SEX-DIFFERENCES IN PULMONARY VASCULAR ENDOTHELIAL DEVELOPMENT: FACTORS THAT INFLUENCE ANGIOGENESIS

by

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ABSTRACT

Extremely preterm birth (<32 weeks gestation) requires life-saving treatments, specifically mechanical ventilation with oxygen therapy, which creates a hyperoxic environment within the still-developing lung. This leads to disrupted alveolarization, increased inflammation, tissue damage, and disrupted angiogenesis, which can all contribute to developing bronchopulmonary dysplasia (BPD). Despite the uniform treatment approach on extremely preterm infants, BPD is a disease with a significant sexual dimorphism where males are disadvantaged compared to their female counterparts. Although mechanisms behind this sexual dimorphism are poorly understood, sex differences in angiogenesis have been identified as one possible source of the male disadvantage in BPD.

Proper lung development in the alveolar stage heavily depends on pulmonary angiogenesis, a complex process of forming new blood vessels. Recent studies have shown significant sex differences in endothelial cell expression profiles, behavior, and angiogenesis. Sex differences can arise from intrinsic (chromosomal) or extrinsic sources. Sex hormones are a commonly studied extrinsic factor, specifically the male-associated hormone testosterone and the female-associated hormone estrogen. During normal human gestation, testosterone peaks between 10 and 17 weeks while estrogen steadily increases starting at 20 weeks gestation through birth. This estrogen spike is abolished in prematurely born infants. Sex hormones have been implicated in angiogenesis, a complex, multi-cellular process that requires significant changes in cellular behavior and metabolism. Proliferator-activated receptor gamma (PPARγ) is a transcription factor that sits in the center of these complex behavior changes, as well as many other cellular responses. Additionally, PPARγ is a cofactor with estrogen

receptors and has been implicated in BPD and pulmonary hypertension. These factors make PPARγ an ideal target to study in the context of sex differences in pulmonary angiogenesis related to BPD. This approach has not previously been taken.

This dissertation is divided into three main aims that characterize the sexual dimorphism in pulmonary angiogenesis and the factors contributing to these differences.

In Aim 1, we characterized the sexual dimorphism in pulmonary angiogenesis, specifically identifying a sex phenotype. Pulmonary angiogenesis was assessed *in vitro* using a bead sprouting assay with pooled male or female human pulmonary microvascular endothelial cells in standard (sex-hormone containing) and hormone-stripped medium. We identified a sex-specific angiogenesis phenotype, specifically that male HPMECs produce fewer but longer sprouts than female HPMECs. This male phenotype was also sex hormone-sensitive, while the female phenotype was not. The sex-specific secretome could also influence the sex phenotype in a sex-specific way. Both male and female HPMECs secrete factors that increase female HPMEC sprout length, which is abolished when sex hormones are present. Taken together, these results demonstrate that the pulmonary endothelial cell phenotypes are influenced by sex hormones and sex-specific secreted factors in a sex-dependent manner.

In Aim 2, we identified sex differences in the proliferative capacity of pulmonary endothelial cells and investigated the role sex hormones play in angiogenesis. Using a Boyden chamber assay, we found there is no sexual dimorphism in HPMEC migration, a cellular process that was also found to be sex hormone insensitive. Pulmonary endothelial cell proliferation was sexually dimorphic, female HPMECs were significantly more proliferative than male HPMECs, but this process

was also sex hormone insensitive. These findings suggest that the source of the sexual dimorphism in HPMEC proliferation is intrinsic to the cells. We used a bead sprouting assay to assess sex hormone influence on angiogenesis directly. Female HPMECs produced more sprouts when exposed to estradiol (E2), while male HPMECs produced fewer. This is an interesting finding that warrants further investigation. In contrast, dihydrotestosterone (DHT) treatment resulted in robust and significant increases in angiogenic properties in both male and female HPMECs. While both male and female HPMECs responded positively to DHT, the female response was attenuated compared to the male response. Taken together, sex hormones heavily influence angiogenesis, and the magnitude of the response may be due to intrinsic sex differences.

In aim 3, we characterized pulmonary endothelial cell metabolism, determined the expression profile of PPARγ, and identified the role PPARγ plays in pulmonary angiogenesis. Using the seahorse assay, we found a sexual dimorphism in the total ATP production where male HPMECs produce significantly more ATP/min/cell compared to female HPMECs. Interestingly both male and female HPMECs generate their ATP equally from glycolysis and mitochondrial sources. Further, almost no ATP was generated from FAO, possibly due to a lack of substrate in a hormone-free medium. We also found that these metabolic profiles are estrogen insensitive in male and female HPMECs. Next, we found a sexual dimorphism in PPARγ expression using western blotting, where female HPMECs had a higher PPARγ expression than males. BPD is associated with decreased expression of PPARγ. Male HPMECs exposed to PPARγ agonist upregulated glycolysis for ATP production. Both PPARγ agonist and antagonist significantly increased angiogenesis in both male and female

HPMECs. These findings demonstrate that PPARγ is involved in pulmonary angiogenesis and could provide a therapeutic target in the context of BPD.

In summary, the work in this dissertation includes a thorough investigation into the sex differences in pulmonary angiogenesis, the role sex hormones play in this process, and how cellular metabolism in the context of PPARγ influences pulmonary endothelial cell biology as it relates to angiogenesis.

Chapter 1

INTRODUCTION

1.1 Introduction to the importance of sex-differences research

The National Institute of Health (NIH) instituted a new policy in the 1990s focused on increasing representation of women in research[1]. Prior to this new policy, sex was either excluded from analysis or studies were designed to only use male subjects, biasing any findings[1]. This bias had implications across research areas, especially as it relates to human disease, as evidence was emerging that diseases manifestation and incidence was not consistent in men and women[1]. Just a few examples of diseases that had reported sexual dimorphisms included, but is not limited to, ischemic stroke, Alzheimer's, autoimmune diseases such as multiple sclerosis and lupus, and various cardiovascular diseases[1]. Eventually, this change in policy, and the initial findings in research that implemented these changes, would lead to the NIH instituting considering sex as a biological variable (SABV) as a policy in research and clinical care[1]. This policy change has resulted in a robust, new focus on sex differences research with profound implications in human diseases.

1.2 Sex hormones and their role in development

Sex differentiation in development is due to both intrinsic (chromosomal) and extrinsic sources. Sex hormones, specifically female associated estrogen and male associated testosterone, heavily influence sexual differentiation throughout development[2,3]. The placenta allows sex hormones to pass between the pregnant

person and the fetus, as well as being a site of sex hormone metabolism, further demonstrating the importance of balanced hormone states for a developing fetus[4–8]. There exist important windows for fetal circulating hormone levels to be increased in order for proper development to occur[2,3]. Fetal testosterone in male fetuses is highest around 10-17 weeks gestation, drastically dropping by 24 weeks gestation where low levels will be maintained through to delivery[2,3] (**Fig. 1.1**). Both males and females will have similar fetal circulating estrogen levels, which steadily increase starting at 20 weeks of gestation and peaking around delivery[2,3] (**Fig. 1.1**).

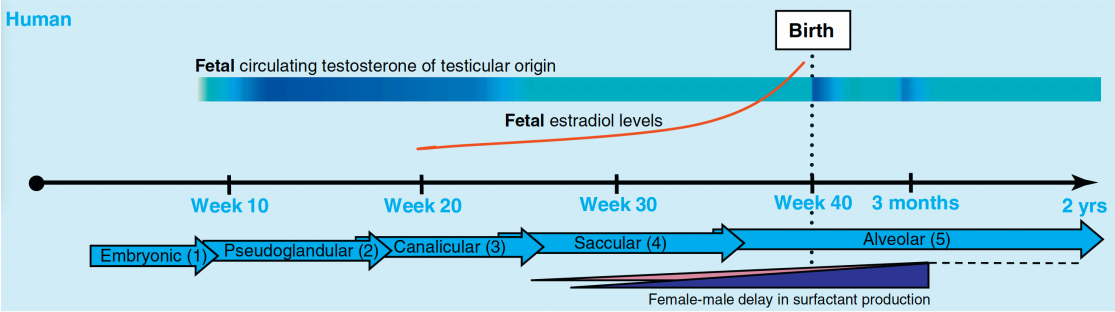


Figure 1.1: Fetal hormone cycling in normal human gestation. Bottom shows timeline of surfactant production, stages of lung development, and a time-line of the weeks of gestation. Top portion show cycling of the fetal sex hormones testosterone and estradiol. Light blue denotes low levels and dark blue denotes high levels. Adapted from Seaborn et al. (2010) Trends Endocrinol. Metab.[3]

Sex hormones can signal through both genomic and nongenomic signaling pathways and are all derived from cholesterol[2,9–13]. There exists multiple enzymatic steps in the metabolism of sex hormones that either allow for easy conversion into the other sex hormones or serve as rate limiting steps so that only one specific sex hormone is present[12]. In the case of testosterone, the aromatase Cytochrome P450

(CYP) 19A1 can, in an irreversible process, metabolize testosterone into β-estradiol (E2) while the presence of 17β-hydroxysteroid dehydrogenase 2 (17β-HSD2) allows for the conversion of testosterone into androstenedione, which can be converted by the same aromatase CYP19A1 into estrone, a female associated estrogen hormone[12]. Cells use 5α-reductase to irreversibly convert testosterone into dihydrotestosterone (DHT), the bioavailable derivative of testosterone that cannot be converted into a female associated sex hormone[2,12]. Testosterone has a single receptor, androgen receptor (AR), that acts as a nuclear factor upon stimulation with testosterone, translocalizing to the nucleus and activating gene expression[2,14].

Similar to testosterone, estrogen is generated from cholesterol and its expression is controlled through rate limiting reaction steps[2,12]. Testosterone can be used to generate E2 through the aromatase CYP19A1 enzyme, or generated from estrone by 17β-hydroxysteroid dehydrogenase 1 (17β-HSD1)[12]. It is important to note that once estrone or E2 is generated, these hormones cannot be converted back into testosterone due to the irreversible conversion by CYP19A1[12]. While all 4 of the estrogen derivatives (estrone, E2, estriol, and estretrol) can bind to the estrogen receptors, E2 is the most abundant circulating form of estrogen[15]. Unlike testosterone, estrogen receptor signaling involves two receptors, estrogen receptor α (ERα) and estrogen receptor β (ERβ)[2,3,12,15]. These receptors will translocate to the nucleus upon binding with their ligand and stimulate or repress gene expression as a result[2,3]. Interestingly, ERα and ERβ antagonize one another[2,3].

1.3 Lung development

The lung is a complex organ that consists of branched airways united with blood vessels for the purpose of gas exchange[2,16,17]. During normal human

development, the lung goes through five major stages: embryonic, pseudoglandular, canalicular, saccular, and alveolar[2,16,18](**Fig. 1.2**). Key developmental transitions occur at these stages. The primary right and left buds form from the foregut endoderm during the embryonic stage (3-7 weeks gestational age)[2,16]. These buds continue to grow and an airway tree is established through branching morphogenesis during the pseudoglandular stage (5-17 weeks gestational age) of lung development[2,16,19]. The pseudoglandular stage is also where lung cellular differentiation starts, giving rise to tracheal cartilage, smooth muscle, and blood vessels[2,16]. Further cellular differentiation, specifically in the epithelium, and continued epithelial branching are the hallmarks of the canalicular stage (16-29 weeks gestational age)[2,16]. At this stage, capillary networks are forming around the distal epithelial airspaces while epithelial differentiation is giving rise to alveolar epithelial cells as mesenchyme cells begin thinning[2,16]. The saccular stage (24-38 weeks gestational age) marks the end of branching morphogenesis and the appearance of saccules at airway ends, each sack being surrounded by capillaries[2,16]. Further epithelial cell differentiation occurs during the saccular stage, which gives rise to type 2 alveolar epithelial AT2 cells, and subsequently surfactant production[2,16]. Alveolarization (32 weeks gestational age through adolescence) is the final lung developmental stage and is evident by the formation of alveoli surrounded by a capillary network[2,16]. This final stage of lung development is heavily dependent upon pulmonary endothelial cells undergoing angiogenesis, the process of making new blood vessels from existing blood vessels[20]. These processes are a result of coordinated spatial biochemical, biophysical, and cellular interactions[21–24]. Disruptions in this process result in congenital conditions and preterm birth associated complications including bronchopulmonary dysplasia[25–27].

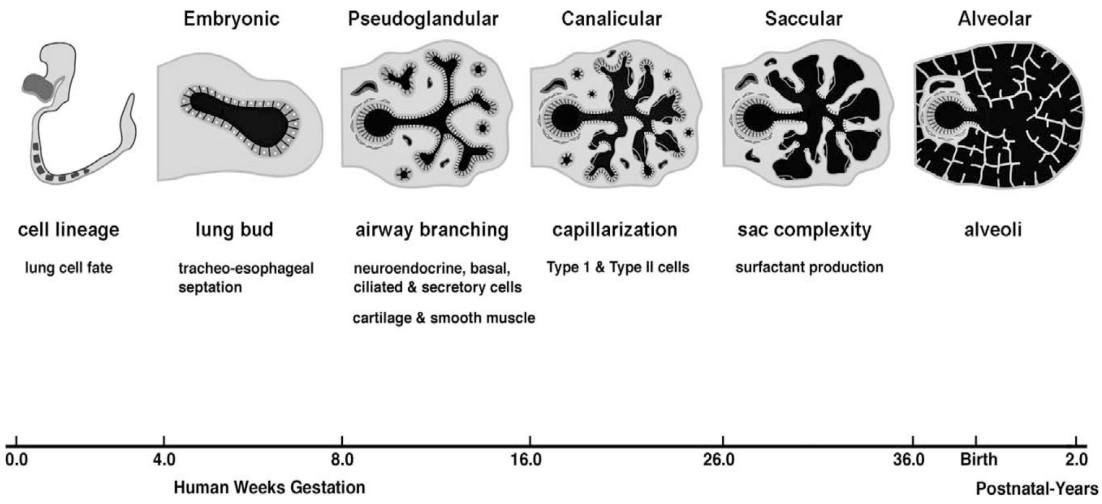


Figure 1.2: Stages of human lung development. Bottom is a timeline of a normal human gestation/postnatal years and top are the morphological changes that occur tat this various stages of lung development. Adapted from Kimura and Deutsch (2007) Pediatr. Dev. Pathol.[28]

There are striking sex differences in lung maturation[29–31]. These differences in lung development can be detected as early as 16 weeks gestation with fetal mouth movements, which are more frequent in female fetuses than male fetuses[32]. When looking at histological index of maturity in the lung, more specifically the development of mature lung structures, female fetuses have a higher index compared to male fetuses between 20 and 32 weeks gestational age[30,32]. Another area of lung development that demonstrates sex differences is in the transition between canalicular and saccular stages, specifically the point where surfactant production begins[32]. Between 30-40 weeks gestational age, when assessing surfactant production by amniotic fluid levels of surfactant phospholipids, female fetuses are 1.2-2.5 weeks ahead of male fetuses using this marker of pulmonary maturity[32]. At birth, while males tend to have larger lungs with more respiratory bronchioles compared to females, the

inverse is true about alveolar numbers per unit with females having a larger number of alveolar numbers compared to males[32].

1.4 Premature birth and bronchopulmonary dysplasia

Preterm birth is defined as delivery before the 36th week of gestation, with extremely preterm birth being infants born before the 32nd week of gestation[33,34]. In the case of extremely preterm infants, this corresponds to the end of the canalicular and beginning of the saccular stage of lung development, when surfactant production begins[2,16,32]. Extremely preterm infant lungs are not expected to perform gas exchange functions during this developmental stage, and as such life-saving measures must be taken for these infants to survive. These therapies, specifically mechanical ventilation with high oxygen, on the still developing lung causes tissue damage and increases inflammation, which in turn disrupts alveolarization and angiogenesis[33–37]. Combined, these factors contribute to the development of bronchopulmonary dysplasia (BPD), which is a debilitating, life-long diseases characterized by impaired alveolarization and vascular rarefication of the lung[33–35,38] (**Fig. 1.3**).

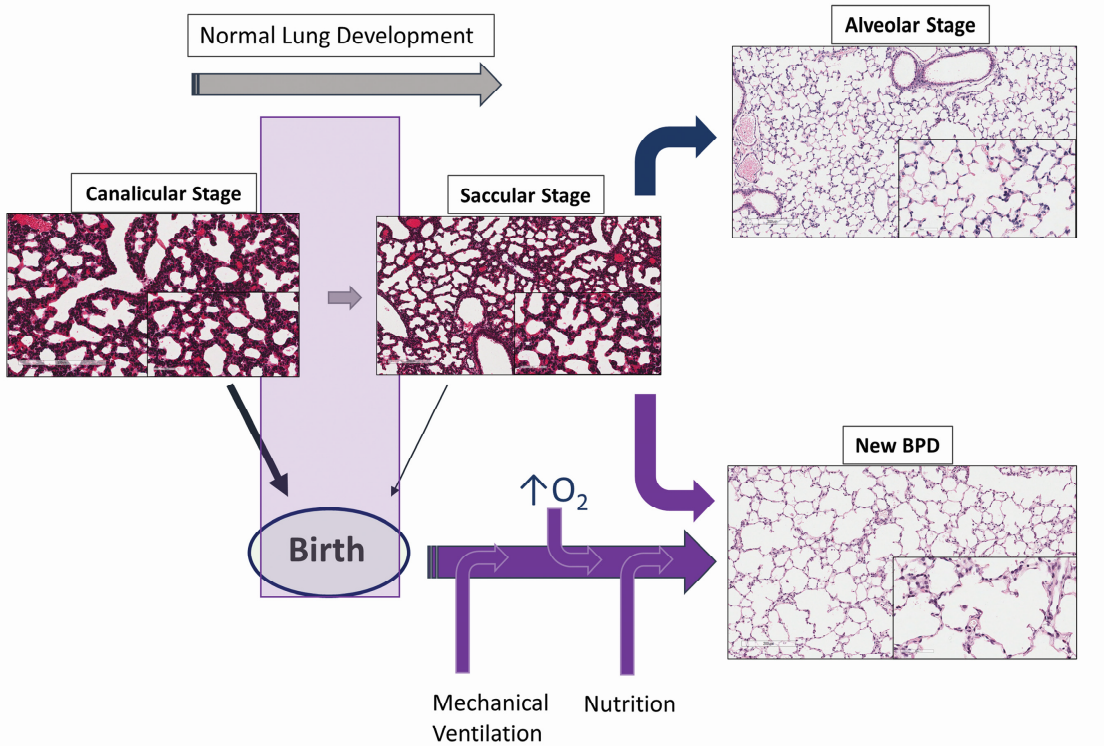


Figure 1.3: Histology of BPD. Histological samples of mouse lung in normal development (left) and in a BPD mouse model (bottom right). Top right is a normal mouse lung in the alveolar stage to compare the vascular rarefication and alveoli simplification in the mouse BPD lung. Adapted from Kumar (2017) Children.[Open Access] [39]

Preterm delivery also disrupts hormone signaling for the fetus, specifically the E2 spike that is supposed to continue through to normal delivery[2–4,40,41]. In fact, a single day after preterm delivery corresponds with a 100-fold decrease in E2 in the infant[40,41]. This is also the time period in which female infants have a higher histological index of maturity in the lung and females start producing surfactant earlier[2,30,32]. All these factors likely play a role in reported sexual dimorphisms in lung disease incident rates among preterm infants, including BPD. Despite these reported

sex differences in lung development, the direct contributing factor that relates to the sexual dimorphism in BPD is still not fully understood.

1.5 Angiogenesis

Pulmonary angiogenesis is critical for proper alveolarization. Blocking angiogenesis in the alveolar stage of lung development results in a simplified histological profile of the alveoli in a rat model[20]. This relationship between pulmonary angiogenesis and alveolarization has been investigated in multiple models, with disrupted angiogenesis contributing to impaired alveolarization[37,42,43]. BPD is characterized by both impaired alveolarization and disrupted vascularization, dysregulated pulmonary angiogenesis could be the link between these processes[35,37,38]. Further, reduced and dysmorphic vascular growth along with downregulation of angiogenic factors have been identified alongside decreased alveolarization, directly, in the context of BPD[37,42,44–46].

Angiogenesis is a complex, multi-cellular process where an endothelial cell will respond to a pro-angiogenic factor and form a new sprout from an existing blood vessel, typically a capillary[47–49]. Upon stimulation from a pro-angiogenic factor, such as vascular endothelial growth factor (VEGF), the responding endothelial cell will undergo a phenotypic switch ang gain a migratory behavior with the induction of filopodia so that this cell, known as the tip cell, can begin to migrate through the VEGF gradient[48] (**Fig. 1.4**). Canonically, this is achieved through VEGF binding with VEGF receptor 2 (VEGFR-2), which activates Notch signaling[48–50]. Notch in the tip cell will bind with delta-like ligand 4 (Dll4) in neighboring endothelial cells, which will cause a phenotypic switch and upregulate proliferation[48]. These proliferative neighboring endothelial cells will form what is known as the stalk and begin sprout

elongation[48]. Together, the tip cell and stalk cells form a new sprout that will generate a new blood vessel once it connects with another blood vessel. These mechanisms have been identified through the use of two-dimensional and three-dimensional *in vitro* and *in vivo* model systems[51–55].

Angiogenesis can be studied *in vitro* in a multitude of ways, from directly studying specific aspects of angiogenesis independently to looking at angiogenesis as a whole. In the case of looking at individual components, proliferation and migration assays are frequently used[56]. The tubal formation assay is one of the simplest angiogenesis assay, but is still only accounting for a few of the processes required for angiogenesis[56–58]. Specifically, tubal formation assay capture the ability of endothelial cells to coalesce into networks but the process is 2-dimensional and sprouts are not produced[56]. The most complex and comprehensive 3-dimensional *in vitro* angiogenesis assay is the bead sprouting assay, where collagen beads are coated in a monolayer of endothelial cells and embedded into a fibrin clot where sprouts will form[56,59]. While the bead sprouting assay is more representative of angiogenesis, the tubal formation assay is typically favored in large part because the 2-dimensional nature of the assay simplifies analysis; a major limitation of the bead sprouting assay is the increased challenges with 3-dimensional sprouting analysis.

1.5.1 Sex-differences in endothelial cells and angiogenesis

Sex differences in lung angiogenesis have been identified, and more importantly, linked to BPD[2,29,38,60,61]. These differences in angiogenesis are likely due to the fundamental sex differences in endothelial cells; findings that are relatively recent for the field[61–65]. Endothelial transcriptomics studies revealed striking differences in gene regulation of human umbilical vein endothelial cells (HUVECs)

where, upon stimulation with shear stress, only 72 genes were shared between male and female HUVECs in being up or downregulated out of the total 2249 genes shown to change[62]. Specifically, female HUVECs up- or downregulated a total of 2006 genes and male HUVECs did the same for 171 genes that didn't share any overlap with the opposite sex[62]. At baseline condition, many of the dysmorphic genes identified in this study were related to cellular metabolism, specifically lipid related metabolism[62]. These transcriptomic differences translated to functional differences, specifically female HUVECs had a higher survival rate in long-term starvation conditions and female HUVECs had a more abundant number of loops in a tubal formation assay, indicating female HUVECs had a higher angiogenic capability compared to male HUVECs[62].

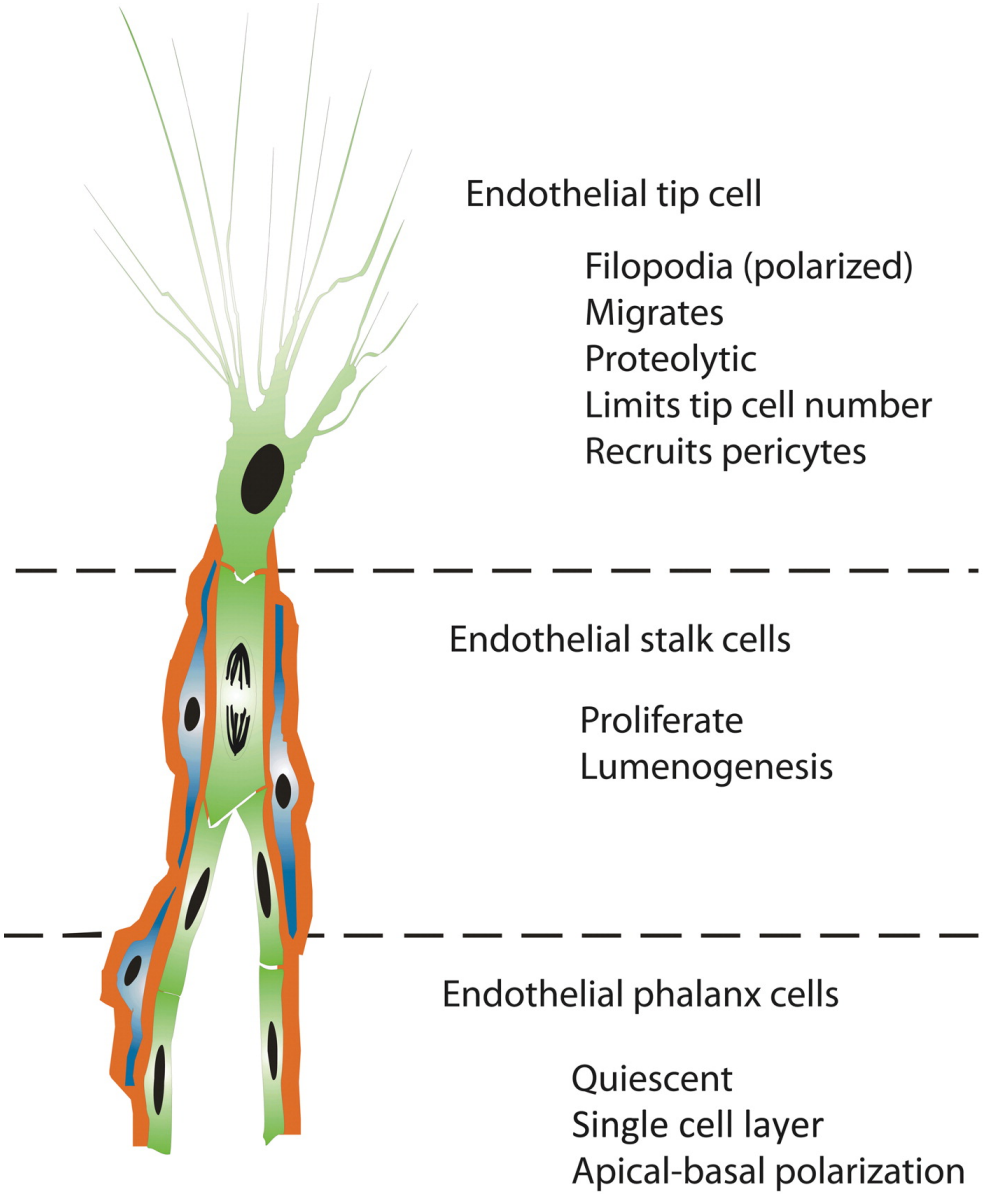


Figure 1.4: Sprouting angiogenesis: endothelial specialized phenotypes arise in sprouting angiogenesis. Top is the tip cell that gains a migratory phenotype, middle are stalk cells that gain a proliferative phenotype, and bottom are phalanx cells which are quiescent endothelial cells. Adapted from Siemerink et al. (2012) J. Histochem. Cytochem.[66]

Sex differences in endothelial cell biology as it relates to BPD have also been reported, specifically that there is a sexually dimorphic response in endothelial cells in response to hyperoxic exposure[61]. Female human pulmonary microvascular endothelial cells (HPMECs) were able to preserve cell viability as well as proliferation when exposed to hyperoxia while male HPMECs were not[61]. Additionally, the exposure to hyperoxia resulted in increased expression of mesenchymal markers and suggested male HPMECs was moving towards endothelial mesenchymal transition[61]. Following this study, it was identified that a key Dll4 regulator, micro RNA (miR) 30-a was sexually dimorphic in HPMECs where female HPMECs had a higher expression, which was shown to further increase after exposure to hyperoxia[59]. Both of these studies used a bead sprouting assay of angiogenesis and found that female HPMECS, under normoxic conditions, had a greater cell migration and sprouting extension than male HPMECs, confirming data previously reported in HUVECs[59,61]. Due to the crucial role angiogenesis plays in proper lung development, these reported sex differences in pulmonary angiogenesis, and the link to BPD warrant a more focused study on the mechanisms behind the sexual dimorphism in pulmonary angiogenesis.

1.6 Endothelial cell metabolism

Control over angiogenesis has canonically been attributed to the drivers of angiogenesis, pro-angiogenic factors such as VEGF. A new area of research has shifted that focus to the cellular process that powers angiogenesis, endothelial cell metabolism[67]. Endothelial cells are uniquely positioned in a high oxygen environment due to their barrier function in blood vessels, and surprisingly majority of their ATP is generated through anaerobic metabolism[49]. It's estimated that at least 75% of

endothelial cell ATP is generated in glycolysis, specifically the metabolism of glucose into pyruvate[49,68]. Glycolysis is so central to endothelial function that blocking it results in cell death[49]. This endothelial cell glycolysis focus is also evident in the number of mitochondria present in the cytoplasm, constituting around 2-5% of the cytoplasmic volume[69]. Microvascular cells, such as those in capillary beds, are reported to be slightly more glycolytic than other endothelial cells *in vitro* and under baseline conditions[49]. These metabolism conditions in endothelial cells change as the function of endothelial cells change.

1.6.1 Glycolysis: Meeting the high energy demands of migration

Endothelial cell stimulation by a pro-angiogenic factor triggers a signaling cascade to initiate sprouting, and part of this signaling cascade is linked to cellular metabolism[49,67]. The initial stage of this is Dll4-mediated Notch signaling. In parallel with this signal cascade, VEGFR activation will upregulate glucose transporter type 1 (GLUT-1), a glucose transporter that allows endothelial cells to uptake glucose without expending energy, as well as 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3), a glycolytic enzyme that generates the product fructose-2,6-bisphosphate[49]. This product is important for activating phosphofructokinase-1 (PFK-1), a rate-limiting enzyme in glycolysis[49]. In this way, pro-angiogenic stimulation of endothelial cells directly upregulate glycolysis by increasing the activity of PFK-1, a necessary step for the phenotypic switch that must occur for the formation of a tip cell[49] (**Fig. 1.5A**).

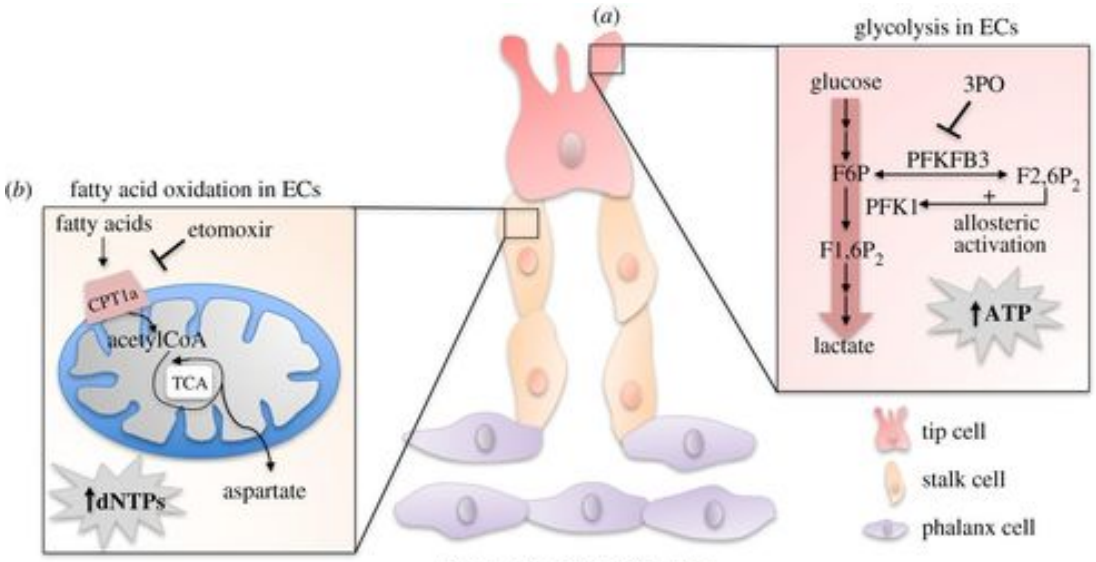


Figure 1.5: Endothelial cell metabolism in angiogenesis. An overview of cellular metabolism in tip vs. stalk endothelial cells. A) shows how glycolysis is upregulated in tip cells through PFKFB3 producing the ligand for PFK1, B) shows how FAO is used in stalk cells to generate dNTPs. In each case, there are inhibitors listed to show external ways to regulate these processes, specifically 3P0 will inhibit PFKFB3 and down regulate glycolysis while etomoxir will inhibit CPT1a and downregulate FAO. Adapted from Draoui et al. (2017) R. Soc. Open Sci. [Open Access] [70]

Tip cell upregulation of glycolysis is also due, in part, to constraints that migrating places on the cytosol[67]. Specifically, thin filopodia are a high energy structure that cannot accommodate bulky mitochondria[67]. Actin-myosin contraction requires high levels of ATP consumption, which can be achieved by the relocation of glycolysis associated enzymes to the lamellipodia and filopodia so that the ATP generated by glycolysis is directly at the site of high energy demand[67]. The exclusion of mitochondria, inclusion of glycolysis enzymes, and increased glucose transport into the cell through GLUT-1 are all necessary for efficient migration in endothelial cells[67]. This complex signal cascade is also mirrored in stalk cells, though the metabolic

pathways are slightly different than in the tip cell. This is also due to the constraints that rapid cell proliferation places on the stalk cells.

1.6.2 Fatty acid oxidation: Importance during sprouting and in injury response

Akin to the energy demands migration places on a tip cell in angiogenesis, proliferation also places a high energy demand on stalk cells that are rapidly moving through the cell cycle[49,67]. Upon VEGF stimulation, proliferator-activated receptor-γ coactivator-1α (PGC-1α; encoded by the gene *PPARGC1A*) is activated, which results in increased mitochondrial biogenesis and an increase in oxidative metabolism[49]. Further, VEGF activation induces a long-chain fatty acid receptor, fatty acid binding protein 4 (FABP4), potentiating fatty acid uptake[49]. Additionally, VEGFR activation of Dll4 results exposure in stalk cells to Notch 1 intracellular domain (NICD) which is known to reduce PFKFB3 activity, and thereby inhibiting glycolysis[49]. Taken together, this results in decreased glycolysis and increased fatty acid oxidation (FAO). This metabolism switch has been demonstrated to be imperative to endothelial cell proliferation because blocking FABP4 alone will attenuate both proliferation and angiogenesis[49]. While energy demands of a dividing cell are high, the ATP generated in FAO does not appear to be the sole reason for its upregulation in stalk cells[67,71].

The initial finding that FAO served a purposed outside ATP generation was discovered by blocking carnitine palmitoyltransferase 1 (CPT1a), the enzyme that is responsible for shuttling fatty acids into the mitochondria and represents the rate-limiting step of FAO, which resulted in stunted sprouts in HUVECs but did not cause an ATP imbalance[71]. Instead, this study found that the fatty acid carbons were the primary carbon source for the tricarboxylic acid cycle (TCA) as labeled carbons from fatty acids appeared as TCA intermediates as well as appearing in amino acids[71]. The

most surprising finding was that knocking down CPT1a resulted in significant decreases of intracellular deoxyribonucleotides, a critical component needed for DNA replication in the S phase of the cell cycle (**Fig. 1.5B**)[71]. While glycolysis can compensate for the loss of FAO source of deoxyribonucleotide precursors, in endothelial cells this is still not sufficient and proliferation is hindered, resulting in attenuated sprout elongation in HUVECs[71]. Taken together, FAO upregulation is key to the phenotypic switch that occurs in endothelial cells that will become the stalk of a new sprout.

FAO control is also important in the context of BPD as it has been shown that FAO is downregulated in the HUVECs of patients who developed BPD[72,73]. In studies that looked at the effect of increasing FAO in pulmonary endothelial cells that are exposed to hyeroxia, it was found that the damage from that high oxygen state was mitigated[72,73]. Interestingly, enhancing FAO in a mouse model of BPD decreased pulmonary endothelial cell apoptosis as well as decreased the presence of simplified alveoli and increased vascularization of the lung[73]. These findings directly connect endothelial cell metabolism to BPD, both indirectly as it relates to vascularizing lung tissue and directly as a means to mitigate the damage life-saving therapies place on the still developing lung of premature infants.

1.7 PPARγ, master transcription factor with a sexual dimorphism

As endothelial cells respond to pro-angiogenic factors and major signaling cascades begin to cause cell phenotype changes, PGC-1α is activated and begins a new cascade in stalk cells[49]. As the name implies, this transcription factor is a coactivator with peroxisome proliferator-activated receptor-γ (PPARγ), a transcription factor that is central to many cellular processes across many cell types, including

endothelial cells[74,75]. PPARγ, like other PPARs, is associated with it's role in lipid and glucose homeostasis, but is also central to angiogenesis[76]. Establishing PPARγ animal models was challenging as a lack of PPARγ expression is embryonic lethal in mice due to failure of trophoblast differentiation and impaired placental vascularization[77]. Established conditional knockout mouse models of PPARγ report significant vascular defects as a result[77]. In line with these other reports, a study performed in mouse and human pulmonary microvascular cells revealed that loss of PPARγ resulted in significant angiogenesis and migration defects, demonstrating the critical role PPARγ plays in endothelial cell fucntion[76]. Additional to the role PPARγ plays in controlling cell proliferation, in conjunction with PGC-1α, PPARγ has also been connected to mitigating inflammation[76].

PPARγ also has implications in various lung diseases, specifically BPD and pulmonary hypertension (PH)[78–80]. It has been shown that PPARγ levels are decreased in infants who develop BPD[73,80]. The upregulation of PPARγ in the pulmonary lung is important in the context of BPD due to the many ways PPARγ can mitigate the complications of ventilation. Specifically, PPARγ directly reduces inflammation through inhibition of nuclear factor kappa B (NF-κB), nicotinamide adenine dinucleotide phosphate (NADPH), proinflammatory adhesion molecules such as intracellular adhesion molecule 1 (ICAM-1) or vascular cell adhesion molecule 1 (VCAM-1), and inhibits chemokine genes[80]. Taken together, the role of PPARγ in mitigating inflammation, maintaining metabolic homeostasis, and increasing angiogenesis all demonstrate the importance this transcription factor plays in BPD.

1.7.1 PPARγ is sex-specific and sex hormone sensitive

The importance of PPARγ in BPD can be well appreciated by its involvement in many processes that are central to BPD, but PPARγ could also play a role in the sexual dimorphism observed in BPD. There is a reported PPARγ sexual dimorphism in adipose and follicular helper T-cells, specifically that female cells tend to have a higher expression compared to males[81–83]. Interestingly, exposure of adipose cells to E2 can increase the expression of PPARγ[83] and similarly, male follicular helper T-cell exposure to E2 significantly increases expression of PPARγ, abolishing the sexual dimorphism that previously existed[81]. The presence of DHT, alternatively, had no influence over expression level of PPARγ in adipose cells[83]. When using physiological doses of E2, PPARγ expression is potentiated and multiple co-regulated genes between estrogen receptors and PPARγhave been identified[84]. This link in the sexual dimorphism and hormone sensitivity of PPARγ make it an ideal target of interest for the study of sex differences as they relate to BPD, especially in the context of the reports of lower PPARγ expression in BPD.

1.7.2 Rosiglitazone: a therapeutic target

As a result of the role of PPARs on cellular metabolism, a class of PPAR ligand drugs known as thiazolidinedione derivatives (TZD) were synthesized and tested in the context of managing diabetes[85]. Rosiglitazone is one TZD compound that has high specificity for PPARγ, which was approved for use in humans under the brand name Avandia, following clinical trials related to diabetes in 1999[86]. This makes the therapeutic application of findings as they relate to PPARγ more applicable given there already exists an FDA approved agonist for PPARγ on the market.

The goal of this dissertation is to characterize sex differences in angiogenesis, identify key regulators of that sex differences, and provide support for new directions and therapeutic targets for pre-clinical research around the sex dimorphism in BPD.

Chapter 2

SEX-RELATED EXTERNAL FACTORS INFLUENCE PULMONARY VASCULAR ANGIOGENESIS IN A SEX-DEPENDENT MANNER

This chapter is adapted from the following manuscript: Hayward-Piatkovskyi, B; Gonyea, CR; Pyle, SC; Lingappan, K; Gleghorn, JP. "Sex-related external factors influence pulmonary vascular angiogenesis in a sex-dependent manner." *Am J Physiol Heart Circ Physiol* 324(1):H26-H32 (2023)

2.1 Introduction

Bronchopulmonary dysplasia (BPD), a chronic lung disease characterized by impaired alveolar development and vascular rarefaction, is a disease with a prominent sexual dimorphism where male sex is considered an independent risk factor[29,36,38]. Alveolar development cannot occur properly when pulmonary angiogenesis is inhibited[59,61,87,88]. While there exists a sexual dimorphism in angiogenesis, investigation of the underlying mechanisms that lie inherent to the lung endothelial cells, or the role sex hormones may play in these differences, have yet to be identified.

Work done on sex differences in the vascular niche during development has shown striking transcriptomic differences between female and male endothelial cells that contribute to differential cellular responses[2,62]. Sex hormones from maternal and fetal origin are crucial in development, directly impact angiogenesis, and are modified by preterm birth[2]. Estradiol, integral to organ development in both sexes but commonly associated with females, has been shown to enhance endothelial cell proliferation and migration[89]. Further, estradiol upregulates VEGF (vascular endothelial growth factor) expression in endothelial cells, subsequently increasing angiogenesis[90]. Dihydrotestosterone, a derivative of testosterone associated with

males, has also been reported to converge on VEGF signaling through the androgen receptor, stimulating endothelial cell proliferation[91,92]. Few studies highlight the intersection of chromosomal and sex hormone influences on cellular sex phenotypes[93].

Accounting for the influence of sex hormones in *in vitro* models is critically important as standard culture practices contain physiologically relevant concentrations of sex hormones in the fetal bovine serum (FBS)[2,4,94–97]. Most standard culture mediums contain phenol red indicator, a weak estrogen receptor agonist[98,99]. As such, standard culture medium exposes cells to sex hormones, and an alternative medium is needed to delineate the role of sex hormones in sex-specific signaling. Using phenol red free medium supplemented with charcoal-stripped FBS serves as a hormone-free medium (HFM) for cell culture[91,95].

Cells also possess distinct secretomes, a profile of secreted factors, that have recently been shown to exhibit sexual dimorphism in male and female endothelial cells[63]. In combination, several studies have established that the chromosomal sex of the cell determines both its secretome and how it responds to soluble external signals[62–65].

Despite these previous findings, a focused study on the underlying sex differences in lung-specific endothelial cell angiogenesis and the exogenous factors that govern these differences *in vitro* have not been previously reported. We hypothesize that sex chromosomes mediate differences in angiogenesis in human pulmonary microvascular endothelial cells (HPMEC). Further, we hypothesize that

sex-specific exogenous factors, such as sex hormones or sex-specific secretome, will have a sex-specific influence on angiogenesis.

2.2 Materials and methods

2.2.1 Cell culture

HPMECs (ScienCell, Carlsbad, CA; female lots: 17799, 17807, 15900; male lots: 11816, 11422, 16021) were cultured on fibronectin-coated plates (2 μg/cm^2) in standard Endothelial Cell Medium (SM, ScienCell) at 37°C supplemented with 5% CO_2. SM was supplemented with 5% FBS, endothelial cell growth supplement (ScienCell), and 1% penicillin/streptomycin (ScienCell). Cultures maintained in HFM were grown in phenol red free Endothelial Cell Medium (ScienCell) supplemented the same as SM but with 5% charcoal-stripped FBS (HyClone, Logan, UT). Individual donors were equally combined to generate male or female pooled HPMECs and grown to near confluence before experimental use (passages 4-6).

2.2.2 Angiogenesis assay

Angiogenesis was determined using a 3D fibrin gel bead assay as previously described[59,61]. Collagen-coated cytodex-3 micro-carrier beads (Sigma-Aldrich, St. Louis, MO) were coated with male or female HPMECs at 20,000 cells per 750 beads, incubated for 4 hours at 37°C with periodic agitation then statically overnight. Beads were resuspended in 2 mg/ml fibrin (Millipore, Burlington, MA) gels supplemented with 0.15 U/ml of aprotinin (Sigma-Aldrich) at 250 beads/ml. Gel cultures were maintained in SM or HFM for four days with daily medium changes. Percentage of beads to produce at least one sprout captured the cell's ability to respond to pro-

angiogenic factors. Number of sprouts per bead captured cell-to-cell coordination. Maximum length of sprouts captured cell proliferation. Lengths were determined in Image J by tracing the sprout from the edge of the bead to the tip of the sprout.

2.2.3 Conditioned medium

Conditioned medium experiments tested the influence of sex-specific secretomes on angiogenesis. SM or HFM media was collected from male or female monolayer cultures (~80% confluent). Conditioned medium was centrifuged (300 g for 10 minutes) to pellet cellular debris, collected, and stored at 4°C for a maximum of 2 days. Cells were seeded into an angiogenesis assay and were maintained in the conditioned medium with daily media changes for four days. These experiments were performed in parallel with male and female HPMECs angiogenesis assays in SM and HFM to serve as controls.

2.2.4 Immunofluorescent imaging

HPMECs were fixed, stained, and imaged as previously described[100,101] in 4% paraformaldehyde (Thermo Scientific, Waltham, MA) with 0.1% Triton-x-100 (Thermo Scientific) for 2 hours at 4°C. Cells were counterstained with phalloidin-554 (Cell Signaling, Danvers, MA) and Hoechst (Invitrogen, Waltham, MA) overnight at 4°C. Images were taken using an epifluorescent microscope (Zeiss, Oberkochen, Germany).

2.2.5 Statistical analysis of control and conditioned media experiments

Significance was determined using Student's t-test. The percentage of beads that produced a minimum of one sprout was determined by averaging across wells, while sprout length and number of sprouts were analyzed by averaging across beads.

2.3 Results

2.3.1 Role of sex hormones in angiogenesis:

Angiogenesis was quantified in male and female HPMECs using a bead sprouting assay to establish baseline sex differences (HFM) and identify differences controlled by sex hormones (SM). Sprouts are here defined as multiple cells in a continuous line from bead to sprout tip (**Fig. 2.1a)**. These data show that at baseline, there is no sex difference between male (64%) and female (82%) HPMECs in their ability to produce sprouts (**Fig. 2.1b**). However, when sex hormones are present, male HPMECs (55%) were less likely to produce sprouts. The ability to produce sprouts was unchanged in female HPMECs. Female HPMECs had more sprouts (3.1) per bead compared to male (2.5) (**Fig. 2.1c**). When male HPMECs were exposed to sex hormones, the average number of sprouts decreased to 2.0 sprouts. In comparison, female HPMECs did not have a significant decrease (2.6 sprouts). Sprout elongation requires that stalk cells gain a proliferative phenotype, a process coordinated by the tip cell. Male HPMECs produced longer sprouts (99.7 μm) compared to females (64.9 μm), a difference that was abolished when sex hormones were present (**Fig. 2.1d**). Male HPMEC sprout lengths decreased to 70.1 μm. In contrast, female HPMECs sprout length was not significantly different (88.5 μm) in the presence of sex hormones.

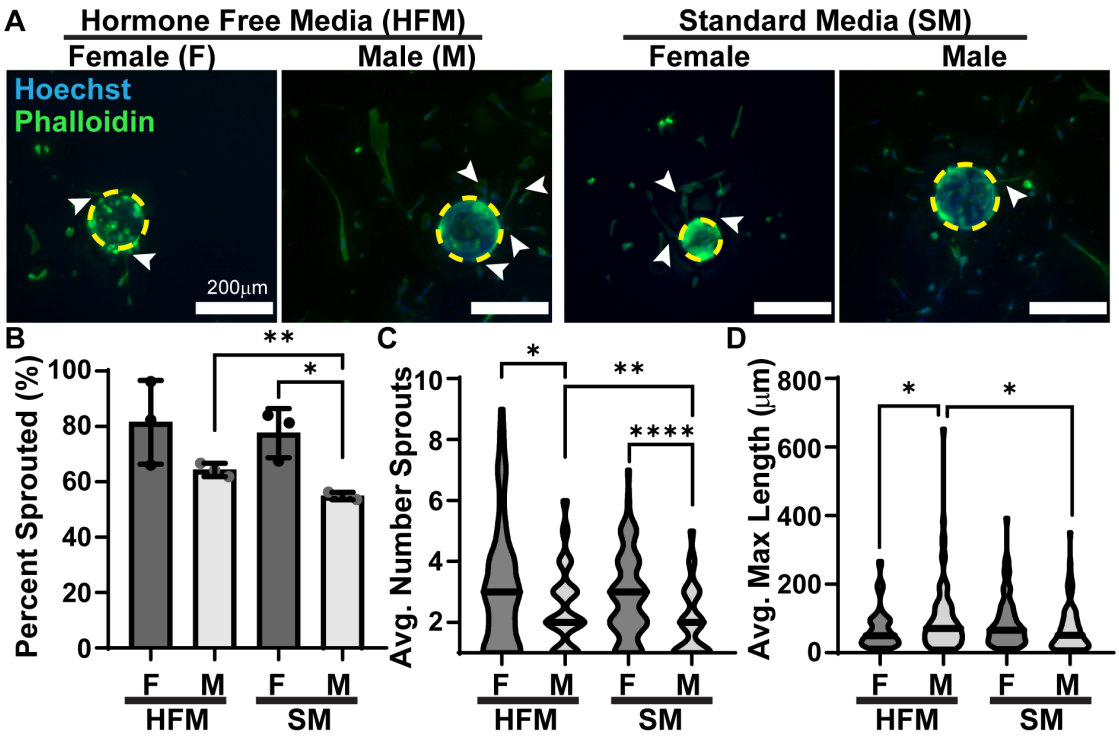


Figure 2.1: Male HPMECs produce fewer, but longer, sprouts compared to female HPMECs. A) Male and female coated beads cultured in HFM and SM. Dashed yellow circles outline beads, arrow denotes sprouts. B) Percentage of beads that produced at least one sprout, mean ± SD, n = 3 wells. C) Average number of sprouts per bead, n = 72-86 beads. D) Average maximal length of sprouts per bead, n = 72-86 beads. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

2.3.2 Influence of sex-specific cell secretions on angiogenesis:

To test the influence of sex-specific secretomes, angiogenesis was assessed in the presence of conditioned media. HFM was conditioned by either male or female HPMECs grown in a monolayer and subsequently used to stimulate angiogenesis in male and female HPMECs (**Fig. 2.2a**). HPMECs cultured in a fibrin clot with un-conditioned HFM served as a control. Neither male- nor female-conditioned media had a significant influence on the ability of male or female HPMECs to produce

sprouts, with 60.0-88.2% beads with sprouts across conditions (**Fig. 2.2b**). Female-conditioned media had no significant effects on the average number of sprouts in male (2.2 sprouts) or female (3.1 sprouts) HPMECs. While male-conditioned media did not significantly affect female HPMEC average number of sprouts (3.6 sprouts), it significantly decreased the average number of male sprouts (2.1) (**Fig. 2.2c**).

The average maximal length of sprouts in male HPMECs remained constant in all three conditions (99.7-105.4 μm). In contrast, female HPMECs had dramatically longer sprouts compared to the control (64.9 μm) compared to female (151.1 μm) or male (178.3 μm) conditioned media (**Fig. 2.2d**). This increase in length in the female HPMECs is ~2.5 times longer than controls.

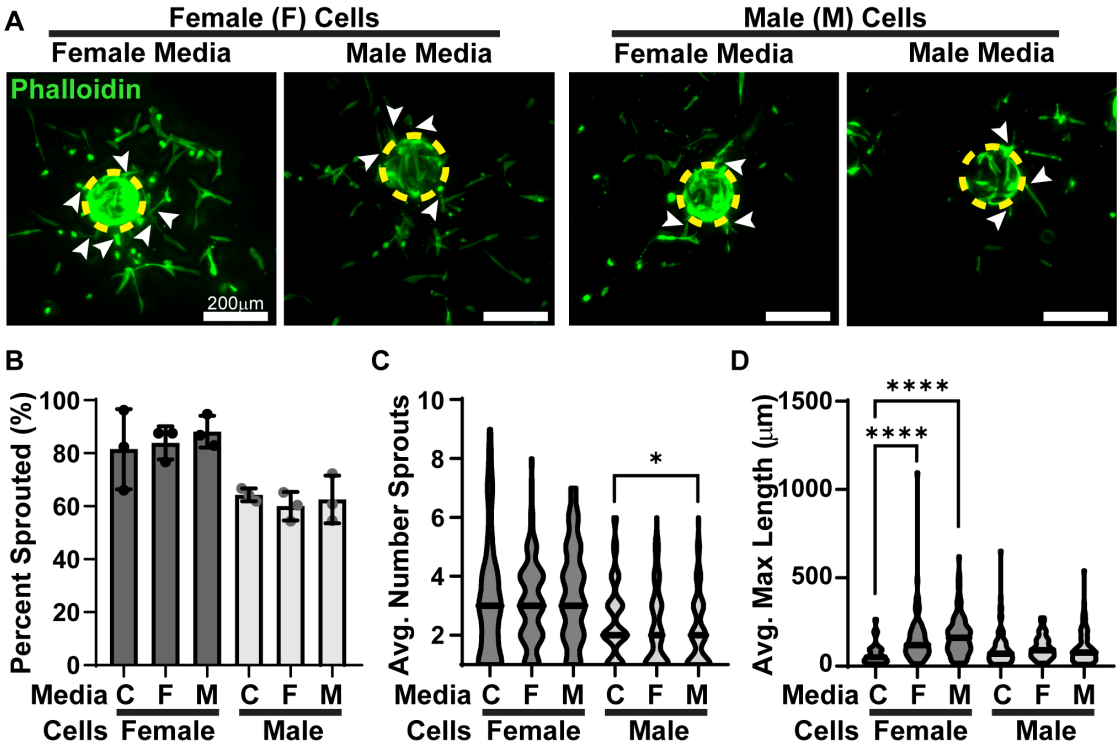


Figure 2.2: HPMEC secretome influences angiogenesis in a sex-dependent manner. A) Male and female coated beads cultured in HFM conditioned by male or female HPMECs. Dashed yellow circles outline beads, arrows denote sprouts. B) Percentage of beads that produced at least one sprout, means ± SD, n = 3 wells. C) Average number of sprouts per bead, n = 72-104 beads. D) Average maximal length of sprouts, n = 72-104 beads. C denotes control, * $p < 0.05$ and **** $p < 0.0001$ compared to the sex-matched control.

2.3.3 Influence of sex hormones on cell secretions on angiogenesis:

The presence of sex hormones can influence the secretome of cells. To test the influence of sex hormones on sex-specific secretions, we conditioned sex hormone containing SM with male or female HPMECs grown in a monolayer and subsequently used it to stimulate angiogenesis in male and female HPMECs (**Fig. 2.3a**). HPMECs cultured in un-conditioned SM served as a control. Neither male- nor female-conditioned media in the presence of sex hormones had a significant influence on the

ability of female (60-84%) or male (54-81%) HPMECs to produce a sprout (**Fig. 2.3b**). These conditioned medias also did not influence female (2.2-3.3) or male (1.8-2.5) average number of sprouts (**Fig. 2.3c**).

The presence of sex hormones with male- and female-conditioned media abolished the HFM secretome response of female HPMECs having longer sprouts. Female HPMECs in conditioned SM (89.6-110.8 μm) had lengths comparable to controls (**Fig. 2.3d**). Female HPMECs secrete factors that male HPMECs responded to in the presence of sex hormones, producing longer sprouts (107 μm) than controls (69.8 μm). In the presence of sex hormones, male HPMECs secretions had no significant influence over maximal sprout length in male HPMECs (92.8 μm).

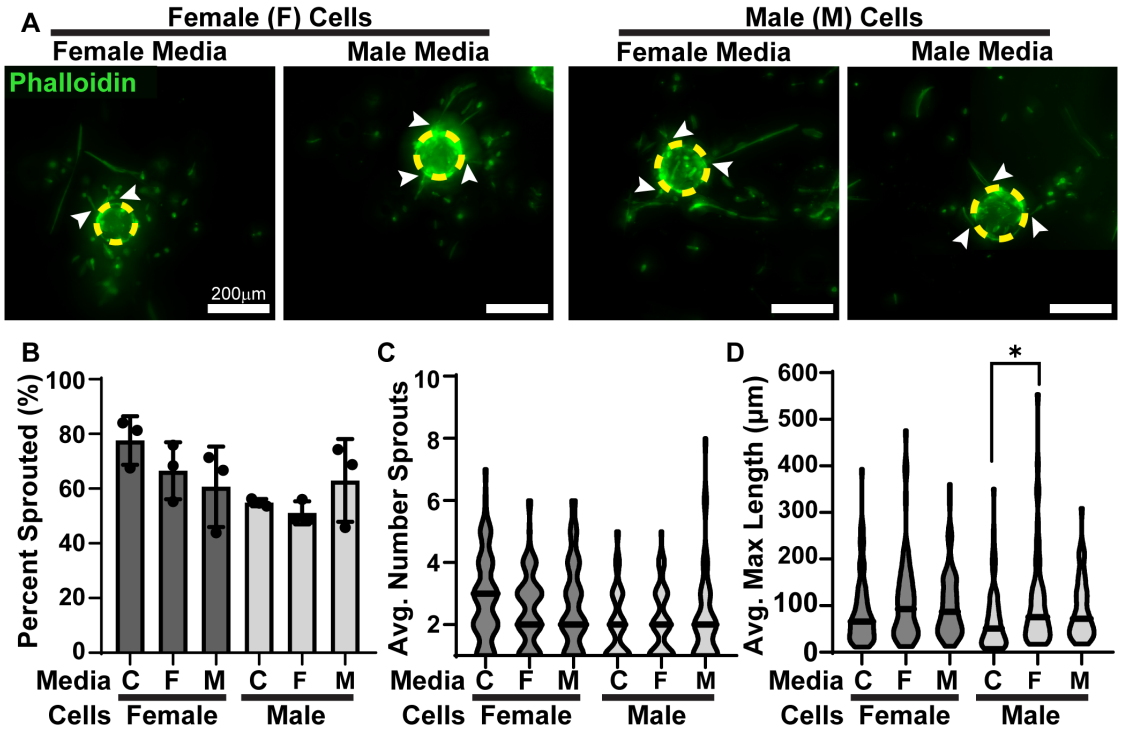


Figure 2.3: HPMEC response to the secretome is sex hormone dependent. A) Male and female coated beads cultured in SM (hormone containing) conditioned by male or female HPMECs. Dashed yellow circles outline beads, arrows denote sprouts. B) Percentage of beads that produced at least one sprout, means ± SD, n = 3 wells. C) Average number of sprouts per bead, n = 59-86 beads. D) Average maximal length of sprouts, n = 59-86 beads. C denotes control, * $p < 0.05$ compared to the sex-matched control.

2.4 Discussion

Sex differences are prominent in pulmonary angiogenesis, a critical process for proper lung development[29]. In this study, we characterized sex differences of human pulmonary microvascular endothelial cells. Our objective was to identify sex differences in 3D angiogenesis and determine the role of exogenous factors in this process.

In the absence of sex hormones, male and female HPMECs were equally likely to produce at least one sprout in response to pro-angiogenic factors. This demonstrates that both male and female lung endothelial cells respond to proangiogenic factors regardless of chromosomal identity. Both estrogen and testosterone play a role in sex-specific signaling and are expected to have some role in the observed phenotype of the HPMEC response to sprouting. When sex hormones were present in the medium, angiogenic sprouting was inhibited only in male HPMECs, with a lower percentage of beads containing sprouts. Therefore, we hypothesized that the inherent ability to respond to a pro-angiogenic factor by male and female HPMECs is the same; however, when exogenous sex hormones are present, tip cell formation is inhibited in male cells but not in female cells. Identifying which sex hormone is responsible for this inhibition in male pulmonary endothelial cells, as well as the underlying mechanism of that inhibition, should be pursued.

Once an endothelial cell responds to a pro-angiogenic factor, a complex signaling cascade inhibits neighboring cells from responding[50,102–104]. This is canonically achieved through Delta like ligand 4 and Notch signaling[50,102]. In the absence of sex hormones, female HPMECs produced more sprouts per bead than male HPMECs **(Fig. 2.1c)**. This suggests that the area of inhibition over neighboring cells becoming tip cells is increased in male HPMECs. The presence of sex hormones did not influence the number of sprouts per bead in female HPMECs but did have a further inhibitory effect on male HPMECs, with fewer angiogenic sprouts per bead observed.

Angiogenesis requires not only the formation of a tip cell but also the coordination of a proliferative stalk[47,104]. Contrary to the tip cell behaviors, in the absence of sex hormones, male HPMECs had longer sprouts compared to female HPMECs **(Fig 2.1c)**. Combined, this represents a male and female phenotype where males produce fewer but longer sprouts compared to female HPMECs (**Fig. 2.4a**). The functional advantage of one phenotype over another is unclear. However, it is likely that the ability to change between different phenotypes in response to stressors such as hyperoxia and inflammation, which are superimposed onto the requirements of the developing and growing lung, may underpin functional outcomes. Consistent with the other parameters of angiogenesis, the sexual dimorphism of maximum sprout length was abolished when male and female HPMECs were exposed to sex hormone-containing medium. Female HPMECs were unaffected by the presence of sex hormones, while male HPMECs exhibited decreased sprout length, further demonstrating that the presence of sex hormones had an overall inhibitory role in male angiogenesis (**Fig. 2.4b**).

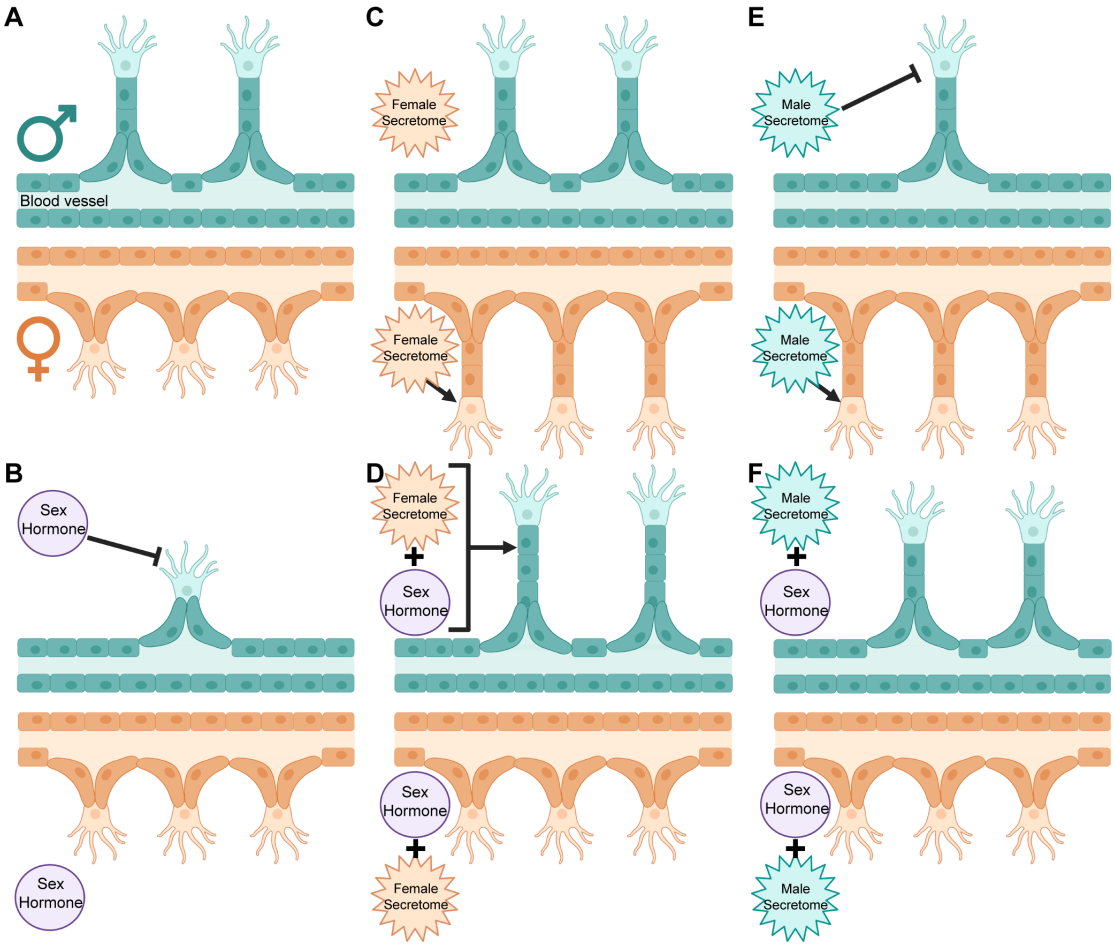


Figure 2.4: Sex-specific angiogenesis phenotype and the influence of exogenous factors. A) Baseline phenotype: male HPMECs produce longer but fewer sprouts than females. B) Male HPMECs produce shorter and fewer sprouts in the presence of sex hormones. C) Female HPMECs produce longer sprouts in the presence of a female secretome. D) Sex hormones and a female secretome increase sprout length in male HPMECs but eliminate changes from the female secretome alone in female HPMECs. E) Male HPMECs produce fewer sprouts while female HPMECs produce longer sprouts in the presence of a male secretome. F) Sex hormones in combination with the male secretome nullify the effects of either source independently, with male and female angiogenesis resembling the baseline phenotype. Created in Biorender.com

To address the secretome, we performed a bead sprouting angiogenesis assay with media conditioned by male or female HPMECs in the absence of exogenous sex hormones. The ability of male and female HPMECs to respond to pro-angiogenic factors and produce at least one sprout remained unchanged when male and female HPMECs were exposed to male or female conditioned media **(Fig 2.2b)**. This was consistent even when sex hormones were present in the conditioned medium (**Fig. 2.3b**). Interestingly, conditioned media from male HPMECs contain factors that decreased the number of sprouts from male but not female HPMECs **(Fig 2.2c)**. The presence of sex hormones abolished this further decrease in sprout number of male HPMECs in response to male conditioned media (**Fig. 2.3c**). In contrast, both male and female HPMECs secreted factors that stimulated the elongation of sprouts in female HPMECs but had no influence on male HPMECs **(Fig 2.2c)**. Again, the presence of sex hormones in the conditioned media abolished this effect on female HPMECs with the average maximal sprout length similar to control cells (**Fig. 2.3c**). In contrast, male HPMECs had increased maximal sprout lengths when exposed to the secretome of female HPMECs in the presence of sex hormones. Together, this demonstrates that male and female HPMECs not only have distinct secretomes but that these secretomes produce a response that is dependent upon both the presence of sex hormones and the sex of the receiving cells (**Fig. 2.4c-f**).

Chapter 3

ENDOTHELIAL CELL SEX DETERMINES MAGNITUDE OF RESPONSE TO SEX HORMONES IN ANGIOGENESIS

This chapter is adapted from the following manuscript in preparation: Hayward-Piatkovskyi, B; Clark, C; Gleghorn, JP. "Endothelial cell sex determines magnitude of response to sex hormones in angiogenesis." (2023) *in preparation*

3.1 Introduction

Bronchopulmonary dysplasia (BPD) is a chronic lung diseases that is characterized by impaired alveolarization and vascular rarefication and primarily affects prematurely born infants[2,35,36]. BPD is associated with a significant sexual dimorphism where males are far more likely to develop BPD compared to their female counterparts, even when accounting for gestational age and birth weight[2,29,36,38]. It is well established that male and female lungs are histologically distinct at 32 weeks of gestation or younger. In particular, female lungs have been shown to produce surfactant earlier than male lungs and the lung structures in females mature earlier, which results in a lower histological index of maturity in male infant lungs compared to female infant lungs[30,38]. This could be partly due to the overall inhibitory role androgens play in early lung development[29,38]. Sex differences have been identified in pulmonary vascular angiogenesis[59,61,105], a process that is necessary for proper alveolarization[20]. We recently characterized a sex phenotype in pulmonary vascular angiogenesis where males produce fewer but longer sprouts[105]. We further identified a role for sex hormones in this sexual dimorphism[105]. We are continuing to build from

our previous work to tease out specific contributions of hormones to this interesting discovery.

Angiogenesis is a complex, multi-cellular migration process that is essential for proper lung development through the alveolarization stages[20]. Upon stimulation from a pro-angiogenic factor, such as vascular endothelial growth factor (VEGF), a responding endothelial cell will undergo a phenotype switch and gain a migratory phenotype to become what is known as a tip cell[47,48]. Neighboring endothelial cells will respond by adopting a proliferative phenotype and become what are known as stalk cells[47,48]. Sprout elongation can occur through this active proliferation in the stalk cells. The ability for the tissue to undergo angiogenesis heavily relies on the ability of endothelial cells to upregulate migratory behavior as well as increase the rate of proliferation. Our recent findings identified a sex phenotype in angiogenesis where males produced fewer but longer sprouts compared to female human pulmonary microvascular endothelial cells (HPMECs), which indicates that there may be sex differences in both the proliferation and migration phenotypes of pulmonary endothelial cells[105]. Other work on human umbilical vein endothelial cells (HUVECs) have demonstrated sexual dimorphisms in angiogenesis using a tubal formation assay[62,65]. A further look into proliferation and migration found that female HUVECs were both more proliferative and migratory than male HUVECs[65]. In the case of HUVECs, these features of angiogenesis were also sex hormone sensitive, specifically to estrogen[65]. These findings support a more detailed look at angiogenesis in pulmonary endothelial cells. Understanding the role hormones play in endothelial cell

phenotypes as well as pulmonary angiogenesis are critical as sex hormones are an important part of human development, and the hormone state in premature infants is unique.

Sex hormones are known to influence normal human development throughout gestation[2,3]. There are key windows of time in normal human gestation where sex hormones are up- and down-regulated. Testicular testosterone in male fetuses is upregulated between 10 and 17 weeks gestation, with a substantial decrease after 24 weeks of gestation, where levels remain low through to birth[2,3]. In contrast, fetal estradiol, which is present in male and female fetuses, steadily increases at 20 weeks gestation through to birth, where it will peak[2,3]. This estrogen spike is not experienced in infants who are born prematurely, with a drop in estrogen in the order of 100 fold within the first day post preterm delivery[2,4,40,41].

These fluctuations in hormone levels throughout gestation, or the abrupt disruption in levels, are especially pertinent given the role sex hormones have been demonstrated to play in angiogenesis[90,106,107]. In a rabbit model, treatment with estradiol substantially increased blood vessel density[90]. Further, estradiol has been found to increase both HUVEC proliferation and migration[108]. In contrast, androgens, such as testosterone and its derivative dihydrotestosterone (DHT), are associated with an overall inhibitory effect on lung development[2]. With respect to angiogenesis, the literature is unclear on the overall role testosterone may play as there are reports of both beneficial and detrimental effects[109]. One study[110] found that testosterone decreased both HUVEC proliferation and migration. Our own studies indicated sex

hormones could impact the male angiogenesis phenotype where they produced even fewer and shorter sprouts, while female HPMECs were insensitive to sex hormones[105].

These previous findings demonstrate that there are striking sexual dimorphisms in angiogenesis, though the specific influence of exogenous factors still remains unclear as it relates to the pulmonary vasculature. We hypothesize that there is a sexual dimorphism in pulmonary endothelial behaviors and that sex hormones play a role in sex differences in angiogenesis. Our findings in this study support this hypothesis as female HPMECs have a higher proliferation rate, and both male and female HPMECs respond to E2 and DHT in angiogenesis. Surprisingly, the strongest angiogenic response is the positive influence of DHT on both male and female HPMECs, which represents a novel avenue of research as it relates to BPD.

3.2 Materials and methods

3.2.1 Cell culture

Human pulmonary microvascular endothelial cells (HPMEC, ScienCell) were cultured on fibronectin-coated plates (2 μg/cm^2) in hormone free endothelial cell media (phenol-red free ECM, ScienCell) supplemented with 5% charcoal-stripped fetal bovine serum (HyClone), endothelial cell supplement (ScienCell), and 1% penicillin-streptomycin (ScienCell). Cultures were incubated in a humidified chamber 37° C supplemented with 5% CO_2. Three male (lots 11816, 11422, 16021) and three female (lots 17799, 17807, 15900) donors were used for all assays and were grown to near confluence (90-95%) before experimental use (passages 4-6).

3.2.2 Treatments

One molar stocks of E2 (MP Biomedicals) and DHT (Cayman Chemical) were prepared in dimethylsulfoxide (DMSO, Sigma-Aldrich) and stored at -20° C. Working solutions of 1000x (1-10 μM) were prepared in DMSO and similarly stored at -20° C between uses. Controls were treated with DMSO (final well concentration of 0.1%) to account for solvent effects.

3.2.3 Direct cell count proliferation assay

Direct cell counts over 4 days was used to determine proliferation rates of male and female HPMECs cultured in hormone free medium. The total number of cells were counted using the NovoCyte flow cytometer (Agilent). Cells were seeded onto fibronectin coated (2 $\mu g/cm^2$) 24-well plates in triplicate at a density of 7500 cells per cm^2 and cultured in hormone free medium with media changes every 24 hours over the course of 4 days. On the day of counting, cells were washed 1x in phosphate buffered saline (PBS, Corning) and subsequently dry trypsinized with 0.05% trypsin-EDTA (Corning). Trypsin was added to wells, allowed to incubate for 10 seconds, and then removed. After about 30 seconds, cells were quenched with complete hormone free medium and transferred to tubes where they were immediately counted on the flow cytometer.

3.2.4 CyQUANT proliferation assay

The influence of sex hormones on proliferation was assessed using CyQUANT (Invitrogen) to quantify cell number. Cells were seeded onto fibronectin coated (2 $\mu g/cm^2$) 96-well plates in triplicate at a density of 10,000 cells per cm^2 and cultured in hormone free medium supplemented with either DMSO (0.1% final concentration), E2 (1-10 nM final concentration), or DHT (1-10 nM final concentration). At the time of

seeding, a standard curve of cells was plated and immediately placed at -80° C. Medium was changed every 24 hours on the cells in the proliferation assay over the course of 4 days. On the day of collection, plates were inverted to remove media from the wells before the plates were placed at -80° C. All samples were given at least 24 hours at -80° C before the CyQUANT quantification, which was performed following the manufacturer's instructions. Briefly, on the day of quantification plates were thawed and then incubated with 1x lysis buffer containing the CyQUANT dye at room temperature for 15 minutes and then fluorescent intensity was determined at 480nm/520nm excitation/emission on a H1 Synergy microplate reader (Gen5 3.11). Cell number was determined using the linear equation of the standard curve with the y-intercept being set to the fluorescent intensity of the blank.

3.2.5 Migration assay

Near confluent monolayers (90-95%) of HPMECs were washed 1x in starvation medium (basal phenol-red free ECM medium (ScienCell) supplemented with endothelial cell supplement (ScienCell), 1% penicillin-streptomycin (ScienCell), and 0.2% bovine serum albumen (Fisher BioReagents) and then starved for 24 hours prior to seeding into the QCM Chemotaxis Cell Migration Assay, 96-well (8 μm) fluorimetric (Sigma-Aldrich). Starved HPMECs were seeded into transwells at 50,000 cells per well in starvation media that was treated with DMSO (0/1% final concentration), E2 (10 nM final concentration), or DHT (10 nM final concentration). At the time of seeding, a standard curve of cells were plated and immediately placed at -80° C. The outer well of transwells contained complete, serum containing hormone free medium supplemented with DMSO (0.1% final concentration), E2 (10 nM final concentration), or DHT (10 nM final concentration). Cells were incubated in these

transwells for 16 hours at 37° C. Following the manufacturer instructions, at the end of the 16 hours, the transwell inserts were inverted to remove non-migrated cells and the inserts were incubated in the kit provided detachment buffer for 30 minutes at 37° C with periodic gentle agitation. Samples were combined with 4x lysis buffer (1x final concentration) containing CyQUANT dye and incubated at room temperature for 15 minutes. Standard curve samples were thawed and similarly incubated in detachment and lysis buffer. Fluorescent intensity was determined at xx/xx excitation/emission using the H1 Synergy microplate reader (Gen5 3.11) and cell number was determined using the equation generated from a linear fit to the standard curve with the y-intercept being set to the fluorescent intensity of the blank.

3.2.6 Angiogenesis assay

A three-dimensional (3-D) fibrin gel bead assay was used to assess angiogenesis as previously described[58,59,61,105]. Briefly, near confluent monolayers of male or female HPMECs were incubated at 40,000 cells per 750 collagen-coated cytodex-3 microcarrier beads (Sigma Aldrich) for 4 hours at 37° C with periodic agitation before static overnight incubation to generate cell coated beads. A 250 beads/mL suspension in 2 mg/mL fibrin (Millipore) and 0.15 U/mL of aprotnin (Sigma-Aldrich) was gelled with 0.625 U/mL of thrombin (Cayman Chemical) for 20 minutes. These gels were maintained in hormone free medium supplemented with DMSO, E2 (1-10 nM), or DHT (1-10 nM) over 4 days with media changes every 24 hours.

3.2.7 Immunofluorescent staining and imaging

Fibrin gels containing HPMEC coated beads were fixed, stained, and imaged as previously described[59,61,105] in 4% paraformaldehyde (Thermo Scientific) with 0.1% Triton-X-100 (Thermo Scientific) for 2 hours at 4° C. Phalloidin-554 (Cell Signaling) and Hoechst (Invitrogen) were used as counterstains with an overnight incubation at 4°C. Images were taken on an epifluorescent microscope (Zeiss).

3.2.8 Statistical analysis

Data are expressed as the means ± standard deviation (SD). A comparison of nonlinear fit test was performed on proliferation assay data and significance was determined at a $p < 0.05$. When null hypothesis was not rejected in this test, one curve was used to represent all data. For migration data, a one-way ANOVA was performed and significance was determined at $p < 0.05$. Normality tests were performed on sprout percentage, sprout number, and sprout length data. Sprout percentage data passed normality tests but had variable standard deviations and as such, were analyzed using Brown-Forsythe and Welch ANOVA where significance was determined at $p < 0.05$. Finally, both sprout number and sprout length did not pass the normality test and as such, were analyzed using the Kruskal-Wallis test where significance was determined at $p < 0.05$. All statistical analysis was preformed using GraphPad Prism software.

3.3 Results

3.3.1 Angiogenesis: A combination of proliferation and migration

Proliferation was assessed using direct cell counts on single donors of male and female HPMECs over 4 days of growth. HPMECs were cultured in hormone free

medium, with daily media changes throughout the experiment. Female HPMECs had a doubling time of just over 21 hours while male HPMECs had a doubling time of just over 25 hours (**Fig. 3.1A**). This demonstrates a sex difference in proliferation rate where female HPMECs proliferate faster than male HPMECs, specifically having a proliferation rate that is approximately 3.7 hours faster than male HPMECs. Migration was assessed using a transwell system where 5% FBS served as the chemotactic agent to stimulate HPMEC migration. While male and female HPMECs responded to the stimulated conditions, there were no observed sex differences in the capacity for migration (**Fig. 3.1B**). There was a significantly higher number (3460 cells) of male HPMECs that migrated compared to the unstimulated male HPMEC control (1820 cells). Similarly, there was a significantly higher number (4487) of female HPMECs that migrated compared to the unstimulated female HPMEC control (2227).

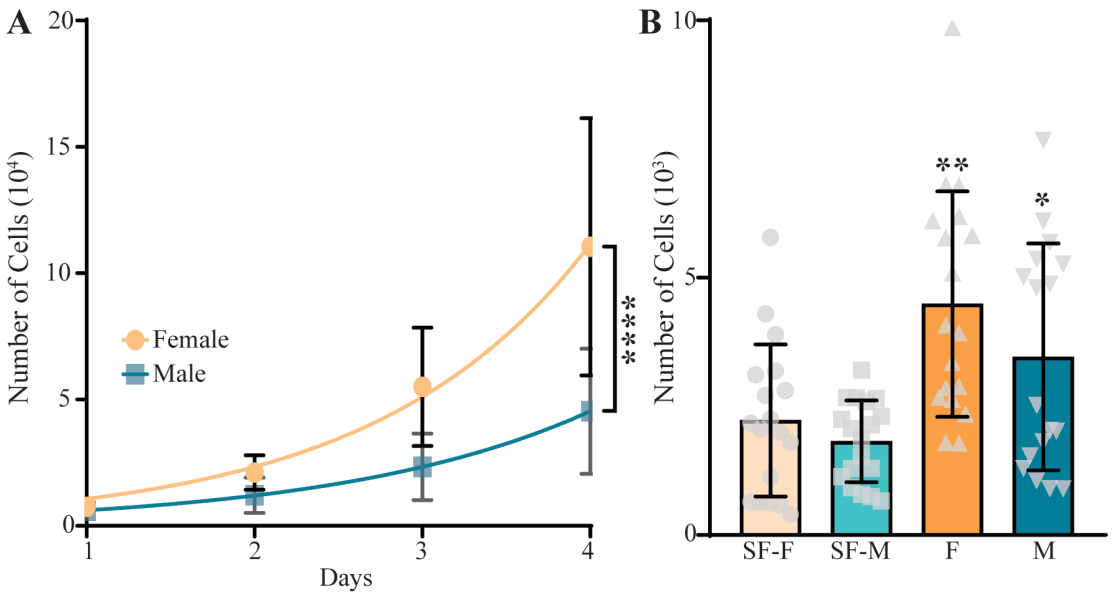


Figure 3.1: Female HPMECs proliferate faster than male HPMECs but both male and female HPMECs are equally migratory, A) Male and female HPMEC proliferation over 4 days when cultured in hormone free medium. n = 9 per sex, statistical analysis: comparison of nonlinear fit, Female R^2 = 0.6855, Male R^2 = 0.5436; B) Male and female HPMEC migration in 16 hours when cultured in hormone free medium. n = 17-18 per sex, statistical analysis: One-way ANOVA. SF-F: serum free female, SF-M: serum free male, F: female, M: male. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$

3.3.2 Female HPMEC proliferation and migration are sex hormone insensitive

Proliferation was assessed over 4 days in a 96-well plate using the CyQUANT DNA dye proliferation kit. Doubling times across E2 conditions ranged from 1.939-2.468 days with no significant differences in the curves and so the null hypothesis was rejected and a single curve was used to represent the proliferation data (**Fig. 3.2A)**. This demonstrates that female HPMEC proliferation is insensitive to E2. Doubling times across DHT conditions ranged from 1.895-2.445 days with no significant differences in the curves, so the null hypothesis was also rejected and a single curve

was used to represent the proliferation data (**Fig. 3.2B**). Finally, female migration was assessed in the presence of E2 (10 nM) or DHT (10 nM). Control female HPMECs had an average of 4487 cells migrate while female HPMECs stimulated with E2 had 4468 and DHT had 4314 cells migrate (**Fig. 3.2C**). This data demonstrates no significant differences in the number of migrated cells in the migration assay.

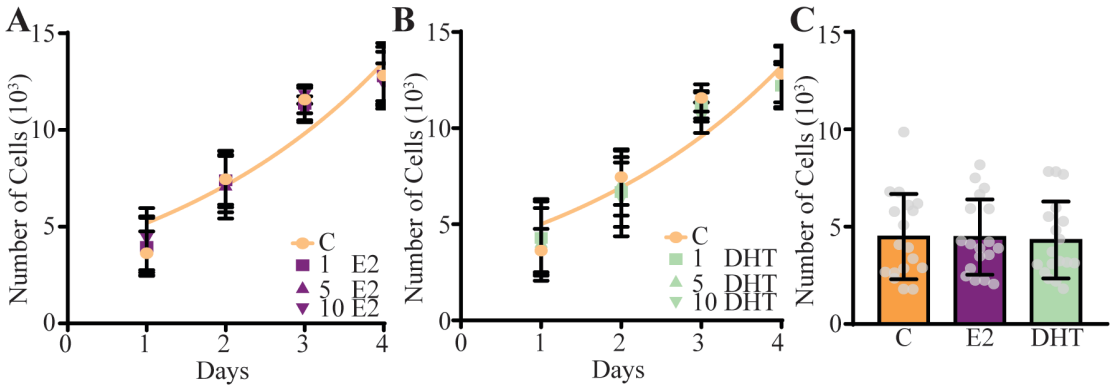


Figure 3.2: E2 and DHT do not influence female HPMEC proliferation or migration
A) Female HPMEC proliferation over 4 days when cultured in hormone free medium and exposed to increasing doses of E2 (1-10 nM). n = 5, statistical analysis: comparison of nonlinear fit, one curve for all datasets $R^2 = 0.804$; B) Female HPMEC proliferation over 4 days when cultured in hormone free medium and exposed to increasing doses of DHT (1-10 nM). n = 5, statistical analysis: comparison of nonlinear fit, one curve for all datasets $R^2 = 0.7873$; C) Female HPMEC migration in 16 hours when cultured in hormone free medium and exposed to E2 (10 nM) or DHT (10 nM). C: DMSO control. n = 18

3.3.3 Male HPMEC proliferation and migration are sex hormone insensitive

Similar to the female HPMECs, single donor male HPMEC proliferation was assassed over 4 days using the DNA dye CyQUANT proliferation assay. Doubling times across E2 conditions for male HPMECs ranged between 2.843-3.548 days with no significant differences between the curves, so one curve was used to represent the

proliferation data (**Fig. 3.3A**). Similarly, the doubling times across DHT conditions for male HPMECs ranged between 2.908-3.778 days, again with no significant differences between the curves and so one curve was used to represent the proliferation data (**Fig. 3.3B**). Finally, migration was assessed for male HPMECs in the presence of E2 (10 nM) or DHT (10 nM). Control male HPMECs had an average of 3460 migrated cells while E2 stimulated male HPMECs had an average of 3224 cells and DHT stimulated had 2152 cells migrated (**Fig. 3.3C**). There were no significant differences between these means.

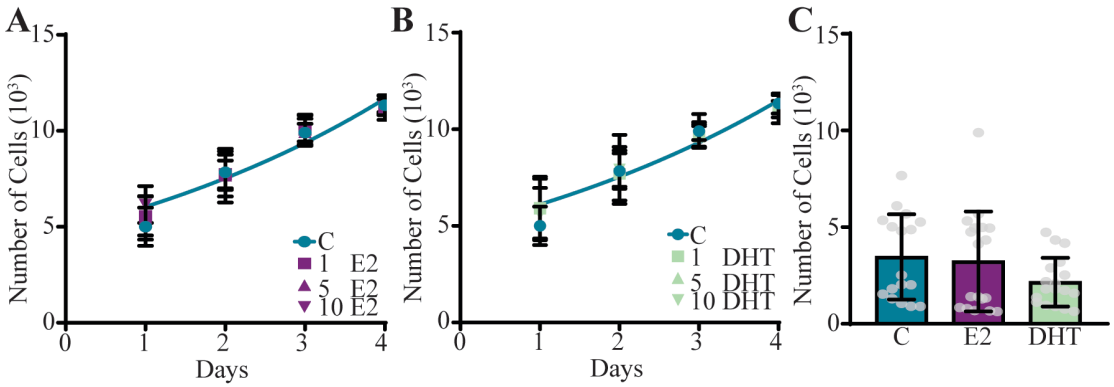


Figure 3.3: E2 and DHT do not influence male HPMEC proliferation or migration A) Male HPMEC proliferation over 4 days when cultured in hormone free medium and exposed to increasing doses of E2 (1-10 nM). n = 4, statistical analysis: comparison of nonlinear fit, one curve for all datasets $R^2 = 0.8515$; B) Male HPMEC proliferation over 4 days when cultured in hormone free medium and exposed to increasing doses of DHT (1-10 nM). n = 4, statistical analysis: comparison of nonlinear fit, one curve for all datasets $R^2 = 0.8038$; C) Male HPMEC migration in 16 hours when cultured in hormone free medium and exposed to E2 (10 nM) or DHT (10 nM). C: DMSO control. n = 17

3.3.4 Female HPMECs respond to E2 stimulation in angiogenesis

Angiogenesis was assessed using a bead sprouting assay in hormone free medium that was supplemented with DMSO (0.1%) or E2 (1-10 nM) to assess the role E2 plays on female HPMEC angiogenesis. There is no influence from E2 on the likelihood of female HPMECs to produce at least one sprout, with all conditions having between 78.4-88.42% beads with at least one sprout (**Fig. 3.4A**). Interestingly, exposure to E2 does influence female HPMEC sprout length. The average number of sprouts in the solvent control DMSO (0.1%) condition was 2.2 sprouts per bead (**Fig. 3.4B**). This number increases to 2.863 sprouts per bead in as little as 1 nM of E2. Average sprout number was also significantly increased in 5 and 10 nM of E2, being 2.539 and 2.636, respectively. In contrast to the significant influence on sprout number, there were no significant differences in the maximum sprout length in response to E2 stimulation (**Fig. 3.4C**). The average max sprout length values ranged from 130.2-145.2 μm across all conditions.

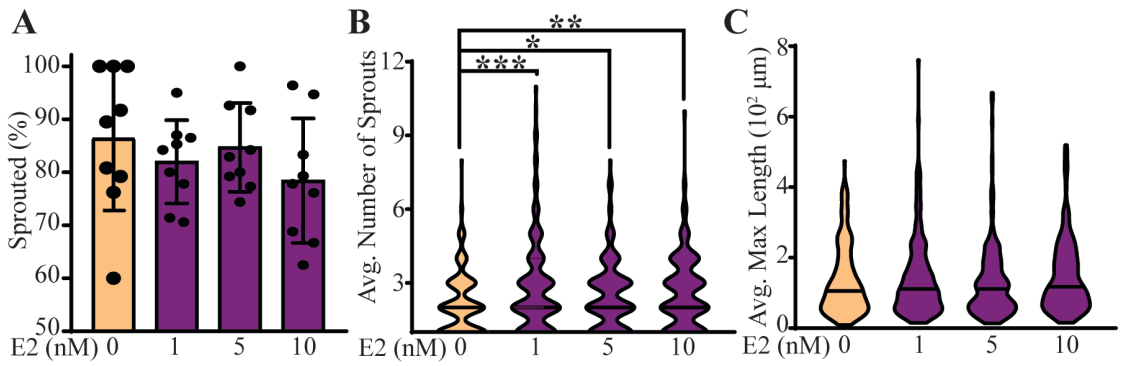


Figure 3.4: E2 increases number of sprouts in female HPMECs, A) Percentage of female HPMEC coated beads that produced at least one sprout when cultured in hormone free medium and stimulated with either DMSO (0.1%) or E2 (1-10 nM), means ± SD, n = 9 wells; B) average number of sprouts per bead in female HPMECs, n = 184-219 beads; C) average maximal length of sprouts in female HPMECs, n = 184-219 beads. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

3.3.5 E2 decreases sprout number in male HPMECs

The influence of E2 was also assessed in male HPMECs using the bead sprouting assay for angiogenesis. Male HPMECs were also cultured in hormone free medium supplemented with either DMSO (0.1%) or E2 (1-10 nM) over the course of 4 days in the bead sprouting assay. Similar to the female response, male HPMECs did not have a significant response to E2 as it relates to the likelihood of producing at least one sprout (**Fig. 3.5A**). The average sprout percentage ranged between 82.11-85.52% of beads producing at least one sprout. Of the beads that produced at least one sprout, male HPMECs in the control condition that were treated with DMSO (0.1%) had an average of 3.136 sprouts per bead (**Fig. 3.5B**). There were no significant differences in average number of sprouts exposed to 1 nM (2.968) and 10 nM (2.908) of E2, while there was a significant decrease in the number of sprouts exposed to 5 nM of E2 (2.379). Similar to the female HPMEC response, there were no significant changes to

the max sprout length in male HPMEC exposed to E2 (1-10 nM) (**Fig. 3.5C**). Across all conditions, the average sprout lengths range from 120.2-159.1 μm.

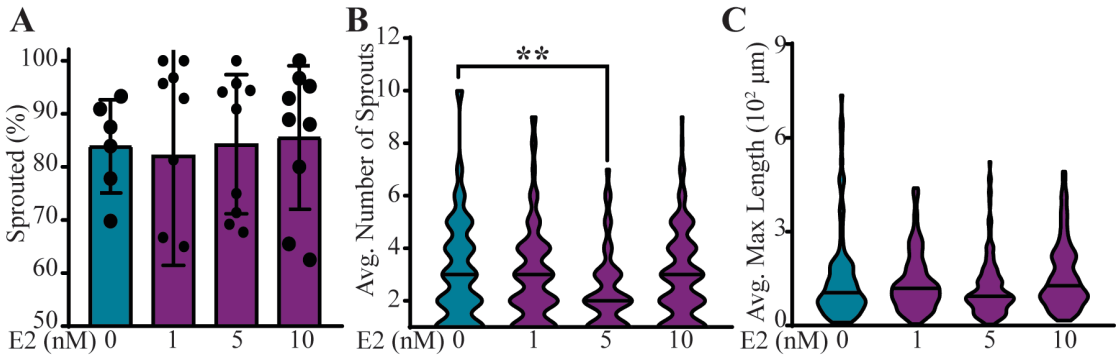


Figure 3.5: 5 nM of E2 causes a significant decrease in sprout number in male HPMECs A) Percentage of male HPMEC coated beads that produced at least one sprout when cultured in hormone free medium and stimulated with either DMSO (0.1%) or E2 (1-10 nM), means ± SD, n = 6-9 wells; B) average number of sprouts per bead in male HPMECs, n = 155-184 beads; C) average maximal length of sprouts in male HPMECS, n = 155-184 beads. ** $p < 0.01$

3.3.6 Female HPMECs have a significant increase in sprout number and length in response to DHT

The influence of DHT was assessed in female HPMECs using the bead sprouting assay of angiogenesis. Female HPMECs were exposed to either DMSO (0.1%) or DHT (1-10 nM) over 4 days before angiogenesis was assessed. There was no significant influence from DHT over the likelihood of female HPMECs to produce at least one sprout, all conditions ranging between 86.38-88.85% beads with sprouts (**Fig. 3.6A**). As already reported, the average number of sprouts for female HPMECs was 2.194 sprouts per bead. The presence of DHT at any dose caused a significant increase in the average number of sprouts, jumping to 3.368 (1 nM), 3.866 (5 nM),

and 3.639 (10 nM) sprouts per bead in DHT treated conditions (**Fig. 3.6B**). The average maximal length of female HPMEC sprouts in the control condition was 136.4 μm (**Fig. 3.6C**). A low dose of DHT, 1 nM, did not result in a significant change in average sprout length (161.3 μm). Higher doses of DHT, however, did result in significant increases in maximal sprout lengths in female HPMECs, specifically 5 nM and 10 nM DHT treated conditions had 249.2 and 216.5 μm sprouts, respectively.

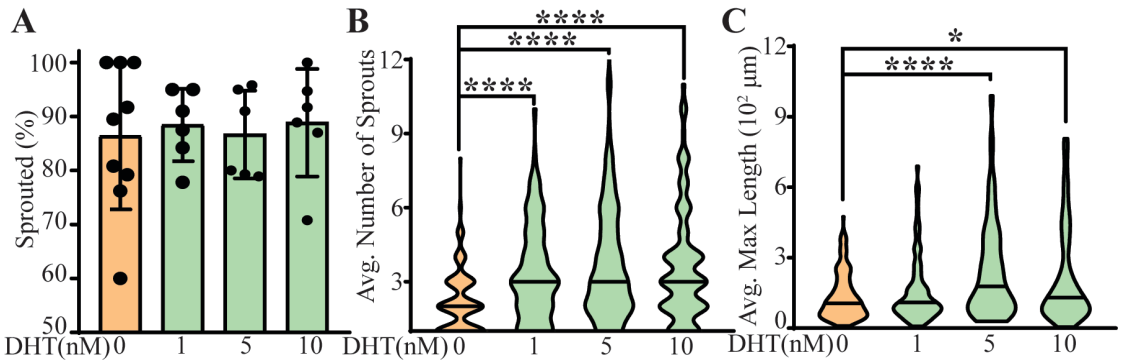


Figure 3.6: DHT significantly increases sprout number and length in female HPMECs A) Percentage of female HPMEC coated beads that produced at least one sprout when cultured in hormone free medium and stimulated with either DMSO (0.1%) or DHT (1-10 nM), means ± SD, n = 6-9 wells; B) average number of sprouts per bead in female HPMECs, n = 95-217 beads; C) average maximal length of sprouts in female HPMECs, n = 95-217 beads. * $p < 0.05$, **** $p < 0.0001$

3.3.7 DHT significantly increases male HPMEC angiogenic response

The influence of DHT was also assessed on male HPMEC angiogenesis ability using the bead sprouting assay. Single donor male HPMECs were cultured in hormone free medium and exposed to DMSO (0.1%) or doses of DHT (1-10 nM) over 4 days before angiogenesis was assessed. All doses of DHT had significant increases in the likelihood of beads to produce at least one sprout (**Fig. 3.7A**). Specifically,

percentages jumped from 83.87% (DMSO) to 99.45% (1 nM DHT), 100% (5 nM DHT), and 96.17% (10 nM DHT). Similarly, there is a significant increase in the average sprout number in male HPMECs treated with DHT (**Fig. 3.7B**). Sprout number in control (DMSO) was an average of 3.136 sprouts per bead and increases to 4.908 (1 nM DHT), 5.214 (5 nM DHT), and 5.258 (10 nM DHT) sprouts per bead in the DHT conditions. Equally impressive are the similarly significant increases in average maximum sprout length after DHT stimulation. DMSO control male HPMECs had an average maximum sprout length of 149.4 μm, which is increased to 265.3 μm in just 1 nM of DHT (**Fig. 3.7C**). 5 nM of DHT resulted in a similar sprout length to 1 nM of DHT, around 266.6 μm in length. Interestingly, 10 nM of DHT produced the longest sprout length at 349.1 μm, which is significantly greater than both the control and 5 nM DHT treated conditions.

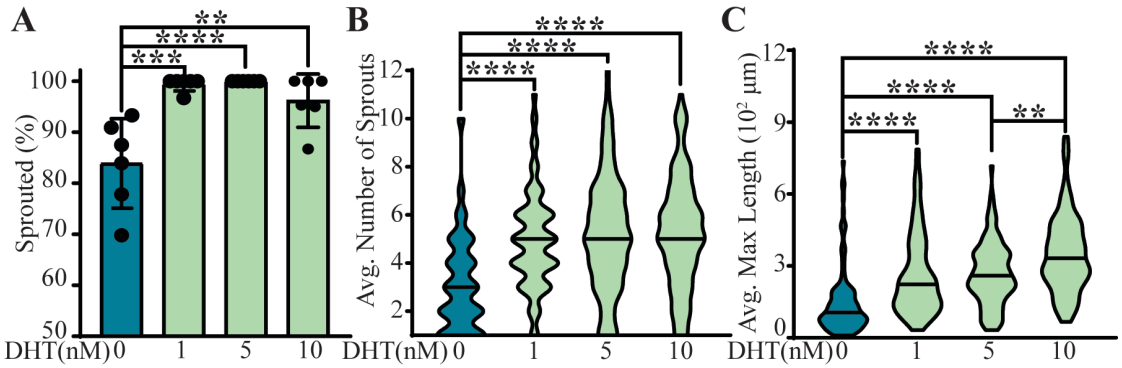


Figure 3.7: DHT significantly increases the ability to sprout, number of sprouts, and length of sprouts in male HPMECs A) Percentage of male HPMEC coated beads that produced at least one sprout when cultured in hormone free medium and stimulated with either DMSO (0.1%) or DHT (1-10 nM) means ± SD, n = 6 wells; B) average number of sprouts per bead in male HPMECs, n = 112-153 beads; C) average maximal length of sprouts in male HPMECs, n = 112-153 beads. ** $p < 0.01$, **** $p < 0.0001$

3.4 Discussion

Angiogenesis is a complex, multi-cellular process that is responsible for vascularizing new tissue, which includes pulmonary tissue[20]. The two major components of angiogenesis are proliferation, the phenotype present in the stalk of a sprout, and migration, the phenotype present in the tip of the sprout[47,48]. Female HPMECs are significantly more proliferative than male HPMECs in a proliferation assay, which serves as a measure of the maximum potential of cells to proliferate. The rate limiting step in sprout elongation is proliferation[76], this data suggests that males will hit this restriction before female HPMECs. Under normal baseline conditions, based on our previously reported data, male and female HPMECs have comparable sprout lengths so this suggests that under normal conditions, in the absence of sex hormones, female HPMECs at least are not hitting their maximum proliferation rate in the stalk[105].

Interestingly, the rate of proliferation in both male and female HPMECs is insensitive to sex hormones. This indicates that the sex difference in proliferation is intrinsic to cells. This can be inferred due to the lack of response to sex hormones, as outlined in approaches to studying sex differences[1,111]. Future work on pulmonary endothelial cell proliferation should be focused on sex chromosomal effects for an identification of the source of the sexual dimorphism in proliferation[111]. Another factor that should be considered for our reported findings is that charcoal-stripping of FBS strips out hormones as well as fatty acids[112]. The lack of fatty acids is especially important to consider due to the role they play in endothelial cell proliferation, specifically that fatty acid oxidation (FAO) is upregulated in endothelial cells as a way to efficiently produce deoxyribonucleotides[49,71]. This highlights the consideration of the fatty acid content of hormone free medium in sex differences studies.

Endothelial cells are responsible for the barrier function in blood vessels and under normal conditions, have a very low propensity towards migration as they remain in a quiescent state[49]. A migratory phenotype can be activated in endothelial cells through exogenous stimulation. In the context of angiogenesis, this takes the form of a proangiogenic factor[49], in an *in vitro* model of migration, this can simply be through serum stimulation. We identified endothelial cell migration as a sex-independent factor as both male and female HPMECs respond with an equal amount of cell numbers. This response is also sex hormone independent. We wanted to establish any baseline sex differences in migration, but it is important to note that sex differences may arise as a result of a sex dimorphism in response to the stimulating factor. Specifically, there could be a sex difference in migration when stimulated by vascular endothelial cell growth factor (VEGF) as it has been reported that female human umbilical vein endothelial cells (HUVECs) have increased migration when stimulated by VEGF compared to male HUVECs[113]. Future work to understand sex differences in cellular migration should include a panel of proangiogenic factors.

E2 is generally thought to be proangiogenic, which is in part due to the link that has been reported between E2 and VEGF[89]. VEGF and VEGF receptors have been shown to have increased expression in endothelial cells after stimulation with E2[89]. Further, E2 has been reported to significantly increase both Notch and delta like ligand 4 (Dll4) activation in HUVECs, which are also involved in VEGF signaling and control angiogenesis[106]. We found the female HPMECs had no change in sprout length as a result of E2 treatment (**Fig. 3.8A**), which is consistent with other findings in HUVECs[106]. Surprisingly, we did see an increase in the number of sprouts in female HPMECs exposed to E2, which is counter to what would be expected given that E2 is

associated with an increase in Dll4[106] which has been reported to decrease number of sprouts[104]. This could be due, in part, to a higher expression of a Dll4 regulator, micro RNA (miR) 30-a which has been reported to be expressed at higher levels in female pulmonary endothelial cells compared to male cells[59]. Male HPMECs had a significant decrease in the number of sprouts at 5 nM E2, which is consistent with what we expected based on the literature. Our data suggests there is something unique about the 5 nM dose of E2 as the 1 and 10 nM dose of E2 had no significant effects in male HPMECs, which we suggest should be followed up in future studies.

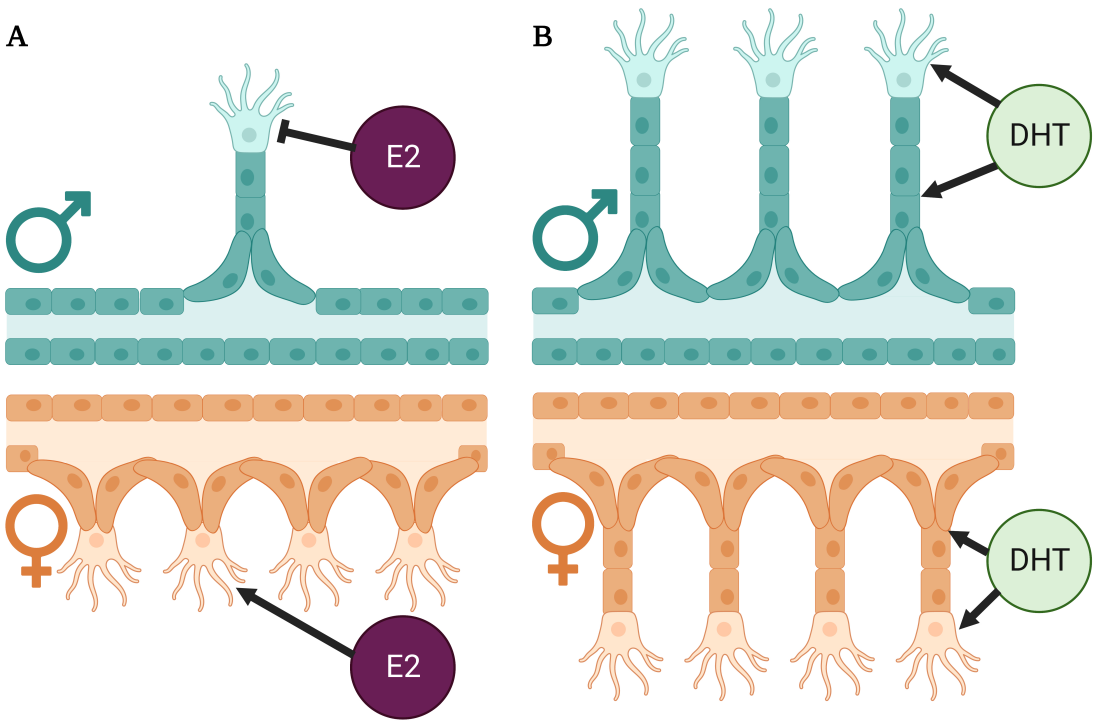


Figure 3.8: Summary of the influence of E2 and DHT on angiogenesis A) influence of E2 on sprouting angiogenesis and B) influence of DHT on sprouting angiogenesis. Top teal represents male response and bottom orange represents female response.

We found that DHT was strongly stimulatory in female and male HPMECs in the angiogenesis response, drastically increasing sprout number and length in male and female HPMECs (**Fig. 3.8B**). Generally speaking, DHT is typically associated with inhibitory effects during development, specifically in the lung[2], though the literature is unclear on the effect as it relates to endothelial cell biology[109,110]. One study has linked DHT to VEGF signaling, but it is dependent upon the sex of the cells, specifically it is only reported to have this link in male endothelial cells[92]. Our data highlights there is a female response in the pulmonary endothelial cells, but our data also supports that the female response is more attenuated than the male HPMEC response. Specifically, female HPMEC maximal sprout length only responded at higher doses of DHT and there was no change in the likelihood of sprouting. In male HPMECs, on the other hand, there was a strong response to the smallest doses of DHT in all parameters of angiogenesis assessed in this study. Further, the maximal sprout length demonstrated a further increase at the highest tested dose of 10 nM DHT. Taken together, these represent important findings on the role of DHT in HPMEC angiogenesis, findings that should be further investigated.

Chapter 4

PULMONARY ENDOTHELIAL SEX DIMORPHISM IN METABOLISM AND THE LINK TO PPARγ

This chapter is adapted from the following manuscript in preparation: Hayward-Piatkovskyi, B; Gleghorn, JP. "Pulmonary endothelial sex dimorphism in metabolism and the link to PPARγ." (2023) *in preparation*

4.1 Introduction

Extremely premature infants account for 2% of all births and requires extensive life-saving therapies, specifically mechanical ventilation with high supplemental oxygen[2,36,38]. The effect of these therapies on the still developing lung results in increased inflammation, tissue damage, disrupted alveolarization, and disrupted angiogenesis, all factors that increase the risk of developing bronchopulmonary dysplasia (BPD)[33–37]. These disruptions in lung development can result in impaired alveolarization and vascular rarefication, further complicating gas exchange in the lung, and are the underlying conditions that result in infants continuing to need oxygen therapy[33–35,38]. While the treatment of prematurely born infants is uniform, males are far more likely to develop BPD compared to their female counterparts even when accounting for age of prematurity and birth weight[2,29,36,38,60]. Sex differences in lung angiogenesis have recently been identified as possible sources of this sexual dimorphism[2,29,38,60,61,105]. Despite these findings, the mechanisms that underlie these differences has only begun to be explored.

The histological index of maturity in male infant lungs at 32 weeks of gestation or younger tend to be lower compared to female infant lungs of the same gestational age[30,38]. These differences may be due, in part, to sex differences in pulmonary angiogenesis, a process that is critical to proper lung development[2,20,42,105]. In fact, if

angiogenesis is inhibited in the lung during the alveolarization stage of lung development, proper alveolarization is abolished[20]. Further, pulmonary angiogenesis has been indicated as having a sexual dimorphism in human *in vitro* models, as well as in mouse and rat *in vivo* models[20,59,61]. In an effort to further characterize these differences, we have previously identified a sex phenotype in pulmonary vascular angiogenesis where male endothelial cells produce fewer but longer sprouts compared to female endothelial cells[105]. Despite these findings, this remains an understudied area of BPD research and further identification of molecular mechanisms behind the sex phenotype are needed.

Angiogenesis is a complex coordination in endothelial cells where a subpopulation must upregulate migratory pathways while another subset must upregulate proliferative pathways[2,105]. These endothelial cell phenotypes can be categorized further by the endothelial cell metabolism, specifically that the migratory tip cells upregulate glycolysis while the rapidly dividing stalk cells upregulate fatty acid oxidation (FAO), a cellular metabolism pathway that is normally downregulated[67,70]. Fatty acid carbons produced by FAO give rise to aspartate, an efficient precursor for nucleotides, and points to the role FAO plays in allowing endothelial cells to quickly generate materials needed for DNA replication[71]. In fact, blocking FAO results in angiogenesis defects, specifically reducing HUVEC proliferation but not migration[71]. Glycolysis can provide an alternative pathway source for nucleotide precursors, but this pathway is far less efficient and drastically reduces the rate of proliferation needed to elongate stalks in angiogenesis[71].

Cellular metabolism is also important for mitigating oxidative stress with FAO being implicated to play a role in mitigating reactive oxygen species (ROS) in the

vasculature[73]. In fact, FAO has been reported to specifically protect the lung vascular endothelial cells from supplemental oxygen-induced hyperoxia[72,73]. The transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) is heavily involved in FAO and is reported to both directly scavenge ROS as well as up- and downregulate pathways to reduce ROS within the cell[80]. Further, PPARγ has been implicated in BPD, specifically it's been reported that those infants who develop BPD have lower levels of PPARγ[80]. Most importantly, PPARγ is an angiogenesis related transcription factor that has been reported to exhibit a striking sexual dimorphism across multiple cell types where females generally have a higher expression and activity of PPARγ compared to males[75,76,81]. In fact, loss of PPARγ activity completely abolishes angiogenesis[76]. It is worth noting that not only does there exist a sexual dimorphism in PPARγ activity, but that this transcription factor is also influenced by the presence of the estrogen and testosterone sex hormone[82,114,115]. Specifically, estrogen can increase PPARγ activity as it is a co-factor while testosterone has been shown to inhibit PPARγ expression[73,82,115]. These findings represent the meticulous work done around PPARγ signaling and these previous findings make PPARγ an attractive molecular target for sex differences in pulmonary angiogenesis as they relate to BPD.

Sex differences have recently been a focus of research in the context of angiogenesis and striking differences have been reported in the literature. Additionally, great advancements have been made in understanding the role of cellular metabolism in angiogenesis as well as the role endothelial metabolism plays in mitigating oxidative stress. Due to the critical role endothelial cell metabolism plays in not only angiogenesis, but also in mitigating oxygen-associated damage, molecular mechanisms that relate to these processes provide an interesting target of

investigation. PPARγ unifies these processes as a sexually dimorphic transcription factor that sits at the center of cellular metabolism, angiogenesis, and ROS mitigation. While there has been extensive work on PPARγ in other cell types, our aim is to provide a focused study on the role PPARγ plays in the sexual dimorphism in pulmonary endothelial cells within the context of cell metabolism and angiogenesis. We hypothesize that male and female pulmonary vascular endothelial cells exhibit sex differences in their metabolism that contribute to the sexual dimorphism that exists in pulmonary endothelial cells angiogenesis. We further hypothesis that these differences are due, in part, to a sexual dimorphism in PPARγ.

4.2 Materials and methods

4.2.1 Cell culture

Human pulmonary microvascular endothelial cells (HPMEC, ScienCell, male lots: 16021, 11816, 11422, female lots: 15900, 17807, 17799) were cultured in hormone free endothelial cell medium (phenol-red free ECM, ScienCell) supplemented with 5% charcoal-stripped fetal bovine serum (HyClone), endothelial cell supplement (ScienCell), and 1% penicillin-strepomycin (ScienCell) on fibronectin-coated plates (2 μg/cm^2). Cultures were maintained at 37° C supplemented with 5% CO_2. All donors were grown to near confluence (90-95%) before experimental use (passages 4-6) and lifted with 0.05% trypsin-EDTA (Corning).

4.2.2 Treatments

Stocks of E2 (1 M, MP Biomedicals), DHT (1 M, Cayman Chemical), rosiglitazone (1 M, Cayman Chemical), and GW 9662 (1 M, Cayman Chemical) were prepared in dimethylsulfoxide (DMSO, Sigma-Aldrich) and stored at -20° C. Working

solutions of 1000x (1-10 μM for E2 and DHT; 0.1-10 mM for rosiglitazone and GW 9662) in DMSO were similarly stored at -20° C between uses, in aliquots to limit freeze-thaw cycles. Controls were treated with DMSO (final well concentration 0.1%) to account for solvent effects.

4.2.3 Western blotting

HPMECs were cultured for 2 days in hormone free medium containing DMSO (0.1%), E2 (1-10 nM), DHT (1-10 nM), rosiglitazone (10 μM), rosiglitazone (10 μM) and E2 (5 nM), GW 9662 (0.1 μM), or GW 9662 (0.1 μM) and E2 (5 nM) with media changes every 24 hours. Western blotting was performed as previously described[116] with slight modifications. Samples were washed with cold phosphate buffered saline (PBS, Corning) and extracted in 1x Laemmli's buffer (250 nM Tris pH 6.8, 40% glycerol, 8% sodium dodecyl sulfate (SDS), 6% beta-mercapto ethanol, 0.05% bromophenol blue). SDS-PAGE was performed on 10% polyacrylamide gels and run using the Quadra Mini Vertical Blotting System (Expedeon, Abcam) using RunBlue TEO-Tricine run buffer (Expedeon). Electrophoresis was performed for approximately 1.5 hours running at 120 V with an ice pack and stir bar in the electrophoresis tank. Proteins were transferred to nitrocellulose membranes (GE Healthcare) for 10 minutes at 25 V using a Power Blotter system (Invitrogen, Thermo Fisher Scientific) in Pierce 1-Step Transfer Buffer (Thermo Scientific). Membranes were blocked in 5% milk (Omniblok, AmericanBIO) for 1 hour at room temperature with rocking before being incubated with primary antibody overnight in 5% milk at 4° C with rocking. Membranes were washed 3x in tris-buffered saline with 0.1% Tween-20 (TBST) for 5 minutes each wash with rocking at room temperature. Membranes were incubated with secondary antibody in 5% milk at room temperature with rocking for 1 hour

before being washed 3x in TBST for 5 minutes for each wash with rocking at room temperature. SuperSignal West Femto Maximum Sensitivity (Thermo Fisher) was used to develop blots which were visualized using the UVP ChemiDocIt (UVP) imaging system. Antibodies that were used: PPARγ (EPR18516, Abcam, 1:1000), β-actin (8H10D10, Cell Signaling, 1:5000), HRP-conjugated anti-mouse or anti-rabbit secondaries (LI-COR, 1:20,000).

4.2.4 Seahorse assay

Metabolic profiling of male and female HPMECs were performed using Seahorse assays (Agilent) as previously described[117,118] and following manufacturers guidelines with slight modifications. Approximately 24 hours prior to the start of metabolic analysis, Seahorse XFe96 Cell Culture Microplates (Agilent) were coated with fibronectin (2 μg/cm^2) and seeded with male or female HPMECs at a density of 40,000 cells per well in hormone free medium that was supplemented with DMSO (0.1%), E2 (5 nM), rosiglitazone (10 μM), rosiglitazone (10 μM) and E2 (5 nM), GW 9662 (0.1 μM), or GW 9662 (0.1 μM) and E2 (5 nM). The Seahorse XFe96 FluxPak (Agilent) was hydrated in 200 μL of molecular water (HyClone) per well overnight at 37° C in an incubator that had no external CO_2. Molecular water on the probe plate was replaced with Seahorse XF Calibrant Solution (Agilent) 1 hour in advance of starting the metabolism assay. HPMECs were washed once with assay media, which was the Agilent supplied Seahorse XF DMEM medium, pH 7.4 supplemented with glucose (10 μM), glutamine (2 μM), and pyruvate (1 μM).

This assay medium was also supplemented with the corresponding drug treatments the cells were previously exposed to in washing and final assay medium.

Cells were incubated at 37° C in a non-CO_2 incubator for 1 hour prior to start of the metabolic profiling. The probe plate was loaded with the Seahorse XF Long Chain Fatty Acid Oxidation Stress Test (Agilent) reagents, resuspended in assay medium so that final well concentrations would be as follows: etomoxir (4 μM), oligomycin (1.5 μM), FCCP (1 μM), and rotenone/antimycin A (0.5 μM) immediately before beginning the assay. Etomoxir inhibits carnitine palmitoyltransferase-1 (CPT1a), therefore blocking the uptake of fatty acids into the mitochondria and preventing FAO. Oligomycin inhibits ATPase activity and therefore blocks any ATP generated in the mitochondria, while FCCP mimics a high energy demand in the cells, stimulating maximal mitochondrial ATP production. Finally, rotenone/antimycin A block electron transporters in the mitochondria, completely blocking ATP generation in the mitochondria due to cellular respiration and therefore any oxygen consumption is due to proton leak and not cellular respiration. Etomoxir, oligomycin, and rotenone/antimycin a were used as Agilent assay suggested concentrations as these concentrations were determined to be effective in optimization experiments (data not shown). Seeding density and FCCP concentration gradients were tested for optimal dosage which was then used in the data reported here.

The Seahorse XFe96 Analyzer (Agilent) was used to perform metabolic analysis and the corresponding Wave software was used for the assay analysis. Calculations for total ATP, the ratio of ATP generated by glycolysis and mitochondrial sources, and the max and spare capacity were generated using the Agilent provided equations within the Wave software. See Appendix A for more equation details. For calculating the percentage of mitochondrial ATP generated in FAO, the total mitochondria ATP generated at baseline and after stimulation with

etomoxir was used. Details of this equation, which was not provided by Wave, is also in Appendix A. Data represent the average of 3 wells per condition for each biological replicate. More specifically, each n contains 3 technical replicates that are averaged to generate that single data point.

4.2.5 Angiogenesis assay

A fibrin gel bead assay was used to assess three-dimensional (3-D) angiogenesis as previously described [58,59,61,105]. Briefly, collagen-coated cytodex-3 microcarrier beads (Sigma Aldrich) were incubated with male or female HPMECs at 40,000 cells per 750 beads for 4 hours with periodic agitation at 37° C and then incubated statically overnight. Beads were resuspended in 2 mg/mL fibrin (Millipore) and 0.15 U/mL of aprotnin (Sigma Aldrich) at 250 beads/mL and gelled with 0.625 U/mL of thrombin (Cayman Chemical) for 20 minutes. These fibrin gels were maintained in hormone free medium supplemented with DMSO (0.1%), rosiglitazone (10 μM), rosiglitazone (10 μM) with E2 (5 nM), GW 9662 (0.1 μM), or GW 9662 (0.1 μM) and E2 (5 nM) over 4 days with media changes every day.

4.2.6 Immunofluorescent staining and imaging

HPMEC coated beads in fibrin clots were fixed, stained, and imaged as previously described . Briefly, gels were fixewd in 4% paraformaldehyde (Thermo Scientific) containing 0.1% Triton-X-100 (Thermo Scientific) for 2 hours at 4° C then counterstained with phalloidin-553 (Cell Signaling, Danvers, MA) and Hoechst (Invitrogen) overnight at 4° C. Images were taken on an epifluorescent microscope (Zeiss).

4.2.7 Statistical analysis

Data are expressed as the means ± standard deviation (SD). A welch's t-test was used for all tests in which 2 means were being compared, specifically for the western blot quantitation, total ATP, max capacity ATP, and spare capacity ATP comparisons that are made between male and female HPMECs or control and E2. A 2-way ANOVA was used for all comparisons related to ATP production rate (%) and mitoATP production rate (%). Western quantitation data where multiple comparisons were made were analyzed using a one-way ANOVA. Normality tests were performed on multiple comparisons data with multiple comparisons that were related to total APT, max capacity total ATP, spare capacity ATP, percentage sprouted, average sprout number, and average maximal length of sprouts. Total ATP, max capacity total ATP, spare capacity ATP, and percentage sprouted passed the normality test and were analyzed using the Brown-Forsythe and Welch ANOVA due to variable standard deviations. The Kruskal-Wallis test was used for the average sprout number and average maximal length of sprouts comparison due to the data showing a non-normal distribution. All significance was determined for $p < 0.05$. All statistical analysis was performed using GraphPad Prism software.

4.3 Results

4.3.1 Baseline HPMEC PPARγ expression and metabolism profile

Western blot analysis was used to assess relative expression levels of PPARγ in male and female HPMECs (**Fig. 4.1A**). Female HPMECs had significantly high expression of PPARγ compared to male HPMECs with females having 0.5853 PPARγ to actin and males having 0.2009 PPARγ to actin (**Fig. 4.1B**). In contrast, male HPMECs had a significantly higher total ATP output, producing an average of 19,116

ATP pmol per minute per cell compared to the 12,567 pmol of APT per minute per cell in female HPMECs (**Fig. 4.1C**). Both the max capacity (**Fig. 4.1D**) and spare capacity (**Fig. 4.1E**) ATP production followed a similar trend. Male HPMEC max capacity of ATP production was 6,316 pmol of ATP per minute per cell and the spare capacity was 4,200 pmol of ATP per minute per cell. Female HPMEC max capacity of ATP production was 3,530 pmol of ATP per minute per cell with a spare capacity of 2,249 pmol of ATP per minute per cell. There were no significant sex differences in the percent ATP production rate attributed to either glycolysis or mitochondrial sources with male HPMECs using glycolysis to generate around 51.6% of the total ATP and female HPMECs using glycolysis to generate around 58.1% of the total ATP (**Fig. 4.1F**). Similarly, the amount of mitochondrial ATP that was generated by FAO was not different between male and female HPMECs (**Fig. 4.1G**). FAO in male HPMECs generated around 1.604% of the total mitochondrial ATP while FAO in female HPMECs generated around 0.6056% of the total mitochondrial ATP.

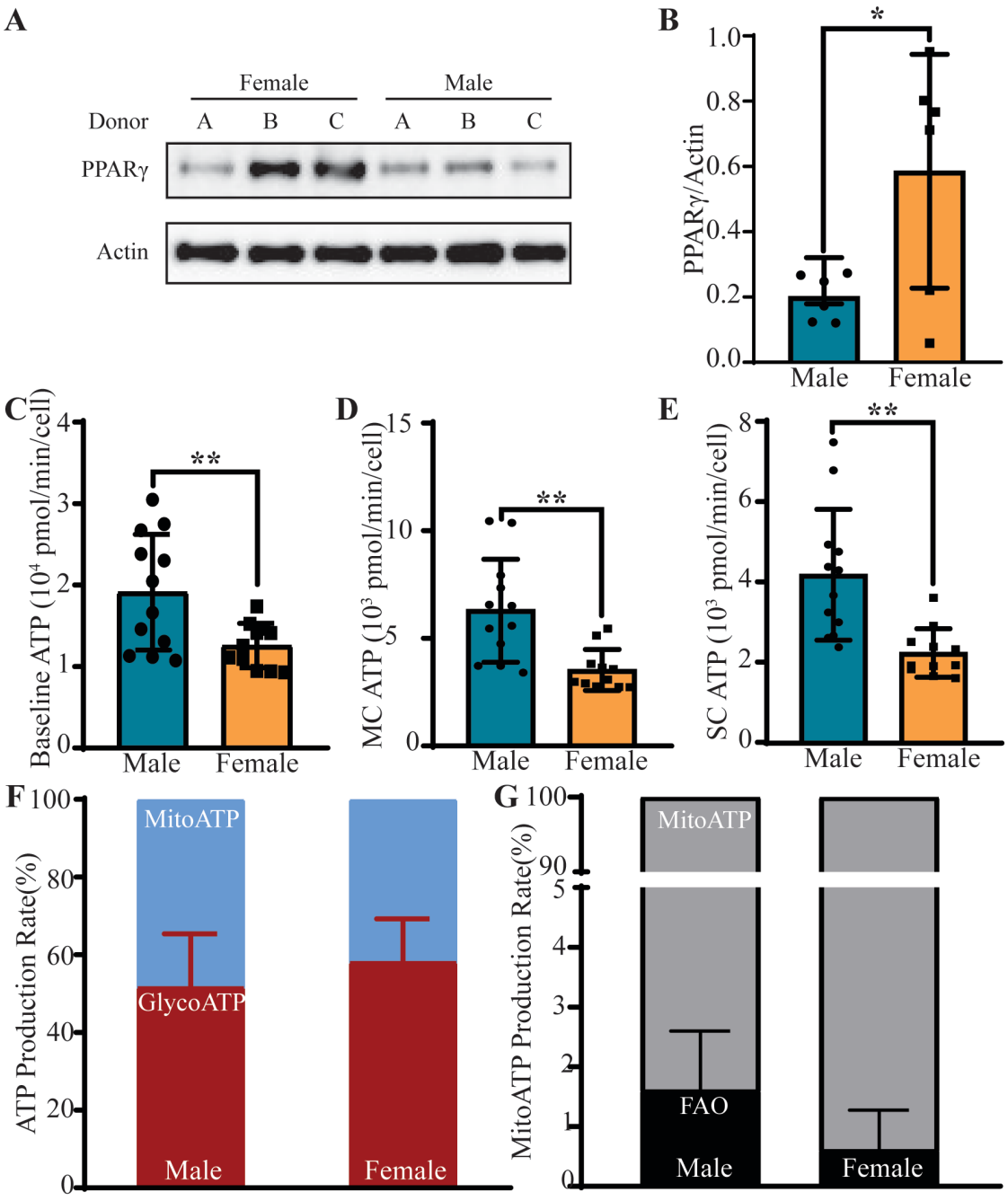

A
Female
Male
Donor
A
B
C
A
B
C
PPARγ
Actin
B
PPARγ/Actin
1.0
0.8
0.6
0.4
0.2
0.0
*
Male
Female
C
Baseline ATP (10⁴ pmol/min/cell)
**
Male
Female
D
MC ATP (10³ pmol/min/cell)
**
Male
Female
E
SC ATP (10³ pmol/min/cell)
**
Male
Female
F
ATP Production Rate(%)
MitoATP
GlycoATP
Male
Female
G
MitoATP Production Rate(%)
MitoATP
FAO
Male
Female

Figure 4.1: Male HPMECs have lower expression of PPARγ but are more metabolically active than female HPMECs, A) Top: representative images of a western blot of male and female HPMECs probed for PPARγ with actin as a loading control, Bottom: quantitation of western blots, n = 6 per sex; B) total baseline ATP generated by male and female HPMECs at baseline, n = 11-12 per sex; C) total maximum capacity ATP generated by male and female HPMECs, n = 11-12; D) total spare capacity ATP in male and female HPMECs, n = 11-12; E) percentage of ATP generated by glycolysis or the mitochondria in male and female HPMECs, n = 11-12; F) percentage of mitochondrial ATP generated by FAO in male and female HPMECs, n = 11-12. * $p < 0.05$, ** $p < 0.01$

4.3.2 Influence of sex hormones on male HPMEC metabolism and PPARγ expression

Changes in PPARγ expression in Male HPMECs exposed to DMSO (control, 0.1%), E2 (1-10 nM), or DHT (1-10 nM) were determined using western blot looking at fold changes as compared to the control (**Fig. 4.2A**). There was no significant change in the expression of PPARγ in response to E2 treatments, where the control was set at 1 and the resulted fold changes were 0.933 (1 nM E2), 0.871 (5 nM E2), and 0.8163 (10 nM E2) (**Fig. 4.2B**). Similarly, there were no significant changes in PPARγ expression as a result of exposure to DHT in male HPMECs, fold changes were 1.274 (1 nM DHT), 1.04 (5 nM DHT), and 0.9678 (10 nM DHT) (**Fig. 4.2C**). Looking at the influence of E2 (5 nM) on total ATP production in male HPMECs, there were again no significant changes due to E2 treatment, average production at baseline was 19,116 pmol ATP per minute per cell and E2 treated was 22,555 pmol ATP per minute per cell (**Fig. 4.2D**). Similarly, there was no influence from E2 (5 nM) on male HPMEC max (**Fig. 4.2E**) and spare (**Fig. 4.2F**) capacity of ATP production. Max capacity at baseline produced and average of 7,802 pmol ATP per minute per cell and E2 treatment produced 8,146 pmol ATP per minute per cell. This resulted in a spare

capacity of an average of 5,245 pmol ATP per minute per cell in controls and 5,485 pmol ATP per minute per cell in E2 treated male HPMECs. There were also no significant changes in the source of ATP with control male HPMECs generating around 51.6% of their ATP from glycolysis and E2 (5 nM) treated male HPMECs generating around 45.56% of their ATP from glycolysis (**Fig. 4.2G**). Similarly, the amount of mitochondrial ATP that was generated from FAO was comparable between control (1.604%) and E2 (6.652%) treated male HPMECs (**Fig. 4.2H**).

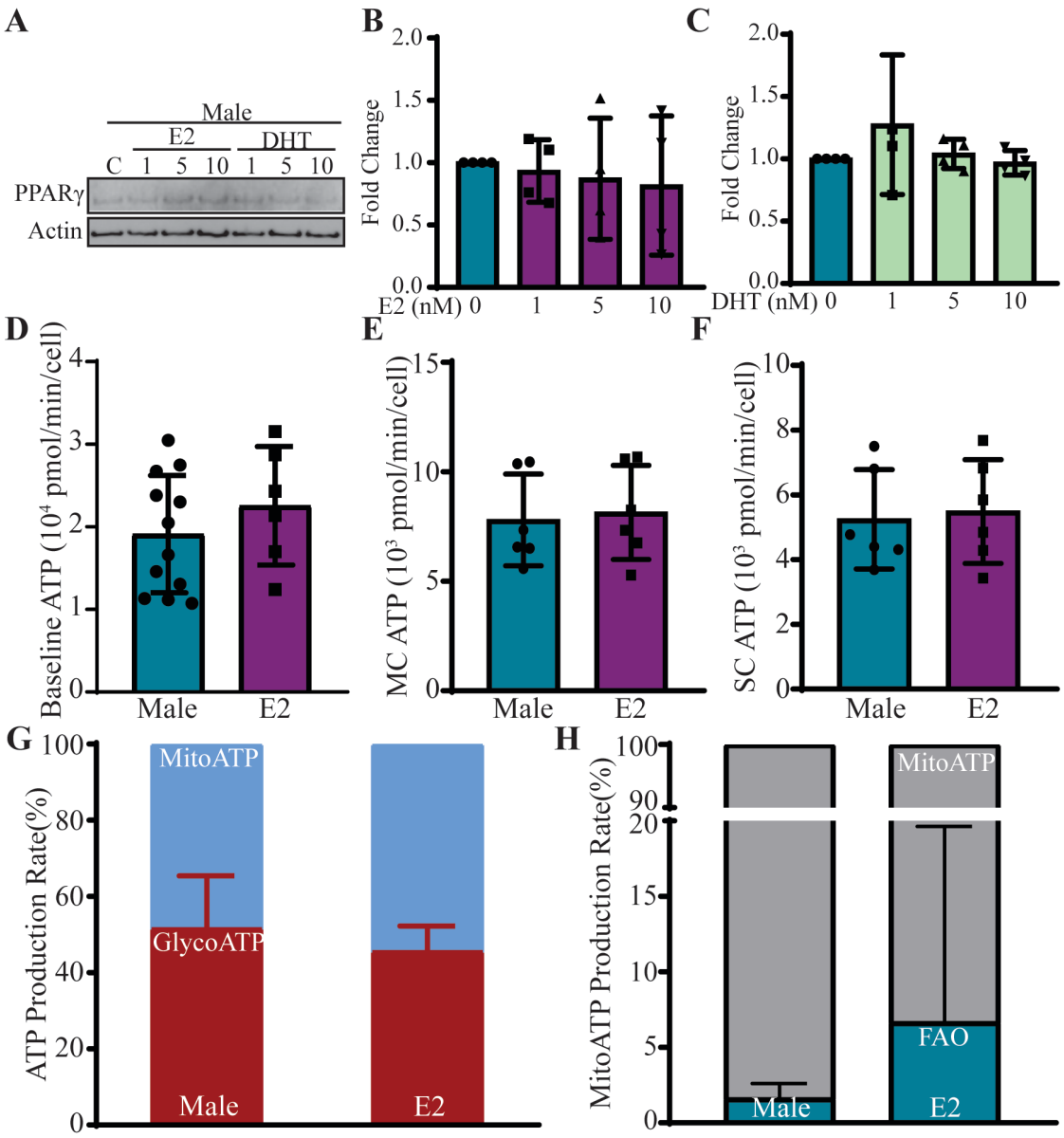

A
Male
E2
DHT
C 1 5 10 1 5 10
PPARγ
Actin
B
Fold Change
E2 (nM) 0 1 5 10
C
Fold Change
DHT (nM) 0 1 5 10
D
Baseline ATP (10⁴ pmol/min/cell)
Male
E2
E
MC ATP (10³ pmol/min/cell)
Male
E2
F
SC ATP (10³ pmol/min/cell)
Male
E2
G
ATP Production Rate(%)
MitoATP
GlycoATP
Male
E2
H
MitoATP Production Rate(%)
MitoATP
FAO
Male
E2

Figure 4.2: PPARɣ expression and metabolic activity in male HPMECs is sex hormone insensitive, A) Top: representative images of a western blot of male HPMECs treated with DMSO (0.1%) or E2 (1-10 nM) and probed for PPARɣ with actin as a loading control, Bottom: quantitation of western blots, n = 4; B) total baseline ATP generated by male HPMECs treated with DMSO (0.1%) or E2 (5 nM), E2 n = 6, control n = 12; C) total max capacity ATP generated by male HPMECs treated with DMSO (0.1%) or E2 (5 nM), E2 n = 6, control n = 12; D) total spare capacity ATP in male HPMECs treated with DMSO (0.1%) or E2 (5 nM), E2 n = 6, control n = 12; E) percentage of ATP generated by glycolysis or the mitochondria in male HPMECs treated with DMSO (0.1%) or E2 (5 nM), E2 n = 6, control n = 12; F) percentage of mitochondrial ATP generated by FAO in male HPMECs treated with DMSO (0.1%) or E2 (5 nM), E2 n = 6, control n = 12

4.3.3 Influence of sex hormones on female HPMEC metabolism and PPARγ expression

Changes in PPARγ expression in female HPMECs were performed similar to the male analysis. Fold changes were determined from western blots of PPARγ that were normalized to actin (**Fig. 4.3A**). DMSO treated female HPMECs served as controls, and fold change was set to 1 for these samples. In response to E2 (1-10 nM) treatment, changes in PPARγ expression in female HPMECs were 0.9707 (1 nM E2), 0.8670 (5 nM), and 0.7651 (10 nM) (**Fig. 4.3B**). In response to DHT (1-10 nM) treatment, changes in PPARγ in female HPMECs were 0.877 (1 nM DHT), 0.7199 (5 nM DHT), and 0.7949 (10 nM DHT) (**Fig. 4.3C**). There were no significant changes in the total ATP in female HPMECs treated with E2 (5 nM) (**Fig. 4.3D**). DMSO (0.1%) treated female HPMECs had an ATP generation rate of 12,567 pmol ATP per minute per cell while E2 treated female HPMECs had an ATP generation rate of 14,478 pmol ATP per minute per cell. Similarly, the max (**Fig. 4.3E**) and spare (**Fig. 4.3F**) capacity in ATP generation in female HPMECs treated with E2 (5 nM) was comparable with the control (DMSO, 0.1%) condition. Baseline max capacity ATP

generation was 3,560 pmol ATP per minute per cell and spare capacity was 2,263 pmol per minute per cell. E2 treated max capacity ATP generation was 3,493 pmol ATP per minute per cell and spare capacity was 2,150 pmol ATP per minute per cell. There were no significant differences in the source of the ATP in female HPMECs with DMSO (0.1%) (58.1%) and E2 (61.96%) treated conditions having comparable percentages of ATP generated by glycolysis (**Fig. 4.3G**). The contribution of ATP from FAO was also comparable between the control (0.6056%) and E2 (0.7928%) treated conditions in female HPMECs (**Fig. 4.3H**).

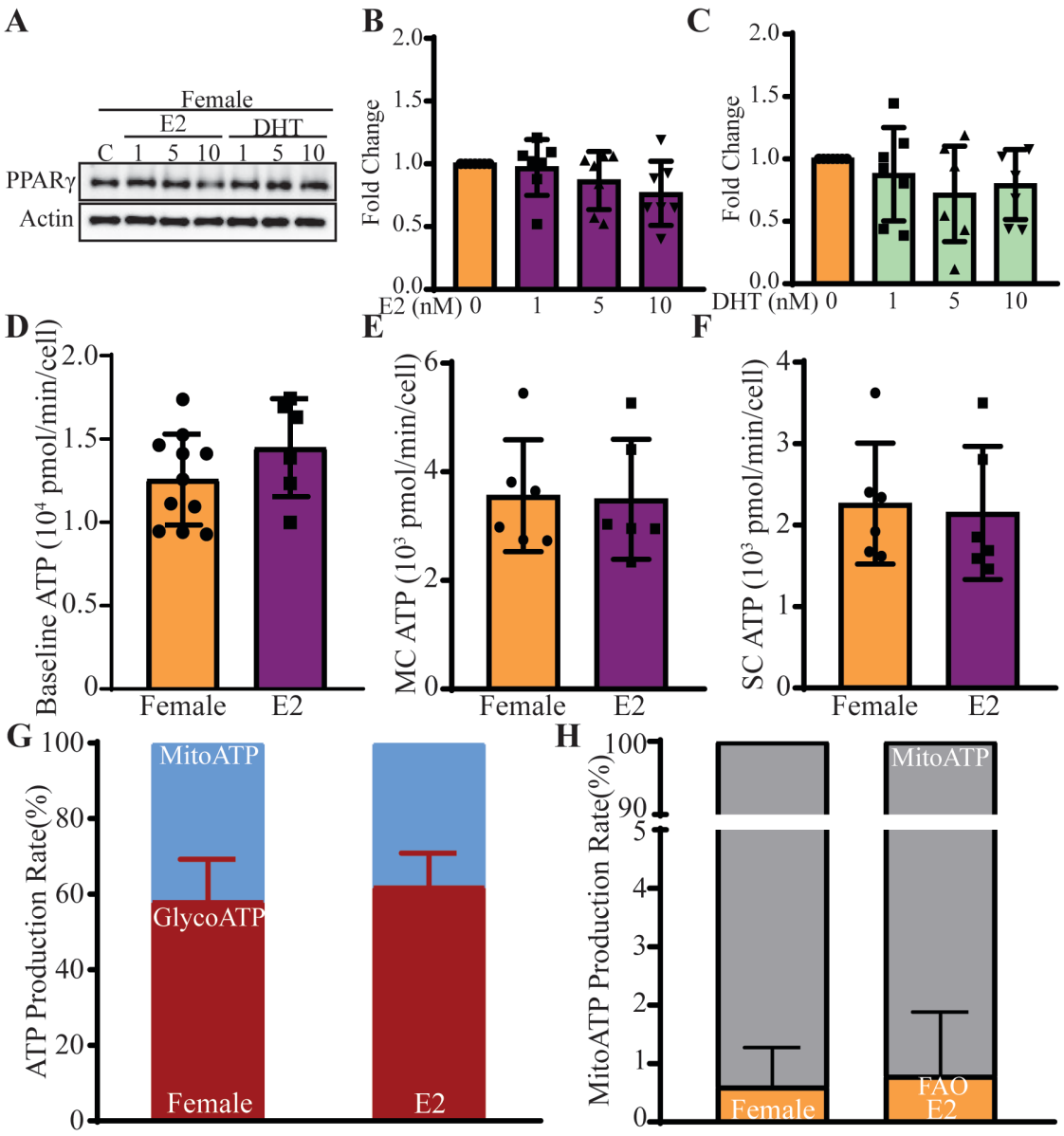
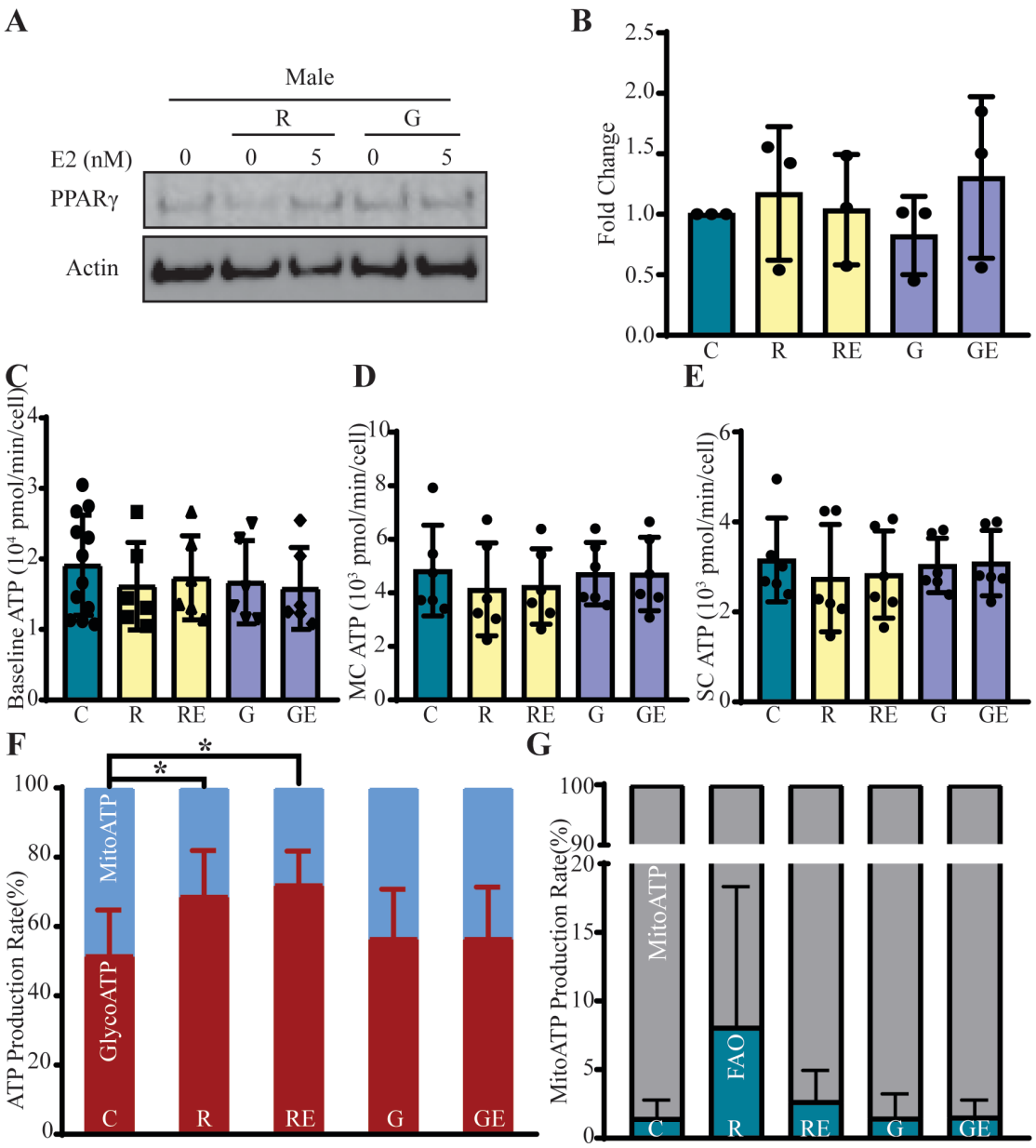

A
Female
E2
DHT
C 1 5 10 1 5 10
PPARγ
Actin
B
Fold Change
2.0
1.5
1.0
0.5
0.0
E2 (nM) 0 1 5 10
C
Fold Change
DHT (nM) 0 1 5 10
D
Baseline ATP (10⁴ pmol/min/cell)
Female E2
E
MC ATP (10³ pmol/min/cell)
Female E2
F
SC ATP (10³ pmol/min/cell)
Female E2
G
ATP Production Rate(%)
MitoATP
GlycoATP
Female
E2
H
MitoATP Production Rate(%)
MitoATP
Female
FAO
E2

Figure 4.3: Female HPMEC metabolic activity and PPARɣ expression are sex hormone insensitive, A) Top: representative images of a western blot of female HPMECs treated with DMSO (0.1%) or E2 (1-10 nM) and probed for PPARɣ with actin as a loading control, Bottom: quantitation of western blots, n = 7; B) total baseline ATP generated by female HPMECs treated with DMSO (0.1%) or E2 (5 nM), E2 n = 6, control n = 11; C) total max capacity ATP generated by female HPMECs treated with DMSO (0.1%) or E2 (5 nM), E2 n = 6, control n = 11; D) total spare capacity ATP in female HPMECs treated with DMSO (0.1%) or E2 (5 nM), E2 n = 6, control n = 11; E) percentage of ATP generated by glycolysis or the mitochondria in female HPMECs treated with DMSO (0.1%) or E2 (5 nM), E2 n = 6, control n = 11; F) percentage of mitochondrial ATP generated by FAO in female HPMECs treated with DMSO (0.1%) or E2 (5 nM), E2 n = 6, control n = 11

4.3.4 Metabolic profile in male HPMECs after modulations in PPARγ activity

PPARγ activity was increased (rosiglitazone, 10 μM) or decreased (GW 9662, 0.1 μM) over 2 days in male HPMECs, in the presence or absence of E2 (5 nM) to assess changes in PPARγ expression relative to actin using western blot analysis (**Fig. 4.4A**). The resulting fold changes were as follows: 1.172 (rosiglitazone), 1.038 (rosiglitazone + E2), 0.824 (GW 9662), and 1.304 (GW 9662 + E2), none of which were significantly different than the DMSO (0.1%) control (**Fig. 4.4B**). The amount of total baseline ATP generated in male HPMECs across conditions was also not significantly different when comparing the DMSO (0.1%) control (19,116 pmol ATP/min/cell), rosiglitazone (16,131 pmol ATP/min/cell), rosiglitazone + E2 (17,318 pmol ATP/min/cell), GW 9662 (16,692 pmol ATP/min/cell), or GW 9662 + E2 (15,806 pmol ATP/min/cell) (**Fig. 4.4C**). Similarly, the max (**Fig. 4.4D**) and spare (**Fig. 4.4E**) capacity in ATP production in male HPMECs were comparable across all conditions. The average max capacity for male HPMECs values were 4,831 pmol ATP/min/cell (DMSO control), 4,130 pmol ATP/min/cell (rosiglitazone), 4,237 pmol

ATP/min/cell (rosiglitazone + E2), 4,716 pmol ATP/min/cell (GW 9662), and 4,703 pmol ATP/min/cell (GW 9662 + E2). The average spare capacity values for male HPMECs were 3,154 pmol ATP/min/cell (DMSO control), 2,750 pmol ATP/min/cell (rosiglitazone), 2,829 pmol ATP/min/cell (rosiglitazone + E2), 3,033 pmol ATP/min/cell (GW 9662), and 3,086 pmol ATP/min/cell (GW 9662 + E2). Increased PPARγ activity in male HPMECs resulted in a significant increase in ATP generated by glycolysis compared to the control (**Fig. 4.4F**). Glycolysis accounted for about 51.55% of the ATP in the control but 68.71% when exposed to rosiglitazone and 72.04% when exposed to rosiglitazone and E2. Inhibition on PPARγ activity did not result in significant changes in ATP generated by glycolysis, resulting in 56.54% (GW 9662) and 56.53% (GW 9662 + E2) of ATP being from glycolysis. While there were some changes when PPARγ activity was changed in glycolysis, there were no significant changes in the amount of ATP generated by FAO with values ranging from 1.444-8.083% (**Fig. 4.4G**).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Male
R
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E2 (nM)
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5
0
5
PPARγ
Actin
B
Fold Change
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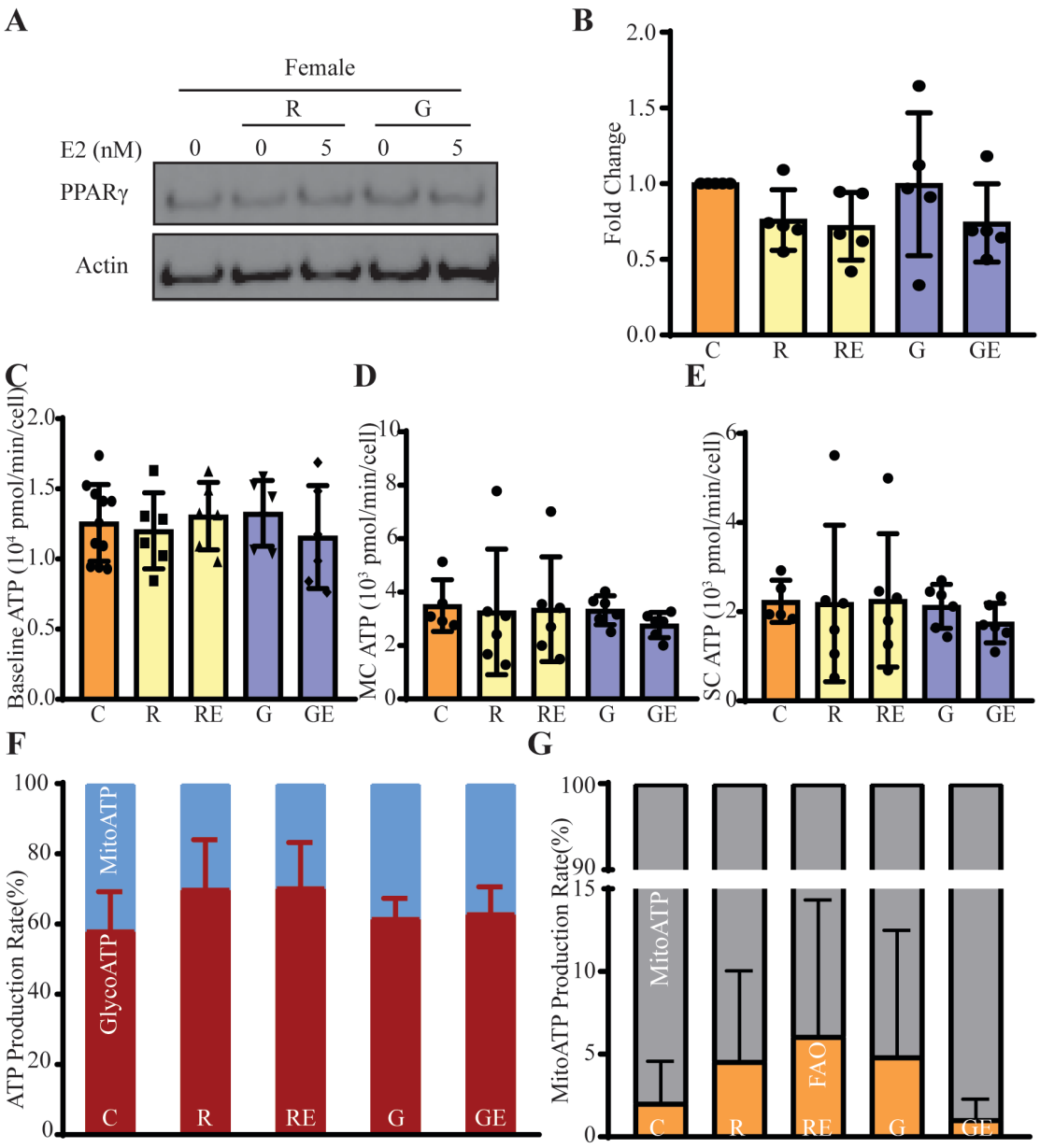
2.0
1.5
1.0
0.5
0.0
C
R
RE
G
GE
C
Baseline ATP (10⁴ pmol/min/cell)
D
MC ATP (10³ pmol/min/cell)
E
SC ATP (10³ pmol/min/cell)
F
ATP Production Rate(%)
MitoATP
GlycoATP
*
*
G
MitoATP Production Rate(%)
MitoATP
FAO

Figure 4.4: An increase in PPARɣ activity modulates causes a shift towards being more glycolytic in male HPMECs, A) Top: representative images of a western blot of male HPMECs treated with DMSO (0.1%), rosiglitazone (10 μM), or GW 9662 (0.1 μM) with or without E2 (5 nM) and probed for PPARɣ with actin as a loading control, Bottom: quantitation of western blots, n = 3; B) total baseline ATP generated by male HPMECs treated with DMSO (0.1%), rosiglitazone (10 μM), or GW 9662 (0.1 μM) with or without E2 (5 nM), treatment n = 6, control n = 12; C) total max capacity ATP generated by male HPMECs treated with DMSO (0.1%) or E2 (5 nM), treatment n = 6, control n = 12; D) total spare capacity ATP in male HPMECs treated with DMSO (0.1%), rosiglitazone (10 μM), or GW 9662 (0.1 μM) with or without E2 (5 nM), treatment n = 6, control n = 12; E) percentage of ATP generated by glycolysis or the mitochondria in male HPMECs treated with DMSO (0.1%), rosiglitazone (10 μM), or GW 9662 (0.1 μM) with or without E2 (5 nM), treatment n = 6, control n = 12; F) percentage of mitochondrial ATP generated by FAO in male HPMECs treated with DMSO (0.1%), rosiglitazone (10 μM), or GW 9662 (0.1 μM) with or without E2 (5 nM), treatment n = 6, control n = 12. C: DMSO control, R: Rosiglitazone, RE: Rosiglitazone + E2, G: GW9662, GE: GW9662 + E2. * $p < 0.05$

4.3.5 Metabolic profile in female HPMECs after modulation in PPARγ activity

In female HPMECs, to assess changes in PPARγ expression as a result of changes in PPARγ activity, cells were exposed to rosiglitazone (10 μM) or GW 9662 (0.1 μM) over 2 days, in the presence or absence of E2 (5 nM) and probed for PPARγ relative to actin using western blot analysis (**Fig. 4.5A**). The resulting fold changes were as follows: 0.7592 (rosiglitazone), 0.7178 (rosiglitazone + E2), 0.995 (GW 9662), and 0.74 (GW 9662 + E2), none of which were significantly different than the DMSO (0.1%) control (**Fig. 4.5B**). Female HPMEC baseline total ATP across conditions was not significantly different when comparing the DMSO (0.1%) control (12,567 pmol ATP/min/cell), all conditions ranging from 11,560-13,254 pmol ATP/min/cell (**Fig. 4.5C**). Similarly, the female HPMEC max (**Fig. 4.5D**) and spare (**Fig. 4.5E**) capacity in ATP production were comparable across all conditions. Female

HPMEC max capacity ranged between 2,765-3,495 pmol ATP/min/cell and the spare capacity ranged between 1,746-2,253 pmol ATP/min/cell. Modulation of PPARγ activity had no impact on the source of ATP from glycolysis, with baseline being 58.07% of ATP generated by glycolysis, rosiglitazone treated being 69.92% (without E2) and 70.32% (with E2), and GW 9662 treated being 61.6% (without E2) and 62.94% (with E2) ATP generated by glycolysis (**Fig. 4.5F**). There were no significant differences in the averages of ATP generated by FAO, all ranging between 1.054-6.082% of ATP generated by FAO (**Fig. 4.5G**).


A
Female
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E2 (nM)
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0
5
PPARγ
Actin
B
Fold Change
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1.5
1.0
0.5
0.0
C
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RE
G
GE
C
Baseline ATP (10⁴ pmol/min/cell)
2.0
1.5
1.0
0.5
0.0
C
R
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D
MC ATP (10³ pmol/min/cell)
10
8
6
4
2
0
C
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RE
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GE
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SC ATP (10³ pmol/min/cell)
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4
2
0
C
R
RE
G
GE
F
ATP Production Rate(%)
100
80
60
40
20
0
MitoATP
GlycoATP
C
R
RE
G
GE
G
MitoATP Production Rate(%)
100
90
15
10
5
0
MitoATP
FAO
C
R
RE
G
GE

Figure 4.5: Modulations in female HPMEC PPARɣ activity do not influence female metabolic profile, A) Top: representative images of a western blot of female HPMECs treated with DMSO (0.1%), rosiglitazone (10 μM), or GW 9662 (0.1 μM) with or without E2 (5 nM) and probed for PPARɣ with actin as a loading control, Bottom: quantitation of western blots, n = 5; B) total baseline ATP generated by female HPMECs treated with DMSO (0.1%), rosiglitazone (10 μM), or GW 9662 (0.1 μM) with or without E2 (5 nM), treatment n = 6, control n = 11; C) total max capacity ATP generated by female HPMECs treated with DMSO (0.1%) or E2 (5 nM), treatment n = 6, control n = 11; D) total spare capacity ATP in female HPMECs treated with DMSO (0.1%), rosiglitazone (10 μM), or GW 9662 (0.1 μM) with or without E2 (5 nM), treatment n = 6, control n = 11; E) percentage of ATP generated by glycolysis or the mitochondria in female HPMECs treated with DMSO (0.1%), rosiglitazone (10 μM), or GW 9662 (0.1 μM) with or without E2 (5 nM), treatment n = 6, control n = 11; F) percentage of mitochondrial ATP generated by FAO in female HPMECs treated with DMSO (0.1%), rosiglitazone (10 μM), or GW 9662 (0.1 μM) with or without E2 (5 nM), treatment n = 6, control n = 11. C: DMSO control, R: Rosiglitazone, RE: Rosiglitazone + E2, G: GW9662, GE: GW9662 + E2. * $p < 0.05$

4.3.6 Changes in male HPMEC angiogenesis when PPARγ activity is modulated

Angiogenesis was assessed using a bead sprouting assay where male HPMECs were exposed to DMSO (0.1%, control), rosiglitazone (10 μM, agonist), or GW 9662 (0.1 μM, inhibitor) with or without E2 (5 nM). The likelihood of male HPMECs to produce at least one sprout per bead was 83.87% (**Fig. 4.6A**). Increased PPARγ activity through rosiglitazone stimulation did not change the likelihood of sprouting in male HPMECs, with the likelihood being 92.27% (without E2) and 95.35% (with E2). Decreased PPARγ activity through GW 9662 stimulation, however, did significantly increase sprouting likelihood in male HPMECs to 98.08% (without E2) and 99.3% (with E2). Interestingly, both increased and decreased PPARγ activity in male HPMECs produced significantly more sprouts, increasing from an average of 3.1

sprouts per bead to 4.7 (rosiglitazone), 4.4 (rosiglitazone + E2), 5 (GW 9662), and 5.6 (GW 9662 + E2) sprouts per bead (**Fig. 4.6B**). Similarly, increased and decreased PPARγ activity also significantly increased the average maximal sprout length from a baseline length of 149.4 μm to 291.8 μm (rosiglitazone), 289.7 μm (rosiglitazone + E2), 274.8 μm (GW 9662), and 278.2 μm (GW 9662 + E2) (**Fig. 4.6C**).

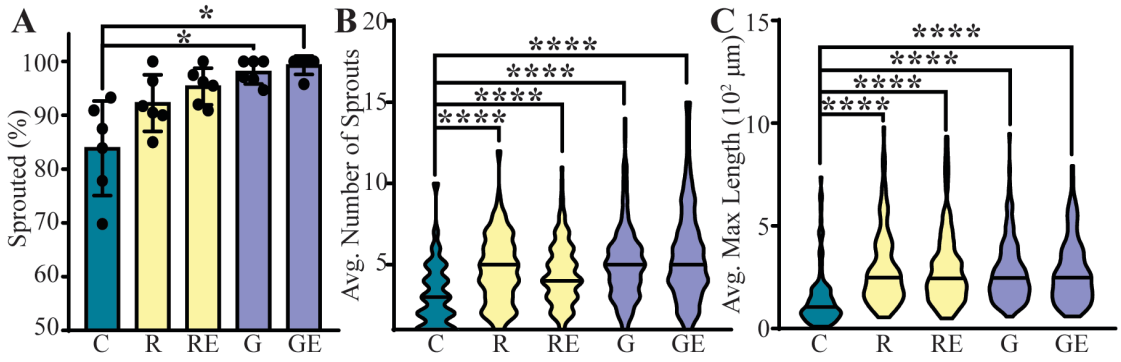

Figure 4.6: Both increases and decreases in PPARɣ activity in male HPMECs significantly increase all angiogenic parameters, A) Percentage of male HPMEC coated beads that produced at least one sprout when exposed to DMSO (0.1%), rosiglitazone (10 μM), or GW 9662 (0.1 μM) with or without E2 (5 nM), n = 6 wells; B) average number of sprouts per bead in male HPMECs treated with DMSO (0.1%), rosiglitazone (10 μM), or GW 9662 (0.1 μM) with or without E2 (5 nM), n = 111-164 beads; C) average maximal length of sprouts in male HPMECs treated with DMSO (0.1%), rosiglitazone (10 μM), or GW 9662 (0.1 μM) with or without E2 (5 nM), n = 111-164 beads. C: DMSO control, R: Rosiglitazone, RE: Rosiglitazone + E2, G: GW9662, GE: GW9662 + E2. * $p < 0.05$, **** $p < 0.0001$

4.3.7 Changes in female HPMEC angiogenesis when PPARγ activity is modulated

Female HPMEC angiogenesis was similarly assessed using a bead sprouting assay where cells were exposed to DMSO (0.1%, control), rosiglitazone (10 μM,

agonist), or GW 9662 (0.1 μM, inhibitor) with or without E2 (5 nM). Modifying PPARγ activity in female HPMECs did not result in any significant changes to the percent of beads that produced at least one sprout, with all conditions ranging from 91.58-93.98% of beads having at least one sprout (**Fig. 4.7A**). Changes in PPARγ activity in female HPMECs did result in significant changes in the average number of sprouts, similar to the response seen in male HPMECs, specifically numbers increased from 2 in the DMSO control to 3.9 (rosiglitazone), 3.4 (rosiglitazone + E2), 3.8 (GW 9662), and 3.3 (GW 9662 + E2) sprouts per bead (**Fig. 4.7B**). Increasing PPARγ activity in female HPMECs also increased the average maximal sprout length from 137.2 μm to 292.6 μm (rosiglitazone) and 252.2 μm (rosiglitazone + E2). Decreasing PPARγ activity in female HPMECs also increased the average maximal sprout length to 301.7 μm (GW 9662) and 252.7 μm (GW 9662 + E2), though the presence of E2 did attenuate the increase, which was significantly smaller than GW 9662 alone (**Fig. 4.7C**).

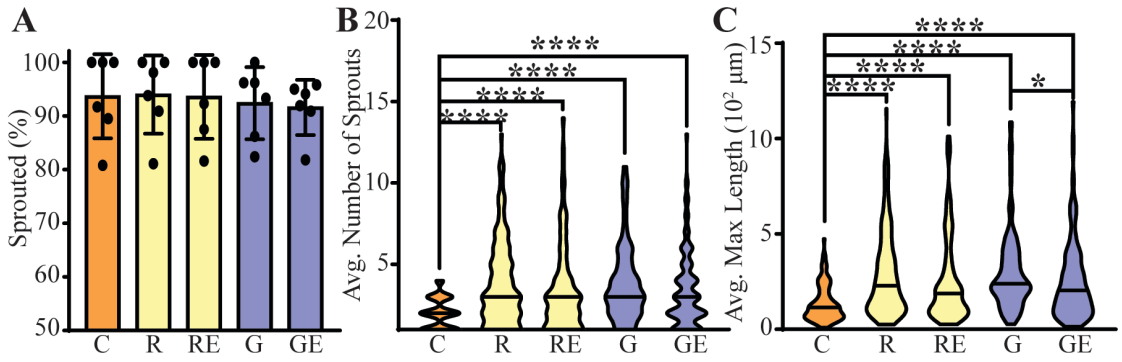

Figure 4.7: Changes in PPARɣ activity in female HPMECs increases sprout number and maximal length, A) Percentage of female HPMEC coated beads that produced at least one sprout when exposed to DMSO (0.1%), rosiglitazone (10 µM), or GW 9662 (0.1 µM) with or without E2 (5 nM), n = 6 wells; B) average number of sprouts per bead in female HPMECs treated with DMSO (0.1%), rosiglitazone (10 µM), or GW 9662 (0.1 µM) with or without E2 (5 nM), n = 112-181 beads; C) average maximal length of sprouts in female HPMECs treated with DMSO (0.1%), rosiglitazone (10 µM), or GW 9662 (0.1 µM) with or without E2 (5 nM), n = 112-181 beads. DMSO control, R: Rosiglitazone, RE: Rosiglitazone + E2, G: GW9662, GE: GW9662 + E2. * $p < 0.05$, **** $p < 0.0001$

4.4 Discussion

Angiogenesis is crucial for proper lung development and vascular dysfunction of the lung is one of the characteristics of BPD[20,37]. Additionally, BPD has a strong sexual dimorphism where male infants are at an increased risk of developing BPD compared to their female counterparts[2]. Foundational transcriptomic data on sex differences in HUVECs identified several sexually dimorphic genes were related to cellular metabolism, specifically lipid and insulin related genes had a higher expression in female HUVECs compared to male HUVECs[62]. Building off of this data, we found that in HPMECs, there is also a sexual dimorphism related to endothelial cell metabolism, specifically that male HPMECs produce a significantly larger amount of ATP per minute than female HPMECs. This dimorphism carries

through from baseline ATP generation into the maximum capacity available to the cells through the electron transport chain.

We found that the source of ATP was comparable between male and female HPEMCs, with equal amounts coming from glycolysis and mitochondrial oxidation. This was a surprising finding as the literature attributes 75-85% of ATP generated in endothelial cells is due to glycolysis, this ratio is often even higher in microvascular cells[49,67]. This data represents a novel finding, especially since this approach of breaking metabolism down in HPMECs has never been reported before. It is also important to note that these studies were also all performed in hormone free medium, which is also an uncommon model to use in endothelial biology research. We also expected to see around 15-20% of the ATP generated from FAO, which should have accounted for most of the mitochondrial ATP, but we found that almost no ATP was generated by FAO. This was surprising, but can be explained by the limitations of using hormone free medium. The charcoal stripping process also removes fatty acids, which explains why FAO was near zero in all conditions[112]. This could also explain the unexpected distribution of ATP generated from glycolysis, the lack of fatty acids would change the sources these cells use to generate both ATP and precursor molecules for cell function. Future studies that focus on FAO changes should include supplemented fatty acids in the hormone free medium to compensate for what is lost in the stripping process.

PPARγ is a transcription factor that is heavily involved in several endothelial cell processes, importantly cell metabolism and angiogenesis, and has been implicated as a key factor in BPD[67,76,80]. There has been a reported sexual dimorphism in the expression levels of PPARγ in other cell types, such as adipose and follicular helper T-cells[81,82], and our data supports that this sexual dimorphism is conserved in pulmonary endothelial cells where females have a higher expression compared to males. Under baseline conditions in the absence of sex hormones, female HPMECs have nearly 3x the expression of PPARγ compared to male HPMECs. In animal models of BPD, there is a reported decrease in PPARγ levels[80]. Administration of PPARγ agonist, rosiglitazone, in these animal models of BPD resulted in increased maturation of the lung[80]. Given the role PPARγ plays in BPD, our reported sexual dimorphism in PPARγ expression should be further investigated as a potential mechanism behind the sexual dimorphism behind the incidence of BPD.

Sex hormones can result in changes to both endothelial cell metabolism [119] and PPARγ expression levels[81,82]. One study reported that E2, but not testosterone, significantly increased the ATP max capacity and a significant increase in the number of mitochondria in human aortic endothelial cells (HAEC), but the sex of the cells was not reported in this study[119]. We found no significant effect from either E2 or DHT on male or female HPMECs in ATP production or source. The mechanism behind this difference provides an interesting new avenue of research as a unique feature to HPMECs but it is important to also note that the study in HAECs used non-stripped

FBS in their basal medium, which contains fatty acids and sex hormones. Future work on the influence of sex hormones on cellular metabolism should include appropriate metabolic substrates. Additionally, we did not find any significant influence over PPARγ expression in male or female HPMECs exposed to E2 or DHT. E2 has been reported to increase PPARγ in male follicular helper-T cells[81,82], abolishing a sex dimorphism, that is not the case in HPMECs as we have reported here.

PPARγ activity can be modulated with the agonist rosiglitazone (brand name Avandia[86]), which is from a class of chemicals known as thiazolidinediones (TZD) and work as a ligand to activate PPARγ signaling[81]. We report that treatment of male and female HPMECs with rosiglitazone did not change the expression levels of PPARγ, which is not unexpected since rosiglitazone serves as a ligand. This data also matches what has been reported about another, similar PPARγ agonist, pioglitazone[81]. Interestingly, treatment with pioglitazone and E2 has been shown to increase PPARγ expression in male follicular helper T-cells[81,82]. We did not see a significant increase in PPARγ expression in either male or female HPMECs when treated with rosiglitazone and E2. This could be due to the dosage of E2, follicular helper T-cell studies were preformed using E2 doses at estrus in a mouse model while the E2 doses we used are benchmarked to smaller doses reported in newborn cord blood[4,97]. Female HPMECs treated with rosiglitazone did not show any differences in their metabolic profile whereas male HPMECs did show a new metabolic change. Increased PPARγ activity in male HPMECs, with and without E2, resulted in an increase in the

percentage of ATP generated by glycolysis, specifically there was a significant increase in the amount of ATP generated by glycolysis. This is likely due to the role PPARγ plays in controlling glucose homeostasis[120].

Through the use of a potent PPARγ antagonist, GW9662, PPARγ activity can be inhibited[121]. Treatment with GW9662 did not change expression levels of PPARγ in male or female HPMECs. This is consistent with our rosiglitazone data and is attributed to the way GW9662 works, specifically that it will covalently and irreversibly in the ligand binding pocket of PPARγ[122]. Further, we did not see any significant changes in the metabolic profile in either male or female HPMECs after treatment with GW9662.

When looking at the influence of inhibiting PPARγ in angiogenesis, we found that the angiogenic parameters were significantly increased. In male HPMECs, it increased the likelihood of sprouting, the average number of sprouts, and the max sprout length. In female HPMECs, it increased the average number of sprouts and the max sprout length. This data indicates that inhibiting PPARγ activity in HPMECs increases angiogenesis. We also looked at increasing PPARγ activity with rosiglitazone which also produced comparable results to PPARγ being inhibited, with significant increases in average number of sprouts and maximal sprout length in both male and female HPMECs. This paradoxical data can be explained by reports in the literature that GW9662, while an inhibitor of PPARγ activity, can act as a PPARδ agonist[122], which is also expressed in endothelial cells and has some shared signaling

pathways with PPARγ[123]. Most importantly, PPARδ has been demonstrated to have strong pro-angiogenic activity and increases endothelial cell proliferation[123,124]. Interestingly, in female HPMECs treated with GW9662 in the presence of E2, there was an attenuated response in sprout length, suggesting sex hormone sensitivity in PPARδ signaling. It has been reported that PPARα is androgen sensitive, and PPARγ is E2 sensitive in follicular help-T cells, while PPARδ has not been reported to have a sexual dimorphism or hormone sensitivity in those same cells[81]. This finding opens a new line of investigation into a possible sex-dependent response to sex hormones in PPARδ signaling. These findings, taken together, demonstrate that there is a sex dimorphism in PPARγ expression in pulmonary endothelial cells and that we can increase angiogenesis in both male and female HPMECs through increasing PPARγ does increase angiogenesis. Concurrently, we have also reported the ability to increase angiogenesis through inhibition of PPARγ due to an off-target effect of the GW9662 inhibitor, opening a potential new avenue of research around PPARδ signaling that has not been previously associated with BPD.

Chapter 5

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Introduction

Across the chapters of this dissertation are three major unifying themes: pulmonary endothelial cells have a sex, the behavior and phenotype of these sex-specific cells can be externally modified, and that the sexually dimorphic transcription factor PPARγ can be modulated to increase pulmonary endothelial angiogenesis. These unifying themes were investigated within the context of the sexual dimorphism that exists in BPD with the goal of discovering underlying mechanism behind sex differences in pulmonary angiogenesis to shed light on novel pathways for future investigation. The significance and future directions of this work will be discussed in this chapter.

5.2 Sex differences in HPMECs: basic differences

Accompanying the NIH policy change aimed to encourage more transparency in research in regards to sex, and treating sex as a biological variable, is the growing field of sex differences research[1]. This policy change is especially important for shedding a light on diseases that have a clinically known sex dimorphism, such as BPD[2,29,36]. Treating SABV has led to important discoveries, specifically that male and female lungs have different histological indexes of maturity at similar gestational ages (<32 weeks)[38]. Building off of this knowledge, there have been multiple reports published related to sex differences in endothelial cell biology[62–64,113], and more specifically differences in angiogenesis[59,61,92], a critical process in the alveolar stage of lung development[20]. While these differences have been identified, the field of sex differences in endothelial biology, and importantly angiogenesis, is still in its infancy.

One of the themes of this dissertation was to characterize the underlying sex differences in pulmonary vascular endothelial cells while controlling for exogenous influences over those characteristics.

In chapter 2, we built off of the previously reported sex difference in pulmonary angiogenesis[61] by taking a more focused look at the details of angiogenesis with a focus on identifying sex differences. To achieve this, we used a bead sprouting assay as our *in vitro* angiogenesis model and expanded the parameters beyond maximal cell distance from the edge of the bead to now include average number of sprouts and percentage of beads to produce at least one sprout. Further, previous work was performed in standard culture medium which contains sex hormones in physiological concentrations[96], and we wanted to determine what a true baseline would be for sex differences. As such, we performed all of our experiments in hormone free medium, which is achieved by using charcoal-stripped FBS and phenol red free basal medium. Our major findings were that males produced fewer but longer sprouts compared to female pulmonary endothelial cells, specifically HPMECs. This finding provides a baseline sex phenotype for angiogenesis. While identifying this sex specific phenotype does provide a baseline for comparison in pulmonary angiogenesis, it is not possible to determine which approach to angiogenesis or why one attribute is prioritized over another in these experiments. The importance of having this baseline phenotype is that it allows for a foundation to compare exogenous factors against, most importantly exogenous sex-associated factors. Shorter but more abundant sprouts may be more advantageous when the lung is being damaged during development, but further research is necessary to confirm this connection. Mechanistically, this question

can be addressed by modulating sprouting phenotype, specifically hindering or increasing sprout numbers, and looking at the incidence of BPD in an animal model.

In chapter 3, we built off of the findings that there is a sexually dimorphic phenotype in pulmonary angiogenesis and aimed to identify sex-differences in underlying processes that contribute to angiogenesis. Specifically, angiogenesis requires that a subpopulation of endothelial cells become migratory[103], what is known as a tip cell, and another subpopulation of endothelial cells become proliferative[47], known as the stalk cells. The sex phenotype in pulmonary angiogenesis that we identified indicated both of these factors could be at play, since males had fewer sprouts this could be the result of a sexual dimorphism related to migration, and males produced longer sprouts, which could be due to a dimorphism in proliferation.

As part of this dissertation, we performed a migration assay in hormone free conditions and reported no sex difference in the migratory phenotype in male and female HPMECs. It is important to note that this response is representative of the maximal migratory capacity of HPMECs to respond to stimulation from hormone free serum, this approach should also be performed using pro-angiogenic factors independently, such as VEGF. The literature supports there may exist a sexual dimorphism in endothelial response to specific pro-angiogenic factors, specifically that male and female HUVECs have a sex difference in their migratory response to VEGF stimulation[65]. This study demonstrated there was increased migration in female HUVECs compared to males, but it is important to note that endothelial cells are organ specific and retain their unique transcriptomic profile which translates into varying endothelial functions based upon their organ of origin[125,126]. Interestingly, this HUVEC study on sex-differences in migration did report that female HUVECs were

more migratory than male HUVECs with just 5% serum stimulation, which further highlights the need to perform these types of studies across different tissue sources of endothelial cells. To tease out these controls over migration and the potential sexual dimorphism that may exist in HPMECs, specifically, serum stimulation should be replaced with specific concentrations of pro-angiogenic factors in the migration assay.

The next major finding in chapter 3 comes from the proliferation assay, which represents the maximum capacity for cellular proliferation in the endothelial cells, the phenotype that is associated with sprout elongation. We expected males to be more proliferative than female HPMECs given that males produced longer sprouts than females in the angiogenesis assay. Surprisingly, the data we reported in this dissertation is that female HPMECs proliferate faster than male HPMECs. This reveals two findings; first that rate of proliferation is not a rate-limiting step in female HPMEC angiogenesis and two, that there are other factors at play that limit the elongation of female HPMEC sprouts. This is likely due to a sexual dimorphism in cell-to-cell coordination that balances sprout elongation against number of responding endothelial cells[103,104]. The reported phenotype in Dll4 defects is that lower Dll4 expression results in higher prevalence of tip cells[103] and by extension, more abundant sprouts[103]. These more abundant number of sprouts were also reported to be shorter[104], consistent with the female phenotype we reported in this chapter. Further support that the female angiogenesis phenotype is Dll4 linked is our own preliminary findings that female HPMECs have a higher expression of miR 30-a, a reported negative regulator of Dll4[59]. These data, and the findings reported in this dissertation, support future work on the role of Dll4 in the sexual dimorphism in human pulmonary angiogenesis differences.

Connecting the sex phenotype in angiogenesis to the reported Dll4 phenotype would require a focused study on Dll4 in HPMECs. We would expect to see male HPMECs having a higher expression of Dll4 given that female HPMECs have a higher expression of miR 30-a[59], which could be tested using western blotting for Dll4 protein levels at baseline. Additionally, previous work further demonstrates that increased expression of miR 30-a in female HPMECs increases sprout length while there is no influence over sprout length in male HPMECs[59]. This study only looked at sprout length but provides a foundation for work that could be explored related to the findings in this dissertation and connecting to the miR 30-a and Dll4 pathway. Using the same lipofectamine transient transfection model of manipulating miR 30-a expression levels, the same characterizations of angiogenesis described in chapter 2 can be applied when analyzing the resulting angiogenesis changes. Similarly, Dll4 expression levels can be manipulated using the same transient transfection tools in HPMECs to characterize angiogenesis changes. Together, these experiments would connect the role Dll4 and miR 30-a have on sex differences in pulmonary angiogenesis.

In chapter 4, we wrapped up our focus on baseline sex differences with a focused look at cellular metabolism. Endothelial cell metabolism represents the engine that powers all endothelial functions, crucially angiogenesis[49,67,70]. There is also literature support for investigating sexual dimorphisms in angiogenesis due to reported transcriptomics data in HUVECs that show females have higher expression of many genes related to metabolism compared to males, many of these genes are related to fatty acid and lipid metabolism[62]. Utilizing the real-time kinetic power of the seahorse assay, we found that male HPMECs are far more metabolically active than female

HPMECs, producing a little over 1.5 times the ATP per minute per cell than female HPMECs at baseline. It's important to note that this rate is normalized to cell number, so despite male HPMECs proliferating slower than females, they are generating significantly more ATP. They also carry a higher capacity for ATP generation, resulting in more spare capacity than female HPMECs. This novel finding suggests either male HPMECs are less efficient at proliferation or this ATP is needed for other processes and provides an interesting new direction for endothelial cell research to uncover what this ATP is being used within. A broad spectrum approach to this could include using labeled substrate sources to identify where the carbons are being used throughout the cells[71].

It has long been established that majority of the ATP generated in endothelial cells come from glycolysis, with around 75-85% of ATP being generated this way, microvascular endothelial cells being on the higher end of the spectrum[49,67]. Interestingly, we found that in HPMECs that only around 50% of the total ATP was generated through glycolysis. Further, we expected a large portion of the mitochondrial ATP to come from FAO, the most commonly used metabolic pathway in endothelial cells for dNTP production and the pathway generally responsible for generating the remainder of the total ATP in endothelial cells[49,67,71]. Surprisingly, we report that almost none of the ATP is generated by FAO in HPMECs. This is likely due to one major limitation in using hormone free medium, importantly that the charcoal-stripping process also removes fatty acids from the FBS[112]. FAO cannot occur without the metabolic substrate, and in light of this finding our data is consistent with our medium being fatty acid deficient. This could also explain the unexpected result of only 50% of the total ATP being generated by glycolysis, but represents an

interesting research question. In other endothelial cells, depletion of fatty acids typically results in cell quiescence, or worse, cell death[71]. HPMECs grown in the absence of sufficient fatty acids remain proliferative and appear to compensate by upregulating other mitochondrial connected metabolic pathways. This is a unique phenotype to HPMECs and warrants further investigation. Further, when using hormone free mediums, special care should be taken around fatty acid content. We further propose future work on pulmonary microvascular endothelial cell metabolism around fatty acid metabolism will provide novel insights into this understudied endothelial cell.

Finally, we looked at a unifying transcription factor, PPARγ to confirm a sexual dimorphism in HPMECs. This transcription factor is critical for angiogenesis[76], regulates endothelial cell metabolism homeostasis[75], and most importantly, has been associated with BPD[80]. Specifically, those infants who develop BPD had lower PPARγ expression in their umbilical cords than those who did not develop BPD[80]. Additionally, PPARγ is known to have a sexual dimorphism in other cells and is estrogen sensitive[81,82]. Our data confirms that a comparable sexual dimorphism in PPARγ is present in HPMECs where female HPMECs have a higher expression, nearly 3 fold higher, compared to male HPMECs. Taken together, the findings in this dissertation represent the first steps in a focused approach to characterize pulmonary microvascular endothelial cell sexual dimorphisms in truly baseline conditions with an emphasis on endothelial cell function. Specifically, factors that contribute to angiogenesis to identify novel routes of investigation in the context of BPD. These novel pathways for future directions could provide novel therapeutic targets to abolish the sexual dimorphism in BPD.

5.3 Exogenous sex-dependent factors on sex-differences

Sex-differences do not just arise from chromosomal sex and sex phenotype can be influenced by exogenous factors, such as sex hormones[1,3] or other secreted factors[63]. After establishing the baseline sex phenotype when exogenous sources of sex-related factors were controlled for, we wanted to characterize which features respond to external stimulus and identify those characteristics that can be controlled exogenously. The second theme of this dissertation is focused around this goal, and as such we looked at the role of sex hormones, specifically E2 and DHT, and sex-specific secreted factors had on HPMEC behavior and function.

In chapter 2, we focused more broadly on sex hormones as a whole, the sex specific secretome, and the intersection of these two. We leveraged the information that standard culture mediums contain physiologically relevant concentrations of sex hormones[96] as a way to initially screen for any signs that sex hormones may play a role in angiogenic differences in HPMECs. Here we report that female HPMECs were sex hormone insensitive and retained the phenotype of having more abundant but shorter sprouts than male HPMECs. Interestingly, male HPMECs responded by having even fewer sprouts compared to baseline and the sex dimorphism in sprout length was abolished. This demonstrates that not only do sex hormones influence angiogenesis, a finding with literature support[90,110], but that the response to sex hormones is sex-dependent. This second part represents a novel finding in regards to sex differences in angiogenesis. Standard culture medium was designed to be a low barrier, initial look at the role of sex hormones on HPMEC angiogenesis and our findings supported a more focused analysis.

In chapter 3, we followed up these experiments with a closer look at angiogenesis, and the individual components, with more control over the type and

concentration of sex hormones present, specifically E2 and DHT. Given our previous finding that there was a sexual dimorphism in proliferation rates, we looked at whether E2 (1-10 nM) or DHT (1-10 nM) influenced male or female HPMEC proliferation. There has been literature support that DHT in the ranges we used in this study resulted in a significant increase in the proliferation rate of HAEC cells, though this study did not report the sex of the cells used[91]. We report here that there was no significant change in these proliferation rates. This suggests that proliferation in pulmonary microvascular endothelial cells is an intrinsic sex difference in HPMECs following the established guidelines on making this distinction[111,127]. Further, this also suggests that this is unique to either microvascular cells or pulmonary microvascular cells, specifically. This raises an interesting question around hormone sensitivity for proliferation in endothelial cells of different organs or vessel type and could provide novel insights into endothelial cell biology. Additionally, we expected to see phenotypic changes in migration when HPMECs are exposed to E2 (10 nM) or DHT (10 nM) based on studies that show DHT downregulates cell migration in HUVECs[110]. We reported that there was no significant influence by E2 or DHT in the male or female HPMEC migratory capacity. Similar to the proliferation rate data, this suggests that migration is sex hormone independent.

As highlighted in the previous section, angiogenesis can be broken down into the major phenotypic aspects, but these are not the only controls over angiogenesis. As such, we also wanted to test if there was any change in angiogenesis as a whole, especially given our initial findings that male HPMECs do respond to sex hormones. Our findings show that E2 significantly increases the number of sprouts in female HPMECs while 5 nM of E2 resulted in a significant decrease in the number of sprouts

in male HPMECs. We expected to see an overall stimulatory, pro-angiogenic effect from E2 given that E2 is reported as being an inducer of angiogenesis[90]. Our reported response even in the female HPMECs was modest and may point to either a minor role of E2 in pulmonary microvascular endothelial cell angiogenesis. The finding of a negative influence over angiogenesis in male HPMECs at a specific dose warrants further investigation. It would be important to follow up these findings with a much more detailed investigation into hormone metabolism of HPMECs, specifically which enzymes related to hormone metabolism are present in these cells[3].

Hormone replacement therapy has been tested clinically in prematurely born infants as a possible treatment to prevent BPD[40,41]. In these clinical trials, there was no effect from the estrogen and progesterone replacement therapy and the risk of developing BPD[40,41]. In the context of these clinical findings, our own estrogen on angiogenesis data was consistent, with only a modest increase in sprout number for female HPMECs and a decrease in male HPMECs exposed to E2. Taken together, this suggests that the prematurity of the infant lung is more of a factor in BPD risk than the absence of an estrogen peak associated with birth in term infants. Our data with the minor E2 effect in pulmonary angiogenesis aligns with this suggestion.

The literature is far less clear on the effect testosterone has on angiogenesis, which conflicting data showing inhibitory[110] and stimulatory[91] effects. One study found that the stimulatory effects of testosterone on angiogenesis was sex specific in HUVECs, specifically that male HUVECs had increased angiogenesis in the presence of DHT[92]. Our data supports that there is a strong, pro-angiogenic response in male HPMECs to the presence of DHT as well as an almost equally strong pro-angiogenic response in female HPMECs. Both male and female HPMECs had significantly more

abundant and longer sprouts compared to control, with males having even longer sprouts at higher doses of DHT. The presence of DHT also increased the percentage of beads that produce at least one sprout. Altogether, this data supports reports of a stimulatory effect of DHT on pulmonary angiogenesis. This is an especially interesting finding given that during normal human gestation, testosterone is down regulated in the fetus around 20 weeks of gestation[3] and has generally been considered inhibitory on lung development[2]. The data reported here suggests there might be a positive role for testosterone signaling in the lung endothelium. Importantly, due to the abolished estrogen spike in infant development due to premature birth, clinical trials have investigated estrogen and progesterone replacement therapy to try and lower BPD incidence[40,41]. Unfortunately, none of these trials provided evidence for this strategy to be incorporated into premature birth clinical care. Our data on DHT does open a new line of research on hormone therapy in the context of BPD. Given the strong positive response in HPMECs, further investigation on DHT signaling in the endothelium of the lung around angiogenesis should be pursued.

In chapter 4, we focused on endothelial cell metabolism with an aim to identify the role, if any, sex hormones might play on ATP output and the source of that ATP. Given the role PPARγ plays in maintaining endothelial cell metabolic homeostasis, we first assessed the effects of E2 and DHT on the expression of PPARγ. It has been reported that PPARγ is a cofactor with E2 and that sexual dimorphisms in PPARγ expression can be rescued with increased E2, specifically that male PPARγ expression in follicular helper-T cells will increase to levels comparable to females[81,82]. We reported that PPARγ expression levels in male and female HPMECs was sex hormone insensitive, neither E2 nor DHT changed the expression level of PPARγ at any tested

doses. This could be due, in part, to the doses chosen by our respective studies. The hormone doses in our studies were based off of the reported range in human umbilical cord blood[4] while the follicular helper-T cell studies used larger doses based off of concentrations found in adult female mice at estrus[82]. Future work could test higher doses to see if the sexual dimorphism can be rescued with higher doses of E2 treatment.

Similar to our PPARγ findings, there were no significant changes in the amount of ATP male or female HPMECs produced after exposure to E2 or DHT. This included the source of the ATP, with all tested conditions being comparable to baseline conditions. This supports sex differences observed in cell metabolism are due to intrinsic differences, but one major limitation to these findings is the lack of fatty acids. As previously mentioned, significant transcriptomics data highlighted sex differences in male and female HUVECs in genes regulating metabolism, specifically many genes related to fatty acid metabolism[62]. These transcriptomics studies were performed on freshly isolated HUVECs that would have been exposed to sex hormones, our findings might be more aligned with this other study if the FAO substrate was available to the HPMECs. This again highlights the limitations of hormone free medium and inclusion of fatty acid supplements for future studies.

In the data from these chapters, we focused on specific endothelial behaviors and pathways to identify sex-specific controls on angiogenesis, but there is also an argument to be made for a broader approach. This area of research is new, and the theme was to identify novel targets for expansion of the field. Another source of influence over sex differences arises from sex-specific secreted factors, another relatively new area of study[63]. In chapter 2, similar to the low barrier, initial

investigation into sex hormones with standard medium, testing secretome effects can be performed with conditioned medium as an initial screen[63]. We reported that in the absence of other exogenous factors, such as sex hormones, that both male and female secreted factors significantly increased sprout length in only female HPMECs, and not male HPMECs, while male HPMECs secreted a factor that only modified the male phenotype, resulting in a significant decrease in sprout number. This demonstrates that there are sex-specific secreted factors in HPMECs and that they influence cells in a sex-dependent manner. Further, we wanted to test if this secretome interaction was also sex hormone dependent and found that in the presence of sex hormones, the effect of the male and female secretome on female sprouting elongation was abolished. Now, a new response appeared where the female secretome on male cells, in the presence of sex hormones, significantly increased sprout length. Further, the inhibitory effect of male conditioned medium on male sprout number was also abolished when sex hormones are present. Together, this data demonstrates that HPMECs do secrete inhibitory and stimulatory angiogenic factors in a sex-specific way and that they also respond to these factors in a sex-dependent way. Further, this process is sex hormone dependent. Further work to identify which secreted factors are responsible for these differences could produce a large array of novel, sexually dimorphic targets in the context of angiogenesis. Identification of novel molecular targets related to sex differences in lung angiogenesis has significant implications for novel therapeutic targets for the treatment of BPD.

5.4 PPARγ and angiogenesis

The transcription factor PPARγ is sexually dimorphic in our HPMECs, as outlined in an earlier section, and could be linked to the sexual dimorphism observed

in HPMEC angiogenesis. Further, PPARγ is an attractive target to study in the context of pulmonary angiogenesis because there is already an FDA approved PPARγ agonist, Avandia[86], which can be clinically leveraged in the context of BPD. To understand the role PPARγ play in HPMEC angiogenesis, with an additional goal of finding a way to increase the male HPMEC angiogenic properties, we used a PPARγ agonist and inhibitor to control PPARγ activity in studies reported in chapter 4. These are ligand pocket binding compounds and did not change expression levels of PPARγ in our cells. It's important to note that PPARγ is a cofactor with E2, and in at least one PPARγ agonist, presence or absence of E2 can produce conflicting results[81,82]. As such, all experiments were performed in the absence and presence of E2 to account for this possible variable. We first focused on any changes from modifying PPARγ activity levels were related to cellular metabolism. There were no significant changes to the total ATP or max capacity of ATP output in male or female HPMECs. When we looked at the ATP source, interestingly, male HPMEC had a significant increase in using glycolysis to generate ATP when PPARγ activity was increased. While PPARγ is often associated with upregulating FAO, which would be a mitochondrial source of ATP, it is also known to regulate glycolysis homeostasis as well[75]. Again, the hormone free medium these experiments were conducted in was fatty acid deprived and the baseline state likely represents HPMEC cell compensation for a lack of fatty acids. These findings that that increased PPARγ activity in males results in increased glycolytic ATP could indicate that PPARγ is returning the male cells to a more balanced metabolic state and regulating homeostasis. Interestingly, female HPMECs did not have a change in the source of ATP. Further studies with the inclusion of fatty acids should be performed to better understand how PPARγ is modifying the

metabolism, and a more focused study around what pathways are being used to generate ATP from the mitochondria in the absence of fatty acids would be a significant contribution to the field.

Finally, PPARγ is known to play a significant role in angiogenesis. In fact, complete knockout of PPARγ in mice is embryonic lethal and conditional knockouts all have significant vascular defects[76,77]. Additionally, inhibition of PPARγ has been reported to inhibit angiogenesis, directly[76]. In our studies, we anticipated a stimulatory effect from increasing PPARγ activity and an inhibitory effect from inhibiting PPARγ activity as it relates to HPMEC angiogenesis. Using rosiglitazone as our PPARγ agonist, we did see a significant increase in sprout number and length in both male and female HPMECs. Further providing support for the positive role PPARγ plays in angiogenesis, and importantly, identifying a novel target to investigate in the context of BPD as a therapeutic target. It has already been reported that BPD is associated with decreases in PPARγ levels[80], this could be more pronounced in males as our data supports that PPARγ is sexually dimorphic. And our studies in this dissertation further support that increasing PPARγ activity can drastically increase pulmonary angiogenesis.

Our findings when inhibiting PPARγ activity, however, were surprising. This also resulted in significant increases in sprout number and length in both male and female HPMECs, on a level comparable to those reported when PPARγ activity was increased. This paradoxical result can be explained by a relatively recent finding that was published about off-target effects of the PPARγ inhibitor we used in these studies, GW9662. While there are a wide range of highly specific agonists to choose from when it comes to PPARγ, there are very few inhibitors available[122]. The PPARγ

inhibitor GW9662 is the most commonly used inhibitor, but it also functions as an agonist for a much less understood PPAR, specifically PPARδ[122]. Endothelial cells have been reported to express all of the forms of PPARs, including PPARδ, and there is some literature support that PPARδ is also a strong regulator of angiogenesis, including increasing endothelial proliferation[123,124].

Our data, taken in the context of this alternative signaling pathway activation, potentially supports this angiogenesis support in HPMECs and provides an exciting new research possibility. There are alternative PPARγ inhibitors that can used in place of GW9662 that are reported to inhibit only PPARγ, reduced angiogenesis would then confirm that PPARγ in HPMECs is directly linked to angiogenesis and that alternative pathway activation is responsible for the increase in angiogenesis from GW9662. One such PPARγ inhibitor, T0070907, covalently binds to PPARγ and the resulting conformational change interferes with the ability of PPARγ to recruit transcription factors to upregulate genes[128]. This mode of action is different than GW9662, which also covalently binds to PPARγ but blocks the ligand binding pocket instead of blocking transcription[129]. To tease out the contributions to angiogenesis by PPARγ and PPARδ, focused studies should also include PPARδ agonists, such as GW501516 or GW610742[130], to confirm increased angiogenesis in HPMECs. Unfortunately, PPARδ is an understudied PPAR and there are no reported antagonists[131], though PPARδ knockdown studies could be performed to demonstrated decreased activity also decreases angiogenesis. A similar approach can also be done to look at PPARγ activity directly, in these focused studies. Applying these mechanistic controls over PPARγ and PPARδ activity can both confirm the crossover in their role for angiogenesis as

well as confirm a novel target for the treatment of BPD, allowing for further preclinical investigation.

Another exciting and novel finding is that our data shows that in female HPMECs, the presence of E2 with GW9662 still produced significantly longer sprouts but this increase was attenuated compared to GW9662 without E2, suggesting PPARδ may also be sex hormone sensitive in a sex-dependent manner. There are no reported sex differences in the expression of PPARδ or reported sex hormone sensitivity[81]. Using the same agonist, antagonist, and knockdown approaches to tease out the role of PPARδ in angiogenesis, the role E2 plays in that signaling can also be deciphered.

5.5 Concluding remarks

In conclusion, this dissertation investigated the sexually dimorphic characteristics in pulmonary microvascular endothelial cells as they relate to angiogenesis and identified multiple, novel findings that provide the framework for new research directions. We found that there is a sex phenotype in pulmonary angiogenesis, where males produce fewer but longer sprouts than females. This phenotype is modified by exogenous sex factors, such as sex hormones or secreted factors, in a sex-dependent manner. As part of these findings, we identified a strong pro-angiogenic effect from DHT in both male and female HPMECs, a connection that has not previously been explored in the context of BPD with the focus of increasing pulmonary angiogenesis. Additionally, we found inverse sexual dimorphisms in pulmonary endothelial cell metabolism and PPARγ, where female HPMECs had a higher expression of PPARγ compared to males but males had a higher ATP production rate. PPARγ is maintains cellular metabolic homeostasis as well as regulates angiogenesis. Increased activity of PPARγ in male HPMECs resulted in

increased ATP produce in glycolysis and was associated with significant increases in angiogenesis in both male and female HPMECs. This highlights the critical role this sexually dimorphic transcription factor plays in pulmonary endothelial cells, and provides a framework for further investigation as a potential BPD therapeutic. This is especially attractive because a PPARγ agonist, brand name Avandia, has already been FDA approved for use in diabetes.

Finally, we reported that inhibition of PPARγ with GW9662 also had a strong, pro-angiogenic effect in both male and female HPMECs. This paradoxical result can be explained by the off-target effect of GW9662, where it acts as an activity increasing ligand for PPARδ, an underappreciated PPAR that also has reported pro-angiogenic effects. We also report that the stimulatory effect of GW9662 was attenuated in female HPMECs in the presence of E2, and the possible connection of E2 sensitivity in PPARδ has never been reported before. This opens the door for additional questioning around PPARδ signaling in pulmonary endothelial cells and another potential molecular target to investigate in the context of BPD.

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APPENDIX A: EQUATIONS

A.1 ATP production rate

Agilent equation:

Eq 1: ATP_{total} (pmol ATP/min) = glycoATP (pmol ATP/min) + mitoATP (pmol ATP/min)

A.2 GlycoATP production rate

Agilent equations:

Eq 2: glycoATP (pmol ATP/min) = glycoPER (pmol H^+/min)

Eq 3: glycoPER (pmol H^+/min) = PER (pmol H^+/min) – mitoPER (pmol H^+/min)

Eq 4: PER (pmol H^+/min) = ECAR (mpH/min) x BF (mmol H^+/L/pH) x Volume (μL) x Kvol

Eq 5: mitoPER (pmol H^+/min) = mitoOCR (pmol O_2/min) x CCF (pmol H^+/pmol O_2)

Eq 6: MitoOCR (pmol O_2/min) = OCR_{basal} (pmol O_2/min) – $OCR_{Rot/AA}$ (pmol O_2/min)

Abbreviations and constants: proton efflux rate (PER), extracellular acidification rate (ECAR), buffer factor (BF, 2.5 mmol H^+/L/pH), Kvol constant factor (1.6), oxygen consumption rate (OCR), and CO_2 Contribution Factor (CCF, 0.61).

A.3 MitoATP production rate

Agilent equations:

Eq 7: mitoATP (pmol ATP/min) = OCR_{ATP} (pmol O_2/min) x 2 (pmol O/pmol O_2) x P/O (pmol ATP/pmol O)

Eq 8: OCR_{ATP} (pmol O_2/min) = OCR_{basal} (pmol O_2/min) – OCR_{Oligo} (pmol O_2/min)

Agilent uses 2.75 pmol ATP/pmol O for the variable P/O in these equations, validation of this can be found in the white papers with assay kits.

A.4 Max and spare ATP production capacity

Agilent provided equations:

Eq 9: $Capacity_{Max}$ (pmol ATP/min) = Max Rate after FCCP (pmol ATP/min) – non-mitochondrial oxygen consumption

Eq 10: $Capacity_{spare}$ (pmol ATP/min) = Maximal respiration (pmol ATP/min) – basal respiration (pmol ATP/min)

A.5 FAO ATP production rate

Eq 11: % FAO ATP = ($MitoATP_{basal}$ - $MitoATP_{etomoxir}$) / $MitoATP_{basal}$

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