxO1 KD also was detected in normal chow-fed mice, indicating that it is not contingent on high fat diet feeding (**Figure S4.2 B,C**). Indeed, after 16-weeks, ~12% of the adipose capillaries in female EC-FoxO1 KD mice were larger than 20 μ m diameter, while males had fewer than 4% vessels of this size (**Figure S4.2C**).

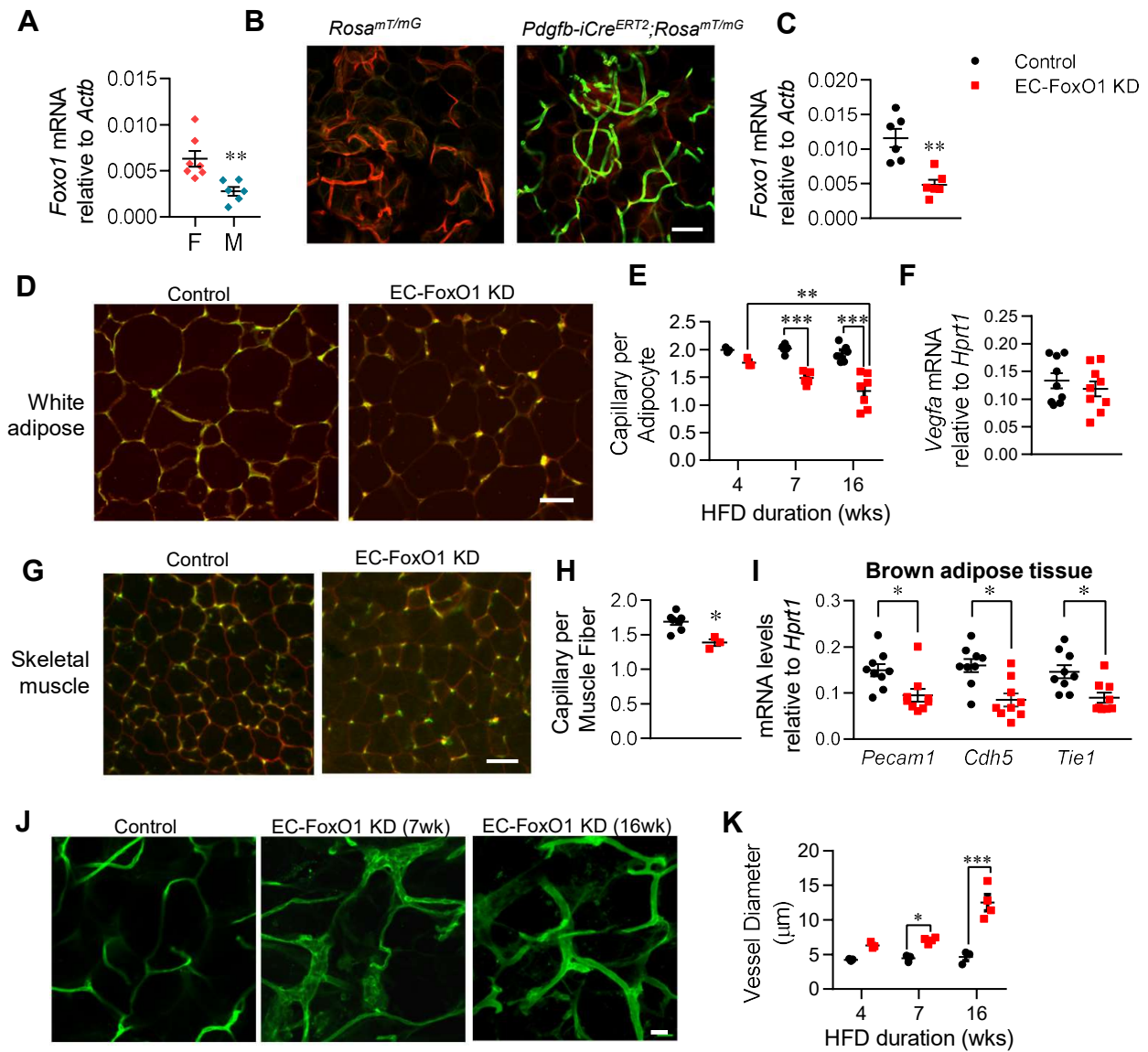


Figure 4.1 Adipose angiogenesis is repressed whereas vessel diameters enlarge following EC FoxO1 knockdown. **A)** *Foxo1* mRNA levels relative to *Actb* from freshly isolated adipose ECs (Female n=7, Male n=6) fed NC diet. **B)** Representative visceral adipose tissue images of tamoxifen-injected *Rosa^{mT/mG}* and *Pdgfb-iCre^{ERT2}; Rosa^{mT/mG}* mice. Green: GFP (expressed in cells with Cre activity); Red: tdTomato (expressed by cells lacking Cre activity). Scale bar = 50 μ m. **C)** *Foxo1* mRNA levels relative to *Actb* ($2^{-\Delta\Delta CT}$) from female EC isolated from white adipose of 7-week HF fed Control and EC-FoxO1 KD mice (n=6). **(D-I)** Control and EC FoxO1 KD mice were fed HFD for 4, 7 or 16 weeks. **D)** Representative images of paraffin-sectioned adipose from 16-week Control and EC-FoxO1 KD HFD female mice. **G.** *simplicifolia* lectin-FITC (green) and Rhodamine Wheat germ agglutinin (red) staining of capillaries and basement membranes, respectively. Scale bar = 100 μ m. **E)** Quantification of capillary to adipocyte ratios. (4-wk n=3, 7-wk Control n=6, 7-wk EC-FoxO1 KD n=5, 16-wk Control n=6, 16-wk EC-FoxO1 KD n=7) **F)** *Vegfa* mRNA levels relative to *Hprt1* from female white adipose tissue fed HF diet for 16 weeks. (n=9) **G)**

Representative images of female EDL muscle stained with *G. simplicifolia* lectin-FITC (green) and Rhodamine Wheat Germ Agglutinin (red) to visualize capillaries and muscle fibers, respectively. **H)** Quantification of female muscle capillary to muscle fiber ratios. (Control n=7, EC-FoxO1 KD n=3) **I)** Endothelial marker mRNA levels relative to *Hprt1* ($2^{-\Delta CT}$) from female brown adipose tissue (16-week HFD). (n=9) **J)** Representative images of female adipose vasculature (stained with lectin) in Control and EC FoxO1 KD mice after 7 or 16 weeks of HFD Scale bar = 50 μ m. **K)** Quantification of vessel diameter of female control and EC-FoxO1 KD mice. (4-wk n=3, 7-wk Control n=3, 7-wk EC-FoxO1 KD n=4, 16-wk Control n=3, 16-wk EC-FoxO1 KD n=4) * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

4.4.3 Reduced capillarization in EC-FoxO1 KD females is associated with adipose tissue expansion and weight gain.

Loss of endothelial FoxO1 induced eventual adipocyte hypertrophy in females. Adipocyte size in perigonadal depots of EC-FoxO1 KD mice trended to decrease after 4-week HFD, but this effect reversed over time, with significant increases in adipocyte size at the 16-week timepoint (**Figure 4.2 A,B**). This adipocyte expansion is a notable distinction from the response of male mice, wherein perigonadal adipocytes of EC FoxO1 KD mice were smaller than those of Control mice after 4 and 16 weeks of HFD (**Figure S4.3A** and (Rudnicki, et al., 2018a)). Increases in adipocyte size were detected even in female mice fed a normal chow diet for 16 weeks (**Figure S4.2 B,C**), indicating that this effect is not dependent on HFD. EC-FoxO1 KD females displayed indicators of adipose dysfunction, including elevated levels of immune cell marker *Itgax* in perigonadal adipose (**Figures 4.2C; S4.3D**) and decreased *Ucp1* transcripts in brown adipose tissue (**Figures 4.2D; S4.3E**), irrespective of diet condition. These unhealthy adaptations of the adipose tissue depots with sustained EC-FoxO1 KD were associated with increased weight gain in female mice, particularly those fed HF diet (**Figure 4.2E**). Perigonadal and brown fat depots were substantially enlarged in EC-FoxO1 KD compared to Control female mice (**Table S4.1**). Fasted plasma glucose levels were elevated in EC-FoxO1 KD compared to Control HF-fed mice (**Figure 4.2 F,I**). Although EC-FoxO1 KD did not exert a significant effect on GTT responses (**Figure 4.2 G,H**), insulin sensitivity was impaired in HF-fed EC-FoxO1 KD mice (**Figure 4.2 J,K**). Increased body weight and elevated fasting plasma glucose levels were detected even in NC-fed EC-FoxO1

KD mice (**Figure S4.3 F,G**) although responses to GTT and ITT were not altered. Overall, these findings suggest that depletion of EC-FoxO1 in females impaired adipose tissue homeostasis and disrupted whole-body glucose metabolism, which was prominent in mice challenged with HF diet. Notably, this contrasts with the improvements in these parameters previously reported in HF-fed

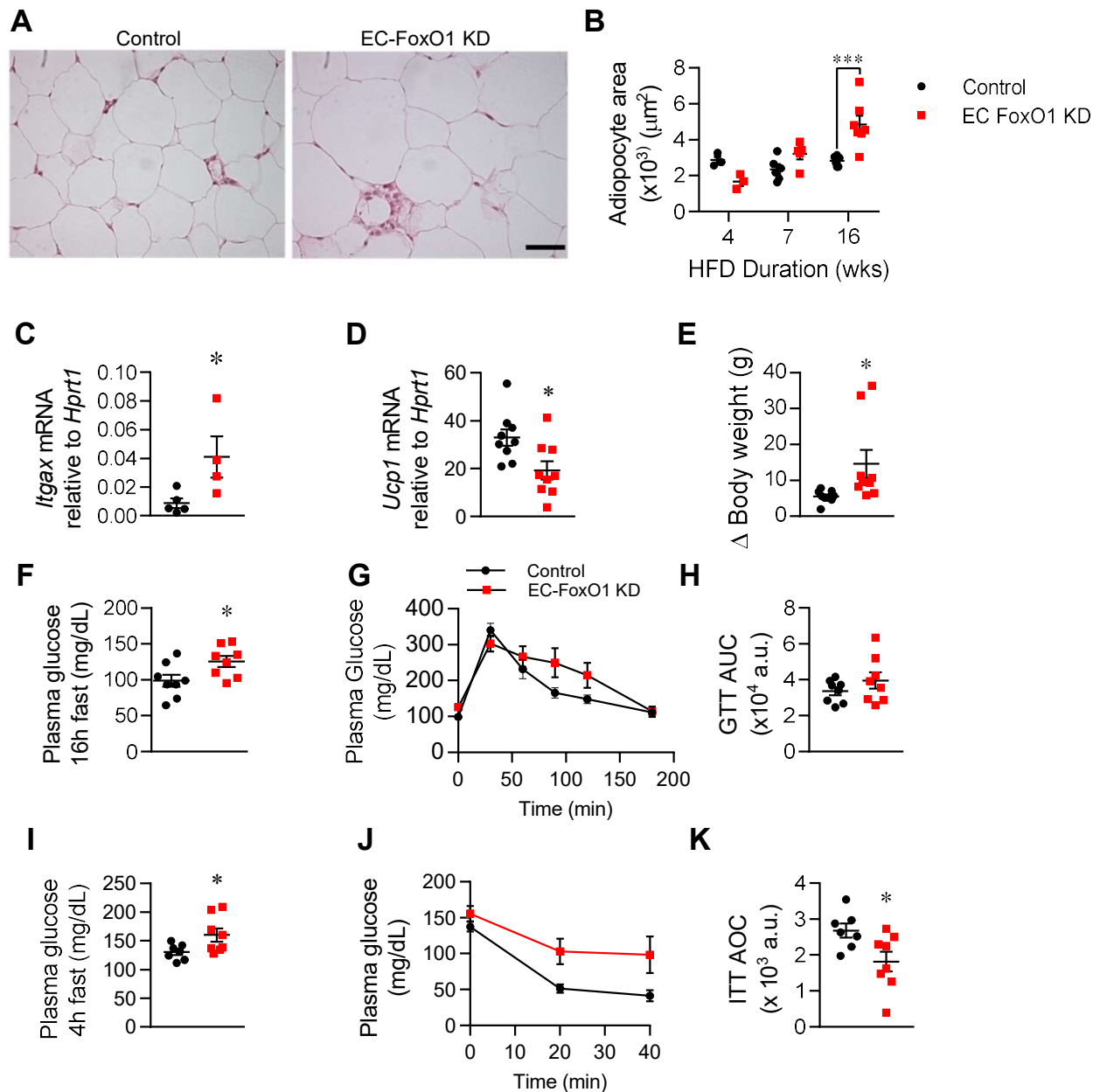


Figure 4.2 EC-specific knockout of FoxO1 causes systemic metabolic disturbances in female mice. **A)** Representative hematoxylin and eosin-stained images of perigonadal adipose tissue from 16-week HF-fed female mice. Scale bar = 50 μ m **B)** Mean adipocyte cross-sectional areas. (4-wk n=3, 7-wk Control n=6, 7-wk EC-FoxO1 KD n=5, 16-wk Control n=6, 16-wk EC-FoxO1 KD n=7) **C)** *Itgax* mRNA levels relative to *Hprt1* ($2^{-\Delta CT}$) from perigonadal adipose tissue of 16-week HF-fed female mice. (n=5) **D)** *Ucp1* mRNA levels relative to *Hprt1* ($2^{-\Delta CT}$) from brown adipose tissue of 16-week HF-fed female mice. (n=9) **E)** Total weight gain over the 16 weeks of HF diet. (Control n=10, EC-FoxO1 KD n=9) **F)** Plasma glucose levels of HF-fed Control and EC-FoxO1 KD mice after 16 hour fasting. (n=8) **G)** Glucose tolerance of HF-fed Control and EC-FoxO1 KD mice was examined by intraperitoneal glucose tolerance test after 15 weeks of HF diet and 16 hr fasting. **H)** Area under the curve. (n=8) **I)** Plasma glucose levels of HF-fed Control and EC-FoxO1 KD mice after 4 hour fasting **J)** Insulin sensitivity of HF-fed Control and EC-FoxO1 KD mice was assessed by intraperitoneal insulin tolerance test after 14 weeks of HF diet and 4 hr fasting. **K)** Area over the curve. (n=8) * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

4.4.4 FoxO1 KD provokes a dysfunctional shift in EC transcriptome

To better understand the cellular mechanisms underlying the sex-distinct effects of EC FoxO1 KD, we conducted transcriptomic profiling on adipose EC from mice that were fed high fat diet for 7 weeks. EC FoxO1 KD resulted in 1303 differentially expressed genes (DEG) in female ECs and 1006 DEGs in male ECs (Adj. P value < 0.05) (**Figure 4.3A**). Principal component analysis (PCA) showed female EC with FoxO1 KD shifted away from the signature of Control female ECs, and closer to that of Control male ECs (**Figure 4.3B**). The influence of EC FoxO1 KD on the distribution of characteristic biological pathways was assessed using gene set enrichment analysis (GSEA), which revealed substantially more biological pathways that were altered by EC FoxO1 KD in female compared to male EC.

To highlight the exaggerated impact of EC FoxO1 KD in females, the expression of core genes comprising the dominant ontologies affected in female EC was depicted by ridge plot, comparing to the expression profile of the same genes in male EC (**Figure 4.3C**) Notably, EC FoxO1 KD caused significant under-representation of proliferation, mitochondrial function and DNA repair gene sets only in females. Furthermore, EC-FoxO1 KD in female mice provoked the

enrichment of pathways relating to reactive oxygen species, senescence associated secretory phenotype (SASP) and cell shape regulation, which were not significantly affected in male ECs. Pathways associated with inflammation and immune responses were enriched in both sexes with EC FoxO1 KD, albeit to a greater extent in females. Translation-associated and ribosome structural genes were significantly repressed in EC-FoxO1 KD versus Control mice of both sexes. Altogether, the transcriptome signatures indicate that the depletion of EC FoxO1 exerts a more robust and broadly disruptive influence on cellular functions in female compared to male ECs.

Considering the extensive literature documenting FoxO1 as a potent repressor of proliferation, we were intrigued that depletion of EC FoxO1 caused suppression rather than enrichment of a proliferative transcriptomic signature. Accordingly, we assessed EC proliferation in perigonadal adipose *in vivo*. Consistent with the transcriptomic profile, EdU incorporation revealed a significantly lower number of proliferating EC in the adipose of female EC-FoxO1 KD mice compared to the Controls after 7-week HFD. In contrast, the EC proliferative index was not affected in male EC-FoxO1 KD mice at this time point (**Figure 4.3 D,E**).

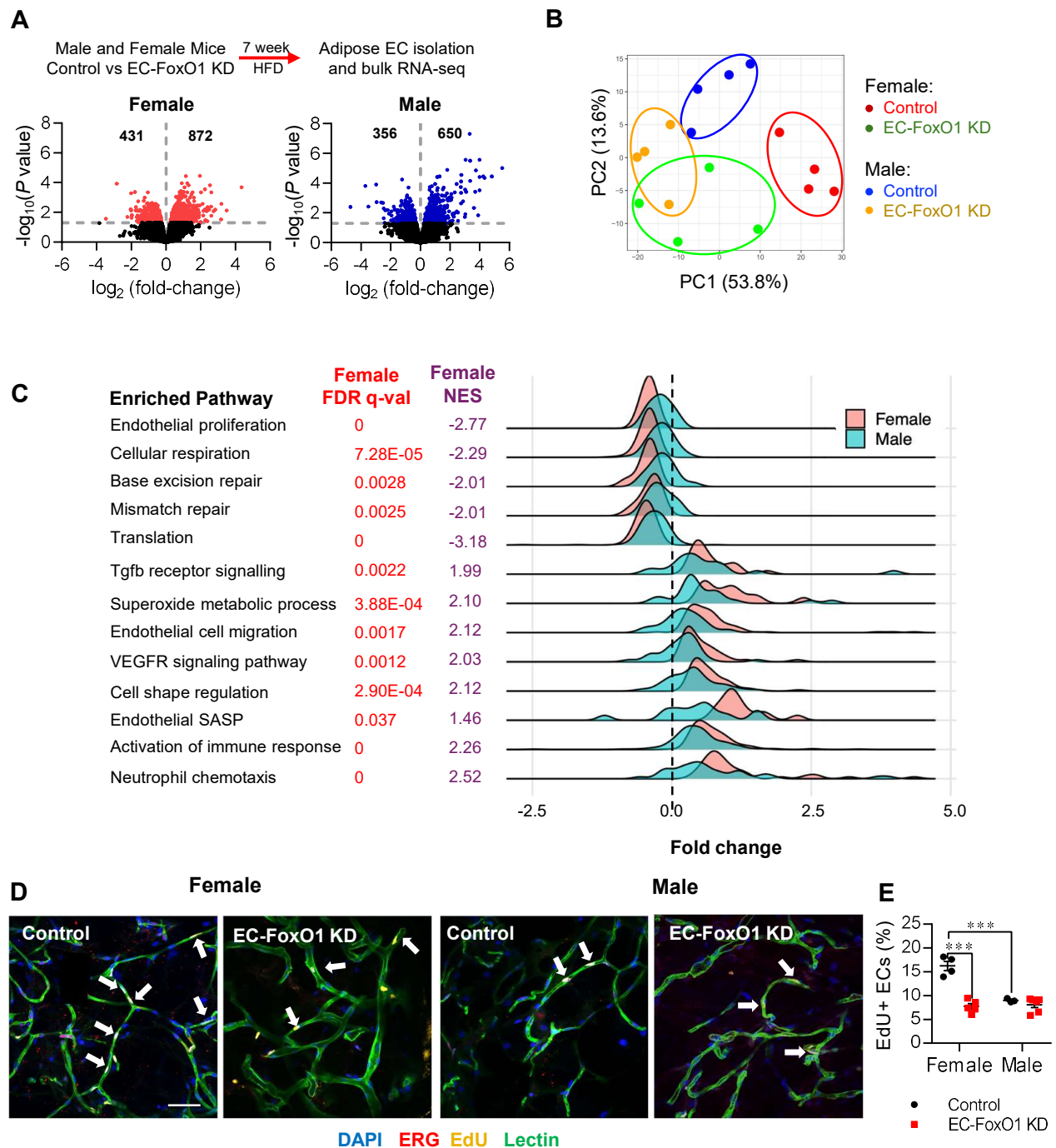


Figure 4.3 Endothelial specific FoxO1 knockout causes transcriptomic shift following 7-week HFD. **A)** Volcano plot illustrating the expression profile of genes following EC-FoxO1 KD in both female and male adipose EC, as determined by RNA-seq (n = 4 mice per group). A total of 1,323 and 1,006 DEGs were identified in the female and male EC, respectively, with an adjusted p value < 0.05 (indicated by the dashed horizontal line). **B)** Principal component analysis (PCA) of male and female control and KD ECs.

Samples are graphed on the PCA plot based on PC1 and PC2. Female DEGs were used to create the PCA. **C)** GSEA was performed on female EC genes to identify pathways altered by FoxO1 deletion along with their corresponding FDR q-values. These pathways' core enrichment genes are compared for male and female EC. Endothelial SASP and Endothelial proliferation pathways were custom as described in iScience, 2023. All other pathways were combined from Gene Ontology Biological Processes and Kegg pathways. **D)** Representative images of visceral adipose tissue and EdU incorporation EC-FoxO1 KD and 7 weeks of HFD. Scale bar = 50 μ m. **E)** EdU-positive cells were quantified as a percentage of total EC number. (Female Control n 4, Female EC-FoxO1 KD n=5, Male Control n=3, Male EC-FoxO1 KD n=6) * P <0.05; ** P <0.005; *** P <0.0005.

4.4.5 Proliferation is repressed by long-term EC-FoxO1 inhibition

Considering that the *in vivo* response to EC-FoxO1 KD reflects a cumulative series of cellular adaptations over multiple weeks, we turned to cultured cells to investigate the direct and cell-autonomous consequences of altered FoxO1 levels on EC phenotype. Importantly, we found that the female ECs maintained higher FoxO1 protein levels than passage-matched male EC even after multiple passages in culture (**Figure 4.4A**). Female EC also exhibited higher transcriptional activity of a forkhead response element luciferase reporter construct versus male EC (**Figure 4.4B**), supporting the role of FoxO1 as a modulator of sex-specific transcriptomes in ECs.

To compare the effects of short-term and long-term FoxO1 inhibition on proliferation, cells plated at low confluency were cultured in growth media for 6 days, in the presence of FoxO1 inhibitor AS1842856 for the entire duration (6d), or for the final 24 hours (1d) (**Figure 4.4C**). Proliferation in EC from both sexes increased following 1d FoxO1 inhibition (**Figure 4.4 D,E**), consistent with prior studies. However, this increase was suppressed in both female and male EC following sustained 6-day FoxO1 inhibition. A similar pattern was detected for mRNA levels of cell cycle marker *Mki67*, which increased following 1 day, but not 6 days, of FoxO1 inhibition (**Figure 4.4F**). In accordance with the decrease of proliferation with 6-day FoxO1 inhibitor, both male and female cultured ECs exhibited increased transcript levels of *Cdkn1a* (p21) (**Figure S4.4A**) as well as a greater intensity of nuclear-localized p21 protein (**Figure 4.4 G,H**). These data provide evidence that sustained FoxO1 inhibition represses EC proliferation in both sexes.

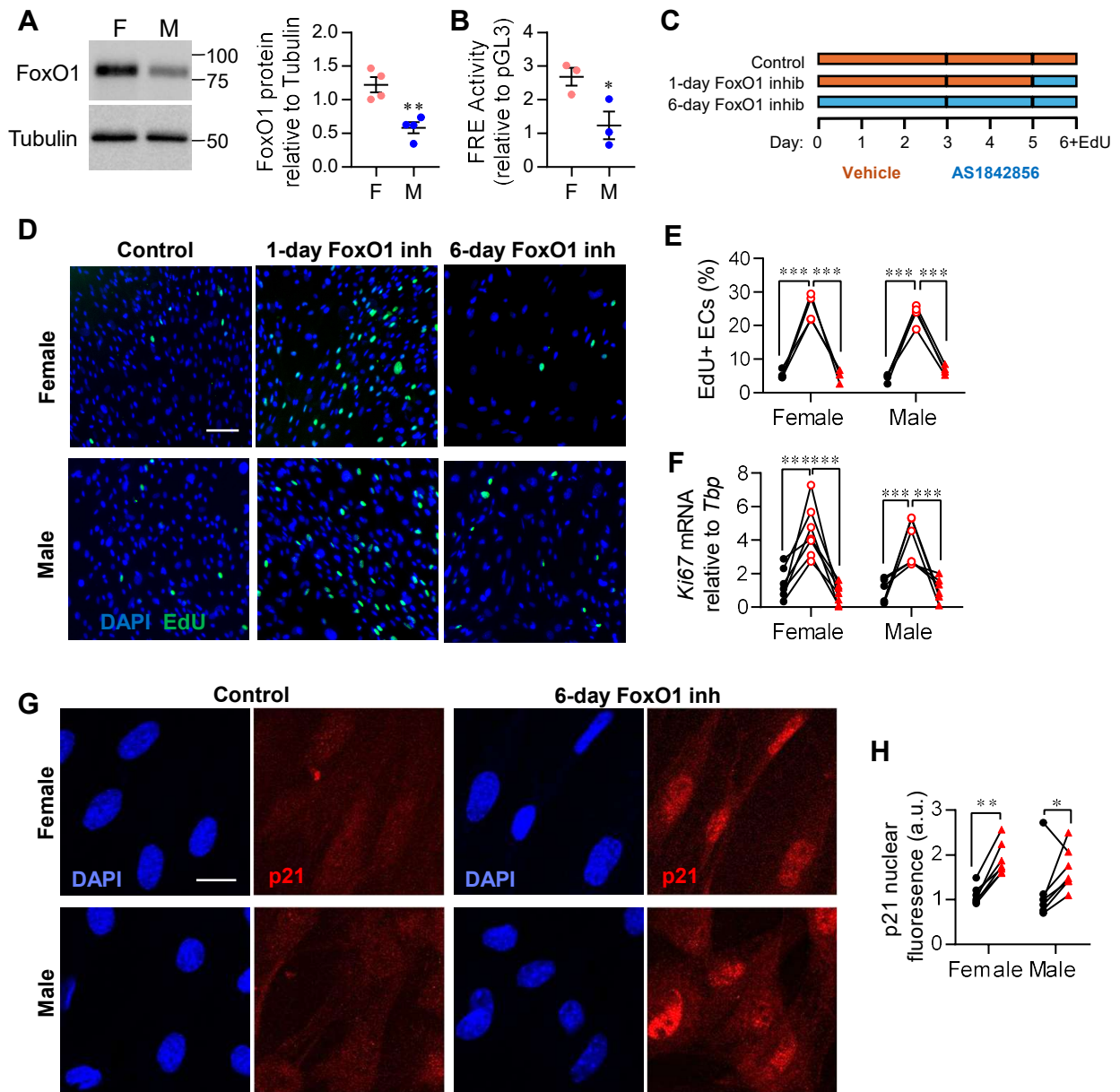


Figure 4.4 FoxO1 inhibition decreases EC cell proliferation and leads to cell cycle arrest *in vitro*. **A)** FoxO1 protein levels relative to Tubulin from cultured adipose ECs (n=4) **B)** Luciferase activity of FoxO-responsive element relative to pGL3 luciferase reporter (n=3) **C)** Schematic of 1-day and 6-day FoxO1 inhibition (AS1842856, 1 μ M) in cultured EC. **D)** Representative images of cultured EC following control (vehicle), 1-day FoxO1 inhibition, or 6-day FoxO1 inhibition, and 4 hours of EdU incorporation. Scale bar = 100 μ m. **E)** EdU-positive cells were quantified as a percentage of total EC number. (n=4) **F)** mRNA level of *Ki67* following FoxO1 inhibitor treatment normalized to housekeeping *Tbp* ($2^{-\Delta CT}$). (Female n=7, Male n=6) **G)** Representative images of 6-day FoxO1 inhibitor-treated male and female EC stained with p21 antibody and DAPI **H)** p21 nuclear fluorescence intensity quantified relative to a female control (n=6). * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

4.4.6 *Tgfb* signaling and a pro-senescent phenotype increases only in female ECs after prolonged FoxO1 signalling disruption

The GSEA analysis indicated enrichment of TGF β signaling in EC from female FoxO1 KD mice, despite previous research showing upregulation of *Tgfb1* mRNA with FoxO1 deletion (Kurakazu et al., 2019; Pan et al., 2023; Ponugoti et al., 2013). Considering that TGF β signaling may promote cell cycle arrest, senescence and inflammation in various contexts, we tested if *Tgfb1* was affected by FoxO1 silencing in cultured EC. Interestingly, *Tgfb1* mRNA were elevated in female ECs but reduced in the male EC after 24 hours of FoxO1 siRNA treatment (which lowered FoxO1 mRNA levels by ~60% amount) (**Figures 4.5A; S4.4B**). Accordingly, nuclear levels of the active phosphorylated form of Smad2, a transcription factor downstream of TGF β receptor (Alk5) signaling, increased only in the female ECs during sustained FoxO1 inhibition (**Figure 4.5 B,C**). Interestingly transcript levels of *Hmgbl*, a protein that can promote *Tgfb1* expression (W. J. Lee et al., 2018), displayed the same sex-divergent response as *Tgfb1*, with elevated levels only in female EC after FoxO1 silencing but decreased in male EC (**Figure 4.5D**). Despite reports that HMGB1 and TGF β 1 both can induce inflammation signaling (Wahid et al., 2021; H. Zou et al., 2021), we could not detect altered expression of other inflammatory response genes in response to FoxO1 siRNA treatment (**Figure S4.4C**). HMGB1 and TGF β 1 also are known to upregulate senescence pathways (Bai et al., 2017; Ueda et al., 2021). We observed that EC nuclear size (which reflects overall cell size (Cantwell & Nurse, 2019; Wu et al., 2022)) increased in both sexes following 6-day FoxO1 inhibition (**Figure 4.5E**). This effect on nuclear size also was detected in female EC that were treated with FoxO1 siRNA (**Figure S4.4D**). Increased cell size may occur due to extended cell cycle arrest and/or senescence. Additionally, no changes in phosphorylation of p70S6K or S6K (effectors of the mTOR protein synthesis pathway) were detected in FoxO1 siRNA-treated cells (**Figure S4.4 E-G**) suggesting that senescence rather than protein synthesis

may cause this increase in cell size. In line with this, we detected an increase in the number of p16-expressing EC, and overall level of nuclear p16, with 6-day FoxO1 inhibition (**Figures 4.5 F-H; S4.4H**), consistent with a shift towards senescence. The increase in p16 was significantly greater in female EC. Thus, our findings support the concept that sustained FoxO1 inhibition exerts a pro-senescent phenotype is induced only in the female EC.

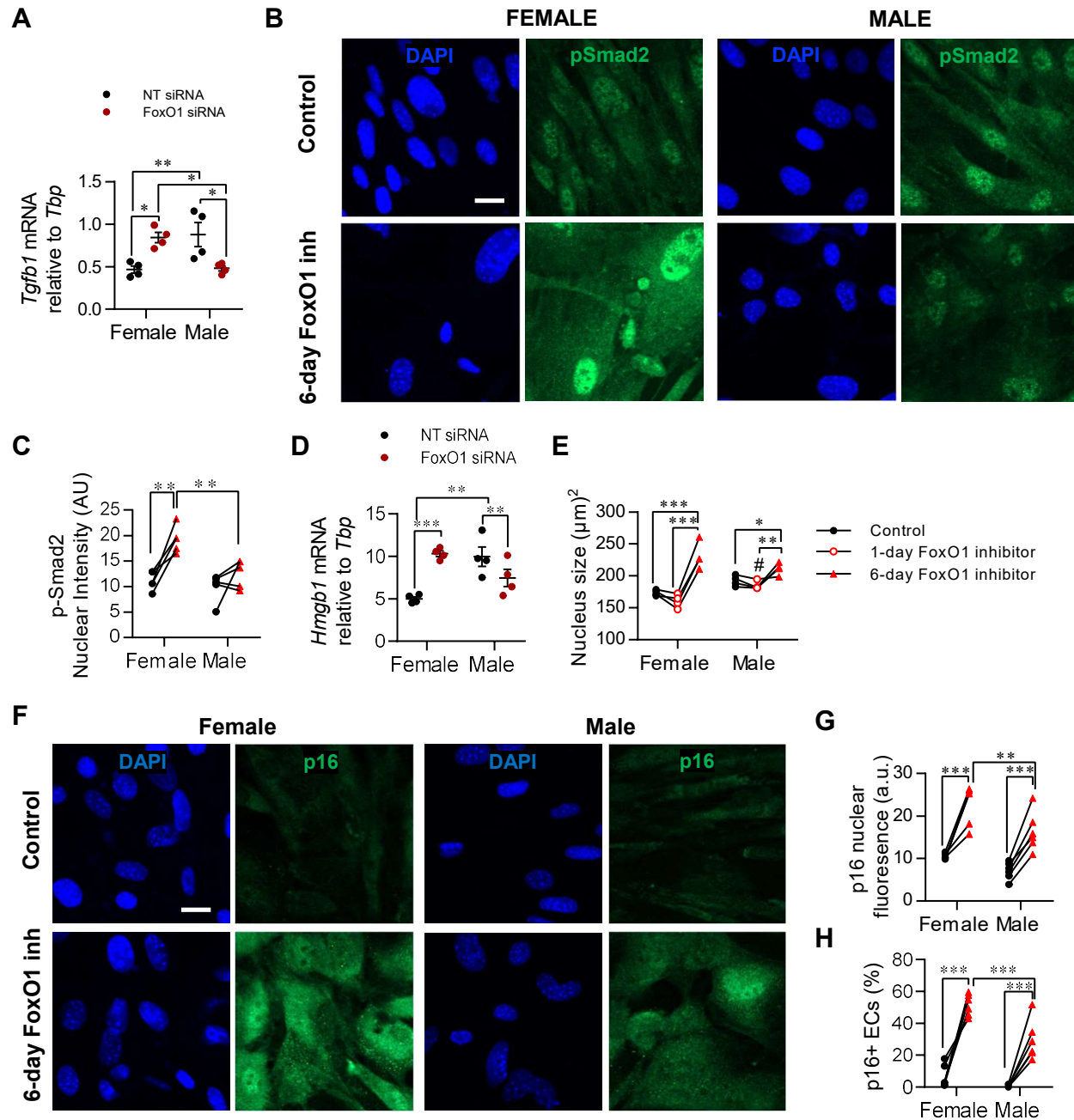


Figure 4.5 Tgfb signaling and pro-senescent phenotype in females with FoxO1 disruption. **A)** mRNA level of *Tgfb1* in cultured adipose EC treated with non-targeting (NT) siRNA or FoxO1 siRNA for 24 hours and 2 hours serum starved. Genes normalized to housekeeping *Tbp* ($2^{-\Delta CT}$). (n=4) **B)** Representative images of 6-day FoxO1 inhibitor-treated male and female EC stained with pSmad2 antibody and DAPI. Scale bar = 20 μ m **C)** pSmad2 fluorescence intensity quantified relative to a female control. (n=5) **D)** mRNA level of *Hmgb1* in cultured adipose EC treated with non-targeting (NT) siRNA or FoxO1 siRNA for 24 hours and 2 hours serum starved. Genes normalized to housekeeping *Tbp* ($2^{-\Delta CT}$). (n=4) **E)** Nuclear size of cultured EC following 1-day and 6-day FoxO1 inhibitor. (n=4) **F)** Representative images of 6-day FoxO1 inhibitor-treated male and female EC stained with p16 antibody and DAPI. Scale bar = 20 μ m **G)** p16 fluorescence intensity quantified relative to a female control. (n=6) **H)** p16-positive cells were quantified as a percentage of total EC number. (n=6). * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

4.4.7 Mitochondrial functions are impaired by sustained inhibition of FoxO1 in female EC.

Considering that cellular proliferation is highly dependent on metabolic flux, we determined if FoxO1 inhibition altered cellular ATP production in cultured EC. Glycolytic ATP production increased in both sexes with 1-day FoxO1 inhibition (**Figure 4.6A**), consistent with previous studies (Rudnicki, et al., 2018a; Sun et al., 2022; Wilhelm et al., 2016) and the increased proliferation seen at this time-point. However, sustained 6-day FoxO1 inhibition decreased glycolytic ATP in both male and female EC (**Figure 4.6A**). Notably, mitochondrial ATP production was suppressed in female EC by both 1-day and 6-day FoxO1 inhibition whereas it was unaltered in male EC (**Figure 4.6A**). Based on the transcriptomic data and the metabolic profile, we expected to detect a concordant downregulation in mitochondrial genes following 6-day FoxO1 inhibition. In contrast, transcripts encoding the mitochondrial electron transport chain components, *Cox7a1*, *Cox7c*, and *CytB*, were elevated in both sexes following 6-day FoxO1 inhibition (**Figure S4.5A**) Because mitochondrial function is strongly dependent on the extent of its network organization (Glancy et al., 2020), we visualized mitochondria in control and FoxO1 inhibitor treated cells. Mitochondria appeared as distinct fused networks in Control cells (**Figure 4.6B**). In females, these networks were disrupted following 6-day FoxO1 inhibition, as indicated by more diffuse staining and a decrease in mitochondrial network lengths. In contrast, the

mitochondrial network lengths increased in male EC with FoxO1 inhibition (**Figure 4.6C**), further reinforcing the evidence that FoxO1 disruption effects are sex specific.

Excessive production of superoxides, and concomitant oxidative stress, may occur due to disruption of mitochondrial structure (Z. Zhang et al., 2023). Indeed, enrichment of superoxide metabolism ontologies was detected in the transcriptome of adipose ECs from female FoxO1 KD mice (**Figure 4.3C**). The antioxidant genes *Prdx3* and *Gpx8* were upregulated in response to 6-day FoxO1 inhibition (**Figure S4.5B**), consistent with a cellular response to elevated ROS. Due to the challenges of direct detection of ROS, we assessed cellular levels of 4-hydroxy-2-nonenal (4HNE)-protein adducts, which is a long-lasting marker of lipid peroxidation. Levels of 4HNE were minimally detectable female EC under control conditions but increased substantially following 6-day FoxO1 inhibition (**Figure 4.6 D,F**). Interestingly, male EC displayed higher basal levels of 4HNE but FoxO1 inhibition did not alter 4HNE levels.

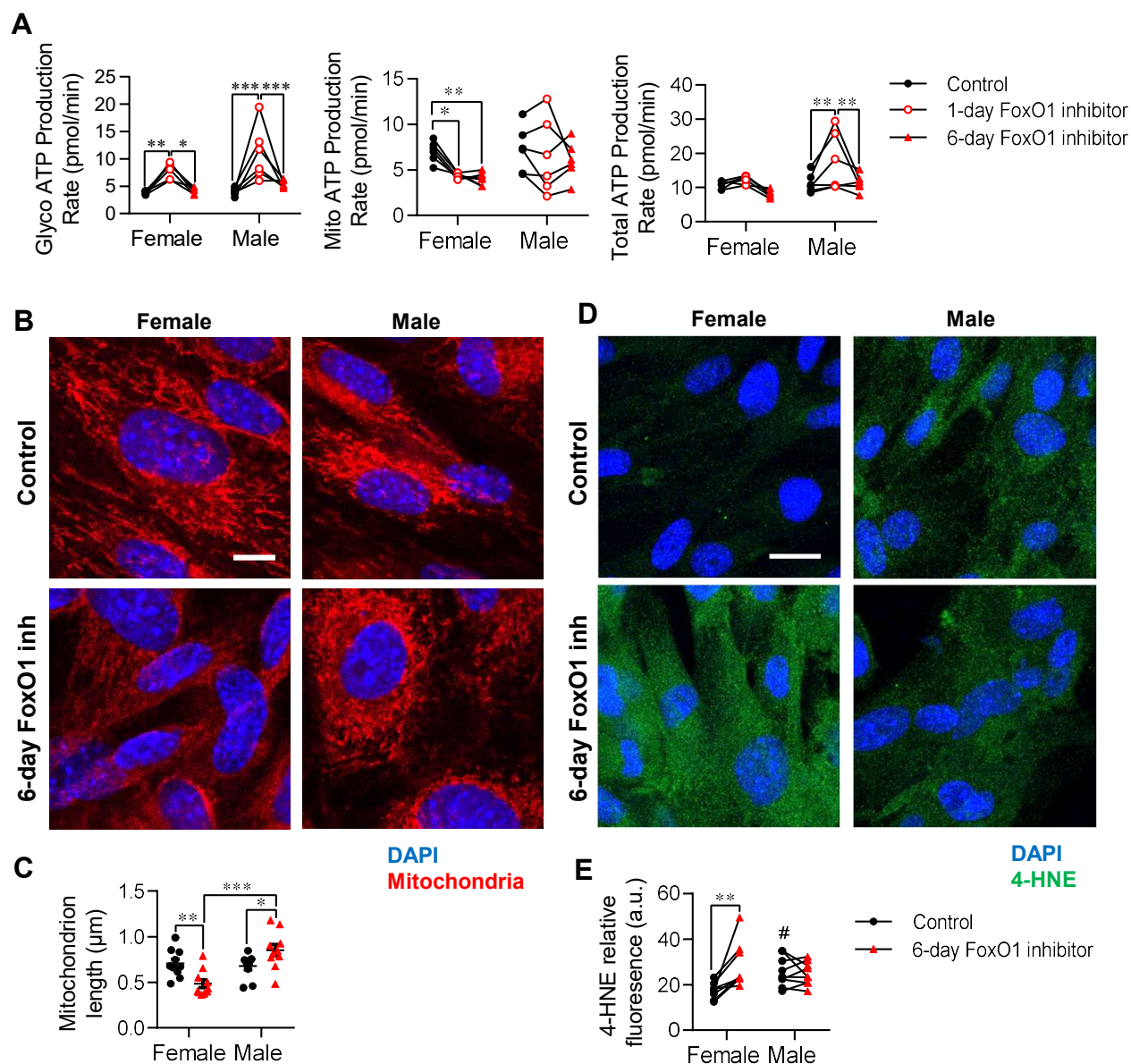


Figure 4.6 FoxO1 inhibition disrupts mitochondrial function in female EC. Cultured adipose EC were treated with FoxO1 inhibitor (AS1842856, 1 μM) or vehicle for 1 or 6 days. **A**) Mitochondrial, glycolytic and total ATP production rates were assessed using Seahorse and normalized to DNA content. (n=6) **B**) Representative images of EC following 6-day FoxO1 inhibition, stained with MitoBrilliant (red) and DAPI (blue). Scale bar = 10 μm . **C**) Quantification of mitochondrion lengths (n=10). **D**) Representative images of cultured EC following 6-day FoxO1 inhibition, stained with 4-HNE (green) and DAPI (blue). Scale bar = 20 μm . **E**) Quantification of relative intensity of 4-HNE staining. (Female n=8, Male n=9). * P <0.05; ** P <0.005; *** P <0.0005; # P <0.05 vs Female Control.

4.4.8 Long term FoxO1 inhibition evokes DNA damage through mitochondrial ROS in female EC.

Transcript levels of base excision and mismatch repair ontologies were significantly downregulated in the EC transcriptome signatures from female EC-FoxO1 KD mice, but not males (**Figure 4.3C**). In line with this, nuclear levels of the tumour suppressor p53, which is activated in response to DNA damage, were elevated in both female and male EC following 6-day FoxO1 inhibition in cultured EC (**Figure 4.7 A,B**). Interestingly, sustained FoxO1 inhibition in cultured EC caused an increase in expression of the DNA repair gene *Gadd45a* expression in both female and male ECs, but a greater increase in the males suggesting a greater DNA-damage repair mechanism in the absence of FoxO1 (**Figure 4.7C**). Although Gadd45 is a prototypic transcriptional target of FoxO1, it is also regulated by other factors such as the tumor suppressor p53.

Next, we assessed evidence of DNA damage as indicated by γ H2AX immunostaining (**Figure 4.7D**). Notably, the number of γ H2AX⁺ cells increased in female but not male ECs following 6-day FoxO1 inhibition (**Figure 4.7E**). Female EC also exhibited a greater portion of cells with extensive γ H2AX foci (10 or more per cell) following FoxO1 inhibition. Similarly, FoxO1 siRNA treatment (3 days) induced higher γ H2AX protein levels in female compared to male EC (**Figure S4.6 A-C**). Moreover, FoxO1 inhibition in led to more γ H2AX fluorescence in both sexes, but to a greater extent in female ECs (**Figure 4.7F**). We also noted that nuclear size positively correlated with the extent of γ H2AX foci per cell (**Figure S4.6D**).

We postulated that excess mitochondrial ROS production in female EC might be an upstream instigator of the accrual of DNA damage. We tested if co-treatment of cells with the mitochondrial superoxide scavenger MitoTEMPO could mitigate the deleterious effects of FoxO1 inhibition in female ECs. MitoTEMPO, which was effective in lowering ROS levels in cells treated

with the oxidant menadione (**Figure S4.6E**), did decrease the extent of γ H2AX foci in FoxO1 inhibitor-treated female EC to almost baseline levels (**Figure 4.7G**), and tended to lower the intensity of γ H2AX fluorescence (**Figure 4.7H**). There was also a trend for nuclear size to decrease with MitoTEMPO (**Figure S4.6F**). In contrast, MitoTEMPO co-treatment did not rescue the effects of FoxO1 inhibition on basal mitochondrial ATP production (**Figure S4.6G**) nor did it rescue proliferation in FoxO1 inhibitor-treated EC (**Figure S4.6H**). Thus, mitochondrial ROS may underlie some but not all the adverse effects downstream of sustained FoxO1 KD or inhibition (**Figure 4.7I**).

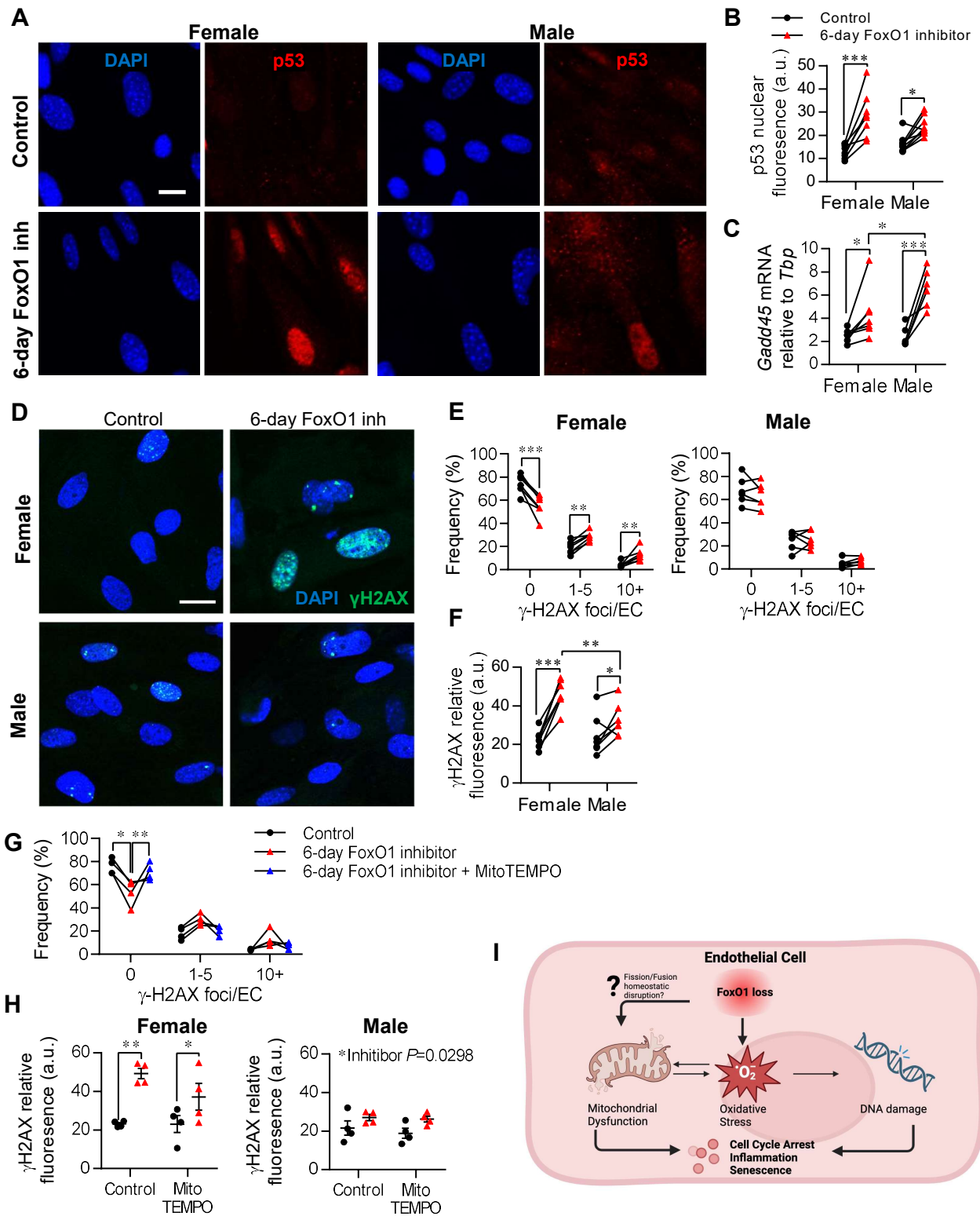


Figure 4.7 FoxO1 disruption increases nuclear size and DNA damage marker γ H2AX in female EC. **A)** Representative images of 6-day FoxO1 inhibitor-treated male and female EC stained with p53 antibody and DAPI. **B)** p53 fluorescence intensity quantified relative to a female control. (Female n=8, Male n=9) **C)** mRNA level of *Gadd45* following FoxO1 inhibitor treatment normalized to housekeeping *Tbp* ($2^{-\Delta CT}$). (Female n=7, Male n=6) **D)** Representative images of 6-day FoxO1 inhibitor EC stained with DAPI and γ H2AX. Scale bar = 20 μ m. **E)** Quantification of γ H2AX foci per EC from **D**. (Female n=7, Male n=6) **F)** γ H2AX nuclear fluorescence intensity (n=7). **G)** Cultured ECs were treated with FoxO1 inhibitor in combination with the antioxidant MitoTEMPO for 6 days, then γ H2AX foci per EC female were quantified (n=4). **H)** γ H2AX nuclear fluorescence intensity following MitoTEMPO treatment (n=4). **I)** Summary schematic of FoxO1 signaling disruption within female EC. * P <0.05; ** P <0.005; *** P <0.0005. Figure was created in Biorender.

4.5 Discussion

Female mice exhibit resistance to obesity-related metabolic dysfunction, a phenotype previously attributed to preserved endothelial cell (EC) health and sustained angiogenesis during perigonadal white adipose tissue expansion (Rudnicki, et al., 2018b; Rudnicki et al., 2023). Here, we establish FoxO1 as a cornerstone of that phenotype. This concept is based on the findings that EC-specific FoxO1 knockdown in female mice resulted in an unexpected, marked reduction in capillary number across multiple tissues and aberrant vessel morphology, concomitant with metabolic disturbances in response to high-fat diet. Mechanistically, both pharmacological inhibition and genetic deletion of FoxO1 in female EC resulted in reduced mitochondrial ATP production, elevated oxidative stress and lipid peroxidation, and DNA damage accumulation. These cellular stress responses were accompanied by increased EC size, dysregulation of TGF β signaling and the emergence of a pro-senescent phenotype. Taken together, these findings position FoxO1 as a central transcriptional regulator of health and stress resilience in female EC under metabolic challenge.

Although FoxO1 is a well-known gatekeeper of endothelial quiescence and its deletion is commonly associated with increased endothelial proliferation and angiogenesis, our data challenge this model in a sex-specific context (Ding et al., 2023; Furuyama et al., 2004; S. Y. Kim et al., 2016; Niimi et al., 2020; Ogunsina et al., 2023; J.-H. Paik et al., 2007; Rudnicki, et al., 2018a; Scallan et al., 2021; Shi et al., 2018; Wilhelm et al., 2016). In female mice, FoxO1 knockdown suppressed angiogenesis, as evidenced by a progressive decline in capillary number within the perigonadal adipose tissue between 4- and 16-weeks post-knockdown. This angiogenic impairment occurred alongside paradoxically increased vessel diameter, suggesting a time-dependent deterioration of vascular architecture. Supporting this, we found that short-term FoxO1

inhibition in cultured EC transiently increased proliferation in both male and female EC, in line with published observations. However, sustained FoxO1 inhibition upregulated cell cycle inhibitors p21 and p53, halting proliferation in EC from both sexes. This finding is consistent with the work of Dharaneeswaran et al, who reported G1 cell cycle arrest in human umbilical vein EC following FoxO1 silencing (Dharaneeswaran et al., 2014).

Despite the sex-independent effects detected in vitro, only female EC exhibited a sustained decline in proliferation and capillary maintenance over time in vivo. This disparity suggests a heightened reliance on FoxO1 in female EC to sustain both metabolic and structural homeostasis. Supporting this, we found that female EC express higher levels and transcriptional activity of FoxO1 than male counterparts, despite an intrinsically greater proliferative capacity. Moreover, in vivo deletion of FoxO1 elicited a distinct dysfunctional shift in the female EC transcriptome, uniquely marked by mitochondrial dysfunction, DNA damage and oxidative stress accumulation and ultimately a pro-senescent phenotype. These detrimental impacts of FoxO1 deletion in female EC could be explained, at least in part, by their higher reliance on the mitochondria, as well as their higher mitochondrial content, as we along with others have reported that female cells display more mitochondria and oxidative phosphorylation machinery, and higher mitochondrial membrane potential than their male counterparts (Damacena de Angelis et al., 2022, Rudnicki et al., 2023). Notably, short-term FoxO1 suppression impaired mitochondrial ATP production selectively in female EC, and long-term inhibition of FoxO1 further led to mitochondrial fragmentation, a structural hallmark associated with reduced ATP efficiency and increased superoxide production (Bhatti et al., 2017; H. Chen et al., 2005; Machiela et al., 2020). These mitochondrial changes were accompanied by increased oxidative stress and accumulation of the DNA damage marker γ -H2AX. The extent of oxidative stress and γ H2AX foci in female ECs was alleviated by the mitochondrial-

specific antioxidant mitoTEMPO, providing support for the concept that mitochondrial dysfunction contributed largely to the ROS and DNA damage during sustained FoxO1 inhibition.

Given the well-established roles of DNA damage and mitochondrial dysfunction in the induction of cellular senescence (Martini & Passos, 2023; Rodier et al., 2009; Shreeya et al., 2024; Tripathi et al., 2021), it is not surprising that FoxO1-deficient female ECs developed a pro-senescent phenotype. This was characterized by the activation of SASP-associated pathways *in vivo* and increased nuclear p16INK4 and cell enlargement *in vitro*. Increased cell size has been linked to increased vascular diameter (Klems et al., 2020), as well as to cellular senescence, enhanced susceptibility to DNA damage and permanent cell cycle exit (Khurana et al., 2023). Interestingly, mitoTEMPO did not rescue EC proliferation, suggesting that mitochondrial ROS-mediated damage is necessary but not sufficient to drive the senescent phenotype. Consistently, we detected numerous pro-senescence factors (such as TGFb, Notch) to be selectively upregulated in female cells with FoxO1 inhibition, potentially amplifying the senescent transition. Further work is needed to elucidate underlying mechanisms. Nonetheless, the amplified accumulation of mitochondrial dysfunction, oxidative stress, and DNA damage in FoxO1-deficient female EC likely underlies their impaired angiogenic capacity and contributes to the broader metabolic deterioration observed in the female mice.

More broadly, our findings reinforce the concept that impaired vascularization of adipose and skeletal muscle tissues contributes causally to obesity-related metabolic dysfunction. EC-FoxO1-KD female mice exhibited increased adiposity and glucose intolerance, phenotypes that stand in sharp contrast to those of male counterparts, where EC-FoxO1 knockdown enhanced tissue capillarization and improved metabolic outcomes. Notably, the EC transcriptome of FoxO1-deficient females shifted to resemble that of Control males, who are more vulnerable to HFD-

induced vascular and metabolic dysfunction. These observations support a model in which FoxO1 acts as a sex-specific regulator of EC resilience and vascular health, and by extension, of systemic metabolic homeostasis.

In summary, our study uncovers a sex-biased requirement for FoxO1 in EC, with female EC relying more heavily on this transcription factor to preserve mitochondrial integrity, prevent senescence, and maintain angiogenic potential. Together, these findings reveal significant roles for FoxO1 in coordinating cellular and architectural aspects of angiogenesis in females and advance our understanding of sex-specific endothelial biology, highlighting the need for sex-informed approaches in the study and treatment of cardiovascular and metabolic diseases. While our study focused the contribution of FoxO1 signaling to the maintenance of a healthy EC phenotype in females under obesogenic conditions, these insights may have broader implications for understanding sex differences in vascular aging and disease susceptibility. Future studies should aim to elucidate the upstream regulators of FoxO1 expression in female EC, and to define the molecular circuits linking sex hormones, metabolic cues, and endothelial homeostasis.

4.6 Supplementary

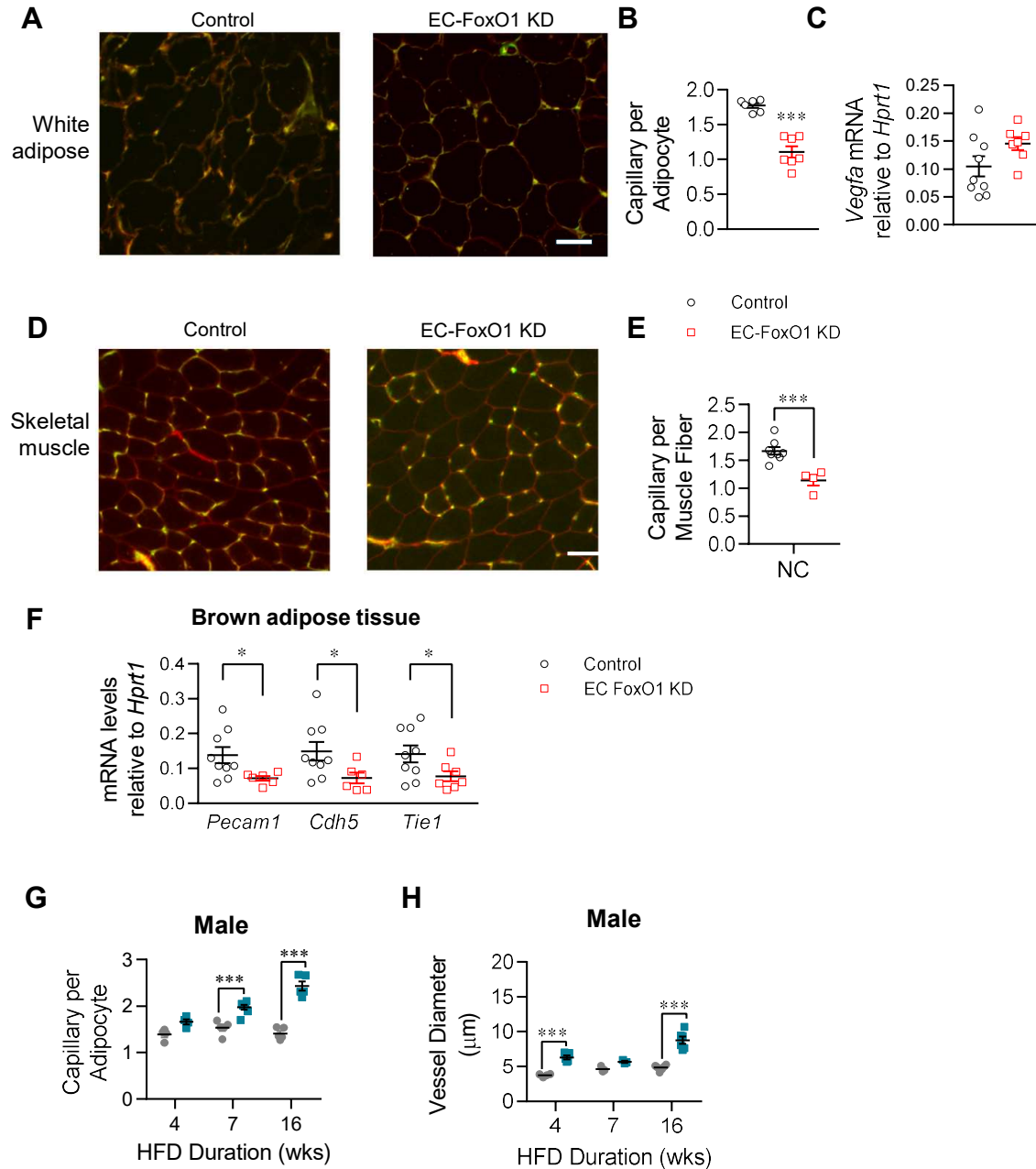


Figure S4.1 Capillary content decreases with EC FoxO1 knockdown in NC-fed female mice. Control and EC FoxO1 KD female mice were fed NC for 16 weeks following tamoxifen injection. **A)** Representative images of paraffin-sectioned adipose from 16-week Control and EC-FoxO1 KD HFD female mice. *G. simplicifolia* lectin-FITC (green) and Rhodamine Wheat germ agglutinin (red) staining of capillaries and basement membranes, respectively. Scale bar = 100 μm . **B)** Quantification of capillary to adipocyte ratios. (Control $n=6$, EC-FoxO1 KD $n=7$) **C)** *Vegfa* mRNA levels relative to *Hprt1* ($2^{-\Delta\text{CT}}$) from perigonadal adipose tissue. (Control $n=9$, EC-FoxO1 KD $n=7$) **D)** Representative images of female EDL muscle stained with *G. simplicifolia* lectin-FITC (green) and Rhodamine Wheat Germ Agglutinin (red) to visualize capillaries and muscle fibers, respectively. Scale bar = 100 μm , and **E)** quantification of capillary to fiber

ratios. (Control n=8, EC-FoxO1 KD n=4) **F**) Endothelial marker mRNA levels relative to *Hprt1* ($2^{-\Delta CT}$) from female brown adipose tissue. (Control n=9, EC-FoxO1 KD n=6) **G** and **H**) Quantification of male HFD capillary to adipocyte ratio (4-wk n=4, 7-wk Control n=5, 7-wk EC-FoxO1 KD n=7, 16-wk n=5) and adipose capillary diameters (4-wk Control n=4, 4-wk EC-FoxO1 KD n=5, 7-wk n=3, 16-wk n=6) in HFD-fed mice (16 week data from Rudnicki et al, eLife 2018). Scale bars in A, D = 50 μ m. * P <0.05; ** P <0.005; *** P <0.0005.

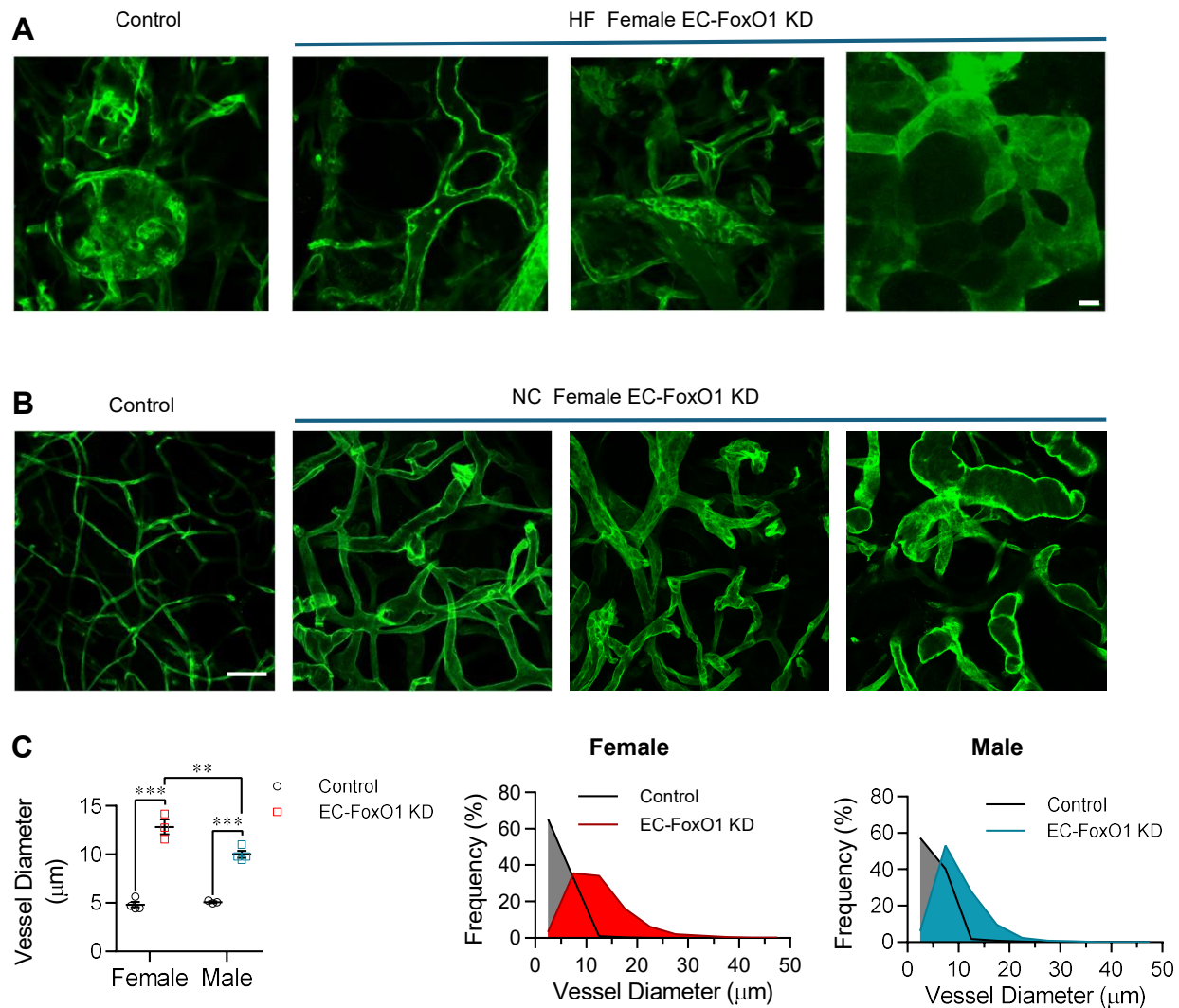


Figure S4.2 Adipose vessel abnormalities and diameter enlargement following EC FoxO1 knockdown are diet independent. **A**) Representative images of female adipose vasculature (stained with lectin) following EC FoxO1 KD and 16 weeks of HF diet. **B**) Control and EC FoxO1 KD mice were fed NC for 4, 7 or 16 weeks. Representative images of female adipose vasculature (stained with lectin) following 16 weeks of NC diet. Scale bar = 50 μ m **C**) Quantification of vessel diameter of NC-fed female and male mice 16 weeks post EC-FoxO1 deletion. (F Control n=3, F EC-FoxO1 KD n=4, M Control n=4, M EC-FoxO1 KD n=3). * P <0.05; ** P <0.005; *** P <0.0005

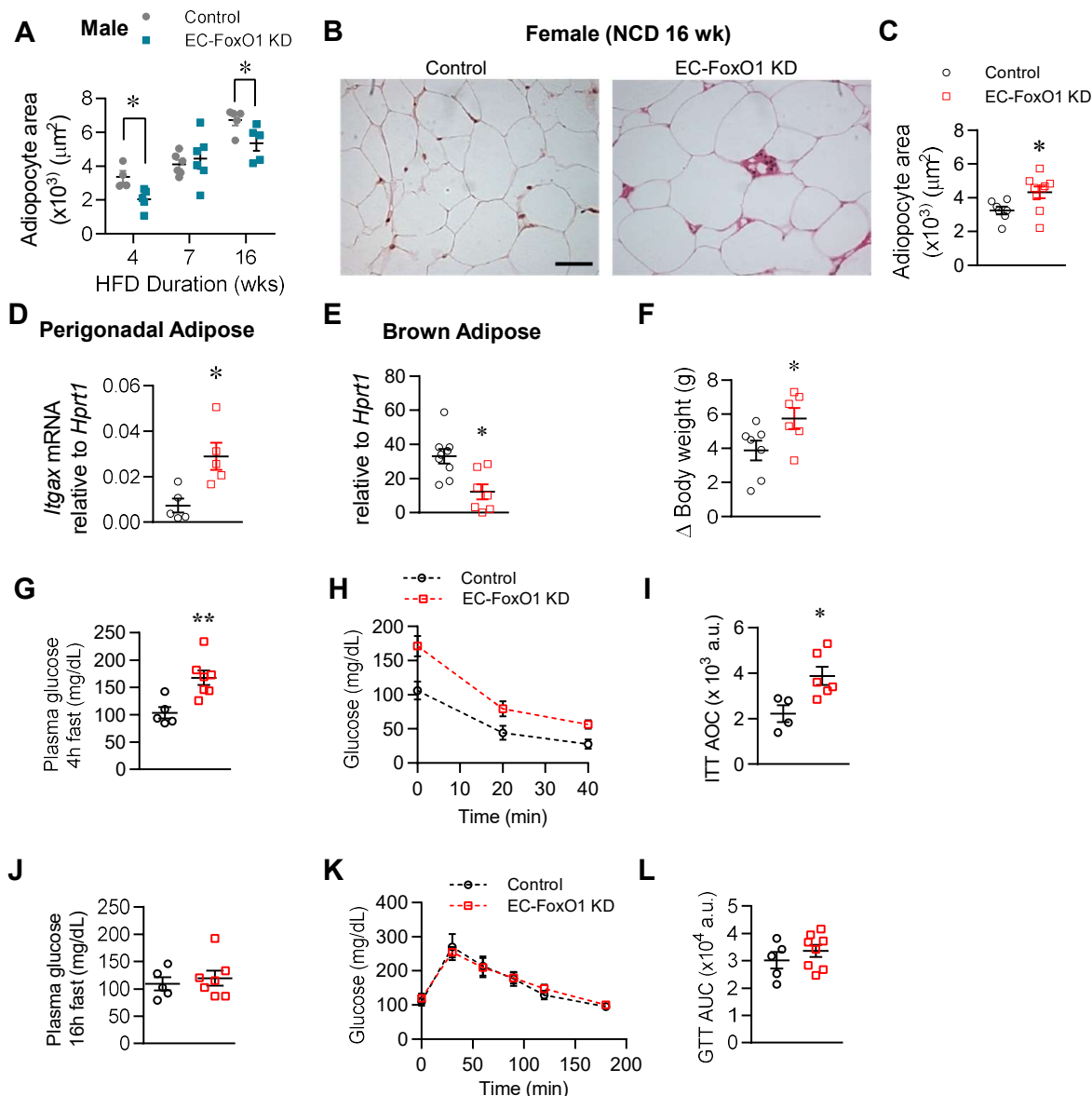


Figure S4.3 EC-specific FoxO1 knockdown changes in whole-body metabolism in NC-fed female mice. **A)** Mean adipocyte cross-sectional areas of HF-fed male mice (16-week data as reported in Rudnicki et al, eLife). diameters (4-wk Control n=4, 4-wk EC-FoxO1 KD n=5, 7-wk n=6, 16-wk n=5) **B)** Representative hematoxylin and eosin-stained images of perigonadal adipose tissue from 16-week NC-fed female mice. Scale bar = 50 μm **C)** Mean adipocyte cross-sectional areas of 16-week NC-fed female mice. (Control n=7, EC-FoxO1 KD n=9) **D)** *Itgax* mRNA levels relative to *Hprt1* ($2^{-\Delta\text{CT}}$) from epididymal adipose tissue of 16-week NC-fed female mice. (n=5) **E)** *Ucp1* mRNA levels relative to *Hprt1* ($2^{-\Delta\text{CT}}$) from brown adipose tissue of 16-week NC-fed female mice. (Control n=9, EC-FoxO1 KD n=7) **F)** Total weight gain over the 16 weeks of NC diet. (Control n=7, EC-FoxO1 KD n=6) **G)** Plasma glucose levels of NC-fed Control and EC-FoxO1 KD mice after 16 hour fasting (Control n=5, EC-FoxO1 KD n=7) **H)** Glucose tolerance of NC-fed Control and EC-FoxO1 KD mice was examined by intraperitoneal glucose tolerance test after 15 weeks of NC diet and 16 hr fasting. **I)** Area under the curve. (Control n=4, EC-FoxO1 KD n=6) **J)** Plasma glucose levels of NC-fed Control and EC-FoxO1 KD mice after 4 hour fasting (Control n=5, EC-FoxO1 KD n=7) **K)** Insulin sensitivity of NC-fed Control and EC-FoxO1 KD mice was assessed by intraperitoneal insulin tolerance test after 14 weeks of NC diet and 4 hr fasting. **L)** Area over the curve. fasting (Control n=5, EC-FoxO1 KD n=8). * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

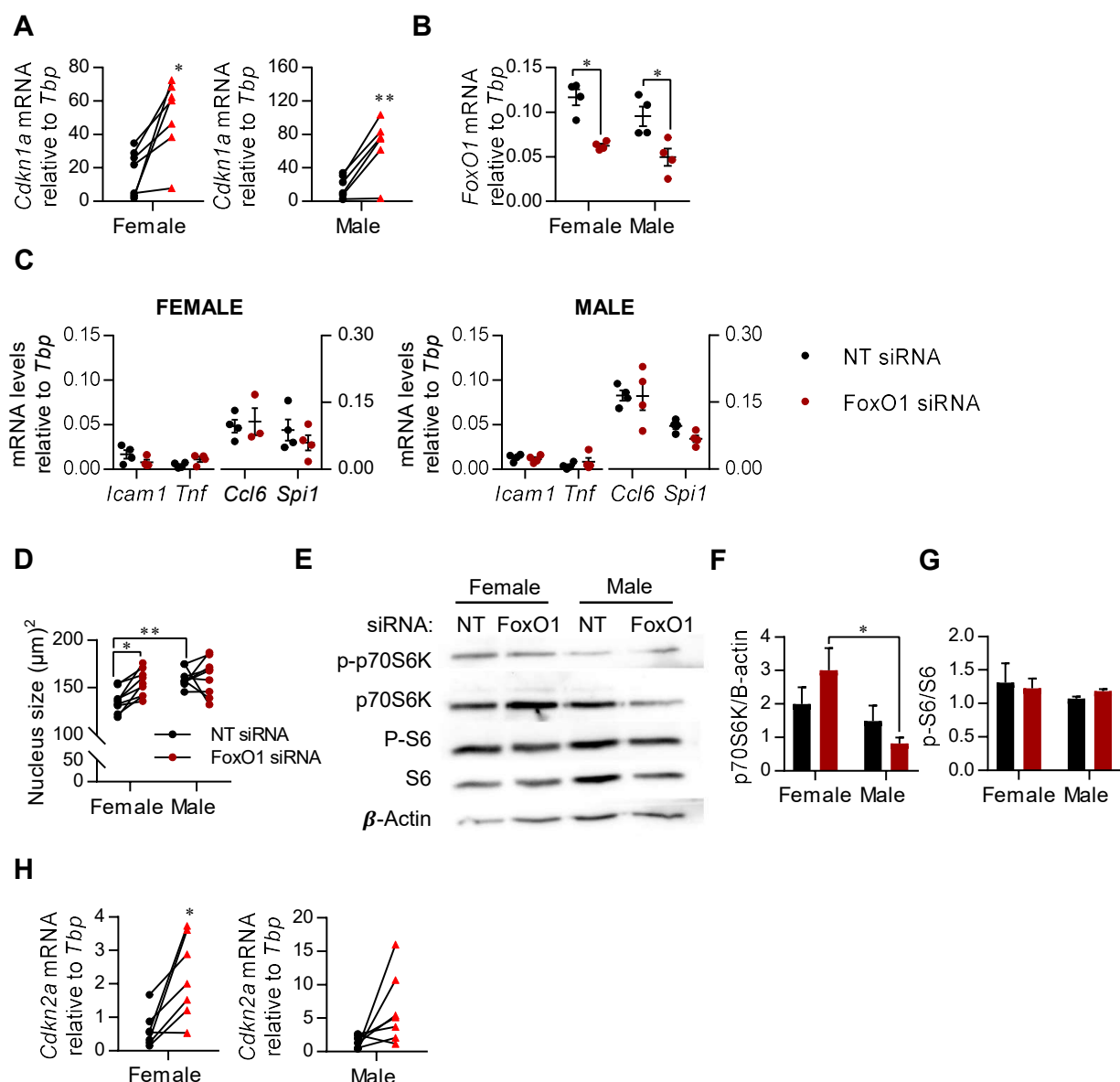


Figure S4.4 FoxO1 inhibition induces cell cycle arrest and a senescent profile in female EC. **A)** mRNA level of *Cdkn1a* following 6-day FoxO1 inhibitor treatment normalized to housekeeping *Tbp* ($2^{-\Delta\text{CT}}$). (Female n=7, Male n=6) **B)** mRNA levels of *FoxO1* in cultured adipose EC treated with non-targeting (NT) siRNA or FoxO1 siRNA for 24 hours and 2 hours serum starved. Genes normalized to housekeeping *Tbp* ($2^{-\Delta\text{CT}}$). (n=4) **C)** mRNA levels of inflammatory genes in cultured adipose EC treated with non-targeting (NT) siRNA or FoxO1 siRNA for 24-48 hours. Genes normalized to housekeeping *Tbp* ($2^{-\Delta\text{CT}}$). (n=4) **D)** Nuclear size of EC treated with either NT or FoxO1 siRNA for 48 hours (n=9). **E)** Western blot of EC treated with non-targeting (NT) siRNA or FoxO1 siRNA for 72 hours levels. **F)** Protein quantification of p70S6K relative to β -actin. (n=3) **G)** Protein quantification of phosphorylated S6 relative to total S6. (n=3) **H)** mRNA level of *Cdkn2a* following 6-day FoxO1 inhibitor treatment normalized to housekeeping *Tbp* ($2^{-\Delta\text{CT}}$). (n=7). * $P<0.05$; ** $P<0.005$

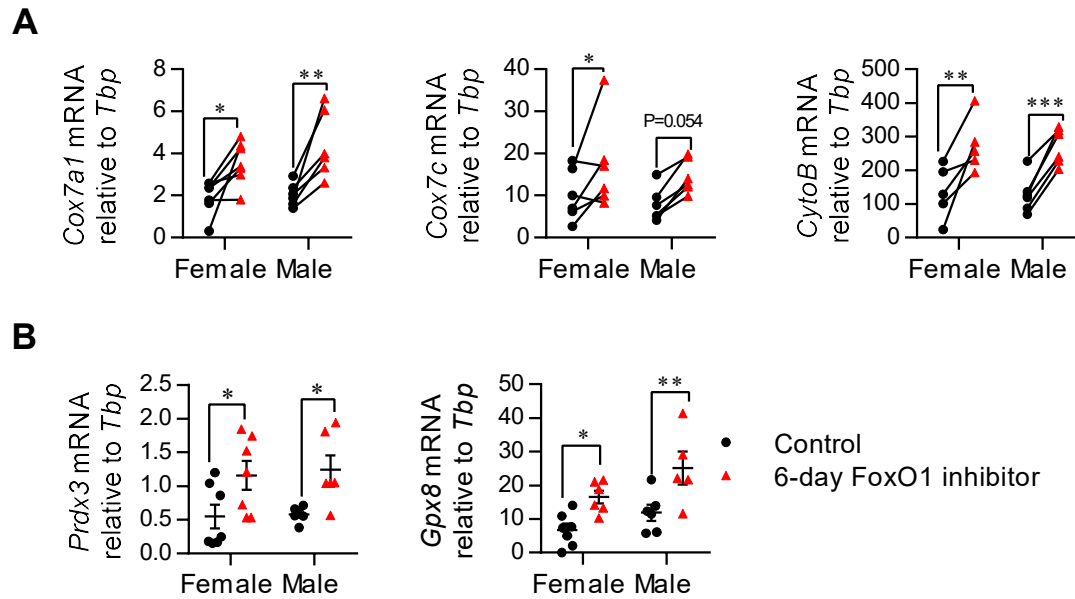


Figure S4.5 Mitochondrial and antioxidant gene levels are affected by FoxO1 inhibition. **A)** mRNA level of mitochondrial genes *Cox7a1*, *Cox7c*, and *CytoB* following 6-day FoxO1 inhibitor treatment, normalized to housekeeping *Tbp* ($2^{-\Delta CT}$). (Female n=5-7, Male n=6) **B)** mRNA level of antioxidant genes *Prdx3* and *Gpx8* following 6-day FoxO1 inhibitor treatment normalized to housekeeping *Tbp* ($2^{-\Delta CT}$). (Female n=6-7, Male n=6). * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$

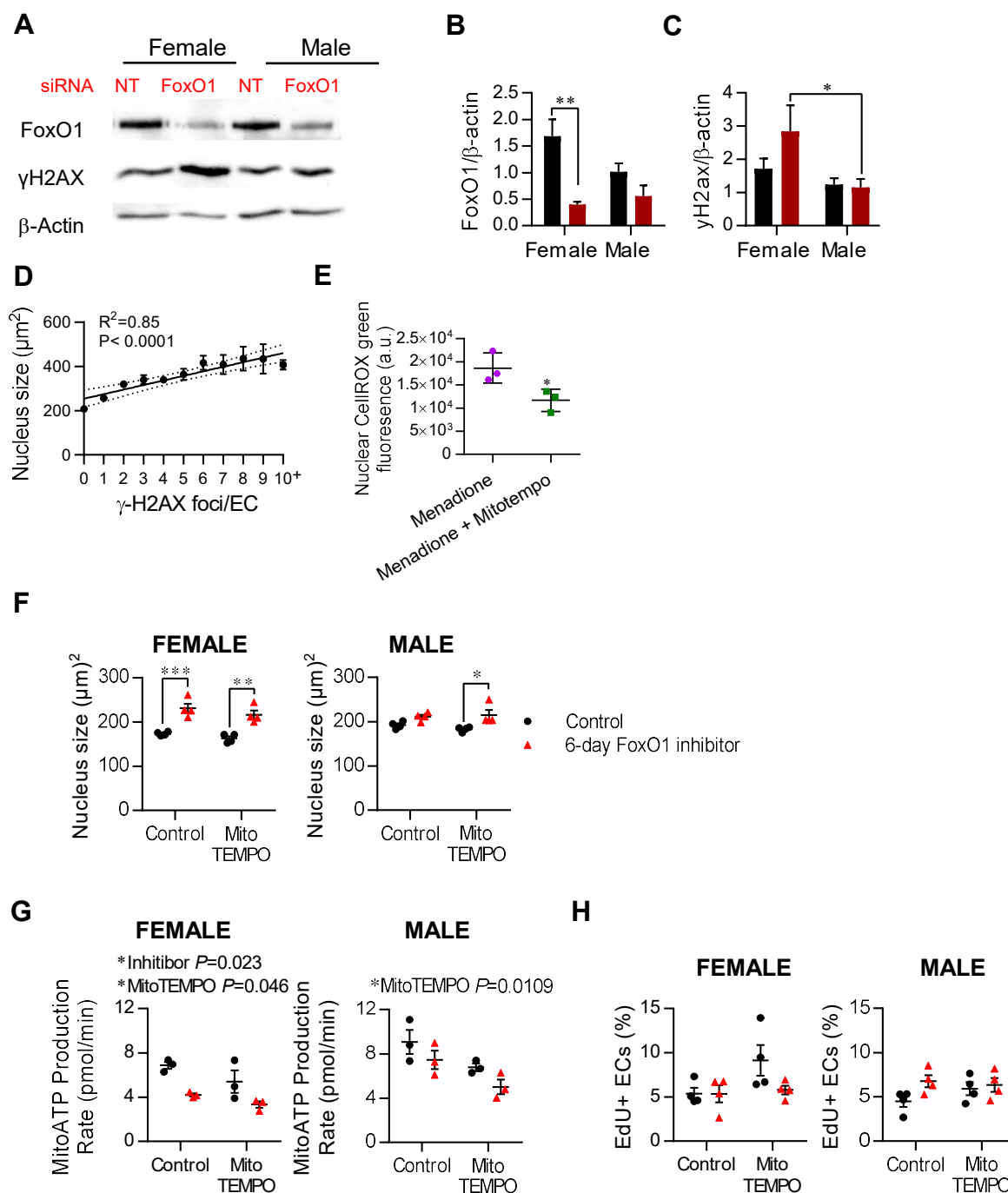


Figure S4.6 DNA damage increase after FoxO1 silencing in female EC. **A)** Western blot of EC treated with non-targeting (NT) siRNA or FoxO1 siRNA for 72 hours. **B)** Protein quantification of FoxO1 relative to β -actin. (n=4) **C)** Protein quantification of γ H2AX relative to β -actin. (n=5) **D)** γ H2AX foci number in the nucleus relative to nuclear size (n=12) **E)** CellROX green (Invitrogen #C10444) fluorescence in EC following control or 24-hour pre-treatment with antioxidant MitoTEMPO (20 μM) and 1 hour treatment with menadione (100 μM). (n=30) **(F-H)** Cultured ECs were treated with FoxO1 inhibitor in combination with the antioxidant MitoTEMPO (20 μM) for 6 days. **F)** Nucleus size quantification following FoxO1 inhibitor and MitoTEMPO treatments. (n=4) **G)** Mitochondrial ATP production using Seahorse, normalizing to DNA content. (n=3) **H)** Proliferation quantification of EC treated with 6-day treatment with FoxO1 inhibitor and MitoTEMPO. (n=4). * $P<0.05$; ** $P<0.005$; *** $P<0.0005$.

Table S4.1 Tissue weights after 16 weeks of NC or HF diet – female mice

	NC		HF	
	Control	EC-FoxO1 KD	Control	EC-FoxO1 KD
Final body weight (g)	26.4 ± 0.7	30.0 ± 2.0	31.2 ± 1.4	39.4 ± 5.2#
Body weight gain (Wk 14-0) (g)	4.2 ± 0.6	5.6 ± 0.8	6.4 ± 1.0	15.6 ± 4.4*
pgWAT (g)	0.9 ± 0.1	1.6 ± 0.2*	1.2 ± 0.2	2.8 ± 0.6**
rWAT (g) \$	0.4 ± 0.06	0.9 ± 0.2	0.6 ± 0.1	1.2 ± 0.3
Subcutaneous (g)	0.9 ± 0.3	1.7 ± 0.3	1.4 ± 0.2	4.3 ± 1.6
BAT (mg)	128.8 ± 5.8	202.3 ± 13.2*	109.3 ± 12.4	203.5 ± 21.6***
Liver (g)	1.0 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	1.4 ± 0.2*
Heart (mg)	100.2 ± 3.6	107.7 ± 6.6	114.2 ± 6.8	117.6 ± 7.9
Gastrocnemius (mg)	96.2 ± 3.2	96.9 ± 4.6	102.0 ± 4.6	100.9 ± 5.6
Soleus (mg)	5.8 ± 0.3	8.2 ± 0.6	6.7 ± 0.3	7.3 ± 0.5
Tibialis anterior (mg)	37.0 ± 1.1	32.3 ± 3.2	37.6 ± 1.9	37.4 ± 1.7
Extensor digitorum longus (mg)	9.4 ± 0.8	8.6 ± 0.7	8.9 ± 0.4	9.1 ± 0.4
Plantaris (mg)	11.1 ± 1.1	12.9 ± 0.7	11.3 ± 0.5	13.2 ± 0.4

Data are expressed as mean ± S.E.M., n=5-9 per group (Subc. control group only n=3)

\$ Main effect of genotype; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs Control group;

$P < 0.05$ vs NC group of same genotype; 2-way ANOVA with Sidak's post-hoc

Table S4.2 List of Taqman Primer/Probe sets

Target Gene	Assay Code
Foxo1	Mm00490671_m1
Actb	Mm04394036_g1
Hprt1	Mm00446968_m1
Tgfb1	Mm01178820_m1
Tbp	Mm00446973_m1
Hmgb1	Mm04205650_gH
Pecam1	Mm00476712_m1
Cdh5	Mm00486938_m1
Tie1	Mm00441786_m1
Vegfa	Mm00437306_m1
Itgax	Mm00498701_m1
Mrc1	Mm00485148_m1
Kdr	Mm01222421_m1
Ucp1	Mm01244861_m1
Cdkn1a	Mm01303209_m1
Icam1	Mm00516023_m1
Tnf	Mm00443258_m1
Ccl6	Mm00436446_g1
Spi1	Mm01270605_g1
Cdkn2a	Mm00494449_m1
Cox7a1	Mm00438297_g1
Cox7c	Mm01545088_g1
Mt-cytb	Mm04225271_g1
Prdx3	Mm00545844_m1
Gpx8	Mm00510156_m1

Chapter 5: Discussion

Chapter 5.1 Overview of key findings

The goal of this dissertation was to uncover the underlying mechanisms responsible for the higher angiogenesis observed in female adipose tissue and to determine whether this sex-specific characteristic could be attributed to intrinsic differences in endothelial cells. In Chapter 2, I established the presence of sex differences in ECs of the adipose tissue both *in vivo* and *in vitro* that help explain the higher female vascularization. Female ECs had a proliferative phenotype, even outside the adipose environment, while male ECs lacked this proliferative signature and instead had a more dominant inflammatory and dysfunctional profile. In Chapter 3, I used a mouse gonadectomy model to further investigate the role of sex hormones on adipose vasculature and endothelial proliferation and metabolism. Interestingly, I found that loss of sex hormones did not impair adipose tissue vascularization or impact EC proliferation and metabolism long-term, suggesting that the angiogenic and EC sex differences reported are independent of sex hormones. In Chapter 4, I found that the anti-angiogenic transcription factor FoxO1 is upregulated in the female EC and contributes to EC health by preventing DNA damage, mitochondrial dysfunction and its presence having an overall positive effect on cell cycle regulation in a sex-specific manner. Overall, this work highlights intrinsic sexual dimorphism of ECs, that may extend past the context of obesity and give insight on sex-specific progression of various pathologies (**Figure 5.1**). Moreover, this dissertation also provides data supporting a protective role of FoxO1 in female ECs, outside its widely known role of an endothelial-quiescent factor.

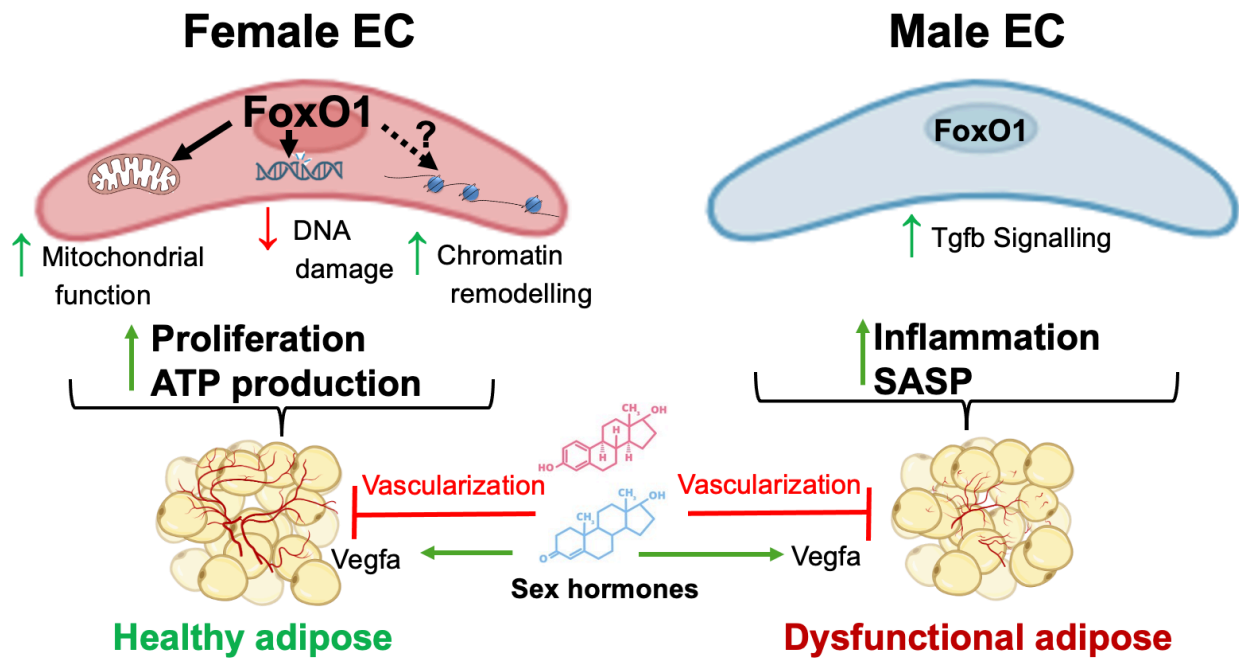


Figure 5.1 Summary highlighting endothelial sex differences in the adipose tissue. Female ECs exhibited higher proliferation and ATP production compared to male ECs, which may be in part due to their higher FoxO1 levels. In female ECs, FoxO1 contributed to their healthier phenotype by maintaining mitochondrial structure and function and preventing DNA damage. Female ECs were enriched in chromatin remodelling pathways compared to the males, and further analysis is required to understand if FoxO1 contributes to this phenotype. In contrast, male ECs had higher inflammation and senescence associated secretory phenotype (SASP) during high-fat feeding, demonstrating a more dysfunctional phenotype where the role of FoxO1 signalling was reduced compared to those of females. These endothelial sex differences help to explain the higher angiogenesis in the female adipose tissue, independent of sex-hormones. In the absence of gonadal sex hormones, adipose angiogenesis was higher in both male and female adipose tissue, despite repression of *Vegfa*. Figure was created in Biorender.

Chapter 5.2 Novel Contributions to the Field

This dissertation presents novel evidence that advances the field of vascular biology by

1. Describing sex-specific differences in adipose ECs
2. Demonstrating that sex hormones exert an anti-angiogenic effect within adipose tissue
3. Showing that long-term inhibition of FoxO1 is detrimental to EC function, despite its widely accepted role in maintaining EC quiescence

5.2.1 Sex differences of adipose EC proliferation and metabolism

Sex is often overlooked as a variable when studying adipose tissue vascularization, which can lead to incorrect assumptions when assessing the link between obesity and adipose angiogenesis. The work presented in this dissertation is the first to describe proliferative sex differences in the ECs of the adipose tissue, which contribute to the understanding of sex differences in metabolic morbidities associated with obesity and overall adipose health. Although several groups described higher female EC proliferation (Boscaro, Trenti, et al., 2020; Fadini et al., 2008; Lorenz et al., 2015; Y. Zhang et al., 2018), none of those studies provided evidence that this sexual dimorphism extended outside *in vitro* conditions or in the context of a pathological progression, such as obesity or aging. In Chapter 2, I demonstrated that proliferation is higher in female cultured ECs and data from Chapter 3 provided a mechanistic explanation of this elevated proliferation by showing increased ATP production in female adipose EC, which may sustain increased proliferation. The higher angiogenic capacity of adipose EC in females during HF feeding is corroborated by a shift in ATP production, where females adopt a higher glycolytic phenotype potentially explaining their higher proliferation, compared to males where they shift to a higher mitochondrial reliance in response to a HF diet. Furthermore, I showed the higher proliferative capacity of females *in vivo*, where the coordination of EC proliferation with adipocyte

hyperplasia is an important factor for healthy adipose expansion and preventing metabolic diseases (Corvera & Gealekman, 2014; Lemoine et al., 2013). The elevated proliferation correlates with the enriched chromatin remodelling signature, as this is a characteristic of proliferating cells; however this may also be due to higher FoxO1 levels in the female ECs, since FoxO1 has been shown to alter chromatin accessibility (Miyamura et al., 2024), which can potentially give rise to the differentially regulated genes between female and male ECs. It is also possible that chromatin remodelling is caused by sex hormones, as estradiol is known to induce changes in chromatin accessibility (D. Chen et al., 2021). The enhanced capacity of chromatin remodelling in females compared to male ECs may enable greater adaptability under pathogenic conditions and contribute to the higher resilience of females by upregulating genes relating to mitochondrial function, antioxidant activity, protect against DNA damage.

5.2.2 Sex Differences of Adipose EC Inflammation and Dysfunction

My dissertation also showed that female ECs have less of a dysfunctional phenotype compared to males (presented by a hyper-response to inflammatory stimuli). Both *in vitro* and *in vivo* assessments demonstrate that females are more resistant to stimuli that may trigger a chronic inflammatory phenotype, which in turn contributes to dysfunctional adipose tissue. Herein, I provide evidence in Chapter 4 of FoxO1 in the female ECs being a potential contributor to the resilient female phenotype, as FoxO1 depletion led to upregulation of inflammatory pathways *in vivo*. A proinflammatory profile is a distinct feature of senescent ECs (Giuliani et al., 2023), and data from Chapter 2 showed that the signature of male ECs included both inflammatory pathways and SASP. The higher levels of FoxO1 in the female ECs may contribute to their reduced inflammation compared to the males and can therefore be linked with lower probability of them becoming senescent and expressing the SASP profile that the males ECs do. Based on my findings,

it is possible that FoxO1 prevents inflammation through its role in ROS maintenance and prevention of mitochondrial dysfunction in the female ECs, and that the higher levels in the female ECs are driving this protective phenotype.

FoxO1 may have opposite effects in female and male ECs that lead to the higher baseline inflammation of the male ECs. Data from Chapters 2 and 4 show that Tgfb1 is upregulated in the male ECs and part of various inflammatory pathways, yet silencing of FoxO1 led to reduced levels of Tgfb1 in males. Silencing of FoxO1 led to opposite results in the female ECs, with upregulated Tgfb1 after only 24 hours of FoxO1 suppression, likely eliminating the possibility of external signals (eg. inflammatory mediators) causing this increase in Tgfb1 expression. Therefore, it is probable that FoxO1 loss presents in ECs in a sex-specific manner, resulting in Tgfb1 signalling to be upregulated in the female ECs. This disparity can contribute to differences in cell-cycle progression, but also in inflammation. Findings in Chapter 2 also showed this inflammatory profile in aging male ECs only, but absent in the females, demonstrating that my dissertation extends outside the scope of the adipose and obesity but also throughout different tissues and different pathologies. Thus, my findings substantiate the observation of higher angiogenesis in the female adipose tissue by demonstrating that female ECs exhibit greater proliferative capacity and reduced inflammation, which are traits that may underlie sex-specific vascular responses across a broader range of pathologies beyond obesity. This introduces the critical variable of endothelial sex differences in understanding disease progression and could guide more effective, personalized prevention and treatment strategies.

5.2.3 Female sex hormone presence is not required for increased vascularization

This dissertation is the first to reveal that gonadal sex hormones, long recognized for their pro-angiogenic and endothelial proliferative effects (H. Liu et al., 2018; Seo et al., 2004; Sobrino et al., 2009), can paradoxically inhibit adipose tissue angiogenesis, challenging existing paradigms and opening new avenues for research. Considering the strong angiogenic role of estrogen (H. Liu et al., 2018), I proposed that it may be one of the factors contributing to the increased female proliferation and metabolism relative to males. Although the data here show similar effects of gonadectomy as other groups (higher adipose mass, impaired glucose sensitivity), one of the most intriguing findings was the higher adipose vascularization following the ovariectomy when challenged with a HF diet. Moreover, the same effect was observed in males following castration despite lower VEGFA in the adipose environment of these ECs in both sexes. It is possible that the higher angiogenesis in the absence of sex hormones was due to FoxO1 pathways being affected by the lack of estrogen, as estrogen has been shown to change FoxO1 expression and alter its transcriptional activity (Yan et al., 2019; J. Zou et al., 2016). However, treatment of ECs with estradiol did not show an increase in FoxO1 levels (Appendix). Loss of sex hormones may alter FoxO1 signaling in adipose ECs, potentially relieving inhibitory effects on angiogenesis that have not previously been explored, and thereby enhancing angiogenic responses. Furthermore, these data confirm that the greater angiogenic capacity of the female adipose tissue is not dependent on sex hormones.

Although estradiol can increase EC metabolism, I showed that the gonadectomy had no lasting effects on EC ATP production, and that direct estradiol stimulation on ECs is required to increase its mitochondrial activity. However, a novel observation arising from this study was that the diet had a lasting impact on the way ECs produce their ATP in a sex-specific manner. Female

adipose ECs from mice fed a HF diet became more glycolytic while EC from males shifted to favour mitochondrial ATP production. These observations may explain why female ECs exhibit higher proliferation compared to males during HF-feeding and provide a new stratum of knowledge regarding the sex-specific metabolic shifts that ECs adopt in obesogenic conditions. This finding is important as it serves as one possible explanation of why females have healthier adipose expansion during obesity and why CVD presents differently based on sex.

5.2.4 FoxO1 is protective in the females

The observation that female ECs have a healthier phenotype led me to understand what factors contribute to this. One of the genes that is a potential candidate for manipulation is FoxO1, the putative transcription factors that was predicted to be upstream of many of the DEGs relating to pathways relating to proliferation and transcription in female ECs. Indeed, the work from this dissertation demonstrates that female ECs have higher levels of both FoxO1 RNA and protein. Although FoxO1 is well described to induce quiescence in ECs and that its knockdown leads to increased angiogenesis and EC proliferation (Wilhelm et al., 2016), my dissertation is the first to show that FoxO1 acts in a sex-specific manner in ECs. Herein, I show FoxO1 to have a protective role in adipose ECs that helps with proliferation, which is exaggerated in those of the females. Moreover, I show differences in the effect of FoxO1 depending on the duration of the KD or inhibition. With a sustained KD of FoxO1, the female ECs adopt a phenotype similar to that of the control male EC, having increased inflammation and SASP profile, and decreased mitochondrial capacity. Thus, the data reported here could be used as evidence to consider FoxO1 as a therapeutic target, where its maintained levels in the female ECs may potentially combat adipose and obesity-related disturbances, and achieve healthier adipose expansion.

The work done in this dissertation provides a novel role of FoxO1 in ECs, showing its importance to DNA repair, with increased DNA damage following its inhibition. FoxO1 has been shown to aid in DNA repair (Ju et al., 2014), however these observations had not been previously reported in ECs. In contrast, a group had showed that FoxO1 KD is beneficial to DNA repair in ECs, through the upregulation of glycolysis (Sun et al., 2022). Similarly, the role of FoxO1 in oxidative stress regulation is heavily understudied in the ECs, with one group showing its phosphorylation following an oxidative stress insult (Y. Wang et al., 2010), but the majority of studies having been conducted using other cell types (Akasaki et al., 2014; X. Huang et al., 2019). My data show that both *in vivo* FoxO1 KD and *in vitro* sustained FoxO1 inhibition led to oxidative stress in female ECs. These discoveries contribute to the knowledge we currently have about FoxO1, confirming that it has a DNA damage repair and anti-oxidative role in adipose ECs as well. My dissertation is critically important in demonstrating that FoxO1 functions differently in males and females, revealing that previous findings suggesting FoxO1 knockdown improves metabolic disease outcomes may be misleading, particularly because this effect was not observed in female ECs.

Chapter 5.3 Implications of EC sex differences and FoxO1 KD

If sex differences in ECs are also present in vascular beds beyond adipose tissue, they may help explain why females often experience better disease outcomes than males. In arteries and coronary vessels of the heart, a hyper-response to inflammatory stimuli in male ECs can lead to formation of atherosclerotic plaques (C. Zhang, 2008), a condition that ultimately reduces blood flow and oxygen delivery to downstream tissues such as the heart and brain. In extreme conditions, these plaques can trigger heart attacks and strokes. Indeed, comprehensive studies have shown that males have higher incidences of heart attacks, which was shown to be likely independent of sex

hormones (Albrektsen et al., 2016). Although many different lifestyle factors can contribute to this, my data draw attention to the possible contribution of endothelial sex differences. Chapter 2 also provided evidence of sex differences in aging ECs. In this context, EC dysfunction could cause higher susceptibility to tissue wasting and increased frailty due to improper oxygenation of tissues. Aging also causes reduced levels of sex hormones, and this was studied using the gonadectomized mice. In this cohort, we surprisingly found increased vascularization in the adipose tissue, despite downregulation of the pro-angiogenic molecule, VEGFA.

My dissertation investigated the consequences of EC FoxO1 knockdown primarily in the white adipose. However, it is possible that the sex-specific effects following FoxO1 loss described in the adipose tissue ECs also extends to ECs of other tissues. In the muscle, EC-FoxO1 KD led to increased capillary content in the males (Rudnicki, et al., 2018a), whereas in this dissertation, I show that the capillary content in the muscle is reduced with the FoxO1 KD in the females. Dysfunctional ECs present in the muscle can cause poor vascularization and limited oxygen supply, especially in during increased metabolic demand or contexts of obesity. Low EC proliferation in the muscle can also delay injury recovery and impede muscle hypertrophy. Similarly in male brown adipose tissue, EC-FoxO1 KD led to an upregulation of endothelial markers (Rudnicki, et al., 2018a); however, the opposite effect was observed in females, where EC-FoxO1 KD resulted in decreased expression of these markers. In the female brown adipose, I show that this EC reduction was accompanied by reduced levels of its thermogenesis-regulating protein, Ucp1. Long-term FoxO1 KD in the female brown adipose leads to low vascularization, which can impair its metabolic activity and thermogenic function (Shimizu et al., 2014). As a result, Ucp1 expression is ultimately decreased which will likely limit the tissue's ability to generate heat and expend energy (Ricquier, 2011).

This dissertation is the first to demonstrate that sustained EC-FoxO1 KD in females leads to vessel morbidities, such as the formation of abnormally large blood vessels with irregular structures, including sheet-like formations, loops, and bulges. These structural abnormalities suggest that some of these vessels may be non-functional or have reduced blood flow. Such enlarged and disorganized vessels can alter normal hemodynamics by lowering vascular resistance and blood pressure, but they may also increase the risk of vascular complications, such as impaired tissue perfusion or vessel rupture. Abnormal vessels due to sustained EC-FoxO1 loss can have deleterious effects (eg. Hemorrhaging) in other tissues, such as the brain or heart. Thus, tight regulation of endothelial FoxO1 may be necessary across tissues if the adipose EC-FoxO1 KD phenotype occurs in other vascular beds.

Chapter 5.4 Limitations of the studies

5.4.1 In vivo and in vitro models

A key limitation of this dissertation is that all experiments were conducted in a mouse model, and it remains important to determine the extent to which these findings translate to humans. In the mouse model, the *Pdgfb*-Cre knockdown model used was a highly useful tool for knocking down FoxO1 specifically in ECs; however, this KD was not specific to the adipose tissue and was present in all tissues where ECs express *Pdgfb*, such as muscle ECs. Thus, the systemic effects of FoxO1 KD cannot be attributed solely to changes in adipose tissue. It is possible that tamoxifen itself may have influenced the results. To control for potential effects of tamoxifen itself, Cre-negative control mice were also administered tamoxifen, and all analyses were performed at time points well after tamoxifen treatment to minimize its direct influence on the results. Another factor that could contribute to metabolic differences is the time of day at which mice were sacrificed, as their metabolic state varies over time. To minimize this variability, sacrifice timing

was kept consistent within cohorts for Chapters 2 and 4. However, due to the large number of mice in the gonadectomy cohort, it was not possible to sacrifice all mice at the same time. To address this, sacrifice times were randomized across groups, and metabolic data remained consistent within experimental groups.

It is likely that lower muscle vascularization in the female EC-FoxO1 KD mice contributed to impaired insulin tolerance. Analyses similar to those done on the adipose tissue ECs should be performed on the ECs from muscle and brown adipose tissue to detect sex differences and if FoxO1 KD results in changes in their EC phenotype and the tissue functions.

Many of the transcriptomic findings (such as higher female EC proliferation in females, higher inflammation in male ECs, higher inflammation following FoxO1 KD) were assessed in cultured cells as a validation. While many of these findings were substantiated in culture, some of the transcriptomic results were not recapitulated in culture. This may be due to challenges such as not being able to maintain the FoxO1 inhibition in culture as long as the KD was maintained in the animal models. Furthermore, the altered adipose environment resulting from EC-FoxO1 KD likely contributes to the dysfunctional endothelial cell (EC) phenotype observed *in vivo*, but this complex interaction cannot be fully replicated in a cultured setting.

While RNA-seq is a powerful and in-depth technique for capturing genome-wide transcriptional changes, a limitation is that transcriptomic alterations may not necessarily be reflected at the proteomic level, as post-transcriptional, translational, and post-translational mechanisms can significantly influence protein expression and function.

5.4.2 Contribution of diet to sex differences

The *in vivo* EC sex differences reported in chapter 2 were isolated from mice fed a HF diet, and RNA-seq analysis was not performed on ECs originating from normal chow-fed mice. In chapter 3, I presented evidence to show that HFD leads to sex-specific sustained metabolic changes in the ECs. Thus, an unanswered question in this dissertation is the extent contribution of HF diet to the EC sex differences reported, and if different types of diet would lead to different sex differences. Although ECs isolated from normal chow-fed female mice also presented higher proliferation, glycolytic and mitochondrial ATP activity, and FoxO1 levels, *in vivo* proliferative differences were not reported in NC models. Future studies should include RNA-seq of ECs isolated from mice fed a normal chow, and different types of diets such as a high-glucose diet to also investigate if these differentially regulated pathways between males and females would still be observed, or if they are dependent on an obesogenic environment of the cells and therefore seen at a lesser extent.

5.4.3 Gonadectomy cohort did not match previous female vs. male cohort effectively

One of the issues when determining the effect of sex hormones on EC phenotype to address if they contribute to the sex differences observed in the previous cohort is that the two cohorts had many variables that were different, making the comparison difficult. The HF diet used for the RNA-seq cohort had a higher proportion of fat content that could affect EC behaviour. The mice were also a different strain which may be the reason of some variability between the studies. Furthermore, the length of the diet was over double in the gonadectomy cohort, making it challenging to assess EC activity during a more dynamic period of adipose remodelling.

5.4.4 FoxO1 inhibition may not inhibit FoxO1 activity in all contexts

The FoxO1 inhibitor used in the cell culture studies binds to the active form of FoxO1, but not to the Ser256-phosphorylated form, which leads to its exclusion from the nucleus. However, it has been shown in other cells types that the p-FoxO1 can be recruited into the mitochondria where it can cause impaired mitochondrial function and increased apoptosis (S. Chen et al., 2024). Because of this, I cannot exclude the possibility of increased mitochondrial FoxO1 in the female ECs following its inhibition that may lead to the exaggerated dysfunctional phenotype. Future work is needed to determine FoxO1 localization in the ECs in baseline conditions by either immunostained ECs for FoxO1 or perform fractionation of the ECs to separate the cytoplasm, mitochondria and nucleus, and measuring the levels of FoxO1 in each compartment of the EC in control conditions and following FoxO1 inhibition.

5.4.5 Adipose tissue analysis challenges

A potential limitation of the whole-mount analysis is that it does not fully account for the three-dimensional architecture of the tissue, which may confound accurate assessment of blood vessel density by influencing the apparent distribution and visibility of vessels in two-dimensional sections (Rudnicki et al., 2022b). Capturing a z-series to view the three-dimensional structure can provide additional details on the microvascular network and capillary structure that is not captured in two-dimensional sections. However, this assessment does not normalize to adipocyte number and can lead to either over or under estimation of the vascular content depending on different adipocyte sizes (Rudnicki et al., 2022b). Parallel analyses should be done to normalize for adipocyte size changes, such as determining the capillary to adipocyte ratio, as well as assessments of mRNA levels of EC markers, for a more comprehensive picture of capillary content and increasing the reliability of our conclusions.

The potential sampling bias between visceral and subcutaneous adipose tissues could also affect the interpretation of results. In some cases, only perigonadal depots were used for assessments, while in others, subcutaneous and visceral adipose tissues were combined. This merging was primarily done to increase the yield of endothelial cells for downstream analyses. However, it is important to note that different adipose depots exhibit distinct functional and metabolic characteristics. Visceral adipose tissue, including perigonadal fat, has a higher rate of lipolysis and more prone to inflammation, promoting systemic metabolic dysfunction. The subcutaneous fat is generally considered more metabolically protective and has a greater capacity for healthy adipocyte expansion without triggering inflammation. Additionally, these depots may be differentially influenced by sex hormones, with perigonadal depots potentially exposed to higher local concentrations of these hormones, which can contribute to differences in fat accumulation and vascularization. Such depot-specific differences could lead to distinct vascular and metabolic responses.

Chapter 5.5 Future Work

5.5.1 Epigenetic sex differences of ECs

Work presented in Chapter 2 identified DEGs enriched in female ECs from mice exposed to a HF diet or to aging both that relate to chromatin remodelling and DNA conformation change. This suggests that one possible characteristic contributing to the healthier female EC phenotype during obesity is the ability to differentially condense and open chromatin in order to maintain sufficient metabolism for proliferation and thus adipose vascular expansion. Furthermore, FoxO1 is higher in female ECs, and has been known to act as a pioneer factor in multiple cell types including ECs (Miyamura et al., 2024). As a pioneer transcription factor, FoxO1 has the ability to bind to heterochromatin and initiate changes that makes the DNA more accessible to other

transcription factors, priming genes for activation. This complex gene networks involved in angiogenesis, metabolism and inflammation and can lead to sex differences in the ECs. This elevated FoxO1 in female ECs compared to males may aid in the female EC chromatin reorganization when challenged with various stressors, in order to maintain homeostasis and a functional phenotype. To get a better understanding of the epigenetic regulation occurring in the cells and if differences in open vs. closed chromatin exist between female and male ECs, the regions of heterochromatin and euchromatin should be analyzed both in baseline conditions and following FoxO1 KD. To do this, ATAC-seq, which identifies open regions in the chromatin, and which genes are associated with these regions, should be conducted to determine differences between both control male and female ECs, but also following FoxO1 KD to determine to what extent FoxO1 contributes to epigenetic modifications.

5.5.2 Further investigation of endothelial phenotype following sex hormone loss

Chapter 3 focused on examining the effects of the gonadectomy on adipose vasculature and ECs, however we found that gonadectomy had no significant impact on endothelial proliferation after 15 weeks of the diet intervention. We might observe higher proliferation of EC when the adipose tissue is in a more active state of remodeling, which typically occurs earlier during diet-induced expansion as the tissue adapts to increased metabolic demand. Future studies should use a shorter diet duration post gonadectomy to assess changes in EC proliferation and how this might affect the overall adipose vasculature. This can help capture a timepoint where ECs are activated rather than quiescent. A longer diet duration can determine if it is possible that given more time with hormone loss, the decreased VEGFA may be reflected on vascularization and that reduced vascularization may eventually be observed. Additionally, to establish the role of sex hormones in angiogenesis and proliferation, a reversal cohort should be added where the

gonadectomized mice receive hormonal injections to determine if this can revert the gonadectomized phenotype to reflect the intact mice.

One of the most surprising outcomes of this study was the higher angiogenesis in the male and female mice that lacked sex hormones compared to those that were intact. To investigate this, we should further investigate the adipose microenvironment and measure the level of pro-angiogenic genes, such as leptin and adiponectin, which would give us insight if the gonadectomy changed the adipose tissue which indirectly led to higher adipose vascularization, rather than acting directly on the ECs. As mentioned, reversing the gonadectomy using hormonal supplementation may also help uncover if the introduction of sex hormones would lead to lower adipose vascularization. The four-core genotypes model, which separates the effects of sex hormones from sex chromosomes, would be highly beneficial for identifying how the loss of sex hormones specifically influences adipose vasculature. Moreover, to further assess the potential inhibitory role of estrogen on angiogenesis and therefore endothelial proliferation and migration, RNA-seq should be done on the RNA collected from the ECs of the gonadectomy cohort to detect any differentially regulated pathways that may explain the changes observed.

5.5.3 Identifying drivers of higher FoxO1 expression and activity levels in female ECs

A major unresolved question is what mechanism leads to higher FoxO1 expression in female ECs compared to males. I investigated the effect of estradiol on FoxO1 levels and found that FoxO1 levels did not change. Thus, future studies should investigate non-hormonal mechanisms that cause elevated FoxO1 levels, such as assessing chromatin availability around the FoxO1 DNA, and using the four-core model to identify the influence of both sex hormones and sex chromosomes on FoxO1 expression. Furthermore, considering that FoxO1 undergoes post-translational modifications that affect its activity, assessments, such as protein quantification, need

to be made of each modification to elucidate if they vary by sex. This could help explain if the activity levels and roles differ between sexes. If differences in post-translational modifications do exist in the ECs, then the next step would be to determine the localization of FoxO1 in the ECs (as described above through immunostaining and fractionation), as it has been shown in different cell types that mitochondrial localization of FoxO1 can lead to mitochondrial dysfunction (S. Chen et al., 2024). This data may provide valuable information on the regulation of FoxO1 and why its role and activity differ between sexes.

5.5.4 Creating a timeline of events for FoxO1

Groups reported a burst in EC proliferation and higher angiogenesis following FoxO1 knockdown or inhibition (Andrade et al., 2021; Ding et al., 2023; J.-H. Paik et al., 2007; Wilhelm et al., 2016), yet we show novel evidence that EC proliferation decreases and unfavourable events (such as oxidative stress and DNA damage) occur in the female ECs with sustained disruption of FoxO1. Thus, similar to our work in vitro with the 1-day and 6-day inhibition, it would be beneficial to have a cohort of mice that have a FoxO1 KD for only a few days and analyze the transcriptome and proliferative activity of the ECs to determine if these are negatively affected at an early timepoint as well, or follow the anti-proliferative well-established paradigm of FoxO1. Adding multiple time points for *in vivo* KD of FoxO1 would also help uncover at which point there is a shift from favourable events caused by the FoxO1 disruption, to events relating to EC dysfunction. Additional time points of FoxO1 inhibition in vitro should also be added, where characteristics such as mitochondrial structure, ROS levels, and DNA damage are analyzed, similarly to how they were at the 6-day time point. This would give insight into the mechanisms of FoxO1 inhibition and help uncover which event occurs first (eg. oxidative stress that results in mitochondrial damage) or if multiple events occur simultaneously.

Longer FoxO1 KD timepoints extending past 16 weeks, as well as FoxO1 inhibition in cultured ECs over 6 days, should be used to further explore sex differences in mechanism of FoxO1. The longer timepoint helps uncover if male ECs will result in the same fate as the female ECs given enough length of time with FoxO1 loss, or if male ECs are more resilient to FoxO1 loss regardless of the time period. In this *in vitro* model, a rescue experiment should be performed that takes away the FoxO1 inhibitor for a few days following FoxO1 inhibition, which would determine if the effects of FoxO1 loss are permanent and cause irreversible damage to the cells, or if the reintroduction of FoxO1 can reverse the dysfunctional phenotype triggered by FoxO1 loss. A longer-term FoxO1 KD should also be used to uncover if the same morbidities occur *in vivo* as they do *in vitro*, such as DNA damage, EC size increase and mitochondrial fission. It would also be useful to study the functionality of the vessels *in vivo*, and assess the blood flow within them, as well as their permeability and ability to respond to proliferative stimuli, given that the shapes of the vessels become increasingly abnormal in the females with the EC-FoxO1 KD. Lastly, an overexpression model should be used to determine if overexpression of FoxO1 in male ECs can make them behave more similarly to those of females and have lower inflammation and SASP, accompanied by higher proliferation and mitochondrial function.

Appendix A: Further characterization of ECs following FoxO1 loss

Appendix A contains data that has been collected but has not been included in the manuscripts

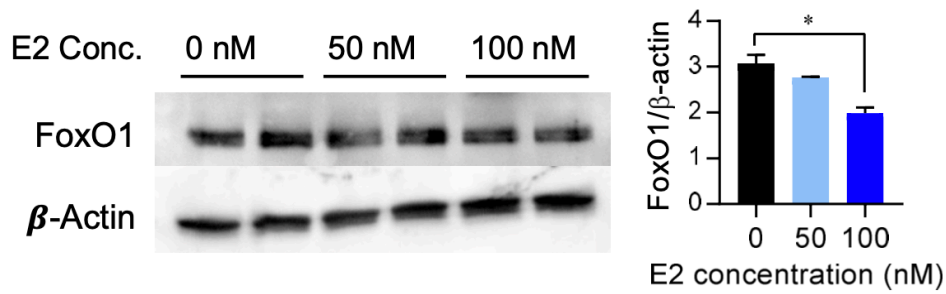


Figure A1. Estradiol effect on FoxO1 levels. To determine if FoxO1 is directly regulated by estrogen cultured P4 male adipose ECs were treated with estradiol (E2) at different concentrations for 20 hours and the FoxO1 protein levels were measured relative to β -actin. (n=3) * $P<0.05$

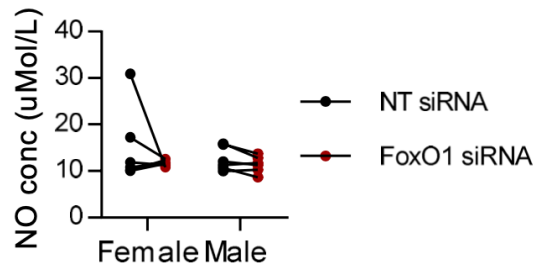


Figure A2. Nitrate/Nitrite concentration of ECs following FoxO1 silencing. Because eNOS mRNA levels were affected by FoxO1 siRNA (see table A1), I tested for differences in NO production. P3 adipose ECs treated with non-targeting (NT) siRNA or FoxO1 siRNA for 48 hours and the media was changed from 20% FBS HG DMEM to 5% FBS HG DMEM after 24 hours. The media was then collected, and total nitric oxide production was quantified using the “Total Nitric Oxide and Nitrate/Nitrite Parameter Assay Kit” (R&D systems #KGE001).

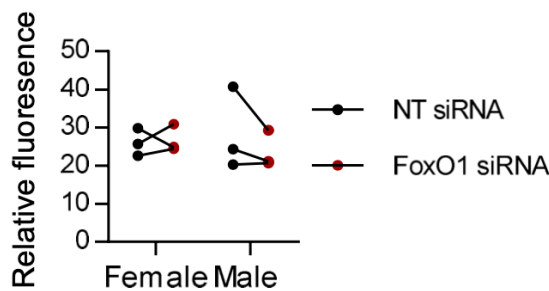


Figure A3. Fatty acid uptake of ECs following FoxO1 silencing. Because adipocyte size increased in the females with FoxO1 KD and there was a trend for fatty acid transport proteins to increase with FoxO1 siRNA (see table A1), I tested for differences in live fatty acid uptake. P3 adipose ECs were plated in a black-walled, clear bottom plate and treated with non-targeting (NT) siRNA or FoxO1 siRNA for 48 hours. They were given 2 μ M BODIPY- C_{12} (Thermo Fisher Scientific, complexed with 1 μ M BSA) for 8 min as described by (Ibrahim et al., 2021).

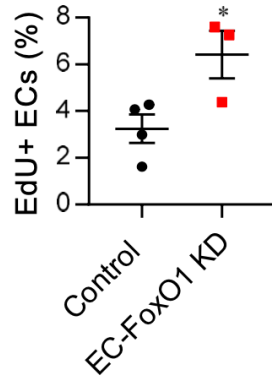


Figure A4. Short term FoxO1 KD leads to increase endothelial proliferation. To test if a proliferative effect could be seen with short duration of EC-FoxO1 KD, I assessed EC proliferation rate after 2 weeks of FoxO1 KD. Female FVB mice were fed a NC for 7 weeks. Mice were injected with EdU two weeks post EC-FoxO1 KD and assessed following 7 weeks of NC. Perigonadal adipose tissue was used for whole-mount imaging analysis to detect proliferating cells. EdU-positive cells were quantified as a percentage of total EC number. (Control n=4, EC-FoxO1 KD n=3). * $P<0.05$

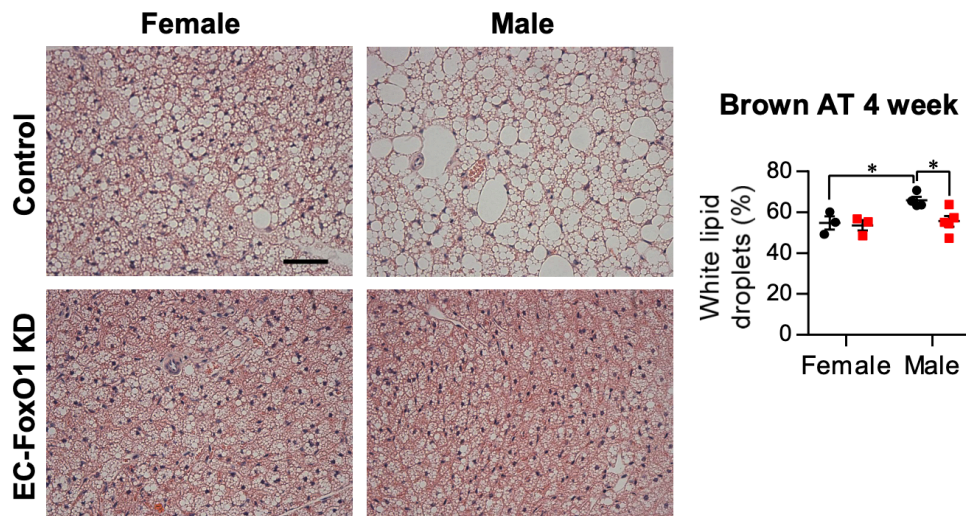


Figure A5. Brown adipose morphology following 4 weeks of FoxO1 KD. Representative hematoxylin and eosin-stained images of brown adipose tissue from 4-week HF-fed female and male mice (Scale bar = 50 μ m) and quantification of lipid droplet area (Female control n=3; Female EC-FoxO1 KD n=3; Male control n=4; Male EC-FoxO1 KD n=5). * $P<0.05$

Table A1. mRNA level of genes in cultured adipose EC treated with non-targeting (NT) siRNA or FoxO1 siRNA as specified. Genes normalized to housekeeping *Tbp* ($2^{-\Delta CT}$).

	Gene	Female				Male			
		NT siRNA x E-3 mean	FoxO1 siRNA x E-3 mean	p-val	n	NT siRNA x E-3 mean	FoxO1 siRNA x E-3 mean	p-val	n
24 hours siRNA	<i>Foxo1</i>	252.80	80.34	0.0042	4	152.40	67.69	0.18	4
	<i>Icam1</i>	16.79	7.79	0.12	4	13.22	11.41	0.90	4
	<i>Nrarp</i>	0.47	2.44	0.58	4	3.12	3.72	0.98	4
	<i>Tnf</i>	5.49	10.97	0.48	4	4.97	8.35	0.74	4
24 hours siRNA + 2 hours serum starved	<i>Foxo1</i>	116.80	62.35	0.028	4	95.36	49.58	0.072	4
	<i>Nos3</i>	5.95	11.98	0.038	4	4.02	1.15	0.39	4
	<i>Dll4</i>	2.23	8.91	0.0096	3	3.78	2.27	0.68	4
	<i>Notch4</i>	22.05	39.07	0.30	4	86.01	9.67	0.017	4
	<i>Nrarp</i>	0.44	0.44	0.99	2	0.21	0.23	0.99	2
	<i>Fabp4</i>	312.80	666.30	0.13	4	723.70	714.70	0.99	4
	<i>Fabp5</i>	25.96	32.68	0.63	4	38.86	30.02	0.46	4
	<i>Hmgbl</i>	5002.00	10300.00	<0.0001	4	9966.00	7452.00	0.0026	4
	<i>Hey1</i>	12.01	16.57	0.61	4	19.22	6.87	0.076	4
	<i>Hes1</i>	2993.00	2803.00	0.79	3	1920.00	1929.00	0.99	4
	<i>Tgfb1</i>	467.80	843.50	0.048	4	879.40	481.00	0.038	4
48 hours siRNA	<i>Foxo1</i>	484.60	245.40	0.036	4	170.50	88.80	0.53	4
	<i>Ccl6</i>	96.78	146.00	0.54	4	164.80	163.70	0.99	4
	<i>Spi1</i>	87.81	73.72	0.78	3	96.98	67.73	0.32	4
	<i>Il1b</i>	0.13	0.32	0.032	2	0.12	0.077	0.38	2
	<i>Tgfb1</i>	2121.00	1709.00	0.64	3	1847.00	1170.00	0.23	4
72 hours siRNA	<i>Icam1</i>	180.30	156.20	0.99	3	x	x	x	x
	<i>Tnf</i>	53.41	25.99	0.33	3	x	x	x	x
	<i>Ccl6</i>	173.60	81.62	0.087	3	x	x	x	x
	<i>Spi1</i>	157.40	83.53	0.65	3	x	x	x	x

Table A2. mRNA level of genes in cultured adipose EC treated with FoxO1 inhibitor (AS1842856, 1 μ M) or vehicle for 6 days. Genes normalized to housekeeping *Tbp* ($2^{-\Delta CT}$).

Gene	Female				Male			
	Control x E-3 mean	6-day inh x E-3 mean	p-val	n	Control x E-3 mean	6-day inh x E-3 mean	p-val	n
<i>Tnf</i>	217.40	290.30	0.88	7	164.30	334.80	0.74	3
<i>Icam1</i>	1.52	1.02	0.99	3	17.63	84.46	0.0041	3
<i>Ccl6</i>	252.70	18.98	0.17	3	269.20	107.70	0.39	3
<i>Tyrobp</i>	2625.00	2691.00	0.99	7	638.10	797.60	0.99	3
<i>Tp53</i>	1234.00	666.40	0.0071	6	394.50	949.30	0.023	4
<i>Piezo1</i>	148.90	1772.00	0.79	3	1805.00	5480.00	0.36	3
<i>Pai1</i>	127.7	129.4	0.97	4	x	x	x	x
<i>Omal</i>	369.00	427.00	0.75	4	x	x	x	x
<i>Nlrp3</i>	78.17	17.45	0.17	4	x	x	x	x
<i>Nos3</i>	23.95	9.06	0.22	4	x	x	x	x
<i>Cyclind</i>	1222.00	2284.00	0.36	3	x	x	x	x
<i>Xrcc6</i>	52.52	63.07	0.68	7	x	x	x	x
<i>Ssbp1</i>	1088.00	1113.00	0.93	7	x	x	x	x

Table A3. List of reagents used in these studies and respective suppliers.

Antibody	Supplier	Catalogue/Clone
Cyclin-dependent kinase inhibitor 1a (p21)	Santa Cruz Biotech	Sc-6246
Cyclin-dependent kinase inhibitor 2a (p16 INK4A)	Cell Signaling	#82172
4-Hydroxynonenal (4-HNE)	R&D systems	#MAB3249
Phosphorylated H2A histone family member X, (γ H2AX)	Cell Signaling	#2577
Phospho-Smad2	Cell Signaling	#3101
Tumor protein p53	Santa Cruz	#sc-6243
FoxO1	Cell Signaling	#2880
Ribosomal protein S6 Kinase beta 1 (p70S6K)	Cell Signaling	#9202
Phosphorylated ribosomal protein S6 Kinase beta 1 (p-p70S6K)	Cell Signaling	#9205
Beta-actin	Santa Cruz	#sc-47778
Cy TM 3 AffiniPure® Goat Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch	#111-165-003
Cy TM 3 AffiniPure® Goat Anti-Mouse IgG (H+L)	Jackson ImmunoResearch	#115-165-003
Fluorescein (FITC) AffiniPure® F(ab') ₂ Fragment Goat Anti-Mouse IgG (H+L)	Jackson ImmunoResearch	#115-096-003
Fluorescein (FITC) AffiniPure® F(ab') ₂ Fragment Goat Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch	#111-096-003
Goat Anti-Rabbit IgG	Jackson ImmunoResearch	#111-035-003
Goat Anti-Mouse IgG	Jackson ImmunoResearch	#115-035-003
Rabbit polyclonal anti-a/b-tubulin	Cell Signaling Technology	#2148
Rabbit monoclonal anti-PFKFB3 (clone D7H4Q)	Cell Signalling Technology	#13123
Rabbit monoclonal anti-Hexokinase II (clone C64G5)	Cell Signalling Technology	#2867
Rabbit monoclonal anti-GAPDH (clone D16H11) XP	Cell Signalling Technology	#5174
Rabbit monoclonal anti-ERG (clone A7L1G)	Cell Signalling Technology	#97249
Rat monoclonal anti-mouse CD31, biotin conjugated (clone MEC 13.3)	BD Biosciences	#553371
Rat monoclonal anti-mouse CD144 (clone 11D4.1)	BD Bioscience	#555289
Goat polyclonal anti-rabbit IgG, Alexa Fluor 647 conjugated	Jackson Immunoresearch	#111-605-0032
Reagent/Kit	Supplier	Catalogue
5-Ethynyl-20-deoxyuridine	Sigma Aldrich	#900584
Azide-fluor 488	Sigma Aldrich	#760765
Azide-fluor 545	Sigma Aldrich	#760757
Copper(II) sulfate	Sigma Aldrich	#451657
L-Ascorbic acid	Sigma Aldrich	#A7506
Recombinant Murine Tumor Necrosis Factor-alpha	Bio Basic Incorporation	#RC234-12
RNeasy Mini Kit	Qiagen	#74106
Click-iT EdU Imaging Kit	Thermo Fisher Scientific	#C10214
Collagenase, Type I	Thermo Fisher Scientific	#17100017

Collagenase, Type I Worthington	Biochemicals	#LS004197
Dynabeads Antibody Coupling Kit	Thermo Fisher Scientific	#14311D
Goat serum	Sigma Aldrich	#G9023
Griffonia (Bandeiraea) Simplicifolia Lectin I (GSL I, BSL I), Fluorescein	Vector Laboratories	#FL-1101
M-MuLV Reverse Transcriptase	New England Biolabs	#M0253
QIAzol Lysis Reagent	Qiagen	#79306
Random Primer Mix	New England Biolabs Incorporation	#S1330S
RNase Inhibitor, Murine	New England Biolabs Incorporation	#M0314S
Rodent Diet With 58 kcal% Fat and Sucrose	Research Diets	#D12331
Rodent Diet With 45 kcal% Fat	Research Diets	#D12451
Rodent Diet With 10 kcal% Fat	Research Diets	#D12450H
Streptavidin Particles Plus - DM	BD Biosciences	#557812
SuperSignal West Pico PLUS Chemiluminescent Substrate	Thermo Fisher Scientific	#34080
TaqMan Fast Advanced Master Mix	Thermo Fisher Scientific	#4444963
VECTASHIELD Vibrance Antifade Mounting Medium	Vector Laboratories	#H-1700-10
Phosphatase inhibitor PhosSTOP	Roche	#4906837001
Complete protease inhibitor	Roche	#11836153001
Tamoxifen	Sigma Aldrich	#T5648
AS1842856 FoxO1 inhibitor	EMD Millipore	#344355
Seahorse XF Real-Time ATP Rate Assay Kit	Agilent	#103592-100
CyQUANT	Thermo Fisher Scientific	#C7026
DharmaFECT	Horizon Discovery	#T-2005-01
ON-TARGETplus Mouse Foxo1 siRNA	Horizon Discovery	#L-041127-00-0005
ON-TARGETplus Non-targeting Control Pool	Horizon Discovery	#D-001810-10-05
MitoBrilliant™ 646	TOCRIS	#7700
MitoTEMPO	Sigma Aldrich	#SML0737
Menadione	Sigma Aldrich	#M5625
CellROX green	Invitrogen	#C10444

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