The Role of FoxO1 and Sex Differences in Endothelial Cells in Response to a DNA-Damaging Agent

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Abstract

Endothelial cell (EC) health can be disturbed in various diseases like aging, obesity, atherosclerosis and type 2 diabetes. Previously, our lab showed that female ECs retain a healthier phenotype than male ECs under obesogenic conditions and that they have higher levels of Forkhead Box O1 (FoxO1) than male ECs. The goal was to investigate whether FoxO1 inhibition enhances EC vulnerability and whether any sex differences occur in response to an oxidant insult. To achieve this, ECs were treated with a FoxO1 inhibitor and treated with cisplatin. FoxO1 inhibition promoted cell cycle arrest and antioxidant induction in both sexes while DNA damage improved only in male ECs. Unperturbed female ECs displayed higher levels of γH2AX compared to male ECs. These data provide evidence that FoxO1 inhibition is potentially protective for the male EC while also revealing a potential sex disparity in the DNA damage response pathway.

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Abbreviations

3PO - 3-(3-Pyridinyl)-1-(4-pyridinyl)-2-propen-1-one

53BP1 - p53-binding protein 1

AKT - Protein kinase B

AS184 - AS1842856

ATP - Adenosine triphosphate

BRCA1 - Breast cancer gene 1

CAT - Catalase

Cdkn1a - Cyclin-dependent kinase inhibitor 1a

Cdkn2a - Cyclin-dependent kinase inhibitor 2a

DAPI - 4',6-diamidino-2-phenylindole

DDR - DNA damage response pathway

DEGs - Differentially expressed genes

DSB - Double stranded break

EC - Endothelial cell

EC-FoxO1 KD – Cre^{+};$FoxO1^{f/f}$

eNOS - Endothelial nitric oxide synthase

FADH2 - Flavine adenine dinucleotide

FoxO1 - Forkhead box O1

FoxO1 Floxed-ECs – Cre^{-};FoxO1

Gadd45α - Growth arrest and DNA damage

GPx - Glutathione Peroxidase

H2AX - Histone 2AX

H2O2 - Hydrogen peroxide

HFD - High fat diet

HR - Homologous recombination

ICAM-1 - Intercellular adhesion molecule-1

IL-1 - Interleukin-1

IL-6 - Interleukin-6

iCreER^{T2} - Inducible form of cre recombinase

IRS-1 - Insulin receptor substrate-1

JNK - c-Jun N-terminal kinase

MRN - MRE11/RAD50/NBS1

mtDNA - Mitochondrial DNA

mtROS - Mitochondrial ROS

NADH - Nicotinamide adenine dinucleotide

NADPH - Nicotinamide adenine dinucleotide phosphate

NFκB - Nuclear factor-κB

NER - Nucleotide excision repair

NO - Nitric oxide

NOX - Nicotinamide adenine dinucleotide phosphate oxidase

NHEJ - Non-homologous end joining

O2- - Superoxide anion

OH - Hydroxyl radical

ONOO - Peroxynitrite

PCNA - Proliferating cell nuclear antigen

P38 MAPK - p38 mitogen activated protein kinase

PFKFB3 - 6-phosphofructo-2-kinase/fructose-2,6-bisphosphotase

PVDF - Polyvinylidene difluoride membrane

Prdx - Peroxiredoxin

Rb - Retinoblastoma

ROS - Reactive Oxygen Species

RPA - Replication protein A

SA - Senescence-associated

SOD - Superoxide dismutase

ssDNA - Single-stranded DNA

TBP - TATA-Box Binding Protein

TNF-α - Tumor necrosis factor-α

TRF2 - Telomeric repeating binding factor-2

Trx - Thioredoxin

VCAM-1 - Vascular cell adhesion molecule-1

VEGF-A - Vascular endothelial growth factor

VEGFR2 - Vascular endothelial growth factor receptor

Chapter 1: Literature Review

1.1 Microvasculature

The vasculature facilitates the transport of oxygen and nutrients throughout all tissue and organs to ensure the survival of cells and removes CO_2 and metabolic by-products from the body (Santamaría et al., 2020; Yuan and Rigor, 2010). Oxygenated blood is moved from the left-ventricle into circulation through the aorta and passed to large-diameter arteries that branch into small arterioles (Miller & Gal, 2017). Arterioles are resistance blood vessels that are encircled by smooth muscle, they can contribute to mean arterial pressure and can control the diameter of the arteriole which is manipulated by the autonomic nervous system. These vessels are responsible for regulating local perfusion of tissue. Their ability to contract and dilate is beneficial as it enables dynamic changes in blood flow based on that tissue's requirements for more nutrients or oxygen (Tucker et al., 2023). Capillaries are the smallest of blood vessels and are responsible for the diffusion of oxygen, nutrients and metabolites to the tissue. One important feature of capillaries is the ability to act as the prominent gatekeeper, enabling the exchange of gases, nutrients and small solutes (Yuan & Rigor, 2010). Following unloading of oxygen at the tissue, the deoxygenated blood begins its transport back to the heart via thin-walled vessels called venules. These venules drain into larger vessels called veins which hold a lot of blood volume at low pressures (L. M. Miller & Gal, 2017; Tucker et al., 2023).

1.2 Muscle Vascularization

Muscle capillarization is essential for providing energy for the muscle and maintaining muscle health by allowing the exchange of gases and nutrients such as carbon dioxide, oxygen and glucose. Skeletal muscle is well vascularized, myofibers are intertwined by a network of blood vessels that enable matching of the constantly changing demand of the muscle (Nederveen et al.,

2021). Higher muscle capillarity provides higher surface area and shorter diffusion distances, both of which support greater delivery of oxygen and nutrients (Chilibeck et al., 1997). Depending on the muscle fiber type the capillary distribution will vary. Type I and type IIa fibers are categorized as slow-twitch oxidative fibers and fast-twitch oxidative fibers, respectively. These fibers consume a lot of oxygen due to a high level of mitochondria; they have the highest number of capillaries surrounding the fiber, with more capillaries surrounding type I fibers than type IIa fibers. Type IIx fibers are classified as glycolytic fast twitch fibers; they have fewer mitochondria and rely less on aerobic metabolism to produce ATP. Thus, type IIx fibers have less capillaries surrounding them compared to type I and type IIa fibers (Ingjer, 1979; Liu et al., 2012).

The demand for oxygen and nutrients increases during exercise. Repeated exercise promotes blood vessel growth so that the elevated oxygen and nutrient demand in the muscle can be satisfied (Latroche et al., 2015; Nyberg & Jones, 2022; Leuchtmann et al., 2020). Angiogenesis, the expansion of capillaries from pre-existing blood vessels (Adair and Montani, 2010), can be stimulated by mechanical stressors such as shear stress and passive stretching of the muscle, however, mechanical stimuli alone are insufficient to induce angiogenesis (Hellsten and Gliemann, 2023). Changes to muscle metabolism such as local hypoxia during exercise or low nutrient availability can provoke angiogenesis (Fraisl et al., 2009; Gustafsson et al., 1999). The vasculature also plays a vital role in muscle regeneration by undergoing angiogenesis which is a necessity for muscle regeneration and recovery subsequent to muscle injury.

VEGF (Vascular Endothelial Growth Factor) -A and VEGF receptor 2 (VEGFR2) are two central proteins involved with angiogenesis. VEGF-A binding to VEGFR2 initiates several intracellular signaling pathways that moderate responses such as endothelial cell (EC) survival,

proliferation and migration (Simons et al., 2016). Mice with muscle specific VEGF-A deletion do not undergo angiogenesis in response to a known angiogenic stimulus like shear stress or exercise (Uchida et al., 2015) (Olfert et al., 2010). These studies provide evidence that VEGF-A is necessary for provoking muscle angiogenesis.

1.3 The Endothelium

The inner wall of all blood vessels contains a single layer of ECs. The endothelium plays a crucial part in regulating vascular tone, the exchange of fluids and solutes, controlling coagulation that limits thrombosis, angiogenesis, and orchestrating inflammatory defense mechanisms (Moncada and Higgs, 2006). Under physiological conditions in a normal adult organism, ECs are quiescent which is characterized by a lack of blood vessel remodeling. Though ECs are not actively growing, they secrete various factors such as nitric oxide and VEGF that aid in the survival of ECs and maintaining a healthy endothelium as well as influencing the function of smooth muscle cells (Apte et al., 2019; Dimmeler and Zeiher, 1999; Ricard et al., 2021). There is structural heterogeneity of ECs throughout the vasculature based on their location. EC phenotype differs depending on the location within the vascular network. For example, ECs from the arterial side are functionally and anatomically different from venous ECs (Chi et al., 2003; dela Paz and D'Amore, 2009). Capillary ECs also are functionally and morphologically dissimilar from artery and vein ECs (Hennigs et al., 2021; Kalucka et al., 2020).

1.4 Cellular Stressors Affecting Endothelial Cells

The chronic inflammatory state that stems from comorbidities of individuals with cardiovascular diseases can play a role in negatively affecting EC health (Castellon and Bogdanova, 2016). Cardiovascular diseases are associated with an increase in production of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, (interleukin) IL-1, IL-6 and

interferon-γ which are released from ECs (Amin et al., 2020). These inflammatory cytokines promote nuclear factor-κB (NF-κB) signaling, which upregulates immune cell adhesion molecules such as intercellular adhesion molecule (ICAM)-1, vascular cell adhesion protein (VCAM)-1 and E-selectin (Theofilis et al., 2021). Consequently, activation of ECs provokes the upregulation of proinflammatory cytokines, chemokines, and adhesion molecules (Sun et al., 2020).

Conditions like obesity, type 2 diabetes, atherosclerosis and aging can contribute to a state referred to as 'endothelial dysfunction' (Giorgi et al., 2018; Masschelin et al., 2020; Nowak et al., 2017; Volpe et al., 2018). Nitric Oxide (NO) is an essential molecule for maintaining the health of ECs; thus, ECs become dysfunctional when they exhibit diminished production of nitric oxide (Hadi et al., 2005). Reactive oxygen species (ROS) are one of the main causes of EC dysfunction as they have the ability to react with NO and reduce its bioavailability (Shaito et al., 2022). Dysfunctional endothelium retains a pro-inflammatory and pro-thrombotic status (Scioli et al., 2020). Thus, EC dysfunction can pave the way to the initiation of various cardiovascular diseases and exacerbate the symptoms of cardiovascular diseases (Sun et al., 2020).

1.5 Reactive Oxygen Species & Oxidative Stress

ROS are oxygen containing molecules that have an unpaired electron in their outer shell. These molecules are highly unstable and reactive due to the unpaired electron that needs to be paired with another electron to stabilize its valence shell (Chaudhary et al., 2023). ROS have the ability to remove electrons from biomolecules (Das and Roychoudhury, 2014; Li et al., 2016). The presence of ROS exerts pleiotropic effects, where physiological levels of ROS are needed for basic cellular processes such as defense against pathogens (Pizzino et al., 2017), insulin signaling (Lennicke and Cochemé, 2021), angiogenesis and cell proliferation (Sies and Jones,

2020). This concept of ROS at physiological levels participating in redox signaling is know as ‘eustress’. On the contrary, supraphysiological levels of ROS can compromise EC health by oxidizing the structural components of proteins, lipids, carbohydrates and DNA, causing damage and thus altering their functions. This can alter basic cellular processes within a cell (Hajam et al., 2022; Pizzino et al., 2017). Oxidative stress occurs when there is excessive production of ROS accompanied by the inability of antioxidant enzymes to efficiently neutralize ROS (Higashi, 2022; Li et al., 2016). In addition, antioxidant enzyme levels may decrease, this leaves the ECs vulnerable to an oxidative insult as they cannot appropriately neutralize the overwhelming levels of ROS that can come about from various diseases (Shafi et al., 2019). Thus, it is imperative that ECs have a sufficient defense mechanism against ROS.

ROS come in various forms in ECs: superoxide anion (O_2^-) can be dismuted to hydrogen peroxide (H_2O_2). NO, a reactive free radical, can react with O_2^- to form peroxynitrite ($ONOO^-$), a highly unstable type of reactive nitrogen species (RNS). H_2O_2, in the presence of transition metals like iron, can form Hydroxyl radical (OH)(Alhayaza et al., 2020; Lyngsie et al., 2018). O_2^- can be formed from various sources within the EC such as the mitochondria, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), uncoupled endothelial nitric oxide synthase (eNOS), and xanthine oxidase (Figure 1).

Mitochondria produce energy in the form of ATP through oxidative phosphorylation. In the process of electron flow, electrons may slip into the matrix of the mitochondria and react with molecular oxygen producing O_2^- (Schulz et al., 2011). In a condition like hyperglycemia, there is an increase in NADH and $FADH_2$ due to increased activity of glycolysis and the citric acid cycle. This overwhelms the electron transport chain, resulting in more electron leakage into

the matrix (Manna and Jain, 2015). Consequently, higher levels of O_2^- are produced within the matrix, forcing the movement of O_2^- into the cytosol of the cell (Schulz et al., 2011).

NADPH oxidases are membrane-bound enzymes that facilitate the reduction of oxygen to superoxide O_2^- by donating an electron to an oxygen molecule (Incalza et al., 2018). Under basal conditions the NOX subunits are not highly activated, thus, not producing O_2^- at supraphysiological levels (Zheng et al., 2022). However, pathological influences such as hyperlipidemia, hyperglycemia and hypertension can rapidly activate NOX2 subunits and cause the overproduction of O_2^- (Drummond and Sobey, 2014; Zheng et al., 2022).

Another source of ROS are nitric oxide synthases. eNOS uses oxygen and l-arginine to produce NO and l-citrulline (Madamanchi et al., 2005). In the absence of l-arginine and/or the co-factor tetrahydrobiopterin (BH_4), eNOS becomes “uncoupled”. In this state, electron flow from the reductase domain of eNOS is shuttled to molecular oxygen instead of l-citrulline giving rise to O_2^- instead of NO. Subsequently, NO and O_2^- can react to produce $ONOO^-$ (Incalza et al., 2018; Schulz et al., 2011; Zheng et al., 2022).

Xanthine oxidase plays a crucial role in purine catabolism (Sabahi et al., 2018) and is an enzyme that forms uric acid. Aging promotes ROS production through xanthine oxidase. For example, plasma extracted from aged individuals displays not only higher levels of xanthine oxidase but it is also associated with increased activity. Similar results are seen in the muscle and aortas of aged mice (Aranda et al., 2007). Xanthine oxidase enzymatic activity orchestrates the oxidation of hypoxanthine to xanthine and then xanthine to uric acid and H_2O_2, causing a flux of electrons that reduce oxygen to O_2^- (Battelli et al., 2014; Berry and Hare, 2004).

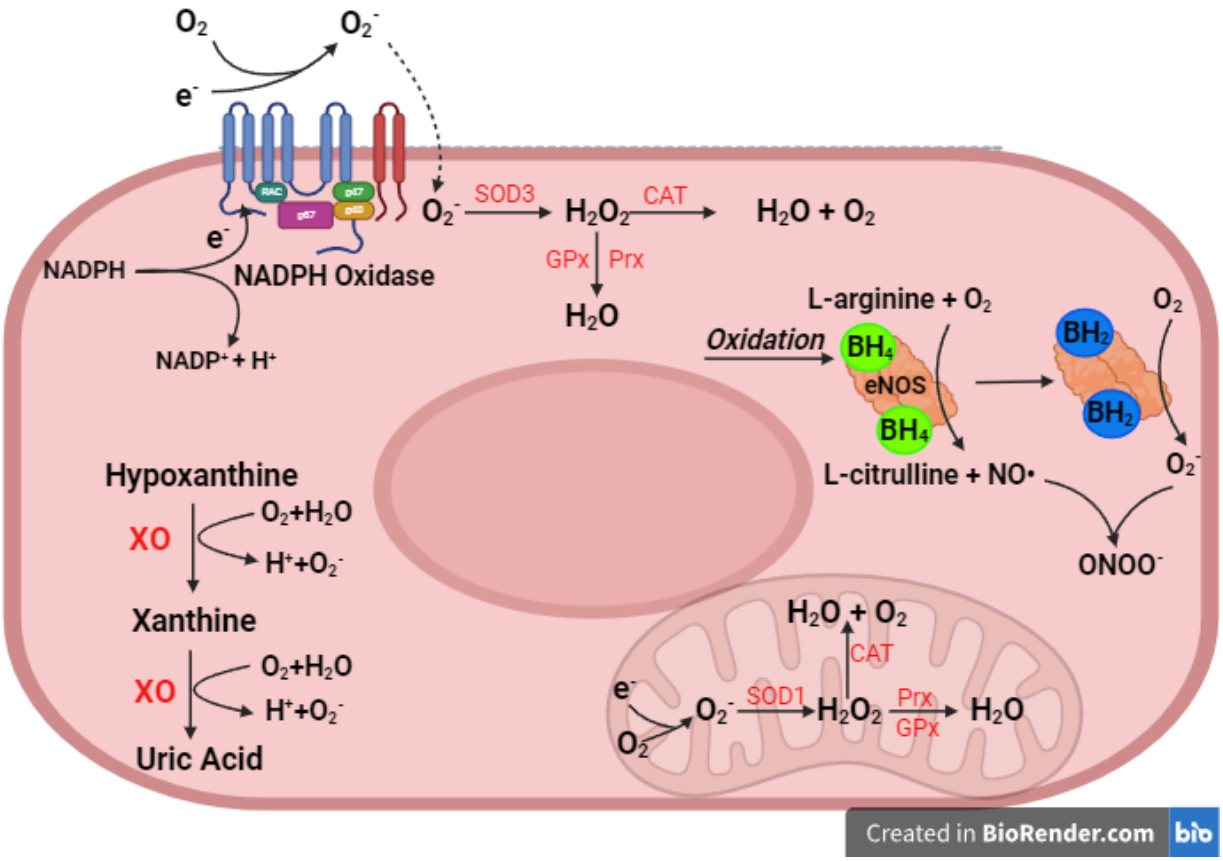

Figure 1: Various sources of EC ROS production and potential scavenging of ROS within the cell.

NADPH oxidase facilitates the oxidation of NADPH to $NADP^+$ and H^+, transferring the electron and reducing molecular oxygen to O_2^-. O_2^- can react with BH_4 oxidizing it to BH_2. Consequently, lack of BH_4 promotes eNOS uncoupling, causing it to produce O_2^- instead of NO. In a state of oxidative stress mitochondrial DNA becomes oxidized leading to defective complex I and complex III; key sites of ROS production. Electrons slip into the matrix, reducing molecular oxygen to O_2^-. Within the cytoplasm, xanthine oxidase catalyzes the oxidation of hypoxanthine to xanthine and then uric acid, simultaneously reducing oxygen to O_2^-. Intracellular O_2^- can be neutralized by SOD3 to H_2O_2, that can be further neutralized to water and oxygen by catalase (CAT). GPx and Prx also can reduce H_2O_2 to water.

1.6 Antioxidant Defense

The ECs rely on proteins called antioxidants to protect themselves from ROS and to maintain cellular redox homeostasis (Ye et al., 2019). Specifically, antioxidants can inhibit the formation of free radicals, direct scavenging, or they can repair the damage on macromolecules by reversing disulfide bonds (Sharifi-Rad et al., 2020; Ulrich and Jakob, 2019). Disulfide bonds are formed between thiol groups of two cysteine residues, and they are indicative of how oxidative

or a reduced the cellular environment is (Bechtel and Weerapana, 2017; Cumming et al., 2004). Examples of antioxidants found in ECs are superoxide (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Lubrano and Balzan, 2015). SOD facilitates the conversion of highly volatile O_2^- to less volatile H_2O_2 (Afonso et al., 2007); CAT neutralizes H_2O_2 producing water and oxygen. GPx oxidizes glutathione and reduces H_2O_2 forming water (Lubos et al., 2011b). In knockout studies where SOD1 and GPx-1 are deleted, mice show increases in muscle oxidative damage. This suggests that muscle cells are more susceptible to oxidation in response to an oxidative challenge, creating a pro-inflammatory environment. (Lubos et al., 2011a; Sakellariou et al., 2018). This highlights the importance of antioxidants for maintaining the proper redox environment, preventing cellular damage.

Peroxiredoxins (Prdxs) are a family of thiol specific antioxidant enzymes. Different isoforms of Prdxs are localized in various cellular compartments such as the cytoplasm, nucleus, mitochondria, endoplasmic reticulum and peroxisomes (Park et al., 2016; Perkins et al., 2015). Particularly, Prdx3 is localized in the mitochondria (Lee et al., 2016), where it neutralizes H_2O_2 so as to prevent its accumulation in the mitochondria that can lead to mitochondrial damage and even cellular damage. When Prdx3 reacts with ROS, the cysteine residues on Prdx3 become oxidized, sacrificing itself rather than important macromolecules from being oxidized and ensuing damage. Consequently, to revert Prdx3 back to its reduced form, thioredoxin (Trx) donates an electron to Prdx3 preparing it for subsequent reactions with ROS (Park et al., 2016). SOD-1 KO mice showed an increase in mitochondrial H_2O_2 generation resulting in impaired contractile properties of skeletal muscle. Conversely, SOD-1 KO mice that also overexpressed muscle specific Prdx3 demonstrated a decline in oxidative damage to contractile proteins and, calcium regulatory proteins, and improved contractile properties of the muscle (Ahn et al., 2022).

This study highlighted the importance of Prdx3 for scavenging and reducing mitochondrial H_2O_2, reversing the detrimental effects of SOD-1 KO.

The transcription factor Forkhead-box class O (FoxO) regulates the production of antioxidant proteins. FoxO has four different isoforms FoxO1, 3, 4, 6 (Salih and Brunet, 2008). They play a role in regulating multiple genes that are involved proliferation, differentiation, cell viability, glucose metabolism, and oxidative stress resistance (Lu and Huang, 2011). However, of these different isoforms, FoxO1 is the most important to biology as it plays a role in the advancement of metabolic disorders in obesity by mediating metabolism and angiogenesis (Rudnicki et al., 2018a). Phosphorylation of FoxO1 by protein kinase B (AKT) at Thr24, Ser256 and Ser319, enhances FoxO1 binding to 14-3-3 chaperone proteins and potentiates its nuclear exclusion, inactivating its transcriptional activity (Klotz et al., 2015; Lu and Huang, 2011). However, when excessive ROS are present, FoxO1 can be phosphorylated by c-Jun N-terminal kinases (JNK) and p38 mitogen activated protein kinase (MAPK) (Klotz et al., 2015), which enables FoxO1 translocation from the cytoplasm to the nucleus. Consequently, FoxO1 promotes transcription of genes involved with cell cycle arrest, ROS detoxification and DNA repair (Ponugoti et al., 2012; Salih and Brunet, 2008; Weng et al., 2016; Xiong et al., 2011). The antioxidants transcribed by FoxO1 are important for the eradication of ROS as well as protecting the cell against ROS induced damage by shifting the cell into a stress resistant state and/or promoting DNA repair (Fig. 2).

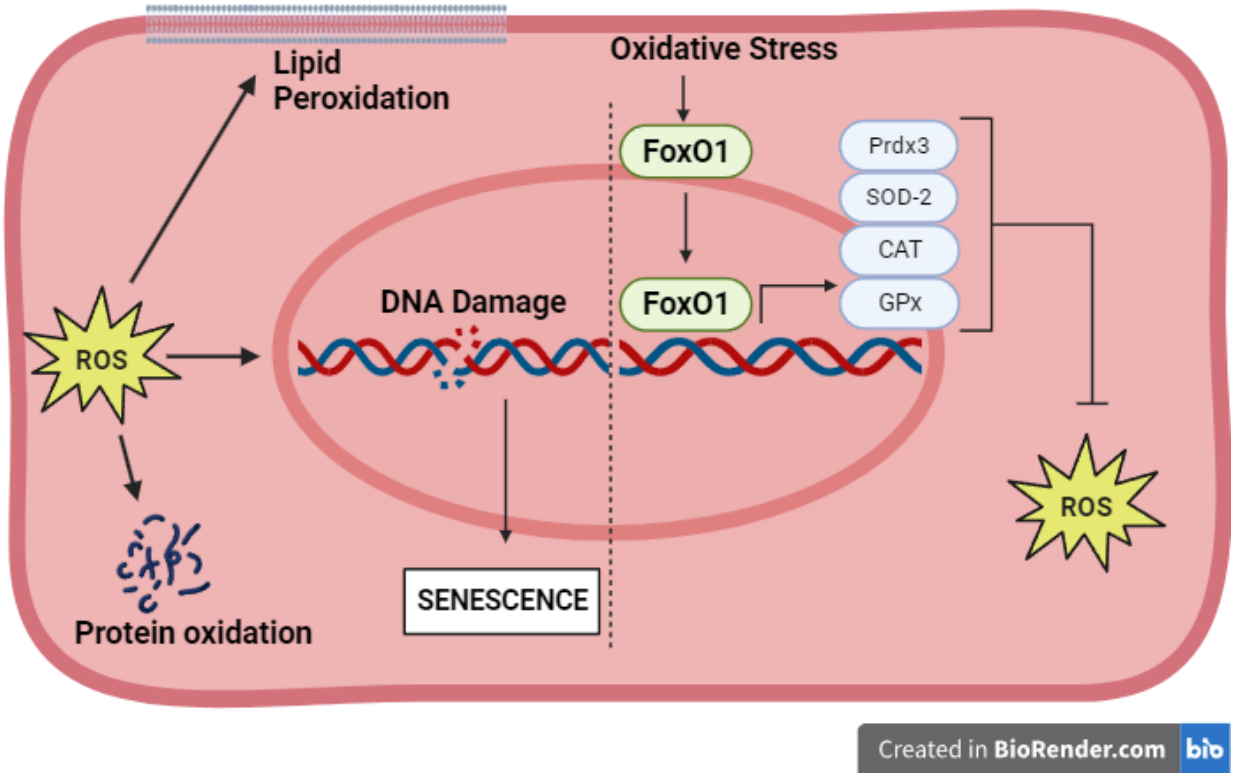

Figure 2: A schematic depicting the role of FoxO1 in response to a DNA-damaging agent. In specific diseases such as obesity, type 2 diabetes, atherosclerosis and aging there is an increase vascular ROS. ROS, an unstable oxygen radical, is highly reactive and can oxidize lipids, proteins and DNA to satisfy its unbound electron. A consequence of DNA damage is senescence, irreversible cell cycle arrest, whereby the EC retains its metabolic activity and develops SASP. FoxO1, a transcription factor that is known to play a role in resisting oxidative stress, translocates into the nucleus facilitating the transcription of antioxidant genes such as, Prdx3, SOD-2, CAT and GPx. These antioxidants neutralize ROS and prevent protein, lipid and DNA oxidation.

1.7 ROS induced DNA damage

ECs that are in a state of oxidative stress can accumulate DNA damage. Consequently, this can give rise to mutations throughout the genome, promoting genomic instability (Han and Kim, 2023; Huang et al., 2022; Rowe et al., 2008). ROS can react with deoxyribose and nitrogenous bases which induces a severe oxidative reaction. The process starts with a free radical abstracting a proton from deoxyribose forming ribose fragments. This gives rise to breaks in the sugar-phosphate backbone, otherwise known as double stranded breaks (DSBs) (Juan et al., 2021; Maynard et al., 2009; Sharma et al., 2016). One well known example of a DNA damage

modification that occurs as a result of oxidative stress is the oxidation of the nucleic acid guanine, which gives rise to a known DNA damage biomarker called 8-oxoguanine. If DNA damage is not sufficiently repaired, the accumulation of mutations in the DNA can lead to chromosome instability, telomere shortening, cancerous growth, cell death or senescence (Barnes et al., 2019; Hakem, 2008; Nikfarjam and Singh, 2023).

1.8 DNA Repair Pathways

The premise of DNA repair is to ensure that any mutations that occur in the genome are not left unresolved as this can lead to disease. Hence, the first step in the DNA repair response is to flag sites of DNA damage by recruiting specialized proteins that act as sensors (Ng and Hazrati, 2022). Next, the sensors recruit other DNA repair complexes to these sites; at the same time, the cell upregulates cell cycle inhibitors and decreases the production of cell cycle activators which is absolutely essential for DNA repair to occur (Cardano et al., 2022). It is in this stage where the cell decides to choose one of the of DNA repair pathways (Chatterjee and Walker, 2017).

The MRN (MRE11/RAD50/NBS1) complex, an important mediator of the DDR pathway, has the ability to choose which DNA repair pathway is best for the damage, either homologous recombination (HR) or non-homologous end joining (NHEJ) (Bian et al., 2019).

1.8.1 Homologous Repair

HR is an error free type of DNA repair that requires a homologous sister chromatid as a recombination template. This type of DNA repair is favoured in the S and G2 phases when there are duplicated chromosomes (Kijas et al., 2015; Qiu & Huang, 2021; Zhao et al., 2020). It is proposed that the MRN complex creates a bridge between DSB breaks and enables DNA end resection, whereby the 5’ ends of broken DNA are cleaved to produce ssDNA overhangs which are crucial for HR DNA repair. Single-stranded DNA (ssDNA) is produced; this acts as a

scaffold for attracting specific HR DNA repair proteins that participate in DNA resynthesis (Her and Bunting, 2018).

1.8.2 Non-Homologous DNA End-Joining

NHEJ DNA repair is error-prone, however, it is faster than HR and is specific for two ended DSBs (Ackerson et al., 2021; Zhao et al., 2020). In contrast to HR DNA repair, NHEJ DNA repair occurs mainly in G1 phase, making it the main repair pathway in mammalian cells (Ackerson et al., 2021; Burma et al., 2006). In fact, there is evidence that NHEJ DNA repair is the first line of defense for repairing DSBs (Beucher et al., 2009; Rothkamm et al., 2003; Shahar et al., 2012). Ku is a heterodimer that binds to DSBs is required for NHEJ as it acts to block end resection, important for preventing HR pathway which forms ssDNA (Ackerson et al., 2021). Similar to the MRN complex, Ku is the first protein to bind to DSBs, this promotes phosphorylation of downstream DNA repair proteins, vital for NHEJ (Burma et al., 2006; Stinson and Loparo, 2021).

1.8.3 Nucleotide Excision Repair

Nucleotide excision repair (NER) is another means by which the cell can remove chemically altered bases and adducts formed from oxidative stimuli (Chatterjee and Walker, 2017). The basis of NER is to remove 12-13 nucleotides; the concomitant opening in the DNA is then filled via a gap-filling repair mechanism where by new nucleotide synthesis occurs using the intact complementary strand as a reference (de Boer and Hoeijmakers, 2000).

1.9 Gadd45α Role in DNA repair

Gadd45α, an 18 kDa protein, is a DNA adaptor protein as it is involved with DNA demethylation during an oxidative insult and plays a role in cell cycle control and DNA repair (Niehrs and Schäfer, 2012). Upon DNA damage from oxidative or genotoxic agents, Gadd45α is promptly

induced by transcription factors such as FoxO1 or p53 (Amin and Schlissel, 2008; Tanaka et al., 2017). G_2 checkpoint activation, which usually occurs after exposure to DNA damaging agents, is compromised in Gadd45α deficient mice. Less induction of cell cycle arrest gives less time for the cell to repair damage to DNA, resulting in genomic instability (Hollander et al., 1999). Accordingly, Gadd45$^{-/-}$ mice displayed an increase in mutation frequency and genomic vulnerability in response to ionizing radiation and chemical carcinogenesis (Liebermann and Hoffman, 2008). The DNA excision repair capabilities of Gadd45α are heightened upon binding to proliferating cell nuclear antigen (PCNA) (Liebermann and Hoffman, 2008). Thus, Gadd45α can interact with the NER complex at sites of DNA adducts or lesions and facilitate the recruitment of DNA repair proteins to the area of damage (Barreto et al., 2007). As a result of its functions, Gadd45α plays an essential role in protecting the genomic stability of the cell.

1.10 DNA Damage Response Pathway

Under conditions where there are sublethal levels of oxidative stress that cause DSBs, the cell's defense mechanism is to enter cell cycle arrest. Subsequently, once the DNA repair machinery restore the DSBs the cell can begin to proliferate again (Chen et al., 2007).

It is widely accepted that DNA damage induced senescence is triggered by the DDR pathway (Cardus et al., 2013). By binding to DSBs, the MRN complex acts as a sensor that signals to the cell that there is DNA damage at that location, as seen in Figure 3 (Kijas et al., 2015). Thereafter, the MRN complex recruits Ataxia Telangiectasia Mutated (ATM) to the site of DSBs, (Zha et al., 2008). Upon its recruitment, ATM is autophosphorylated which activates its kinase ability, promoting the phosphorylation of proteins involved in the DDR pathway. A primary response to DSBs is the ATM-dependent phosphorylation of histone 2AX (H2AX) at serine 139 (Burma et al., 2001), referred to as γH2AX (Marinoglou, 2012). γH2AX acts as a cue

for other DNA repair proteins to be recruited, such as p53-binding protein 1 (53BP1), mediator of DNA damage checkpoint 1 (MDC1), breast cancer gene 1 (BRCA1) and for the assembly of DNA repair complexes (Maréchal and Zou, 2013; Schmitt et al., 2022). Once DNA damage has been tagged as a DSB through the formation of γH2AX foci, the next phase of DNA repair is to inhibit the cell cycle to allow the DNA to undergo repair (Foster et al., 2012). The G1 checkpoint is crucial as it prevents the replication of damaged DNA. Tumor suppressor protein p53 (p53), a transcription factor, is essential for G1/S checkpoint (Pellegata et al., 1996). However, in a senescence context only, ATM phosphorylation of p53 increases the transcriptional activity of p53 (Webley et al., 2000). p53 controls the expression of genes that are also involved with halting cell cycle progression (Kastenhuber and Lowe, 2017). One of these genes, *Cdkn1a,* that belongs to the cyclin-dependent kinase (CDK) inhibitor family, encodes a protein called p21, which mediates G1 growth arrest (Brugarolas et al., 1995; Deng et al., 1995). p21 prevents proliferation through inhibiting CDK4,6/cyclin-D and CDK2/cyclin-E complex assembly, resulting in the hypo-phosphorylation of retinoblastoma protein (Rb). As a result, this retains the transcription factor E2F bound to Rb, restraining the release of E2F to promote the transcription of cell cycle genes, thus blocking G1/S and G2/M transitions (Karimian et al., 2016). Low levels of p21 do not prevent cell cycling, which does not give the cell an optimal environment for DNA repair (Kumari and Jat, 2021; Łukasik et al., 2021). Overall, p21 is crucial for the last step in the DDR pathway stopping proliferation temporarily in response to DNA damage until DNA damage is resolved. In scenarios of proper DNA damage repair, the cell cycle can resume (Hernandez-Segura et al., 2017).

1.10.1 DDR-induced Senescence

Constant bombardment of DNA damage inducing agents that induce sufficient DNA damage or faulty DNA repair promotes chronic DDR pathway activation, consequently, p53 and p21 are persistently being activated leading to premature cellular senescence in healthy ECs (Kumari and Jat, 2021; Yosef et al., 2017). Senescence is characterized by permanent cell cycle arrest while the cell is still metabolically active. This phenotype includes distinct transcriptional changes that results in a robust secretory program comprised of cytokines, chemokines, growth factors, extracellular matrix proteases and ROS (Huang et al., 2022). ECs also experience physical changes; they exhibit a flatter and enlarged appearance when they become senescent (Jia et al., 2019). Some factors that can induce senescence are telomere attrition, oxidative stress, mitochondrial dysfunction, oncogene activation and mitotic stress (Narasimhan et al., 2022). In particular, telomere attrition and damage are the main reasons why senescence is higher in aging individuals (Rossiello et al., 2022). In a cancer context, cellular senescence is protective. When an organism senses aggressive proliferative activity, its first line of defense is to prompt permanent cell cycle arrest, emphasizing the positive role for senescence in preventing tumor development (Schmitt et al., 2022). However, in the context of cardiovascular pathologies there is significant DNA damage done to the endothelium. Thus, if DNA damage is persistent and remains unresolved or repaired incorrectly, DSBs gather and lead to senescence (Wu et al., 2023). The senescence of vascular ECs plays a critical role in the commencement and progression of various cardiovascular diseases (Katsuumi et al., 2018; Minamino et al., 2002; Morgan et al., 2019; Yokoyama et al., 2014).

1.11 Senescence Associated Secretory Phenotype

The SASP acts in an autocrine manner to maintain the senescent phenotype in senescent cells via interleukin-1 (IL-1) and interleukin-6 (IL-6) (Kumari and Jat, 2021; Tasdemir and Lowe, 2013). It was originally thought that senescence was autonomous, that its effects were not able to be propagated to the surrounding tissue and cells. However, there is a collection of evidence that the secreted factors from senescent cells have the capacity to induce senescence in neighboring healthy cells in a paracrine manner (Coppé et al., 2010; Kumari and Jat, 2021; Sun et al., 2022; Tasdemir and Lowe, 2013). (Nelson et al., 2018, 2012). In the context of the vasculature, the senescent signal can be transmitted to perivascular cells or other ECs throughout the vascular network, creating a positive feedback loop.

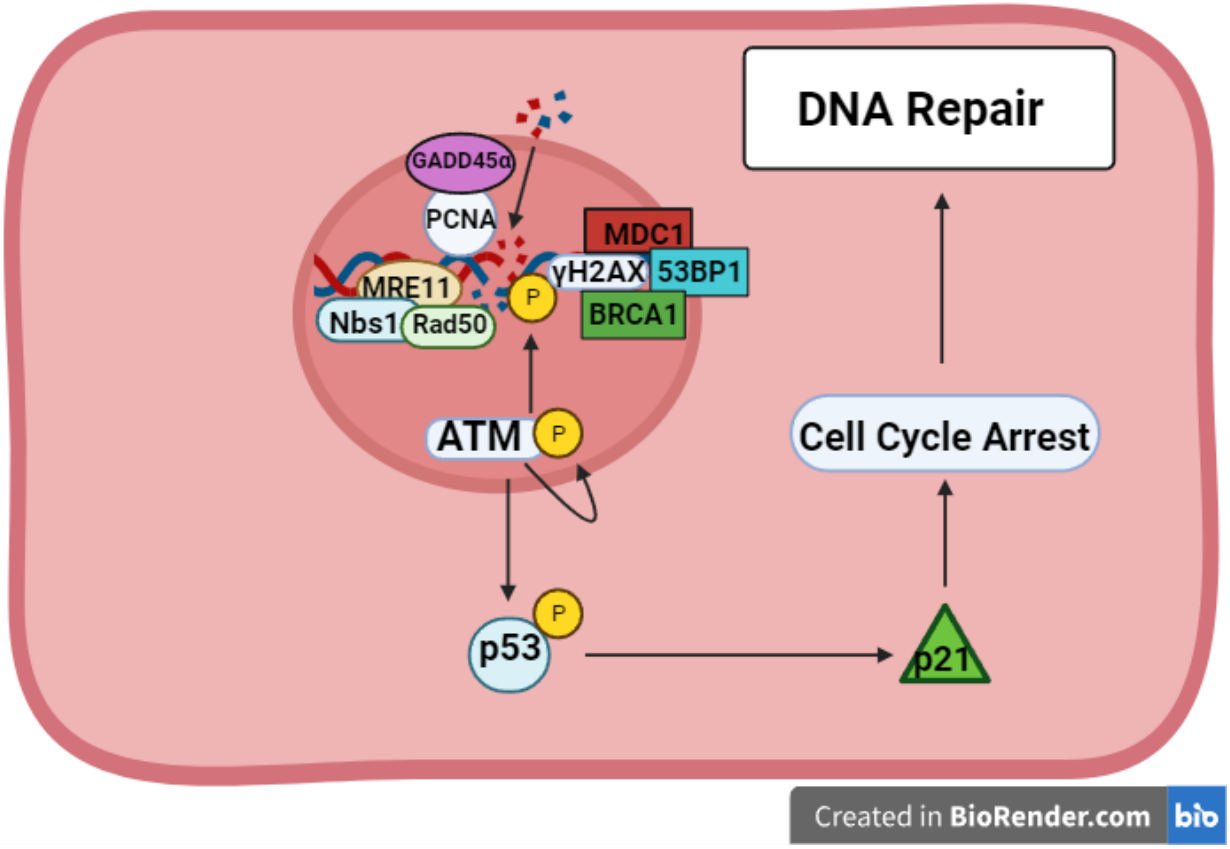

Figure 3: DNA Damage Response Signaling.

DNA damage response signaling is imperative for DNA repair. The MRN complex acts as a sensor as it localizes at DSB and recruits ATM. ATM becomes autophosphorylated and phosphorylates H2AX at Ser139 forming γH2AX, which acts as a platform for recruiting DNA repair proteins such as MDC1, 53BP1 and BRCA1. ATM also phosphorylates and activates p53, transcribing the cell cycle inhibitor p21, facilitating cell cycle arrest. Once the cell cycle is temporarily halted, DSBs can be successfully repaired.

1.12 DNA repair and Glycolysis

Glycolysis is an important metabolic process to convert glucose into pyruvate. During this process, energy is released in the form of adenosine triphosphate (ATP) and NADPH (Shi et al., 2017). 6-phosphofructo-2-kinase/fructose-2,6-bisphosphotases (PFKFBs) are rate-limiting enzymes in the glycolytic pathway. There are four different isoforms of PFKFBs, 1, 2, and 4 being ubiquitously expressed and PFKFB3 being the inducible form. It can be activated in various settings such as, high levels of insulin, hypoxia, extracellular acidosis and a proinflammatory environment (Calvo et al., 2006). Stress stimuli such as H_2O_2, anisomycin, and UV radiation all increase the expression of the *PFKFB3* gene (Gustafsson et al., 2018a; Shi et al., 2017).

Glycolytic ATP is required for HR DNA repair and is necessary for the production of HR DNA repair proteins (Sun et al., 2023). Knockdown of PFKFB3, which inhibited glycolysis, hindered DNA repair activity in ECs subjected to an oxidative challenge. This led to unresolved DSBs and an increase in senescence (Sun et al., 2023). Beyond its role in glycolysis, PFKFB3 rapidly localized with nuclear γH2AX foci that were induced after ionizing radiation. However, this induction and co-localization of PFKFB3 and γH2AX was prevented with ATM. Upon induction of DSBs, PFKFB3 colocalized with DNA repair proteins 53BPI and BRCA1 (Gustafsson et al., 2018b). Similar findings by (Sun et al., 2023) showed that an oxidative challenge with H_2O_2 induced nuclear translocation of PFKFB3 to sites of DSBs, with the overexpression of PFKFB3 prompting foci numbers of ATM and MRN to increase. Inhibition of

PFKFB3 prevented the addition of nucleotides during DNA repair by decreasing the nucleotide supply (Gustafsson et al., 2018b).

1.13 Senescence and the p16 pathway

Senescence is not only regulated by the DDR pathway, it can also be induced by the $p16^{INK4}$/pRb pathway. The *Cdkn2a* gene belongs to the *INK4* gene family. The *Cdkn2a* encodes for p16, another cell cycle inhibitor that controls the G1 to S transition (Safwan-Zaiter et al., 2022). Similar to p21, p16 binds CDK4/6 prohibiting its kinase action, hypophosphorylating Rb. The lack of phosphorylation of Rb prevents the dissociation of E2F from this complex, thus, preventing the transcription of cell cycle genes. The activation of p16 in premature senescence requires oncogenic signaling through the Ras/MAPK pathway (Lin et al., 1998; Ohtani et al., 2001; Serrano et al., 1997). In response to DNA damage, p21 is the primary protein for initiating the senescence program. Although p16 is not highly expressed during this stage, it is essential for subsequent reinforcing and maintaining the growth-arrested state to maintain senescence (Robles and Adami, 1998; Song et al., 2020).

1.14 Pathological Consequences of Senescence

EC senescence is an important factor in the development of cardiovascular diseases as there is evidence that EC senescence precedes and plays a role in causing these diseases. Senescent ECs are involved in metabolic disorders such as hyperglycemia, which can be caused by impaired insulin signaling (Georgieva et al., 2023; Le, 2023).

Under obesogenic conditions where mice were fed a high fat, high sugar diet, ECs from muscle expressed high levels of p53 protein and *Cdkn1a* expression indicative of senescence. Concurrently, GLUT-1 expression was reduced under obesogenic conditions, consequently, this augmented the onset of insulin resistance and reduced glucose transport (Yokoyama et al., 2014).

However, inhibition of EC p53 increased glucose uptake by increasing GLUT-1 expression. Thus, obesity-induced senescence can promote hyperglycemia by reducing EC glucose uptake through the upregulation of p53 and down regulation of GLUT-1 (Bloom et al., 2023a; Yokoyama et al., 2014). Another study induced senescence by deleting a protein involved with telomere capping called telomere telomeric repeating binding factor 2 (TRF2) in ECs. Senescent ECs induced adipocyte dysfunction through the senescence associated secretome. The adipocytes displayed impaired insulin signaling associated with a decrease in insulin receptor substrate-1 (IRS-1). In accordance, senescent ECs can impair adipocyte function and cause whole body metabolic dysfunction (Barinda et al., 2020).

Senescent ECs display a reduction in the expression of eNOS and lower NO production (Bloom et al., 2023a; Matsushita et al., 2001). Limited production of NO is a symptom of EC dysfunction and is accountable for vascular diseases such as diabetes, hypertension, atherosclerosis, aging and heart failure (Bloom et al., 2023a; Matsushita et al., 2001; Sun et al., 2020). Moreover, EC senescence is associated with increased expression of adhesion molecules such as ICAM-1 and VCAM-1 (Gorgoulis et al., 2005; Honda et al., 2021; Sena et al., 2018), increasing immune cell rolling and infiltration, promoting vascular inflammation (Bloom et al., 2023a). Senescent ECs also exhibit decreased integrity of their adherence junctions. Thus, there is increased permeability of the endothelium resulting in the infiltration of LDL cholesterol and immune cells. This can enhance the likelihood of the initiation and development of atherosclerotic plaques within the wall of large arteries (Krouwer et al., 2012).

Another consequence of senescent ECs is an impaired ability to undergo angiogenesis. ECs that undergo replicative senescence from aging can prevent neovascularization (Xiao et al., 2023). Reduced angiogenic potential of senescent ECs can result in impaired recovery from

wound healing, tissue damage, infarction or ischemia. This can lead to other complications especially in older individuals such as ulcers or even tissue necrosis due to the lack of blood flow to the injured areas (Lähteenvuo et al., 2012). Senescent ECs also display a reduction in *VEGF* expression, a protein that is promotes EC survival (Chang et al., 2013; Shibuya, 2011).

Mitochondrial dysfunction can induce EC senescence through oxidative stress. Alterations in mitochondrial function and morphology in ECs have been implicated in obesity and diabetes (Sun and Feinberg, 2021). Senescent ECs have an impaired ability to undergo mitochondrial fission which affects the autophagic process. As a result, mitochondrial turnover is defective ultimately retaining dysfunctional mitochondria in ECs (Lin et al., 2015).

Disturbed flow occurs in atheroprone areas such as arterial bifurcation or curvatures and it may play a vital role in driving senescence in ECs (Warboys et al., 2014). Disturbed flow increased senescence-associated (SA) β-galactosidase+ ECs. Disturbed flow activated the DNA damage response pathway whereby p53 and p21 of ECs increased compared to undisturbed flow. Senescent ECs can potentially be involved in the initiation and progression of atherogenesis (Warboys et al., 2014).

1.15 Sex Differences in ECs their Ability to Resist Oxidative Stress

Men and women are biologically dissimilar from one another, specifically at the cellular level, organs and at the organism level (Ventura-Clapier et al., 2017). Biological sex can be a potential factor for the observed results from a study (Cattaneo et al., 2021; Shah et al., 2014). However, information about the biological sex of tissues or cells is seldom mentioned. Sex differences that are not considered, may limit our understanding of different cellular processes and potential pathogenic consequences that can manifest due to sex (Cattaneo et al., 2021).

1.15.1 Sex Differences in ECs

There is evidence of sexual dimorphisms in ECs. Gene expression analysis of human adult untreated ECs showed 1798 differentially expressed genes (DEGs) between male and females. Of these DEGs, 845 genes were highly expressed in males and 953 were highly expressed in females. In particular, enrichment of the genes highly expressed in females pointed to estrogen responses and genes expressed higher in males showed oxidative phosphorylation pathways (Hartman et al., 2020). Interestingly, our RNA sequencing data showed the opposite and we have seen a higher ratio of oxidative:glycolysis in culture female ECs (Rudnicki et al., 2023). Male and female mice that underwent high fat diet induced obesity demonstrated different angiogenic potential in response to obesogenic signals in perigonadal adipose tissue (Rudnicki et al., 2018b). Several reports have shown that female ECs display a higher pro-angiogenic response, increased expression of angiogenic components such as *Vegfa*, and higher proliferative and migratory capacity compared to male ECs (Addis et al., 2014; Boscaro et al., 2020; Rudnicki et al., 2018b). Furthermore, female ECs also showed an enrichment in genes for superior immune responsiveness compared to male ECs suggesting that they may be better equipped to fend off inflammatory stimuli (Lorenz et al., 2015).

1.15.2 Sex Differences in Oxidative Stress Resistance

The degree to which oxidative stress contributes to the pathology of diseases is variable, however, it could be influenced by sex differences in the buffering capacity of antioxidants (Forman and Zhang, 2021). In general, sex differences in EC oxidative stress resistance are not well elucidated. There is some evidence that females have decreased levels of oxidative stress compared to men. An in vivo study that analyzed urine and plasma samples from healthy young male and females found that males displayed higher levels of oxidative stress biomarkers

compared to females (Ide et al., 2002) (Kander et al., 2017). Blood collected from young healthy females presented higher glutathione levels, GPx activity and a GSH/GSSH ratio compared to males. This suggests that females are better equipped to defend an oxidative insult and are in a greater reduced state than males (Alkazemi et al., 2021). Estrogen signaling may contribute to these differences, as estrogen can increase the expression of antioxidant enzymes (Borrás et al., 2010). Blood extracted from premenopausal women that had hysterectomies exhibited a decrease in glutathione concentration and a decrease in the GSH/GSSG ratio after 30 days. However, estrogen replacement therapy increased glutathione levels back to pre-hysterectomy levels (Bellanti et al., 2013). This emphasizes a potentially crucial role for estrogen in protecting females from oxidative stress through regulating antioxidant levels and providing a reduced redox environment. Vascular ROS was found to be elevated in male rats compared to females at baseline, hypertensive and atherosclerotic conditions (Miller et al., 2007). Female ECs treated with H_2O_2 were more resistant to an oxidative insult than males as there was a greater percentage of apoptosis in the males compared to the females (Norton et al., 2019). (Norton et al., 2020) demonstrated that there was greater EC death in male compared to female mice on a normal diet. In addition, there was a significant increase in basal levels of ROS from males compared to females in intact small resistance vessels (Norton et al., 2020). This may suggest that male vessels may be more susceptible to vascular pathologies.

Transcriptome analysis revealed that there were sex-distinct transcriptomes of ECs from white adipose of mice that were fed a high fat diet for seven weeks (Rudnicki et al., 2023). Male ECs exhibited a pro-inflammatory phenotype, whereby there was a significant enrichment in genes associated to inflammasome and inflammasome assembly. ECs from males had an upregulation of genes that are associated with SASP versus female ECs (Rudnicki et al., 2023).

Altogether, female ECs were able resist the negative consequences of a high fat diet better than male ECs.

Although it has not been reported in ECs, several studies have reported that endogenous DNA damage differs by gender. Female peripheral blood mononuclear cells displayed less DNA damage than males when treated with the mutagenic agent benzopyrene diolepoxide (Slyskova et al., 2011). In agreement with this, another study found that male lymphocytes, under basal conditions, had significantly elevated levels of DSBs than female lymphocytes (Hofer et al., 2006).

The accumulation of DNA mutations may be from impaired DNA replication but more importantly can suggest that there are deficiencies in DNA repair in males compared to females (Broestl and Rubin, 2021; Cardano et al., 2022). One reason for this disparity between sexes could be that there was elevated p21 expression in females versus males across multiple species and tissues (Broestl and Rubin, 2021). Accordingly, another study revealed that when astrocytes undergo DNA damage, females displayed a significant increase in p21 and p16 levels compared to males (Kfoury et al., 2018). This would provide more time for females to repair DNA damage because of the roles p21 and p16 play in cell cycle arrest.

P38 is a protein that is involved with cell survival under oxidative stress conditions (Canovas and Nebreda, 2021). Our lab has previously shown that there were higher levels of phospho-p38 in females compared to males suggesting that female ECs may be superior at sensing and counteracting intracellular stress (Rezvan et al., 2020). Additionally, female ECs displayed significantly higher levels of FoxO1 compared to males (Rudnicki et al., 2023 and Unpublished Data). Due to the role of FoxO1 in upregulating genes that are involved with resisting oxidative

stress, this could be a potential mechanism by which female adipose ECs are more resilient to the detrimental effects of high fat feeding compared to males (Rudnicki et al., 2023).

1.16 Study Rationale

EC health can be compromised by DNA damage in states of disease such as obesity, aging, atherosclerosis, type 2 diabetes (Bloom et al., 2023b; Martinet et al., 2002; Sun et al., 2023; Włodarczyk and Nowicka, 2019). Our lab has previously shown that male and female ECs under high fat conditions behave differently regarding the maintenance of metabolic homeostasis at the adipose tissue and systemic level (Rudnicki et al., 2018b). Recently, RNA sequencing of adipose tissue ECs from mice fed a high fat diet (HFD) reported sex disparities in the EC transcriptional profiles. Male ECs displayed an enrichment in genes related to inflammation, sustained a pro-inflammatory phenotype, possessed an enrichment of genes associated with SASP in response to a high fat diet whereas female ECs preserved a healthier phenotype. However, the extent to which female ECs are protected from oxidative stress, and the underlying mechanisms, remain poorly understood. Considering that FoxO1 protein levels were significantly higher in female compared to male ECs (Rezvan et al.,2020), and that FoxO1 can transcribe anti-oxidant genes, it is possible that FoxO1 contributes to healthier phenotype of female ECs.

1.17 Hypothesis and Objectives

Hypotheses:

1) The extent of oxidative stress induced damage is dependent on the function of FoxO1.
2) Female ECs are superior at resisting oxidative stress compared to male ECs.

Objective 1:

To identify oxidative stimuli that effectively induce DNA damage and senescence in microvascular ECs.

Objective 2:

To investigate whether inhibition of FoxO1 increases the vulnerability of ECs in response to a DNA-damaging agent.

Objective 3:

To identify sex differences in EC behaviour in response to a DNA-damaging agent.

Chapter 2: Methods

2.1 Endothelial Cell Isolation

Microvascular ECs were isolated from the hindlimb skeletal muscles of male and female mice (n=2-4 mice per sex). Muscles were pooled together, mechanically minced with a scalpel, then digested enzymatically with type II collagenase (0.5%, #17101015, ThermoFisher Scientific, USA) and agitated at 37 °C for 1 hour. The resulting mixture was centrifuged for 5 minutes at 4000 x g and the pellet formed was resuspended and passed through a 100 µm cell strainer. The heterogenous mixture of cells that were liberated from the digested muscle was incubated with biotinylated rat anti-mouse CD31 (BD IMag, USA) antibody conjugated to streptavidin magnetic beads (ThermoFisher, Scientific, USA) then separated using a magnet. They were resuspended and plated on a gelatin coated T25 flask and grown in media consisting of: high-glucose (4.5 g/L) DMEM (#19960044, Gibco, USA) supplemented with 20% fetal bovine serum (FBS, #10082147, Gibco, USA), Penicillin-Streptomycin (100µg/mL, 15140122, Gibco, USA), GlutaMAX™ L-glutamine (2 mM, #35050061, Gibco, USA), and Sodium Pyruvate (1 mM, #11360070, Gibco, USA). Cells used for experiments were in passages 2-5 and were passage matched when comparing males and females.

2.2 Cell Culture Experiments

Testing of Agents that Induce DNA Damage:

Male and female ECs were plated in 48-well plates ($20x10^3$ cells per well) in growth media. For treatments, cells received an equal volume of the drug diluent dimethyl sulfoxide (DMSO)

(D2650, Sigma, MO, USA) or treated with an oxidative agent. Cells were subjected to ferric ammonium citrate (FAC; 10 or 100 μM), tumor necrosis factor-α (TNF-α; 5 or 10 ng/ml), H_2O_2 (75 or150 μM), cisplatin (1, 10 or 50 μM), or vehicle (control) for 48 hours prior to RNA extraction.

***Male and Female ECs from FoxO1 Floxed and Knockdown mice treated Cisplatin*:**

The ECs used for this experiment were extracted from male and female Cre^{+};$FoxO1^{f/f}$ (EC-FoxO1 KD) and Cre^{-};FoxO1 (FoxO1 Floxed-ECs) mice (Rudnicki et al., 2018a). The Cre^{+} mice expressed a tamoxifen inducible form of Cre recombinase ($iCreER^{T2}$) controlled by the *Pdgfb* promoter (Claxton et al., 2008). Cre-mediated recombination was induced in 6-week-old mice by 5 consecutive tamoxifen injections, as done previously (Rudnicki et al., 2018a). Two weeks following injections, endothelial cells were extracted for cell culture (as described in 2.1). These cells were treated with vehicle or 10 μM cisplatin for 48 hours prior to RNA extraction.

***FoxO1 inhibition combined with Cisplatin Treatment*:**

$15x10^3$, $20x10^3$ and $200x10^3$ ECs were plated for RNA, immunofluorescence staining and protein extraction in a 48-well plate, 8-well chamber slide and 6-well plate, respectively. FoxO1 inhibition in ECs was carried out using AS1842856 (AS184), a cell permeable drug that blocks the transcriptional activity of FoxO1, which has been successfully used in our lab previously (Rudnicki et al., 2018). ECs were treated with vehicle DMSO, AS184, cisplatin or the combination of AS184 and cisplatin. AS184 (1μM) was added to high-glucose DMEM (10% FBS) 24 hours prior to treatment with cisplatin (10 μM) for 48 hours prior to RNA and protein extraction.

***FoxO1 and PFKFB3 inhibition with Cisplatin Treatment*:**

ECs were plated at a density of $200x10^3$ in a 6 well plate. ECs were treated with vehicle (DMSO), PFKFB3 inhibitor 3-(3-Pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3-PO;10 μM), 1 μM AS184 or a combination of both 3-PO and AS184 for 24 hours. Next, cisplatin (10 μM) was added (to the ECs treated with AS184, 3-PO and the combination) and cells were cultured for 48 hours.

2.3 RNA extraction

ECs were lysed using Qiazol Reagent (#79306, Qiagen, ON, Canada), chloroform was added and centrifuged at 4°C for 15 minutes at 12,000 x g. The subsequent RNA containing aqueous phase of the lysate was added to isopropyl alcohol and centrifuged at 4°C for 20 minutes at 12,000 x g for precipitation of RNA. The resulting RNA pellet was washed with 75% ethanol before being resuspended in 42 μL of RNase-free water. RNA concentrations were analyzed via spectrophotometer.

2.4 RT-qPCR

160 ng of RNA was reverse transcribed to produce cDNA using deoxynucleotide triphosphates (dNTPs) (#N0447S, New England Biolabs, ON, Canada), random primers (#100026484, Invitrogen, CA, USA), oligo deoxythymidines (dT) (#100002344, Invitrogen, CA, 29 USA), RNase inhibitor (#EO038, ThermoFisher Scientific, MA, USA), M-MuLV reverse transcriptase and M-MuLV buffer (New England BioLabs, ON, Canada) via a polymerase chain reaction. This was performed in a Thermal cycler (2720 cycler, Applied Biosystems, CA, USA). The newly formed cDNA was diluted to 3 μg/μL of RNase free water.

Quantitative polymerase chain reaction (qPCR) was carried out by combining 2 μL of cDNA with Taqman® Fast Advanced Master Mix (#4444557, Applied Biosystems, Thermo Fisher Scientific, CA, USA). TaqMan® FAM-Labelled probes *TATA-Box Binding Protein* (*Tbp*),

Cdkn1a, Cdkn2a and *Gadd45* were added to the master mix and cDNA and measured using the PCR Cycler Rotor-Gene Q system (Qiagen, ON Canada).

Relative mRNA levels of the target gene were assessed by calculating the average cycle threshold (C_T) and comparing it to the average C_T value of *Tbp*; the house keeping gene for the same sample. This was determined by the formula $2^{-\Delta CT}$, where ΔC_T = Average C_T (gene of interest) - Average C_T (housekeeping gene).

2.5 Protein Extraction

Proteins were extracted using RIPA lysis buffer (50 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1 SDS%). Phosphatase inhibitor tablets (PhosSTOP™) and protease inhibitor tablets (cOmplete™ Protease Inhibitor Cocktail, Sigma-Aldrich, USA). The wells were scraped, and lysates were centrifuged for 20 minutes at 12,000 x g. The resulting supernatant was collected and stored at -80°C.

2.6 Protein Quantification

Protein concentrations in cell extracts were measured using the bicinchoninic acid assay (BCA) (Pierce, Fisher ThermoScientific, ON, Canada). Protein lysates were pipetted into 96 well plates in triplicates, subsequently, reagent A and reagent B were mixed at a 49:1 ratio respectively and added to each sample triplicate. Serial dilutions of bovine serum albumin (BSA 0-2000 µg/mL, Pierce, ThermoFisher Scientific, ON, Canada) were made to create a standard curve to which the samples were compared to. The absorbance of the samples was analyzed at 562 nm using the Cytation3 microplate reader (Biotek, Vermont, USA).

2.7 Western Blot

Western Blots were conducted using 20 µg of total protein per sample prepared in 4x DTT (Dithiothreitol) (1 M Tris; pH 6.8, 1 M DTT, 40% SDS, 100% glycerol, 1% Bromophenol blue). Subsequently samples were centrifuged briefly, heated to 100°C for 5 minutes and briefly centrifuged again. The lysates were then loaded and separated by size via electrophoresis through 12% SDS-Polyacrylamide gels. Next, proteins were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane (Immobilon P, EMD Millipore, ON, Canada) that was submerged in transfer buffer (25.01 mM Tris-Base, 191.82 mM Glycine) at 100V for 30 minutes using a wet transfer system powered by PowerPac™ HC High-Current Power Supply (Bio-Rad, ON, Canada). Membranes were then blocked in 5% milk diluted in 0.05% Tween Tris-Buffered saline solution (TTBS) with agitation. After blocking, membranes were incubated with primary antibodies overnight at 4°C with agitation. Primary antibodies included phospho-histone H2A.X (Ser139) (1:1000; #9718, Cell Signaling, ON, Canada), FoxO1 (1:1000; #2880, Cell Signaling, ON, Canada), Prdx3 (1:1000; #A3076, ABclonal, MA, USA), β-Actin (1:5000; #sc-47778, Santa Cruz Biotechnology, TX, USA), and p21 (1:1000; #sc-6246, Santa Cruz Biotechnology, TX, USA). All primary antibodies were diluted in a solution that contained 5% bovine serum albumin (BSA) (Bio Basic, ON, Canada) and 0.05% TTBS. The next day, membranes were washed with TTBS and incubated with secondary antibody (1:10000; #115-035-003, peroxidase-conjugated goat anti-mouse IgG; 1:10000; #111-035-003, peroxidase-conjugated goat anti-rabbit; Jackson ImmunoResearch Laboratories Inc., USA), in 5% milk that has been diluted in 0.05% TTBS for 1 hour at room temperature with gentle agitation. The antibodies bound to protein were detected by enhanced chemiluminescence (Pierce, Fisher

Thermoscientific, ON, Canada) using the iBright 1500 Imaging System (#A44114, ThermoFisher Scientific, ON, Canada).

2.8 Immunofluorescence Imaging of DSBs

$20x10^3$ ECs were plated in an 8-well chamber slide coated with type I rat tail collagen (12 $\mu g/cm^2$). The media was aspirated, ECs were washed with PBS and then fixed with 4% paraformaldehyde for 20 minutes. ECs were blocked and permeabilized with blocking solution that consisted of 0.1% Triton-X and 5% donkey serum that was diluted in PBS for 1 hour. The blocking solution was removed, and ECs were incubated with phospho-histone H2A.X (Ser139) (1:400; #9718, Cell Signaling, ON, Canada) for 2 hours diluted in blocking solution. ECs were washed with PBS and incubated with Alexafluor 488-conjugated donkey anti-rabbit secondary antibody (1:200; #711-545-152, Jackson ImmunoResearch Inc., USA) diluted in blocking solution for 1 hour at a concentration of 1:200. Lastly, ECs were subsequently counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 5 minutes. Imaging was performed using a fluorescence microscope using the 10x objective and imaging parameters remained constant between each channel. The number of γH2AX+ nuclei were counted using ImageJ Analysis Software, the percentage of γH2AX+ cells were expressed as (γH2AX/DAPI) x 100. The average of 6 fields of view were taken per well and used to represent the percentage of γH2AX+ cells in each condition.

2.9 Statistical Analysis

Results are presented as Mean ± standard error of the mean (SEM). All *n* were representative of The effects of various oxidant stimuli such TNF-α, Ferric Ammonium Citrate, Cisplatin and H_2O_2 on ECs were measured using a one-way ANOVA. The effects of sex and EC FoxO1 KD on senescence genes *Cdkn1a* or *Cdkn2a* and DNA repair gene *Gadd45* were analyzed using a two-

way ANOVA followed by Sidak post hoc tests. The effects of FoxO1 inhibition and cisplatin experiments for both mRNA expression, protein and γH2AX+ cells were analyzed using a two-way ANOVA followed by Sidak post hoc tests (Prism8; GraphPad Software Inc; La Holla, CA, USA). Male and female γH2AX protein levels and γH2AX+ cells were analyzed by a students *t*-test. In all cases, $p < 0.05$ was considered to represent statistical significance. All data sets were not tested for normality.

Chapter 3: Results

3.1 mRNA Expression of Senescence Markers and FoxO1 in ECs Treated with Various Oxidative Agents

Male ECs were treated with TNF-α, FAC, Cisplatin and H_2O_2 for 48 hours with the goal of inducing DNA damage due to an increase in ROS. I assessed two cell cycle inhibitor genes, *Cdkn2a* and *Cdkn1a,* that are established to increase with senescence, and *Foxo1,* a gene known to transcribe antioxidant proteins. The various doses of TNF-α and FAC did not affect the expression of *Cdkn2a*, *Cdkn1a* or *Foxo1* expression (Fig. 3.1A,B). Cisplatin (10 and 50 μM) induced a significant increase in *Cdkn2a* while 1 μM cisplatin significantly increased the expression of *Cdkn1a*. *Foxo1* mRNA expression was not altered (Fig. 3.1C). Lastly, 150 μM H_2O_2 significantly elevated the expression of *Cdkn1a* mRNA but had no effect on *Cdkn2a* or *Foxo1* mRNA. (Fig. 3.1D). Based on these results, I selected 10 μM cisplatin for the remaining experiments because it caused the greatest changes in gene expression.

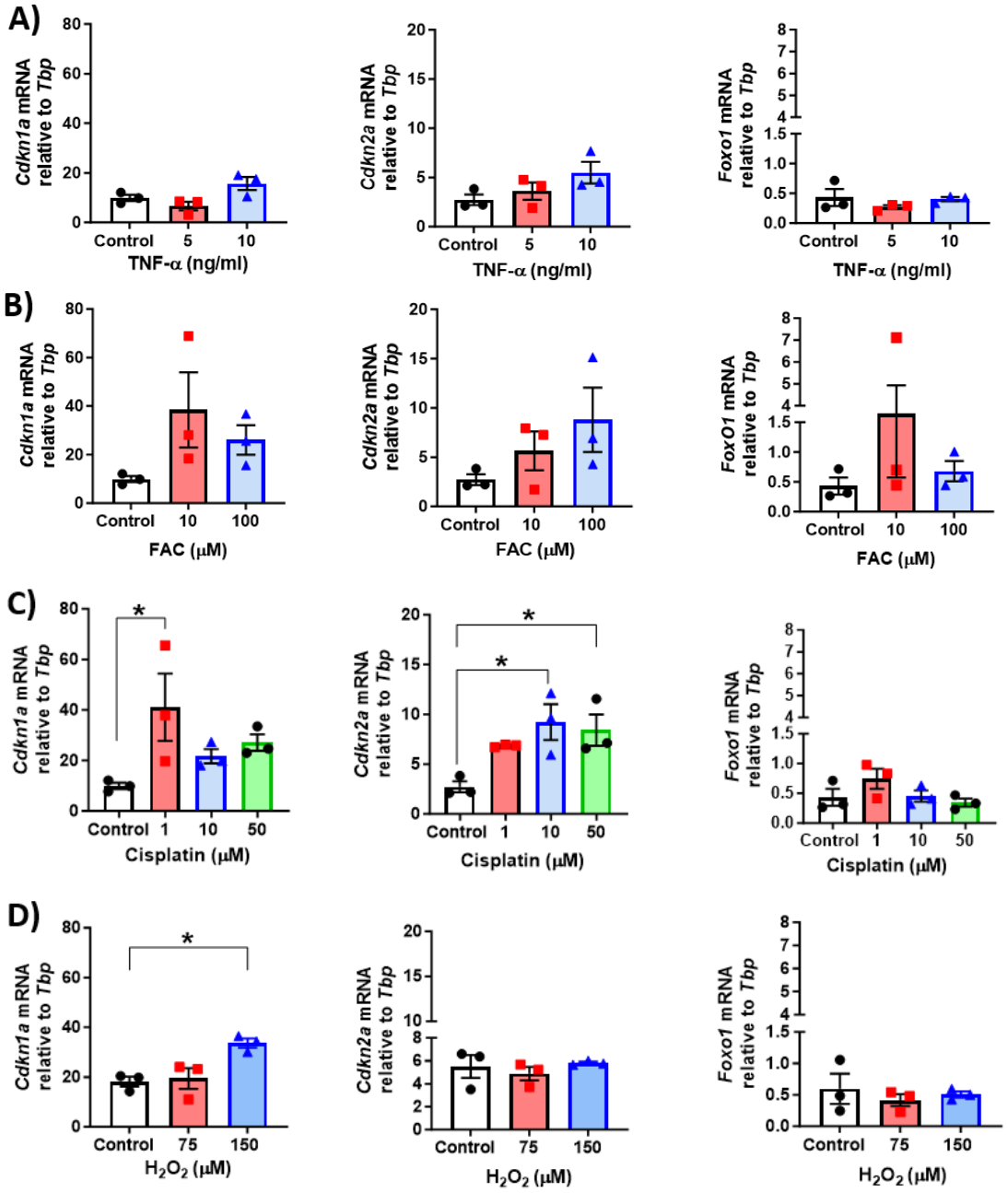

Figure 3.1: mRNA levels of *Cdkn1a, Cdkn2a, Foxo1* in ECs treated with oxidative agents.

Male ECs were cultured in control media or with the addition of **A)** TNF-α (5 or 10 ng/ml), **B)** FAC (10 or 100 μM), **C)** Cisplatin (1, 10 or 50 μM) or **D)** H_2O_2 (75 or 150 μM) for 48 hours prior to RNA extraction and qPCR analysis. The mRNA levels of *Cdkn1a, Cdkn2a,* and *Foxo1* were quantified relative to *Tbp* mRNA, $n = 3$. All data were expressed as mean ± SEM and analyzed using one-way ANOVA with Sidak *post-hoc* analysis when significant effects were detected. When compared to control, *post-hoc* significance was seen in **C)** *Cdkn1a;* 1 μM cisplatin ($P = 0.041$), *Cdkn2a;* 10 and 50 μM cisplatin ($P = 0.016, 0.032$) respectively, **D)** *Cdkn1a*; 150 μM H_2O_2 ($P = 0.018$).

3.2 Sex Differences in EC *Cdkn1a*, *Cdkn2a* and *Gadd45α* levels when challenged with Cisplatin

I next assessed potential sex differences in ECs response to cisplatin. Senescence genes *Cdkn2a* and *Cdkn1a* as well as DNA repair gene *Gadd45* were analyzed. Unexpectedly, there were no significant effects of cisplatin treatment on the expression of these genes in ECs from either sex (Fig. 3.2A,C,D). However, mRNA levels of *Cdkn2a* were significantly lower in female compared to male ECs (Fig. 3.2B). Consistent with the previous results, *Cdkn2a* mRNA expression was lower in female versus male EC-FoxO1 KD in the untreated condition (Fig. 3.2E).

I also used these cells to analyze the effects of the EC specific deletion of FoxO1 on *Cdkn1a, Cdkn2a,* and *Gadd45.* In male ECs, there was a main effect of EC-FoxO1 KD on *Cdkn2a* expression, but not on the levels of *Cdkn1a* and *Gadd45* mRNA (Fig. 3.2H). In female ECs, there was a main effect of EC specific FoxO1 deletion on the expression of *Cdkn2a* and *Cdkn1a* (Fig. 3.2J and K). DNA repair gene *Gadd45* was significantly upregulated in female EC-FoxO1 KD compared FoxO1 floxed-EC when treated with cisplatin (Fig. 3.2L).

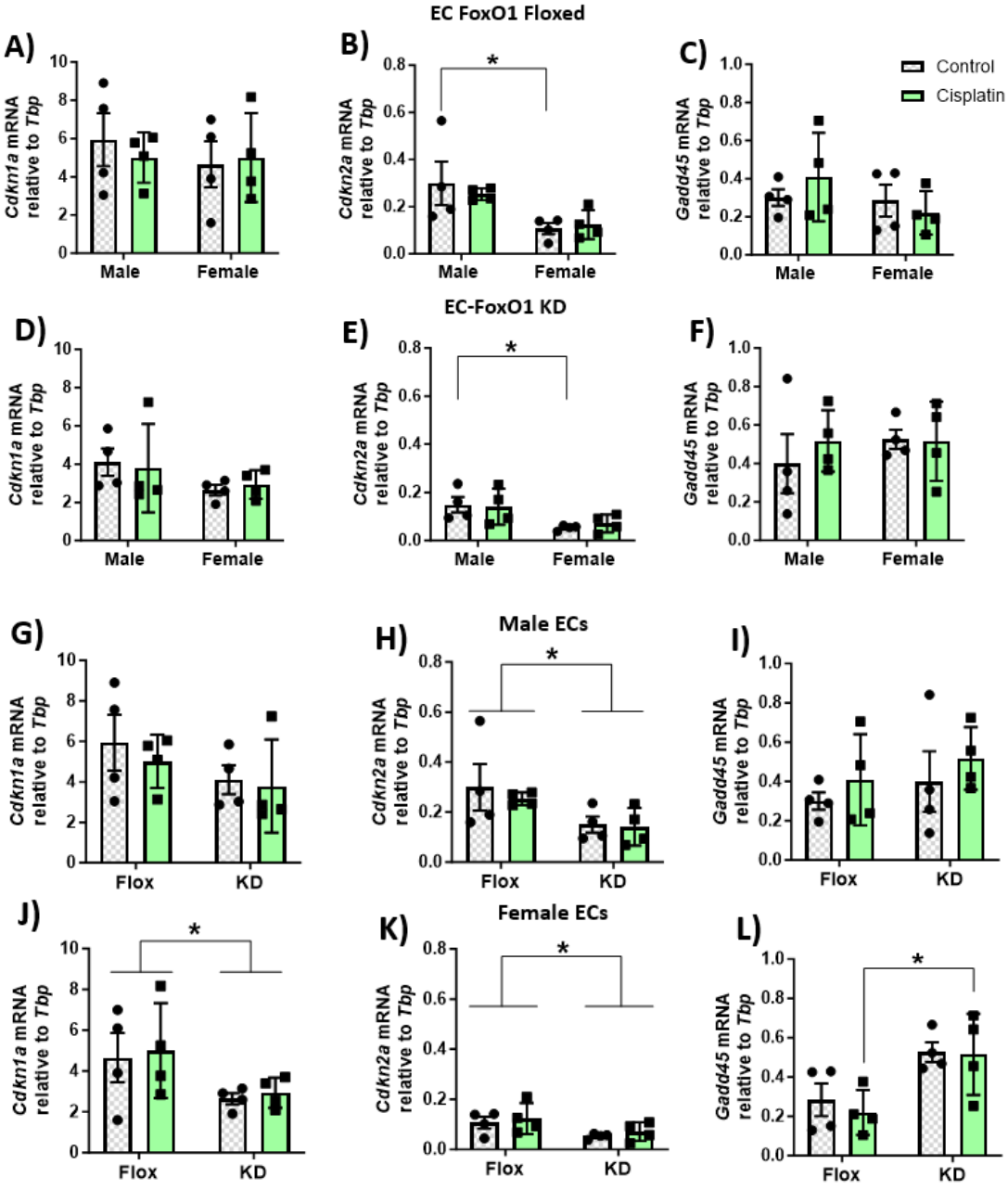

Figure 3.2: The effects of sex and FoxO1 on EC gene expression of *Cdkn1a*, *Cdkn2a*, and *Gadd45* when challenged with cisplatin.

Male and female ECs from FoxO1 floxed and EC-FoxO1 KD were maintained under control conditions or treated with 10 µM cisplatin for 48 hours prior to RNA extraction and qPCR analysis. The mRNA levels of *Cdkn1a, Cdkn2a* and *Gadd45* were quantified relative to *Tbp* mRNA, $n = 4$. Gene expression analysis for **A-C)** Male versus female FoxO1 floxed; **D-F)** Male versus female EC-FoxO1 KD; **G-I)** Male FoxO1 floxed versus KD ECs; **J-L)** Female ECs FoxO1 floxed versus KD ECs. All data were expressed as mean ± SEM and analyzed using two-way ANOVA with Sidak *post-hoc* analysis when significant effects were detected. A main effect of genotype was detected in **H)** ($P = 0.028$), **J)** ($P = 0.036$), **K)** ($P = 0.028$). A significant effect of sex was detected in untreated cells **B)**; *(*$P = 0.038$), **E)**; ($P = 0.049$) **L)** ($P = 0.035$) with Sidak *post-hoc* analysis.

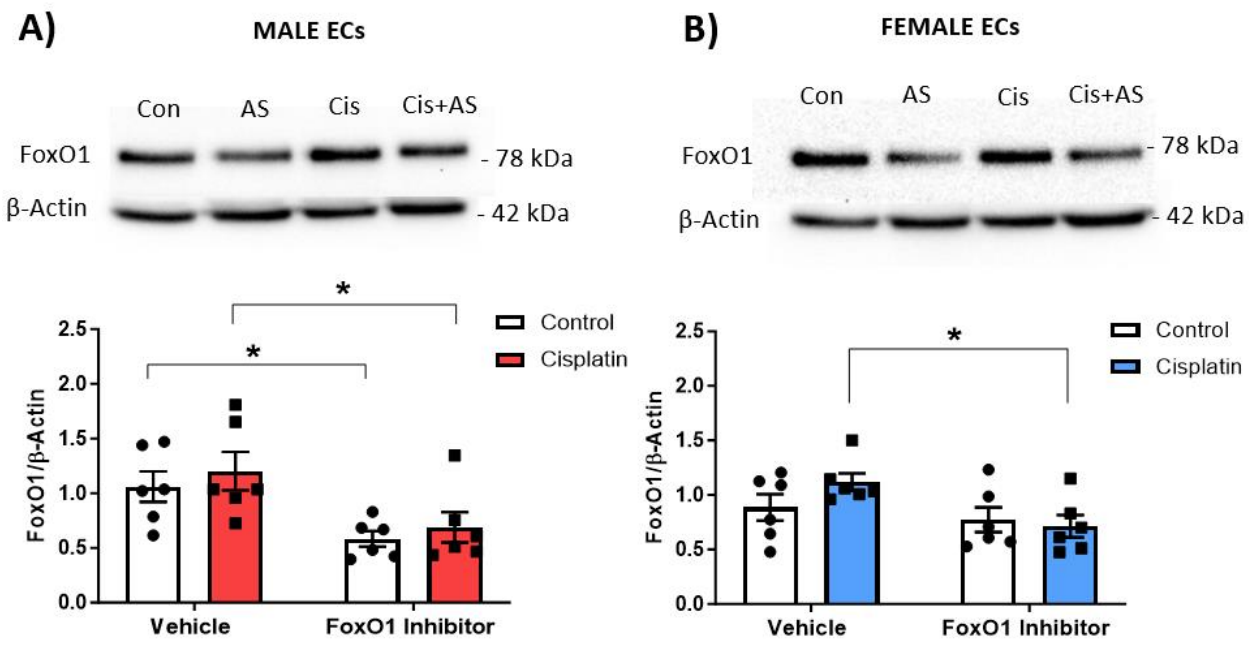

Figure 3.3 Effects of FoxO1 inhibitor AS1842856 on FoxO1 levels.

Western blot analysis of FoxO1 levels in **A)** male and **B)** female ECs. Protein quantification for FoxO1 was normalized to β-Actin, $n = 6$. All data were expressed as mean ± SEM and analyzed using two-way ANOVA with Sidak *post-hoc* analysis when significant effects were detected. A significant effect of FoxO1 inhibition independent of cisplatin **A);** *$P<0.05$ and FoxO1 inhibition in combination with cisplatin **B)**; *$P<0.05$.

3.3 FoxO1 Inhibition Promotes *Cdkn1a* Expression in Male and Female EC

The extent of deletion of FoxO1 deletion in the cultured EC was not able to be verified by genotyping, suggesting a loss of FoxO1-deleted EC. Thus, I conducted further experiments using the FoxO1 specific inhibitor AS1842856 (AS184), to ensure consistent FoxO1 inhibition. In male ECs, FoxO1 protein levels did not change with cisplatin treatment. In addition, FoxO1 inhibition significantly reduced FoxO1 protein levels in male ECs (Fig. 3.3A). Cisplatin had no effect on FoxO1 levels in female ECs. FoxO1 inhibition significantly lowered FoxO1 levels only in ECs treated with cisplatin. (Fig. 3.3B).

In male ECs, the expression of *Cdkn1a* was not affected by the treatment of cisplatin. In contrast, FoxO1 inhibition significantly increased *Cdkn1a* mRNA, independent of cisplatin treatment (Fig. 3.4A). Cisplatin exerted a main effect on increasing *Cdkn2a* expression. FoxO inhibition had no

effect on the expression of the *Cdkn2a* gene (Fig. 3.4B). In female ECs, cisplatin treatment did not affect the expression of *Cdkn1a* or *Cdkn2a*. Similar to the male EC, *Cdkn1a* mRNA significantly increased with FoxO1 inhibition (Fig. 3.4C). There also was a main effect of FoxO1 inhibition ($p=0.0377$) on the expression of *Cdkn2a* mRNA (Fig. 3.4D). These data indicate that FoxO1 inhibition increases the gene expression of *Cdkn1a* in male and female ECs.

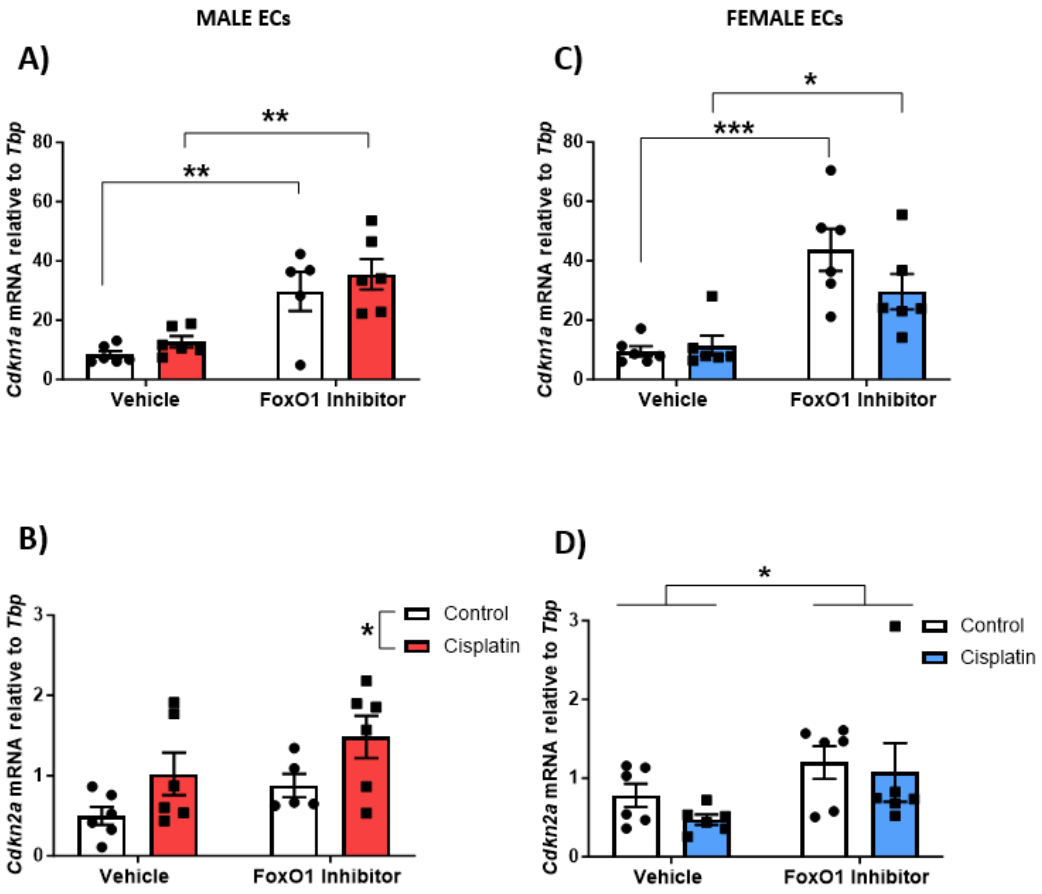

Figure 3.4: Effects of FoxO1 inhibition and cisplatin on mRNA levels of *Cdkn1a* and *Cdkn2a*.

Male and female ECs were cultured under control conditions or treated with FoxO1 inhibitor (AS184; 1 μM) for 24 hours prior to 48 hours cisplatin treatment (10 μM). The mRNA levels of *Cdkn1a* and *Cdkn2a* of male **(A, B)** and female ECs **(C, D)** were quantified relative to *Tbp* mRNA, n = (5-6). All data were expressed as mean ± SEM and analyzed using two-way ANOVA with Sidak *post-hoc* analysis when significant effects were detected. A significant effect of FoxO1 inhibition was detected in **(A, C)**; *P<0.05, **P<0.005, ***P=0.0002 vs. untreated cells. independent of cisplatin. A main effect for cisplatin was detected in **(B)** (P = 0.016). A main effect for FoxO1 inhibition was detected in **(D)** (P = 0.037).

3.4 FoxO1 inhibition increases p21 levels and *Gadd45α* expression

To confirm that the increase in *Cdkn1a* expression was associated with a concomitant increase in protein levels, I measured p21 protein levels in ECs. Cisplatin had no effect on p21 levels in Male ECs. Similar to its gene expression profile, p21 levels were significantly elevated in ECs that were subjected to FoxO1 inhibition, independent of cisplatin treatment (Fig. 3.5A). I examined the gene expression of *Gadd45α*, cisplatin treatment had no effect on *Gadd45α*

expression compared to control conditions. Male ECs subjected to FoxO1 inhibition significantly increased *Gadd45* mRNA levels in cisplatin treated cells (Fig. 3.5B).

Cisplatin treatment also had no effect on p21 protein levels in female ECs. FoxO1 inhibition significantly increased the levels of p21 compared to control, independent of cisplatin treatment (Fig 3.5C). Notably, cisplatin treatment resulted in a significant decrease in p21 protein levels in the presence of FoxO1 inhibition, which was not shown in male ECs. Cisplatin did not alter *Gadd45α* in female ECs. FoxO1 inhibition did however increase the levels of *Gadd45* expression in untreated ECs (Fig 3.5D). Taken together, these data indicate that FoxO1 inhibition results in higher levels of the cell cycle arrest protein p21 in both male and female ECs.

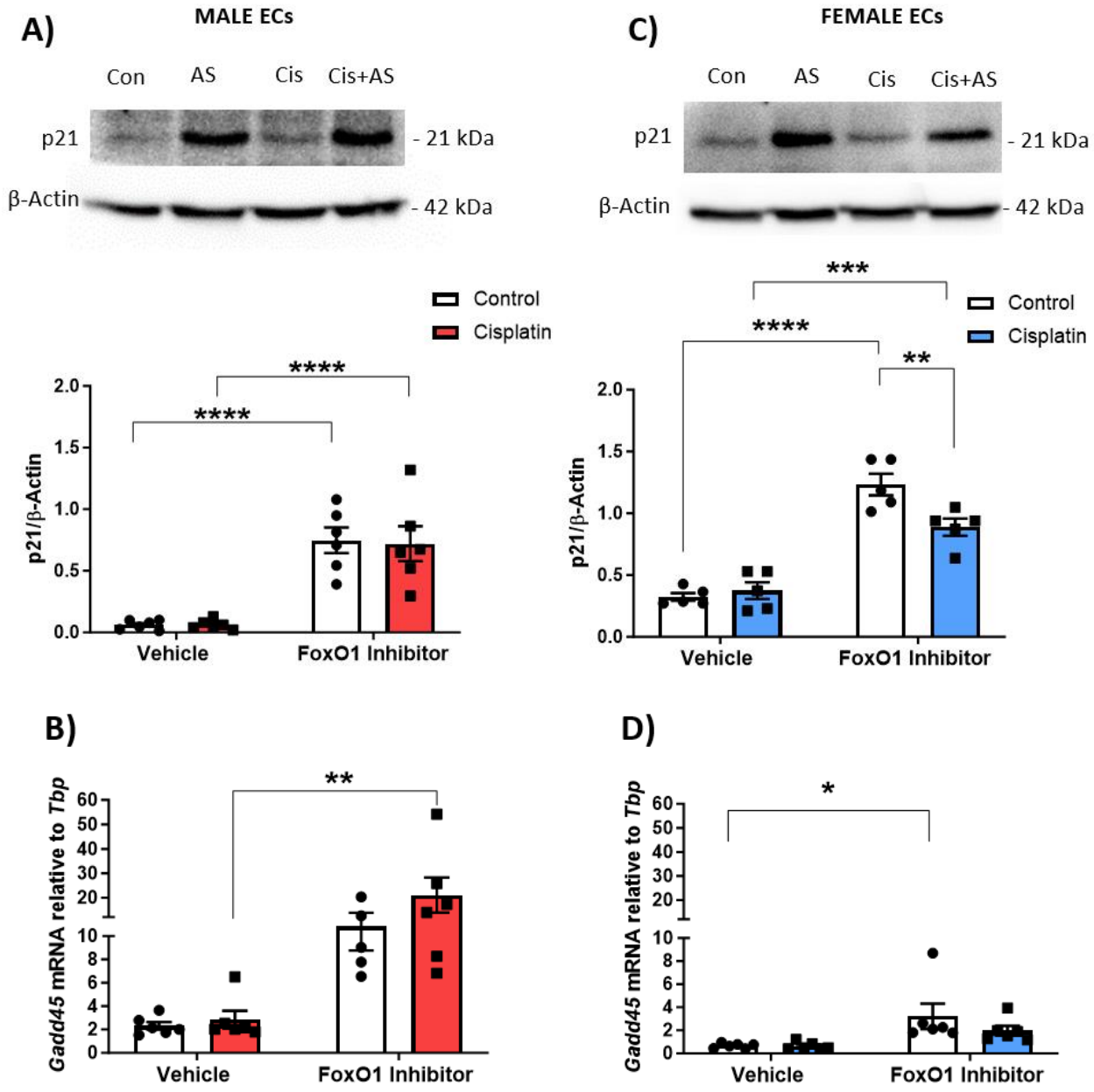

Figure 3.5: The effects of FoxO1 inhibition and cisplatin on p21 and *Gadd45α.*

Representative western blots depicting p21 **(A** and **C)** and gene expression analysis for *Gadd45* **(B** and **D**) in male and female ECs. Protein quantification for p21 was normalized to β-Actin, $n = 6$. The mRNA levels of *Gadd45* were quantified relative to *Tbp*, $n = 5$-6. All data were expressed as mean ± SEM and analyzed using two-way ANOVA with Sidak *post-hoc* analysis when significant effects were detected. A significant effect of FoxO1 inhibition was detected in **(A-D)**; *$P<0.05$, **$P<0.01$, ***$P\leq0.0001$, ****$P<0.0001$. A significant effect of FoxO1 inhibition was detected in cisplatin treated ECs **(B)**; **$P<0.01$.

3.5 The Effects of FoxO1 inhibition on Prdx3

Since FoxO1 is a transcriptional regulator of antioxidant proteins including Prdx3, I tested if FoxO1 inhibition would lower Prdx3 levels in cultured EC, potentially making cells more vulnerable to oxidative damage in the presence of cisplatin. However, in both male and female ECs, FoxO1 inhibition significantly increased the levels of Prdx3, independent of cisplatin treatment. Cisplatin did not affect the levels of Prdx3 in either sex (Fig. 3.6A and B). These results demonstrate that FoxO1 inhibition can promote a phenotypic change in antioxidant capacity in both male and female ECs by increasing Prdx3.

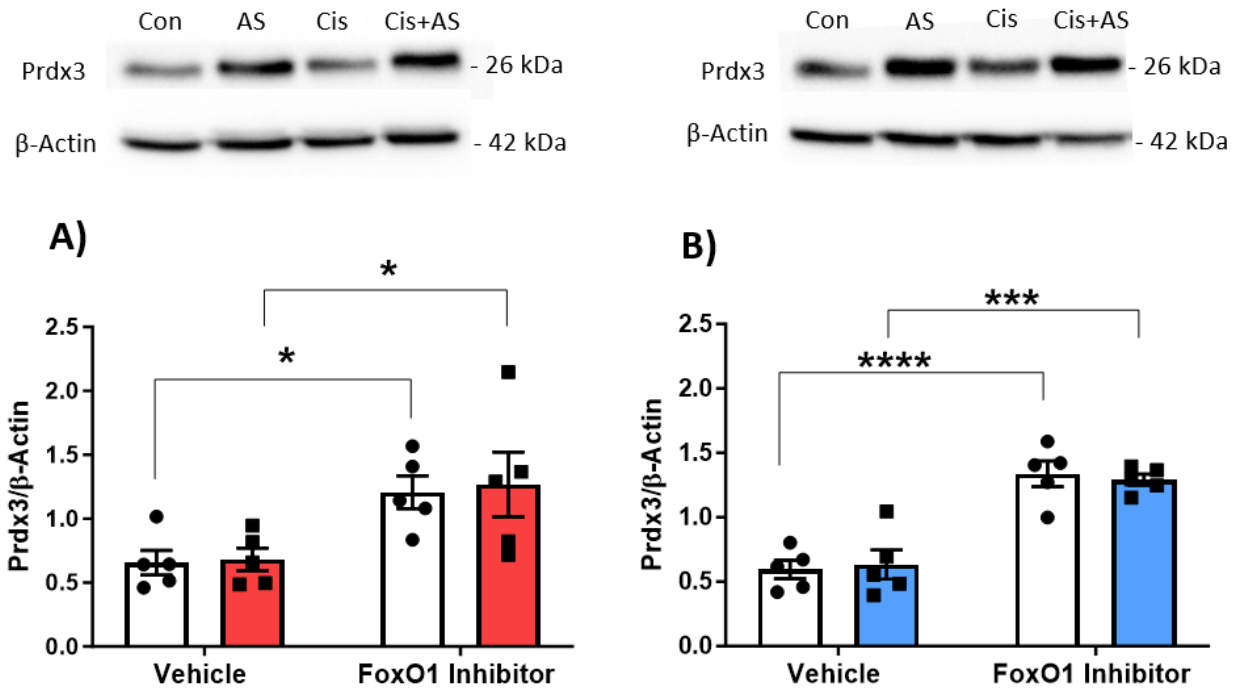

Figure 3.6: Levels of Prdx3 in response to FoxO1 inhibition.
Western blot analysis of Prdx3 protein levels in **(A)** male and **(B)** female ECs. Protein quantification for Prdx3 was normalized to β-Actin, n = (5-6). All data were expressed as mean ± SEM and analyzed using two-way ANOVA with Sidak *post-hoc* analysis when significant effects were detected. A significant effect of FoxO1 inhibition was detected independent of cisplatin **A** and **B)**; *P<0.05, *** P = 0.0001, **** P<0.0001.

3.6 FoxO1 inhibition decreases DNA damage in Male ECs in response to Cisplatin

Next, I examined whether FoxO1 inhibition altered the vulnerability of male and female ECs to cellular damage by cisplatin by measuring γH2AX, a DNA damage marker. By Western blotting, male ECs exhibited a significant increase in γH2AX levels when treated with cisplatin compared to control. Conversely, ECs treated with cisplatin along with the FoxO1 inhibitor displayed a significant decline in γH2AX levels (Fig. 3.7A). Cisplatin did not alter γH2AX levels in female ECs while FoxO1 inhibition significantly reduced the levels of γH2AX treated irrespective of cisplatin treatment (Fig. 3.7B). FoxO1 inhibition, however, reduced DNA damage suggesting that its absence is beneficial to ECs in response to an oxidative insult.

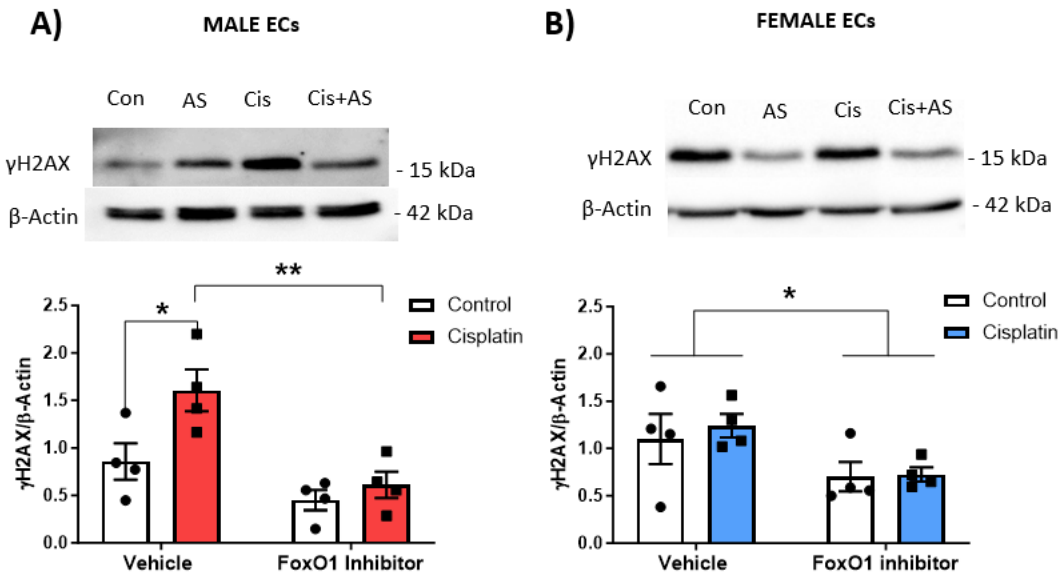

Figure 3.7: γH2AX levels in ECs following treatment with cisplatin.
Representative western blots measuring γH2AX in **A)** male, **B)** female ECs. Protein quantification for γH2AX was normalized to β-Actin, (n = 4). All data were expressed as mean ± SEM and analyzed using two-way ANOVA with Sidak *post-hoc* analysis when significant effects were detected. A main effect for FoxO1 inhibition was detected in female ECs **B)**; $P = 0.019$. A significant effect of cisplatin was detected in male ECs compared to control and for ECs under FoxO1 inhibition treated with cisplatin **A);** *P<0.05, **P<0.005.

These outcomes were confirmed by immunofluorescence staining of γH2AX+ nuclei. In male ECs, cisplatin significantly increased the proportion of γH2AX+ cell nuclei compared to control.

Furthermore, FoxO1 inhibition significantly lowered the number of γH2AX+ cell nuclei in cisplatin treated ECs (Fig. 3.8A). However, female ECs show a different pattern with immunofluorescence imaging compared to the western blot. Cisplatin significantly increased the proportion of γH2AX+ cell nuclei in ECs under FoxO1 inhibited conditions (Fig. 3.9A).

3.7 Sex Disparity in γH2AX levels

While imaging γH2AX+ nuclei, I noticed that there was a tremendous amount of γH2AX+ nuclei of female compared to male ECs under control conditions (Fig. 3.10A), which was statistically confirmed to be true (Fig. 3.10B). Similarly, there was a significantly higher amount of γH2AX protein in female compared to male ECs by Western blot (Fig. 3.10C). These results indicate that there seems to be a basal sex discrepancy in the DDR pathway.

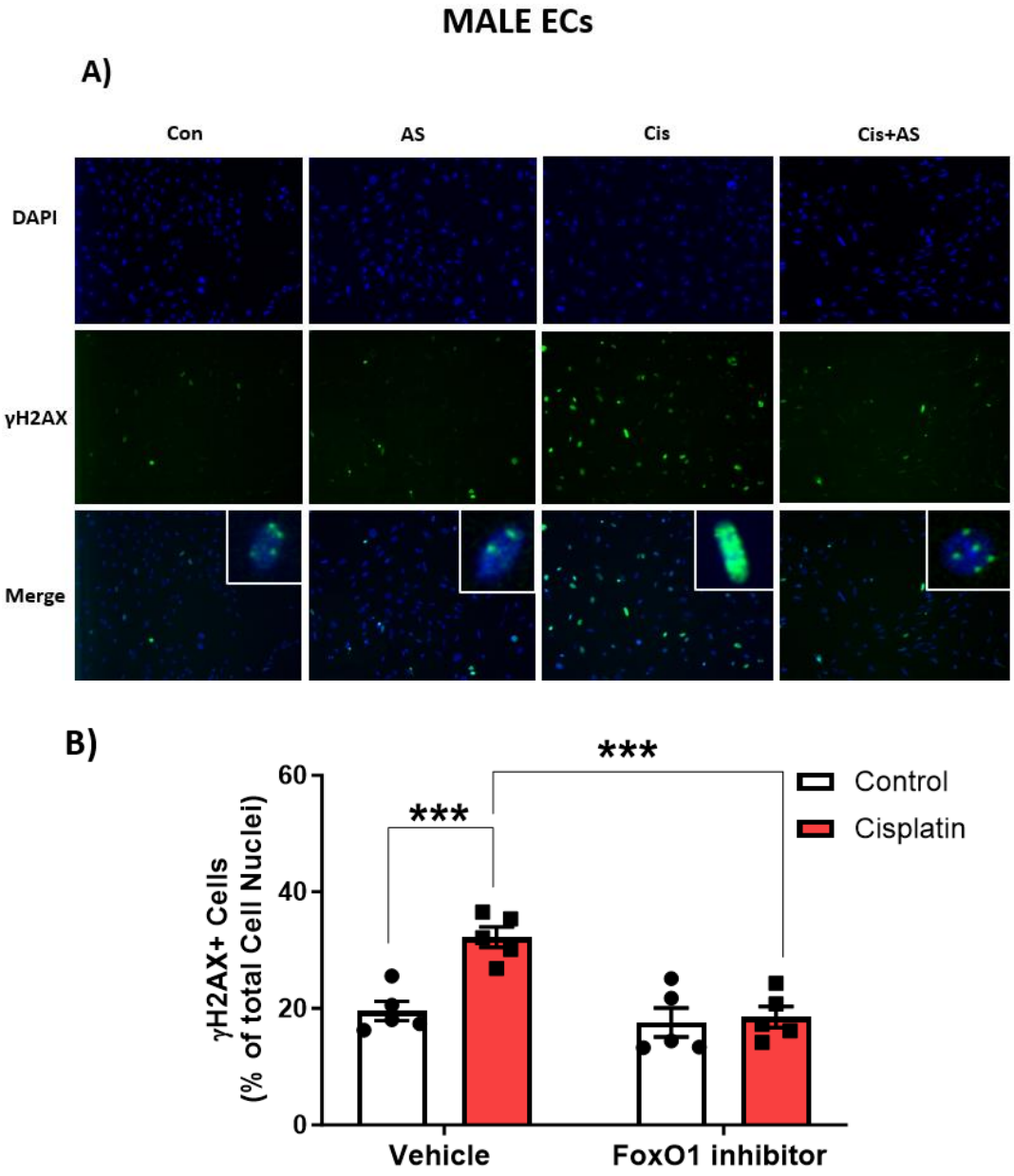

Figure 3.8: Cisplatin-induced increases in γH2AX are reduced by FoxO1 inhibition

ECs were treated with AS184 24 hours prior to being with cisplatin for 48 hours prior to immunostaining for γH2AX. **A)** Representative images of male γH2AX+ ECs under control conditions, AS184, cisplatin or AS184 and cisplatin together. γH2AX+ cells (green). All EC nuclei were stained with DAPI (blue). Inset boxes represent a zoomed image of γH2AX foci in a single nucleus. **B)** γH2AX+ EC nuclei were expressed as a percentage of total EC nuclei count (n = 4). All data were expressed as mean ± SEM and analyzed using two-way ANOVA with Sidak *post-hoc* analysis when significant effects were detected. A significant effect of cisplatin was detected in male ECs compared to control; ***P = 0.0006. A significant effect of FoxO1 inhibition was detected in cisplatin treated cells; ***P = 0.0003.

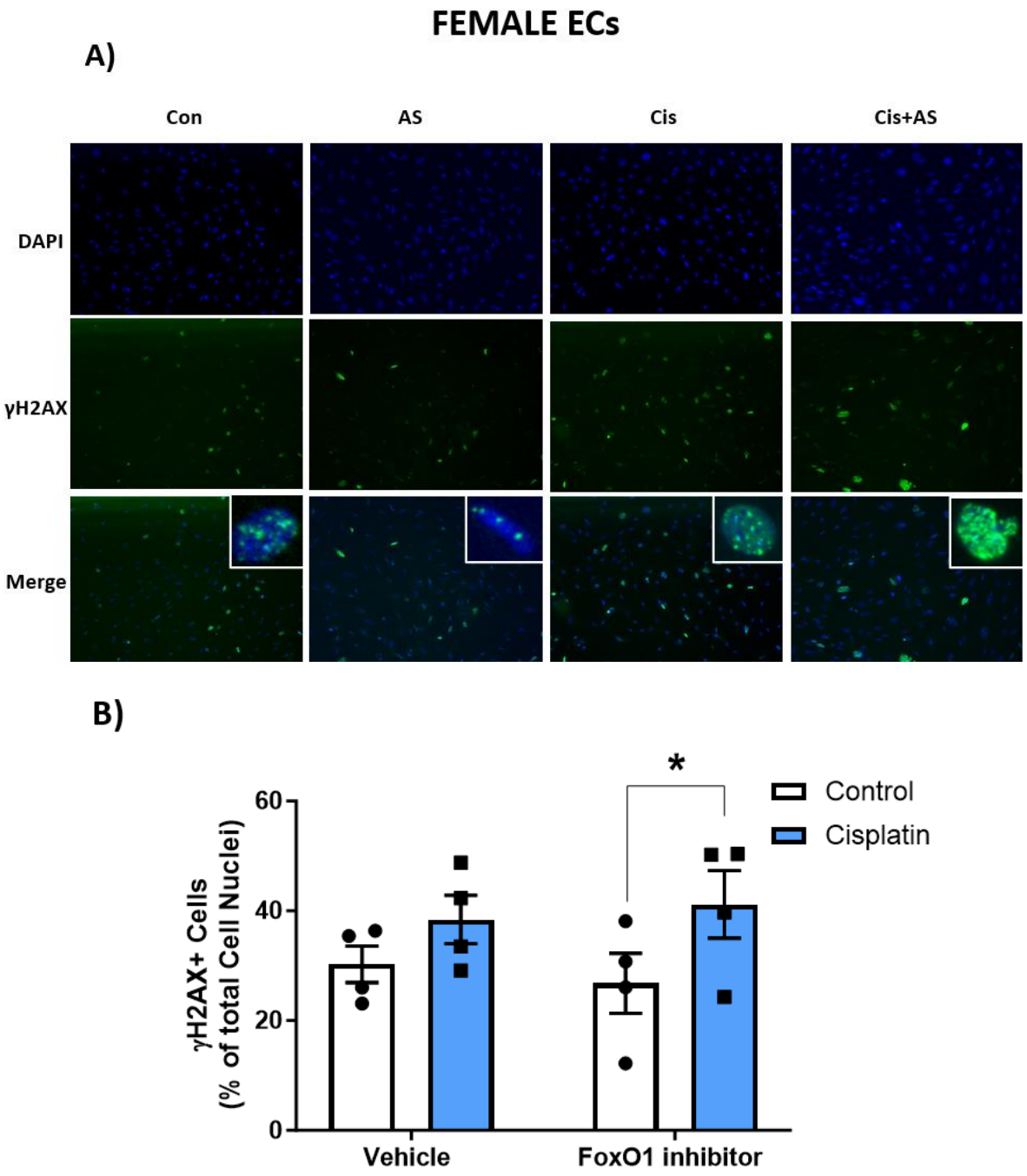

Figure 3.9: γH2AX+ nuclei increase in female ECs in response to FoxO1 inhibition combined with cisplatin.

ECs were treated with AS184 24 hours prior to being with cisplatin for 48 hours prior to immunostaining for γH2AX. **A)** Representative images of female γH2AX+ ECs under control conditions, AS184, cisplatin or AS184 and cisplatin together. γH2AX+ cells (green). All EC nuclei were stained with DAPI (blue). Inset boxes represent a zoomed image of γH2AX foci in a single nucleus. **B)** γH2AX+ EC nuclei were expressed as a percentage of total EC nuclei count ($n = 4$). All data were expressed as mean ± SEM and analyzed using two-way ANOVA with Sidak *post-hoc* analysis when significant effects were detected. A significant effect of cisplatin was detected under FoxO1 inhibition; $*P = 0.028$.

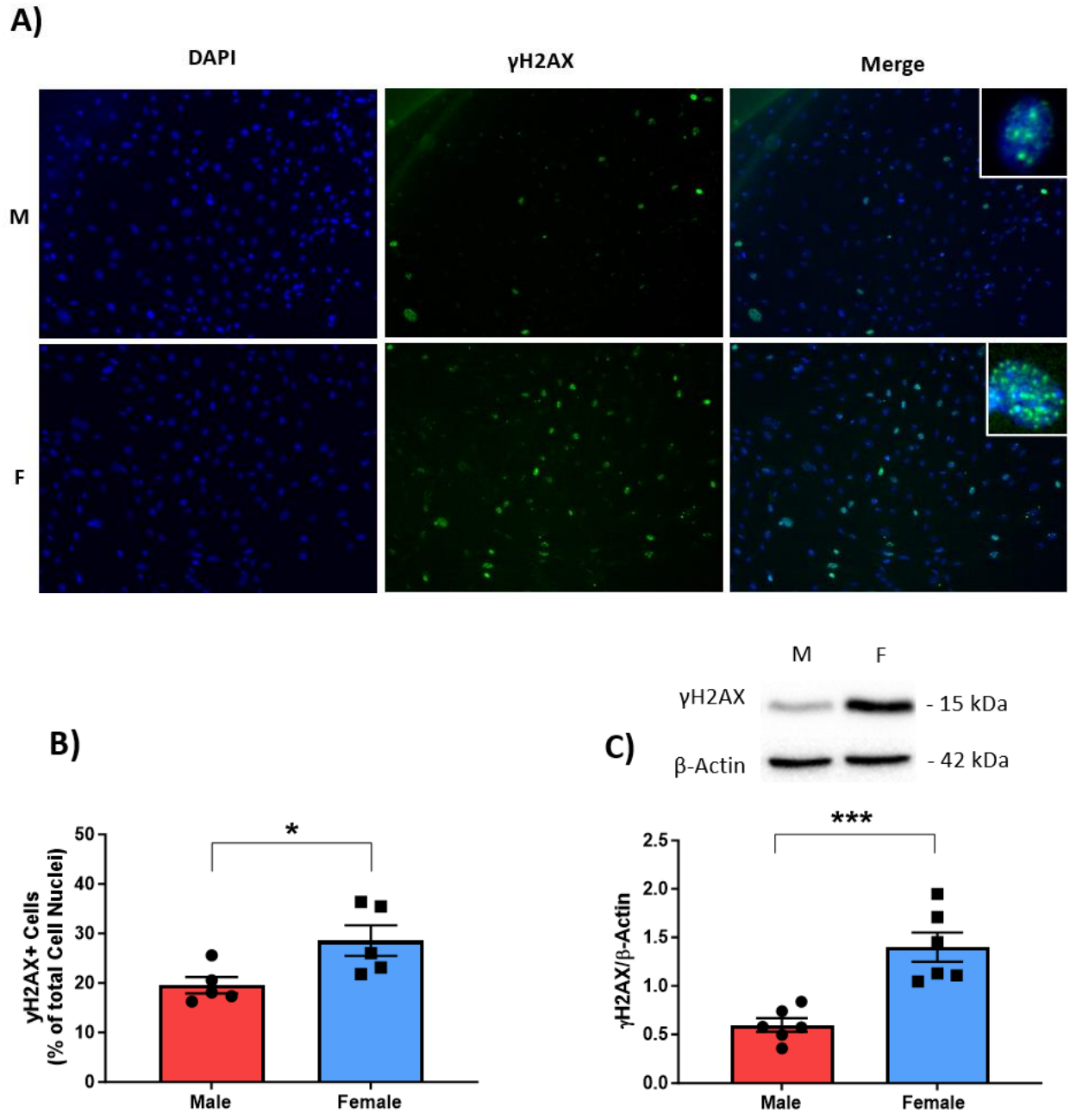

Figure 3.10: Female ECs have higher levels of γH2AX under control conditions versus male ECs.

A) Representative images of γH2AX+ male and female ECs under control conditions. γH2AX+ cells (green). All EC nuclei were stained with DAPI (blue). Inset boxes represent a zoomed image of γH2AX foci in a single nucleus. **B)** γH2AX+ EC nuclei were expressed as a percentage of total EC nuclei count (n = 5). **C)** Representative western blot images of γH2AX+ ECs in male and female ECs. Protein quantification for γH2AX was normalized to β-Actin, (n = 6). All data were expressed as mean ± SEM and analyzed using unpaired Students t test. **B)** A significant effect of sex was detected; *P = 0.044. **C)** A significant effect of sex was detected; ***P = 0.0007.

3.8 Effects of PFKFB3 and FoxO1 inhibition on DNA damage

The effect of FoxO1 inhibition to decrease γH2AX staining in male ECs was counter to my original hypothesis. One potential reason for this could be that DNA repair is fueled by glycolysis (Sun et al., 2023). Thus, the DNA repair abilities of the cell could be enhanced by FoxO1 inhibition because it upregulates glycolysis (Rudnicki et al., 2018a). To test whether the improvement of γH2AX levels with FoxO1 inhibition is mediated by enhanced glycolysis, I treated male ECs with a PFKFB3 inhibitor (3PO) to block glycolysis.

In male ECs treated with 3PO and cisplatin, AS184 increased p21 levels (Fig. 3.11A). γH2AX levels significantly elevated with 3PO and cisplatin compared to control (Fig 3.11B); however, in the presence of 3PO, FoxO1 inhibition did not increase γH2AX levels. 3PO significantly increased Prdx3 levels compared to control. Addition of AS184 significantly increased levels of Prdx3 compared to 3PO with cisplatin (Fig. 3.11C). FoxO1 levels were significantly lowered by AS184 in male ECs treated with 3PO and cisplatin, compared to cells treated with 3PO and cisplatin alone (Fig. 3.11D).

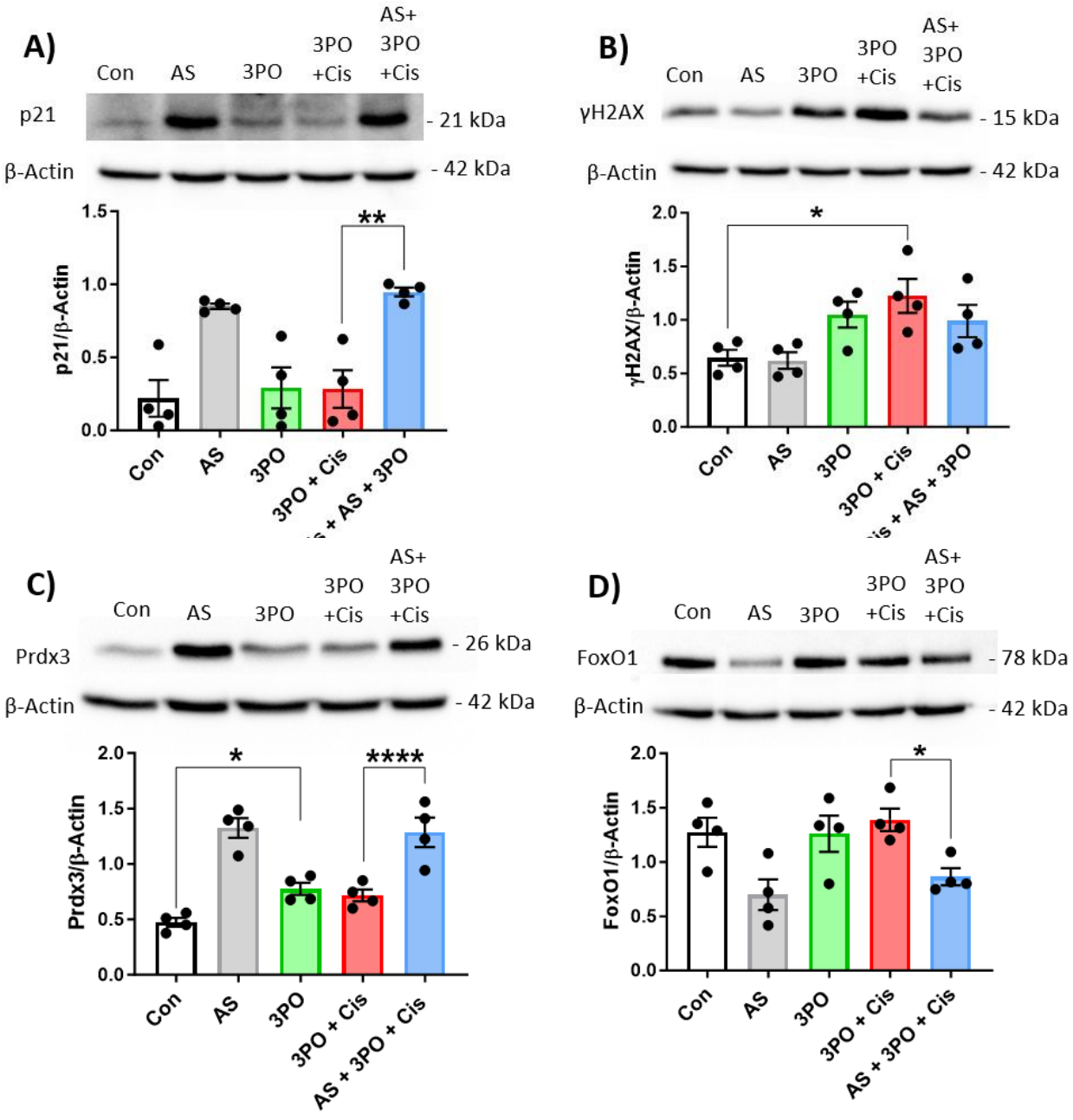

Figure 3.11: Effects of PFKFB3 and FoxO1 inhibition in combination with cisplatin.
Male ECs were treated with 3PO, AS184, or both for 24-hour hours prior to cisplatin treatment for 48 hours. **A-D)** Representative western blot images and quantification of p21, γH2AX, Prdx3 and FoxO1. For all blots, proteins were normalized to β-Actin and data were expressed as mean ± SEM and analyzed using one-way ANOVA with Sidak *post-hoc* analysis when significant effects were detected, $n = 4$. **A)** A significant effect of FoxO1 inhibition in combination with 3PO and cisplatin was detected compared to 3PO and cisplatin alone; $*P = 0.022$. **B)** A significant effect of 3PO and cisplatin was detected compared to control; $*P = 0.012$. **C)** Significant effects of 3PO compared to control, and FoxO1 inhibition in combination with 3PO and cisplatin compared to 3PO and cisplatin alone, were detected; $*P = 0.038$, $***P = 0.0004$. **D)** A significant effect of FoxO1 inhibition in combination with 3PO and cisplatin was detected compared to 3PO and cisplatin; $**P = 0.0011$.

Chapter 4: Discussion

The overall goal of my thesis was to assess potential sex differences in the behavior of ECs in response to a DNA damaging agent. Secondly, I investigated the role that FoxO1 plays in protecting ECs in response to DNA damaging agent. My findings support the potential for sex differences in basal DNA damage susceptibility and the capacity for DNA repair in ECs. I showed that the levels of γH2AX and *Cdkn2a* mRNA are higher and lower in females compared to males, respectively. Furthermore, my data provide evidence that FoxO1 inhibition increases proteins associated with cell cycle arrest (p21) and oxidant protection (Prdx3) in male and female ECs. However, my experiments revealed potential sex-specific variations in the role of FoxO1 within ECs, especially in managing DNA repair and damage, based on the expression pattern of *Gadd45α* and γH2AX in males.

4.1 Sex Differences in Cell Cycle Inhibition and DNA Damage Response in ECs

The experiments that I conducted revealed some EC sex differences in the expression of cell cycle arrest proteins. Gene expression analysis revealed that unperturbed female EC-FoxO1 KD and EC-FoxO1 Floxed ECs had significantly lower level of *Cdkn2a,* which is a cell cycle inhibitor gene that encodes for the protein p16 (Foulkes et al., 1997). This may be in line with previous data our lab has shown wherein female ECs have higher proliferation rates than males (Rudnicki et al., 2023, 2018a). However, this outcome can be perceived through a different perspective. As *Cdkn2a* expression also may be linked to senescence, it could suggest lower levels of senescence in female ECs. This interpretation also aligns our previous findings, wherein female adipose ECs displayed lower expression of SASP related genes compared to male ECs in (Rudnicki et al., 2023). The lower expression of *Cdkn2*a and p21 protein in female ECs, particularly when treated with FoxO1 inhibitor and cisplatin, can imply that they have less time

to undergo DNA repair (Campos and Clemente-Blanco, 2020), which might result in accumulation of DNA damage.

This concept is supported by the higher level of γH2AX in female compared to male ECs, even under basal conditions. γH2AX being a marker of DNA damage, can be looked at through two lenses: 1) it could suggest that females accrue more DNA damage than males or that 2) females can have a lower DNA repair capacity than males under basal conditions. The higher proliferation rates witnessed in female compared to male ECs may lead to replication stress, which in turn induces DNA damage, as has been reported in cancer cells (Zhang et al., 2022). Proliferation triggers replication stress, constitutive phosphorylation of H2AX in untreated cells is generally the result of ROS generated during oxidative phosphorylation (Tanaka et al., 2007). Thus, another potential reason for higher levels of γH2AX in females is that female ECs have greater oxidative phosphorylation than male ECs ((Rudnicki et al., 2023) and unpublished data). However, *Gadd45α* expression appeared lower in females compared to male ECs, suggesting there may be a lower DNA repair capacity of female ECs. There also is some evidence in the literature to indicate lower repair in females. One study suggested that females have a decreased ability to repair DNA damage induced by tobacco (Wei et al., 2000). Another study revealed that base excision repair is lower in females compared to males (Trzeciak et al., 2008). In sum, further research needs to be done to decipher whether the higher level of γH2AX in female ECs is due to being more proliferative than male ECs, or a lower capacity for DNA repair.

4.2 Cisplatin induced *Cdkn2a* expression and DNA damage in male ECs

Cisplatin, a platinum compound, is used as a chemotherapeutic agent for various cancer types. Cisplatin can react strongly with not only nuclear DNA, but also mitochondrial DNA (mtDNA) forming DNA adducts (Kleih et al., 2019). As a result, cisplatin mediates mitochondrial

dysfunction inducing the production of mitochondrial ROS (mtROS) (Lomeli et al., 2017). Cisplatin induced the expression of *Cdkn2a* in male ECs independent of FoxO1 inhibition, however female ECs remained unaffected by cisplatin. This may suggest that male ECs have a heightened capability halting cell cycle progression in response to an insult from a DNA damaging agent like cisplatin than female ECs. Cisplatin increased DNA damage in male ECs, whereas this effect was not seen in female ECs. The effects of cisplatin may have been masked in female ECs due to the high basal levels of γH2AX.

4.3 FoxO1 inhibition promotes a healthier phenotype in male ECs with conflicting effects on phenotype in female ECs

FoxO1 inhibition increased cell cycle gene *Cdkn1a* expression and p21 protein levels in both male and female ECs. I had anticipated that the opposite would occur because FoxO1 generally promotes cell cycle arrest (Tinkum et al., 2013). Although high p21 levels are associated with senescence (Englund et al., 2023), the induction of *Cdkn1a* expression and p21 protein levels with FoxO1 inhibition here may not be associated with senescence but potentially with DNA repair. My results are consistent with a recent publication, which showed that FoxO1 deletion in ECs significantly reduced senescence, increased DNA repair proteins, and decreased DNA damage (Sun et al., 2023).

My finding that FoxO1 inhibition increased *Gadd45α* expression in untreated female ECs and in male ECs treated with cisplatin diverges the current literature on the role of FoxO1. FoxO1 promotes the expression of genes involved in DNA repair such as *Gadd45a* (Ju et al., 2014) (Amin and Schlissel, 2008). A study that silenced FoxO1 in cardiomyocytes under oxidative stress conditions found decreased DNA repair proteins and increased DNA damage (Zhou et al., 2019). The underlying mechanism that resulted in higher *Gadd45α* mRNA in my

study remains to be established. It is possible that the upregulation of *Gadd45α* in FoxO1 inhibited cells may be due to the actions of p53, as it is a known regulator of *Gadd45α* (Kastan et al., 1992; Smith et al., 1994; Zhan et al., 1994). I observed a significant increase in the expression of the *Cdkn1a* gene, also a known target of p53 (Engeland, 2022; Hill et al., 2008), and an increase in p21 levels with FoxO1 inhibition. Thus, I did attempt to probe for p53 protein to identify it as a potential reason for the increases in *Cdkn1a*, p21 and *Gadd45α*, but I did not have success in attaining an analyzable blot due to faulty p53-antibody.

4.4 FoxO1 inhibition decreases DNA damage in male but not female ECs

FoxO1 inhibition significantly decreased DNA damage in male ECs challenged with cisplatin, based on Western blot and immunostaining of γH2AX. Interestingly, the effects of cisplatin and FoxO1 inhibition on DNA damage were not mirrored in female ECs. One potential reason for these disparate outcomes could be that under FoxO1 inhibition in combination with cisplatin, female ECs failed to upregulate *Gadd45α* expression, in contrast to the increased expression seen in male ECs. This could have led to differences in DNA repair. The decreased levels of p21 observed in cisplatin treated female ECs under FoxO1 inhibition might explain why FoxO1 inhibition did not lead to the same increase in *Gadd45α* expression that was observed in male ECs. The lower levels of p21 leave the female ECs in a relative proliferative state, potentially reducing time for the cell to orchestrate a DNA repair response. My data are not consistent with the sex-specific transcriptomic molecular signatures of ECs isolated from adipose tissue of high fat fed mice, wherein male ECs were pro-inflammatory and pro-senescent compared to females (Rudnicki et al., 2023). However, my data from male ECs agree with a study by Sun et al., (2023) where they showed that silencing FoxO1 in HUVECs significantly decreased γH2AX levels following exposure to high glucose and H_2O_2. Future research should be done to delineate

which DNA repair proteins are responsible for decreasing γH2AX under FoxO1 inhibition and whether there is a sex difference in the levels of these DNA repair proteins.

4.5 Effects of FoxO1 inhibition on antioxidant proteins

Because FoxO1 can promote the transcription of antioxidant genes, I hypothesized that inhibition of FoxO1 would decrease antioxidant proteins, leaving cells more vulnerable to oxidative damage. However, in both sexes, FoxO1 inhibition increased the levels of Prdx3. There is evidence that suggests that *Prdx3* is a target gene of c-MYC, whereby the absence of c-MYC reduces *Prdx3* expression by 50% (Wonsey et al., 2002). In turn, FoxO1 represses c-MYC signaling (Wilhelm et al., 2016). Consequently, the increase in Prdx3 in male and female ECs under FoxO1 inhibition may result from an increase in c-MYC signaling. Another possible explanation for this outcome could be that oxidative stress also increases levels of a transcription factor called NF-E2-related factor (NRF2), which can trans-activate the transcription of Prdx3 (Kasai et al., 2020).

Although these data do not fit my hypothesis, they fit with the overall profile of FoxO1 inhibited male ECs. It can be speculated that the increased Prdx3 leads to more mtROS detoxification. This could contribute to the lower γH2AX levels in cisplatin treated male ECs under FoxO1 inhibition. Further work needs to be done elucidating the role of FoxO1 in DNA repair and the molecular reasons as to why its inhibition promotes a decrease in DNA damage.

4.6 Impairing glycolysis in male and female ECs

Conversely, I postulated that FoxO1 inhibition may be beneficial to protecting ECs by upregulating glycolysis. Evidence suggests that DNA repair is dependent on glycolysis and glycolytic enzymes such as PFKFB3 (Gustafsson et al., 2018b; Sun et al., 2023). FoxO1 represses the expression of genes involved with glycolysis (Zhang et al., 2006). Previous data

from our lab show that FoxO1 inhibition in ECs increased the expression of glycolytic genes and promoted the increase in glycolytic enzymes such as hexokinase-2 and PFKFB3 (Rudnicki et al., 2018a). While inhibition of glycolysis with 3PO did increase γH2AX levels in cisplatin treated male ECs, it did not reverse the protective effect of FoxO1 inhibition in male ECs. This suggests that the protective role of FoxO1 inhibition is independent of its effects on glycolysis. My data are not in agreement with Sun et al., (2023), where they saw significant increases in DNA damage and decreases in HR and NHEJ DNA repair when FoxO1 and PFKFB3 was silenced in HUVECs.

4.7 Significance

Through these experiments, I have established several significant differences between male and female ECs, with respect to basal levels of γH2AX and *Cdkn2a* expression that may be indicators of disparate efficacies in DNA damage repair. My data highlight the importance of studying biological effects in both male and female ECs and support future work in determining the potential downstream consequences that can occur because of these differences. My experiments also indicate that the function of FoxO1 is more complex than we thought. Although my experiments do not paint a full picture of FoxO1 functions, my thesis opens new avenues of research regarding the role of FoxO1 in DNA repair. Moreover, my findings shed light on a potential therapeutic approach as FoxO1 inhibition in male ECs promoted positive cellular outcomes, which could be applied to preventing blood vessel damage in response to chemotherapeutic agents delivered intravenously or hyperglycemic conditions that can be caused by obesity.

4.8 Limitations of the study

4.8.1 Cell Culture Conditions

All my experiments were performed *in vitro* where ECs constantly proliferate. *In vivo*, EC in healthy adult vessels are quiescent unless acted upon by pro-angiogenic stimuli (Carmeliet and Jain, 2011). This *in vitro* model may not provide a full explanation for why FoxO1 improves DNA repair as it does not represent the quiescent behaviour of ECs that is present *in vivo*. Taking into consideration that cell cycle inhibition is crucial for DNA repair (Campos and Clemente-Blanco, 2020), this could imply that DNA repair may be activated more quickly *in vivo*. Supplementing that idea, ECs are removed from their microenvironment when cultured, hence they are not exposed to various stimuli such as sex hormones, growth hormones, cytokines, and secretion of various proteins from perivascular cells (Staton et al., 2009). Along with this, all ECs are constantly subjected to shear stress *in vivo*, which can change the way they behave compared to *in vitro* conditions (Staton et al., 2004). Additionally, a confounding factor especially for the DNA damage can be the high glucose media that was used to culture the ECs. As previously mentioned, DNA repair is dependent on glycolysis; thus, this can promote greater DNA repair than typically would be observed *in vivo*.

4.8.2 Cisplatin Treatment

My experiments required the selection of an agent that would induce DNA damage and increase senescence signaling within ECs. Cisplatin is known to induce nuclear DNA damage which can promote senescence (Yimit et al., 2019). Furthermore, cisplatin induces mtDNA damage (Kleih et al., 2019) that in turn should increase ROS production. Based on this knowledge, and my initial experiments, I was surprised by the lack of consistent effect of cisplatin on gene expression of cell cycle inhibitors and anti-oxidant genes. The timing of gene

activation may explain why the expression of these genes were low. It is plausible that these genes peaked prior to the 48-hour time point post cisplatin treatment before returning to baseline levels.

4.8.3 Modifying FoxO1 function

I wanted to use EC from EC-FoxO1 KD mice to test the functional role of FoxO1. However, results from the experiments that involved these ECs were ambiguous because I could not confirm if the FoxO1 deletion was still represented in the population of cultured ECs. To circumvent this problem, I conducted experiments using the FoxO1 inhibitor. Although this pharmacological drug binds to and inhibits the transcriptional activity of FoxO1 (Flores et al., 2023), FoxO1 is still present in the cell. This can potentially mediate other effects within the cell as opposed to its levels being severely reduced. The area of research regarding FoxO1 inhibition promoting healthy cellular outcomes for protecting the cell against a DNA damaging agent, is relatively unexplored. FoxO1 can still be regulated by AKT via phosphorylation on Ser256 (Saline et al., 2019), it is unknown whether phospho-FoxO1 plays any role in DNA repair that could have affected the results. AS184 also binds to and inhibits the transcriptional activity of FoxO3 and FoxO4; however, its affinity for these isoforms is much lower than for FoxO1 (Flores et al., 2023). FoxO3 protein levels significantly decreased in ECs treated with the FoxO1 inhibitor with and without cisplatin. Potentially, this can be a confounding factor for results I observed.

If I were continuing my research, I would use siRNA for FoxO1 or use a model in which the deleted EC FoxO1 gene produced in mice is retained *in vitro*. Complete knockdown of FoxO1 in ECs would ensure that there are no secondary effects that FoxO1 may mediate within the cell. This would provide an enhanced understanding of what the effects of diminished FoxO1

has on the cell. Also, this would be beneficial to observe any differences between FoxO1 inhibition or FoxO1 knockdown. Use of a DNA damaging agent *in-vivo* can also be more representative of EC behaviour as they are in a quiescent state; it would give more insight into the DNA damage response *in-vivo*.

4.8.4 Measurements of RNA versus Protein

The higher levels of mRNA I observed for the genes I measured may not correlate with increased translation and higher protein levels. There are post-translational modifications that also can alter protein levels, thus, mRNA levels cannot fully explain cellular phenotype.

While I attempted to measure p53 protein by Western blot, I think it would be more advantageous to instead assess phospho-p53. Since *Gadd45α* and *Cdkn1a* are influenced by p53, measuring the active form of p53 could offer greater insight into the reason why the levels of these genes are higher with FoxO1 inhibition. I would also like to measure Gadd45α protein levels to see if they correspond with the exceedingly higher expression of *Gadd45α* mRNA in males versus females. This could provide more evidence to decipher whether male ECs have a better DNA repair program than females.

4.9 Future Work

Current literature supports the idea that γH2AX can also be an indicator of replication stress rather than DNA damage, if it is not associated with the DDR pathway protein 53BP1 (Rybak et al., 2016). Thus, to better interpret the sex differences in γH2AX levels, co-staining of γH2AX with 53BP1 should be conducted. Also, it will be valuable to perform western blot analysis on DNA repair proteins such as those from the MRN complex. Considering that the MRN complex is mainly associated with HR DNA repair (Valikhani et al., 2021), proteins involved in NHEJ DNA repair such as DNA-PKs and Ku70/80 should also be assessed. This will help to determine

if the higher levels of γH2AX are potentially due to lower DNA repair capacity, providing further insight into which sex is better equipped for DNA repair.

My results are not in agreement with the currently accepted roles of FoxO1 in DNA repair, cell cycle and oxidative stress resistance (Ebrahimnezhad et al., 2023) and suggest that the function of FoxO1 can compromise the health of ECs. Future experiments that involve overexpressing FoxO1 in the presence of a DNA damaging agent will be vital to form a better understanding of FoxO1 function in EC DNA damage pathways. I postulate that FoxO1 overexpression will promote negative outcomes in the cell, increasing DNA damage. Another potential measurement could involve co-immunoprecipitation to investigate any interactions between FoxO1 and DNA repair proteins. This experiment could involve DNA damage sensors like the MRN complex or downstream proteins in HR DNA repair like BRCA1/2 and replication protein A (RPA), or proteins like 53BP1 that is involved in NHEJ. An interaction between FoxO1 and these DNA repair proteins could suggest that FoxO1 can inhibit these proteins from carrying out their functions in DNA repair processes.

Culturing cells with high glucose media may permit higher levels of DNA repair than could be seen in vivo. We might not get a full understanding of what role FoxO1 inhibition is playing to promote the positive cellular events. For future experiments, I think culturing the cells in low or no glucose DMEM will be beneficial as it minimizes this potentially confounding variable of glycolysis in DNA repair. Under these conditions, cisplatin may be more effective in inducing its detrimental effects, thus providing a deeper understanding of the therapeutic effects of FoxO1 inhibition.

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