THESIS

IMPACT OF TESTOSTERONE ON TROPHOBLAST MITOCHONDRIAL FUNCTION

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ABSTRACT

IMPACT OF TESTOSTERONE
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Several pregnancy disorders involve placental abnormalities, including gestational diabetes mellitus (GDM) (2-10% of pregnancies), preeclampsia (PE) (6-8% of pregnancies), and polycystic ovary syndrome affects (PCOS) (6 to 15% of women in reproductive age), which not only has a negative impact on maternal health but can also lead to birth defects and postnatal health complications. These disorders commonly also present high levels of androgens in maternal blood, accompanied by placental insufficiency. The placenta in these pathologies presents morphological and physiological alterations, including in the trophoblast mitochondria.

The placenta is a multifunctional, transient organ that mediates the transport of nutrients and waste to and from the fetus, gas exchange, and endocrine signaling to maintain maternal and fetal homeostasis. To facilitate these diverse and important functions and enable proper fetal growth and development, the placenta is highly metabolically active and consumes ~40% of the total oxygen. Oxygen is used for the synthesis of ATP in mitochondria, which in turn is mainly used for cholesterol transport and steroidogenesis. The placenta is well recognized as a hormone-synthesizing and secreting organ; however, studies revealed it is a target of these hormones as well and contains receptors for various steroid hormones including androgens.

Placental androgen receptor (AR) is relevant in pregnancy disorders with elevated androgens such as GDM, PE, and PCOS. These are accompanied by placental pathologies that include mitochondrial adaptations that vary according to the stage of the pathology, and in advanced stages when levels of reactive oxygen species (ROS) become too high they can have detrimental effects on the placenta and can even lead to pregnancy loss. Of particular interest here is the recent observation that AR has been identified as a regulator of mitochondrial function in other tissues such as the prostate and cancer. The production of ROS and/or the decrease in antioxidant defenses

are the main mechanisms underlying placental insufficiency in pathologies with elevated androgens. A better understanding of the regulation of androgen signaling in placental mitochondria will lead to new insights and opportunities to understand and treat disorders of pregnancy that affect a significant number of pregnant women.

Studying the human placenta in vivo presents several complications, so it is necessary to use in-vitro models. There are several human trophoblast cell lines available, but none are perfect replacements for the original organ, rather each one has qualities that allow investigators to choose one best suited for their study. The overall goal of our studies is to investigate the role of AR signaling in trophoblast cell mitochondrial respiration. Our hypothesis is that AR signaling regulates mitochondrial oxygen consumption and ROS production.

The first chapter will provide an overview on the role of androgens in placental physiology and pregnancy, and the role of mitochondria in trophoblast cell function. In the second chapter, we present studies aimed at characterizing mitochondrial respiration in existing placental cell lines and elucidating a possible role for AR signaling in mitochondria. Specifically, we first demonstrated the presence of AR in placental mitochondria. Next mitochondrial oxygen consumption and ROS production are characterized and compared using an Oroboros O2K oxygen respirometer in three well-known human (ACH-3P, BeWo, and Swan-71) and one immortalized ovine trophoblast cell (iOTR) line. Finally, ACH-3P cells are selected to test mitochondrial responses to testosterone, mimicking placental pathologies seen in GDM, PE, and PCOS. Our results revealed that both human ACH-3P and Swan-71 cells, as well as the sheep iOTR cells, demonstrated normal oxygen consumption and ROS production following the addition of selected complex protein substrates. Chronic testosterone treatment led to significant increased ROS production in ACH-3P cells, which correlates with what has been observed in term placentas of women with placental hyperandrogenism. In conclusion, the ACH-3P cell line is a good in vitro model to study placental mitochondrial respiration.

Ultimately, the presented data provide new information regarding the possible role of AR signaling in placental mitochondria and will pave the way for future studies aimed at uncovering the mechanism of AR regulation of mitochondrial function in normal and abnormal pregnancies (discussed in Chapter 3).

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CHAPTER I

A POSSIBLE NOVEL ROLE FOR ANDROGEN SIGNALING IN TROPHOBLAST CELLS THROUGH MITOCHONDRIA

I. Introduction

The placenta is a unique, transitory organ that, from its formation after implantation until the moment of delivery, mediates healthy fetal development. The placenta regulates the circulating supply of energy substrates, micronutrients, and waste products dynamically as fetal requirements change throughout pregnancy. Furthermore, the placenta secretes numerous factors into the maternal circulation that alter systemic hemodynamics, stimulate the passage of energy substrates to the fetus, and prevent immunological rejection of the conceptus. When these processes are disturbed, pregnancy disorders such as preeclampsia (PE), gestational diabetes mellitus (GDM), and intrauterine growth restriction (IUGR) can occur, which can jeopardize maternal health and impair fetal development. Moreover, constraints on placental maturation or development that alter the intrauterine environment during embryonic and fetal life may influence the trajectory of cardiometabolic disease risk of the offspring into adulthood (Barker 2007). Therefore, understanding the mechanisms that regulate placental function and the intrauterine environment during pregnancy is of utmost importance for promoting optimal human health and development.

Steroid hormones, including progesterone, estrogen, androgens, and glucocorticoids, play essential roles in orchestrating fetal development. Disturbances in steroid hormone regulation are associated

with clinical complications such as PE, polycystic ovarian syndrome (PCOS), and GDM (Keya SL, 2019). These pregnancy disorders are also accompanied by placental defects that result in impaired fetal development due to IUGR, and excessive production of reactive oxygen species (ROS) by trophoblast mitochondria (Fisher JJ, 2020). Considering the high rate of oxidative metabolism where trophoblast cells consume ~40% of the oxygen used by the gravid (pregnant) uterus (Kolahi 2017) it is not surprising that trophoblast mitochondria play an important role in placental development.

The primary aim of this review is to provide an overview of androgen signaling in the placenta and explore its possible role in trophoblast mitochondria. Based on recent evidence for the presence of androgen receptors (ARs) (Bajpai 2019, Kobayashi 2020) within mitochondria, we postulate that in addition to its role in nuclear genome activation, mitochondria themselves could be a target of AR signaling. First, a brief overview of human placental development will be provided, highlighting key differences in placental physiology across mammalian species. Next, androgen signaling in trophoblast cells during normal and abnormal pregnancy will be discussed. Finally, the implications and regulatory functions of AR signaling on mitochondria will be discussed, with a particular focus on their potential contribution to placental function.

II. Human placental development and function

Women contain a discoid hemochorial placenta, and placentation is broadly characterized by proliferation and differentiation processes predominantly of the trophoblast. This leads to the formation of villous and extravillous structures, and differentiation of newly formed villi that

ultimately causes expansion of the placental mass (Gude 2004). Placental villi anchor the placenta to the uterus and reshape the uterine spiral arteries into low-resistance vessels.

II.A. Early placentation

Four days after fertilization, two cell lineages can be distinguished in the blastocyst, the inner cell mass and the trophectoderm, which will give rise to the embryo and fetal part of the placenta, resp. (Hamilton 1962). Embryo implantation occurs 7-8 days after conception when an embryonic pole has formed. The trophectoderm contains specialized cells called trophoblasts that proliferate and differentiate into two cell lineages, the extravillous cytotrophoblast, and the villous trophoblast. The extravillous cytotrophoblast invades and reshapes the maternal spiral arteries to establish a vascular blood supply for the fetus (Hertig 1956). The villous trophoblast of the placenta is composed of a layer of proliferative villous cytotrophoblast which differentiate into a multinuclear syncytium called syncytiotrophoblast that is in direct contact with maternal blood. The feto-maternal interface is formed by these two cell types, and they are responsible for fulfilling the main functions of the placenta, such as the transport of nutrients, gas exchange, as well as the secretion of immune and endocrine factors in the maternal system.

Placentation involves extensive vasculogenesis, i.e., the formation of new blood vessels from endothelial progenitor cells, starting 3 weeks post conception. Approximately a month after conception, additional blood vessels form first through branching angiogenesis followed by non-branching angiogenesis. Branching versus non-branching angiogenesis generally involves a switch from high levels of Vascular Endothelial Growth Factor (VEGF) and lower levels of Placental Growth Factor (PlGF) to decreasing amounts of VEGF and higher levels of PlGF (Ahmed 2000).

Extensive reviews on human placental vasculogenesis and angiogenesis have been published (e.g., Charnock-Jones, 2004). Endothelial function is critical for regulating vascular tone and placental blood flow between the mom and the developing fetus. Moreover, it has been postulated that mitochondrial dysfunction in vascular endothelial cells contributes to impaired angiogenesis and placental diseases such as preeclampsia (Kluge 2013, Correia 2021).

II.B. Placental mitochondria

The placenta has a high metabolic level so mitochondria are abundantly present and necessary to produce adenosine triphosphate (ATP) to meet the energy demands of trophoblast cells. Mitochondria have their own genome (mtDNA) that codes for 13 proteins in electron transfer complexes involved in oxidative phosphorylation and replicates independently of the host (nuclear) genome (Anderson 1981). It works in conjunction with the nuclear genome to achieve coordinated mitochondrial activities that are essential for several trophoblast functions, including energy production, cell cycle control (apoptosis), calcium storage, and synthesis of steroid hormones (Nunnari 2012). Three of the 4 primary pathways involved in cellular ATP production (glycolysis, pyruvate oxidation, the Krebs cycle, and oxidative phosphorylation) occur in the mitochondria. Pyruvate oxidation occurs in the mitochondrial matrix and transforms pyruvate into acetyl-CoA, which combines with oxaloacetate to form citrate in the first reaction of he Krebs cycle, also known as the tricarboxcylic or citric acid cycle. The Krebs cycle supplies the majority of reducing equivalents for oxidative phosphorylation, whereby NADH and $FADH_2$ carry electrons from oxidized substrates to the mitochondrial respiratory chain, which couples electron transfer to oxygen with ADP phosphorylation through generation of a proton gradient across the inner mitochondrial membrane. Since oxygen is the final electron acceptor in this system,

mitochondria can be a primary producer of cellular reactive oxygen species (ROS) in many cell types. ROS regulate several physiological processes, such as the production and secretion of hormones, cytokines (communication between cells), the binding of G proteins to their receptors, transcription factors, and regulation of transporters and ion channels. ROS production also regulates mitochondrial fission and fusion (Willems 2015) which are important for cellular responses to stress and metabolic demands. However, ROS can also be harmful to cells when produced in large quantities, damaging organelles, and inducing cell death. The oxidative stress generated by the overproduction of ROS is associated with aging and pathologies such as obesity and type 2 diabetes, among others (A. Bindoli 1988).

Pregnancy itself is associated with persistently elevated oxidative stress, but both PE and GDM exacerbate these conditions. GDM leads to increased production of specific mitochondrial ROS in term human placentas (Hastie R 2014). Under conditions of hyperglycemia, glucose first via the glycolytic pathway followed by the Krebs cycle produces excessive NADH, increasing substrate availability for complex I (Wu J et al., 2016). Furthermore, hyperglycemia leads to the accumulation of advanced glycation end products (AGEs) in the placenta, and binding of AGE to its receptors activates the NADPH oxidase pathway, resulting in increased ROS production (Tag X et al., 2015). Mitochondrial respiration is reduced in pregnancies complicated by gestational diabetes mellitus (Muralimanoharan S 2016), as are antioxidant levels (Lappas 2011). Preeclampsia and gestational diabetes mellitus also markedly increase ROS production, which has been associated with damage to mtDNA and impariments in electron transport chain activity, ATP production, and metabolic activity, particularly in ROS-sensitive syncytiotrophoblasts (Vanderlelie 2005).

As described above, the placenta is a heterogeneous tissue, with differences in mitochondria morphology and function between proliferating cytotrophoblast cells and differentiated syncytiotrophoblast cells. Transmission electron microscopy images reveal cytotrophoblast mitochondria as being larger and containing lamellar cristae, whereas syncytiotrophoblast mitochondria are smaller with vesicular-shaped cristae (Martinez F et al., 1997). In addition, respiration rate was also examined in isolated cytotrophoblast cells from term placenta and after in vitro differentiation into syncytiotrophoblast, revealing increased oxygen consumption and resting ATP content in isolated cytotrophoblasts (Kolahi KS et al., 2017). Both baseline glycolysis and lactate production were stimulated by epidermal growth factor signaling through AKT in isolated cytotrophoblast cells. Overall, these data suggest that compared to syncytiotrophoblasts, cytotrophoblast cells are more glycolytically active and have a higher mitochondrial respiration rate (Kolahi KS et al., 2017). Table 1 summarizes reported differences between mitochondria from cytotrophoblast cells and syncytiotrophoblasts.

Table 1. Previously described mitochondrial characteristics comparing cytotrophoblast cells to syncytiotrophoblast.

	Cytotrophoblast cells	**Syncytiotrophoblasts**
Mitochondrial volume	large	small
Cristae	lamellar	vesicular
CYP450scc	low	high
Oxygen consumption rates	high	low
Glycolysis	high	low
Lactate production	high	low

Cardiolipin content	high	low
Apoptosis extrinsic, caspase intrinsic, pro-apoptotic proteins	 yes high	 absent low

References: Bustamante J. et al., 2014; Kolahi KS et al., 2017; Martinez F et al., 1997; Longtine MS et al., 2012; Fisher JJ et al., 2020, Clin Exp Pharmacol Physiol)

Proliferative cytotrophoblast cells differentiate and fuse to form syncytiotrophoblasts. Mitochondrial dynamics regulate many trophoblastic events, including cell proliferation, differentiation, and invasion/migration (Seok et al., 2020). Given the role of mitochondria in intrinsic apoptotic signaling, it is worth mentioning that apoptosis in placental villi is also primarily restricted to cytotrophoblast cells. Studies show that pro-apoptotic proteins, p53, BCL2-associated regulator of apoptosis X, and cytochrome c, are decreased in syncytiotrophoblast relative to the cytotrophoblast cells (Bustamante et al., 2014). In addition, apoptosis is an active process that requires energy, and mitochondria from syncytiotrophoblast appear to have reduced metabolic efficiency and ATP production compared to the cytotrophoblast cells. For example, in human term explants, immunofluorescence staining reveals cytotrophoblast cells undergo caspase-mediated (intrinsic) apoptosis following staurosporin (PKC inhibitor) treatment, but the syncytiotrophoblast does not. (Longtine et al., 2012). Alternatively, the fused syncytiotrophoblast layer covering placental villi is in direct contact with maternal blood and responsible for nutrient and gas exchange, where mitochondrial content is lower, and glycolytic/anaerobic ATP production is primarily used for steroidogenesis. In pregnancy disorders such as preeclampsia, gestational diabetes, polycystic ovarian disorders, and intrauterine growth restriction, abnormal mitochondria are evident through increased oxidative stress, impaired steroidogenesis, and increased apoptosis

(Correia et al., 2021, Longtine et al., 2012, Cuffe et al., 2017, Fisher et al., 2020). Finally, it has been proposed that in order to adapt to different stimuli, mitochondria are constantly changing through mitochondrial fusion and fission pathways (Fisher et al., 2020), where fission is thought to be a way for damaged mitochondria to be removed from cells and fusion of damaged mitochondria is way to preserve mitochondrial respiration (Westermann et al., 2010, Youle at al., 2012). Imbalances in these processes are also associated with placental dysfunction, including preeclampsia and gestational diabetes.

The recent discovery of steroid receptor transport and localization into mitochondria in cancer cells provides for novel potential roles for steroid hormone signaling in mitochondrial function. Of particular interest here is the observation that many pregnancy disorders that results from placental dysfunction not only are accompanied by abnormal mitochondrial respiration and morphology, but also abnormal androgen and androgen receptor levels (Fishe JJ et al., 2020, Kumar et al., 2018).

III. Androgen signaling in trophoblast cells

The placenta releases hormones into the maternal circulation, including androgens, estrogens, and progestogens. These hormones act in an endocrine manner on maternal and fetal tissue, and in a paracrine and autocrine manner within the placenta, affecting proliferation and differentiation of the placental trophoblast cells, the growth, and maturation of placental vascular network, and uterine endovascular invasion by the extravillous cytotrophoblast cells (Maliqueo et al., 2016)

Like progesterone and estrogen, androgen levels increase throughout pregnancy in women and are highest during the final trimester before parturition (Makieva et al., 2014). In addition to being a substrate for estrogen biosynthesis through CYP19A1 (aromatase) and secretion by the placenta, androgens play a role in placental development and function considering the presence of AR in trophoblast cells throughout pregnancy. In situ hybridization and immunocytochemistry techniques localized AR to cytotrophoblast cells, syncytiotrophoblast, extravillous trophoblast cells, as well as stromal and endothelial cells (Hsu et al., 2009) in the hemochorial placenta, suggesting a broader role for AR in placental physiology.

The AR gene resides on the X chromosome and contains 8 exons encoding a 919 amino acid protein. At least 18 different splice variants have been discovered (Lu and Luo, 2013) and recent studies revealed that in addition to full-length AR, three additional transcripts are present in human and sheep placentas. An AR45 (45kDa) variant which contains the DNA-binding hinge and ligand-binding domain, but lacks most of the N-terminal domain and C-terminal domain), and ARV1 (76kDa) and ARV7 (75kDa) variants both containing the N-terminal domain and DNA binding domains (Meakin et al., 2021, reviewed in Parsons and Bouma, 2021). The AR45 variant results from an alternate exon 1b between exon 1 and 2 with unique 7 amino acid long sequence in the N-terminal domain, and is primarily expressed in cardiac tissue (Ahrens-Fath I et al., 2005). AR45 has been detected in a variety of placental mammals, including non-human primates, elephant, dog and pig, but appears absent in mice and rats (Weiss et al., 2007). Its expression in the placenta is noteworthy, as this truncated transcript acts as a dominant negative regulator of the full-length protein *in vitro*, suggesting that AR45 could modulate androgen signaling within the placenta.

III.A. AR in normal pregnancy

Testosterone (T) and dihydrotestosterone (DHT) need to bind to either an intracellular receptor (AR), or to membrane-bound receptors such as G protein-coupled receptor family C group 6 member A GPRC6A (Parsons and Bouma, 2021), to exert cellular responses. When ligands T or DHT bind to AR a conformational change occurs which exposes the nuclear localization signal in the hinge domain, allowing interaction with importin-α which facilitates its nuclear translocation. Once inside the nucleus, ligand-bound AR dimerizes and bind an Androgen Response Element (ARE; AGAACAnnnTGTTCT) in promoter regions of AR-regulated target genes, leading to either activation or suppression of expression.

In the endometrium, androgens are thought to play a role in stromal cell proliferation (Xu J et al., 2015). Androgen and progesterone receptors regulate distinct gene networks and cellular functions in decidualizing endometrium (Cloke et al., 2018). In fact, androgen signaling promotes differentiation of endometrial stromal cells into secretory cells (decidualization) and is necessary for endometrial receptivity and early establishment of pregnancy by regulating endometrial gene expression [(Gibsonet al., 2016) and cell migration and trophoblast invasion (Hirschberg et al., 2021). Finally, testosterone signaling through AR stimulates VEGFA and human myometrial endothelial proliferation (Dietrich et al., 2011).

Like progesterone and estrogen, androgen serum levels increase during, and placental AR is present throughout normal pregnancy, however relatively little is known about the role of endogenous androgen signaling in placental function during pregnancy. Instead, most data

obtained on androgens on placental development and function comes from pregnancy disorders associated with abnormally high levels of androgens such as PE, GDM and PCOS.

III.B. AR in perturbed pregnancy

III.B.I Preeclampsia (PE)

According to the CDC, every year in the United States, 6% to 8% by PE. PE is a primate-specific pregnancy disorder characterized by hypertension and proteinuria that can lead to maternal and fetal morbidity and mortality (Venkatesha et al., 2006). In the case of PE, the placenta is considered the etiology of the pregnancy disorder, since there is a failure in the remodeling of the uterine spiral arteries by the extravillous trophoblast that is essential to promote blood flow to the placenta. This defect in the remodeling of the spiral artery supports the development of pregnancy disorders mediated by the placenta, which has impact on maternal cardiovascular system and, also affects fetal development by causing fetal growth restriction.

Women with PE contain increased serum levels of testosterone (Lan et al., 2020, Kumar et al., 2018). Furthermore, placental AR levels are significantly higher regardless of the sex of the fetus in women with PE (Sathishkumar et al., 2012), and serum testosterone levels are even more elevated in women with PE pregnant with a male fetus (Steier et al., 2002). In fact, females that experienced a PE pregnancy while in utero tend to have higher serum levels of testosterone when they reach puberty and are at increased risk for cardiovascular disease in adulthood (Alsnes IV et al., 2016, Vyes AK et all, 2016). Elevated testosterone during PE is thought to be responsible for systemic hypertension by reducing endothelial NOS activity (Chinnathambi V et al., 2013).

Placentas from women with PE and accompanying preterm delivery have a variety of maternal vascular malperfusion pathologies, including more advanced and hypoplastic villous maturation and decidual vascular abnormalities (Fillion et al., 2021), although a role for androgen signaling in these processes are unknown. Increasing testosterone synthesis through overexpression of CYP11A1 in *in vitro* and *in vivo* studies revealed a significant increase in trophoblast autophagy as well as invasion, and this effect was alleviated using the AR antagonist flutamide (Pan T et al., 2017). This suggest that elevated testosterone and AR signaling are involved with shallow invasion of trophoblast cells and the etiology of PE. Finally, elevated testosterone negatively impacts placental amino acid but not glucose transport in rats by reducing expression of system A amino acid transporters (Slc38a2) (Sathishkumar K et al., 2011). This could explain the observed decreases in fetal weight observed in sheep (Hampton et al., 2004, Cleys et al., 2012), rats (Sun M et al., 2012), and some humans (Sir-Petermann et al., 2005) associated with pregnancies that are accompanied by elevated testosterone levels.

III.B.II Gestational diabetes mellitus (GDM)

In the US, 2% to 10% of pregnancies are affected by GDM (CDC). GDM is defined as diabetes diagnosed during pregnancy. This disorder is associated with adverse pregnancy outcomes, including fetal macrosomia, fetal death, neonatal metabolic disturbances, and other related problems (O'Sullivan JB et al.,1966). During GDM, it is thought that increased maternal glucose but not insulin transfers through the placenta and leads to fetal hyperglycemia and hyperinsulinemia ultimately resulting in increased protein and fat metabolism and macrosomia (Kamana et al., 2015).

Hyperandrogenemia is associated with GDM. Recent reports suggest that the placentas of GDM pregnancies contribute to elevated serum testosterone due to a decrease in the conversion of testosterone to estrogen (Uzelac et al., 2010). In addition, GDM placentas have significantly elevated amounts of AR. Although it is unclear if or how this increase in contributes to the observed placental abnormalities, studies in rat cerebral arteries have demonstrated that testosterone increased vasoconstriction through the thromboxane A2 signaling pathway by increasing endothelial cell thromboxane A2 synthesis (Gonzales et al., 2005). Thromboxane A2 regulates vascular tone in human placentas, and thromboxane A2 receptors are present in placental blood vessels (Walters et al., 1991). Consequently, GDM placentas exhibit a variety of pathological features, including villous immaturity, chorangiosis (extreme villous hypervascularity), and villous fibrinoid necrosis (Daskalakis G et al., 2008). In addition, significant increases in umbilical arterial pulsatility index (increased vascular resistance) have been reported in ischemic villitis (inflammation of chorionic villi) of pregnant women with GDM, with corresponding increases in thromboxane A2 (Saldeen et al., 2002).

III.B.III. Polycystic ovarian syndrome (PCOS)

PCOS, a dysfunctional ovarian disorder associated with hyperandrogenism and anovulatory cycles. The prevalence of PCOS ranges from 6% to 15% in women of reproductive age (Kumarapeli V et al., 2008). PCOS women are at risk of early pregnancy loss (EPL), defined clinically as first trimester miscarriage. EPL occurs in 30 to 50% of PCOS women compared with 10 to 15% of normal women (Gray et al., 2000). Moreover, GDM complicates 40 to 50% of PCOS pregnancies (Veltman-Verhulst SM et al., 2010).

Circulating androgen levels are at least 3-fold higher in women with PCOS, perhaps due in part to increased placental activity of 3B-HSD1 and reduced activity of aromatase, leading to greater levels and secretion of testosterone (Maliqueo et al., 2013). Other pathologies in PCOS include chronic villitis (inflammation) and intervillositis (Palomba et al., 2013). Recently, Risal and colleagues reported female offspring of mothers with PCOS are more likely to develop PCOS, and in mice transgenerational susceptibility to PCOS was observed in offspring of pregnant females injected with dihydrotestosterone (Risal S et al. 2019). These data highlight the potentially detrimental long-term impacts of elevated androgens and androgen signaling on placental function, fetal programming and development, and postnatal health.

A well-established model of developmental programming of PCOS has been developed and studies in sheep, where pre-natal androgenization leads to development of the metabolic, neuroendocrine and reproductive PCOS phenotypes in offspring (Cardoso et al., 2019, Abbott et al., 2019). Placental analysis in this animal model of PCOS revealed significant increases in placental lipid accumulation and collagen deposition (Kelley et al., 2019). In addition, HIF1A and VEGF expression was increased at in placentas at gestational day 90 (term is gestational day 135), and this increase in VEGFA was also observed in our own studies (Cleys ER et al., 2015). Although increased HIF1A would suggest hypoxic conditions, increased VEGFA appears to correlate with the increased vasculature and presence of “type D” placentomes in prenatal androgenized ewes (Becket EM et al., 2014; Cleys ER et al., 2015), which morphological resemble overgrowth of cotyledonary (fetal) tissue. These data indicate that supraphysiological amounts of testosterone, likely through AR (see below), activate angiogenic networks to increase placental vasculature.

III.C. AR in placental angiogenesis

The role of androgens in placental angiogenesis is complex. Chromatin immunoprecipitation studies in sheep revealed that AR binds an ARE in the vascular endothelial growth factor A (VEGFA) promoter suggesting a role for AR signaling in placental angiogenesis (Cleys ER et al., 2015). However, testosterone treatment in rats leads to both up-regulation (e.g., *Nos3, Angptl4, Emcn, Edn1, Flt, sFlt, Cxcr4, Bmp4, Plau* and Il1b) and down-regulation (e.g., *Stra6, Dhcr7, Arid1a, Ccr3, Ptprj, Col1a2, Lef1, Col1a1* and *Mmp2*) of angiogenesis-related genes in the placenta, ultimately resulting in decreased umbilical arterial diameter, total length of vasculature and branching (Gopalakrishnan et al., 2016). Considering the higher-than-normal amount of testosterone, and the potential involvement of AR was not investigated it is unclear how AR signaling contributes to normal placental angiogenesis.

Testosterone signaling in vascular cells involves AR, and AR is present in arterial walls, vascular smooth muscle cells, endothelial cells, macrophages, monocytes thereby having diverse effects on vascular function (Wu FC et al., 2003; Liu PY et al., 2003). At physiological concentrations, testosterone causes vasodilation in isolated human pulmonary arteries (Rowell et al., 2009), but beneficial, neutral and detrimental effects on vascular reactivity have also been reported leading to vasodilation or vasoconstriction via endothelial-dependent and independent mechanisms (Wu FC et al., 2003). Ultimately, functional studies modulating AR function in vivo are necessary to provide insight into the role of AR signaling in placentation.

IV. Androgen signaling in placental mitochondria

Recent studies have demonstrated the presence of nuclear steroid receptors in mitochondria, further expanding the potential impact of steroid hormones on trophoblast cell physiology. Steroid hormones, either through their classical nuclear pathway or directly within the mitochondria, can regulate mitochondrial respiration and oxidative phosphorylation (Kobayashi A et al., 2020). For example, T increases mitochondrial activity and expression of mtDNA-encoded mRNA through AR-mediated upregulation of *Nrf* transcription, which in turn increases expression of TFAM, mitochondrial transcription factor A (Pronsato et al., 2019). In addition, AR binds mtDNA to activate (or repress) expression of core subunits of mitochondrial respiratory complexes (see Table 2).

IV.A Steroid receptors in mitochondria

IV.A.I Glucocorticoid receptor

Glucocorticoid receptors (GR), members of the nuclear receptor superfamily, through the binding of their ligand (cortisol) regulate cell metabolism, immune response, and development. In addition to the classical nuclear mechanism, the presence of GR in the mitochondrial interior has been observed (Scheller et al., 2000). In addition, mtDNA exhibits the glucocorticoid response element (GRE) consensus sequence, particularly for the cytochrome c oxidase I and III genes and the D-loop region of mtDNA (Demonacos C et al., 1995). This would indicate that the GR present in the mitochondria may be regulating mitochondrial transcription independently of their nuclear function. Experiments in which nuclear polymerase was inhibited showed that administration of dexamethasone stimulated the synthesis of several mitochondrial-encoded OXPHOS genes (Psarra et al., 2011). On the other hand, studies in the thymus showed that mitochondrial GR induces

apoptosis after dexamethasone treatment, as mitochondrial import of GR correlates with the release of cytochrome c and the activation of caspase 3 (Talabér G et al., 2009).

IV.A.II Estrogen receptor

Estrogen receptors (ESRs), members of the nuclear receptor superfamily, are highly homologous proteins except for the DNA-binding domains that make ESR2 a poor transcription factor compared to ESR1. In addition to its classic function as a nuclear transcriptional factor, ESR is localized and acts in the mitochondria, regulating apoptosis and reducing ROS by activating the enzyme manganese superoxide dismutase (MnSOD) (Pedram A et al., 2006).

IV.A.III Androgen receptor

Non-genomic actions of androgens have extensively been described and usually refer to rapid mediated effects on intracellular second messenger systems via the plasma membrane. More recently, androgen binding and the presence of AR were demonstrated in mitochondria of prostate cancer tissue and cells (Bajpai P, et al. 2019). The first 36 amino acids of the AR N-terminal domains contain a mitochondrial localization sequence, and the removal of this sequence prevented AR localization into the mitochondria. Within mitochondria, AR regulates the expression of mtDNA-encoded oxidative phosphorylation (OXPHOS) complex subunits (Table 2). AR also controls the translation of genes encoded by mtDNA by regulating the expression of multiple mitochondrial ribosomal proteins encoded by nuclear DNA. Overexpression and deletion experiments of AR in prostate cancer cell lines negatively regulates mtDNA content and OXPHOS complex protein expression, assembly, stability, and activity (Bajpai P, et al. 2019).

Table 2. Mitochondrial genes in the nucleus and mitochondria regulated by AR.

Regulated by AR	
Nucleus	*TFAM (Mitochondrial transcription factor A),* NADH dehydrogenase (NDUFB8), succinate dehydrogenase (SDHA), ubiquinol-cytochrome c reductase (UQCRC2), Cytochrome c oxidase polypeptide 5 (COX 5), ATP synthase F1 subunit alpha (ATP5A)
Mitochondria	*NADH dehydrogenase 1 (MT-ND1), NADH dehydrogenase 4 (MT-ND4), Cytochrome c oxidase subunit II (MT-CO_2)*

Alternatively, AR knockdown in induced pluripotent stem cell (iPSC)-derived motor neurons reduces mitochondrial ATP production, suggesting an essential role for AR in maintaining OXPHOS capacity (Pourshafie N et al., 2020). Similarly, granulosa cells isolated from systemic (global) AR knockout mice have reduced ATP content (Wang RS et al. 2015), further suggesting that AR may be required for mitochondrial ATP production. Therefore, AR signaling appears to be necessary for maintaining mitochondrial function, but excessive activation may disrupt optimal mitochondrial activity. Recently we identified AR in ACH-3P and Swan-71 cell mitochondria, two well-established human trophoblast cells lines (Figure 1). This preliminary data suggests that AR may also directly regulate mitochondrial function in trophoblasts, which could impact ATP-dependent processes such as apoptosis and steroidogenesis.

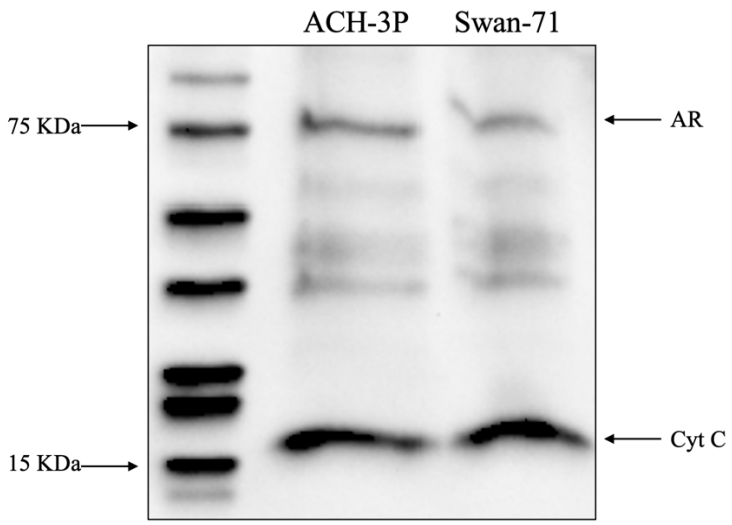

Figure 1: Androgen receptor expression in trophoblasts mitochondria enriched fraction. AR protein (ab74272) expression in ACH-3P and Swan-71 mitochondrial isolates [enrichment confirmed by robust expression of cytochrome c (sc-13156)] lysates by immunoblot.

IV.B. Pregnancy disorders with abnormal androgen levels and mitochondrial dysfunction

Mitochondrial abnormalities have been described in PE placentas. PE placentas are characterized by a higher total antioxidant capacity with higher levels of MnSOD compared to controls (Holland OJ et al. 2018). Excessive mitochondrial ROS production may contribute to increases in antioxidant enzyme expression and oxidative stress in PE placentas and GDM (Holland OJ et al., 2018; Murphy et al., 2018). PE placentas have lower mitochondrial content and a greater abundance of key components of glycolysis, suggesting a shift from oxidative phosphorylation to anaerobic glucose metabolism. Consistent with this finding, markers of mitochondrial biogenesis are reduced in PE, while the regulators of mitophagy, autophagy and mitochondrial fission tend to be higher compared to controls. This suggests that trophoblast mitochondrial stress or damage is occurring in PE placentas, potentially contributing to placental dysfunction in this condition.

In women with GDM greater superoxide dismutase activity was observed in syncytiotrophoblast compared to controls, suggesting chronic elevations in placental ROS production (Fisher et al., 2021). This was associated with greater mitochondrial production of progesterone in placenta in women with GDM, suggesting an impact on the steroidogenic function of mitochondria. Furthermore, reduced expression of mitochondrial respiratory Complexes II and IV components leads to reduced ATP production in GDM placental cells (Muralimanoharan et al., 2016). Interestingly, isolated cytotrophoblast cells from GDM placentas demonstrated decreased oxidative phosphorylation compared to non-GDM placentas indicated by reduced oxygen consumption rates in under basal and substrate-stimulated conditions, however this difference was not observed when GDM cytotrophoblast cells were differentiated into syncytiotrophoblasts (Valent AM et al., 2021). Instead, GDM syncytiotrophoblast exhibited decreased expressed of syncytin-1 (necessary for cell fusion) and hCG. Moreover, mitochondrial fusion factors mitofusin 1, mitofusin 2 and optical atrophy 1 were all significantly lower in placental tissue from GDM women (Kolac UK et al., 2021). These observed changes in mitochondrial function may have diverse impacts on trophoblast that ultimately impact placental function in GDM.

Both GDM and PCOS are often associated with metabolic disease (type II diabetes, obesity), and similar to GDM it is not surprising mitochondrial dysfunction has been reported in women with PCOS. However, very little is known about mitochondrial function in placenta of PCOS women. Recent studies in a rodent model of PCOS indicate increased placental ROS production in animals co-treated with DHT and insulin, and a decrease in mtDNA copy number. However, DHT or insulin treatment individually had no effect on placental ROS, but an increase in mtDNA copy number (Zhang Y et al., 2019). Ultrastructure analysis via transmission electron microscopy

revealed morphological changes in DHT and insulin co-treated animals, with decreased electron density of the mitochondrial matrix, and swelling, blebbing and intracristal dilation. Finally, DHT and insulin co-treatment was reported to increase placental necroptosis, a pro-inflammatory for of cell death (Zhang Y et al., 2020)

Considering the correlation of abnormal androgen signaling and similar placental mitochondrial dysfunction, and the recent observation that steroid receptors, including the AR, it is tempting to speculate that impaired androgen signaling in mitochondria could play a role in the placental pathology and pregnancy complications that accompany PE, GDM and PCOS.

V. Conclusion and Future Directions

Proper placental development and function is critical for normal fetal tissue programming and postnatal health. Pregnancy disorders such as PE, GDM and PCOS are all accompanied by placental dysfunction and is associated with abnormal steroid levels including androgens. Aside from the potential negative impact of elevated androgens on both maternal and fetal tissues, the placenta is a direct target of androgens and contains AR. Therefore, impaired trophoblast cell function due to altered androgen signaling may contribute to placental malformation that can compromise nutrient transport and gas exchange to the developing fetus.

Importantly, most of our understanding of the possible functions of AR in placental physiology comes from *in vitro* experiments in trophoblast cells or *in vivo* studies that employed supraphysiological doses of steroids or synthetic analogs. Based on these studies, AR signaling may impact blood flow to the developing fetus by influencing the development and function of the

placental vascular network. Emerging evidence for a role of ARs in regulating trophoblast mitochondrial function may further explain impacts on placental steroidogenesis and apoptosis. The recent observation of AR in mitochondria further emphasizes the potential impact of androgens on placental biology, particularly given the role of mitochondria in steroidogenesis known to be dysregulated in women with PE, GDM, and PCOS.

There are several investigative avenues that could be pursued to further elucidate the role of steroid hormone signaling in placental function. Development of gene-targeting and gene-editing techniques to manipulate gene function specifically in the placenta can aid in elucidating placental steroid receptor signaling in vivo (Anthony RV et al., 2010; Hord TK et al., 2020). In addition, development of antibodies and agonists/antagonists for the different AR isoforms will further help clarify the functions of these receptors in trophoblast development, differentiation, and function. For example, antibodies that can distinguish between the AR variants will enable a better understanding of their (sub)cellular presence in placental tissues or used to inhibit their function. Finally, the presence of both AR within mitochondria greatly expands their potential impact in trophoblast cells. Future studies aimed at preventing mitochondrial localization by mutating or deleting the mitochondrial localization signal could aid uncovering the role for mitochondrial AR signaling. Combined with recent developments using placental specific gene targeting approaches, there are exciting opportunities for delineating the independent and interactive functions of placental AR signaling emanating from cytoplasmic/nuclear and mitochondrial compartments on pregnancy and fetal development.

The overall goal of this thesis is to provide novel insight into the possible role of AR signaling in trophoblast cells through their action on mitochondrial function. We hypothesize that AR are present in trophoblast mitochondria and that testosterone treatment decreases mitochondrial oxygen consumption and increases ROS production. Ultimately, our reported findings will provide novel insight into the mechanism of trophoblast mitochondrial dysfunction in pregnancy disorders associated with hyperandrogenism.

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CHAPTER 2

IMPACT OF TESTOSTERONE TREATMENT ON TROPHOBLAST MITOCHONDRIAL FUNCTION

Summary

Proper placental development and function are critical for pregnancy maintenance and maternal, fetal, and postnatal well-being. The placenta is comprised of trophoblast cells that are rich in mitochondria, which play important roles in placental energy metabolism, physiology and pathophysiology. Given the logistical and ethical limitations of studying the placenta during pregnancy, investigators frequently use trophoblast cell lines to model placental biology *in vitro*. Characterization of these cell lines is important for selecting those best suited to the processes and hypotheses being investigated. Herein, we evaluated mitochondrial function in three human trophoblast cell lines (ACH-3P, BeWo and Swan-71) and an immortalized ovine trophoblast cell line (iOTR) using high-resolution fluorespirometry. ACH-3P exhibited the highest rates of mitochondrial oxygen consumption and respiratory control of the 4 cell lines tested, and robust expression of androgen receptor protein found to co-localize with mitochondria by immunoblot and immunofluorescence. Pregnancy disorders such as pre-eclampsia, gestational diabetes, and intrauterine growth restriction are associated with high androgen levels, so the effects of testosterone exposure on ACH-3P mitochondrial function was tested. Acute administration of testosterone (10 uM) tended to decrease rates of mitochondria respiration and increase release of reactive oxygen species (ROS; H_2O_2), while chronic (7 days) of testosterone exposure (10 uM)

markedly increased mitochondrial content, respiration rate, and ROS release. Taken together, these studies establish ACH-3P as a robust cell line for studying trophoblast mitochondrial function and provide foundational evidence for deleterious effects of androgen exposure on placental mitochondrial ROS production, with potential relevance to several pregnancy disorders.

I. Introduction

The placenta is a highly dynamic and metabolically active transitional organ with significant energy requirements necessary for nutrient transfer, hormone synthesis, and fetal growth. It uses a variety of substrates to meet its energy needs and has a higher rate of oxygen (O_2) consumption than many other fetal and adult tissues. Indeed, O_2 consumption by the human or ovine term placenta accounts for 40 percent of the total O_2 consumption by the gravid uterus (Carter et al., 2000). Placental function, therefore, is critically dependent on mitochondria for energy (ATP) production, as well as steroidogenesis and regulation of apoptosis (Miller et al., 2013).

Oxidative phosphorylation (OXPHOS) is the primary mechanism of mitochondrial ATP production and cellular aerobic metabolism, but also produces reactive oxygen species (ROS) as a byproduct. Under normal homeostatic conditions, most mitochondrial ROS are neutralized by endogenous cellular antioxidant systems. However, when ROS production exceeds the capacity of antioxidants, oxidative stress occurs, which can alter cellular processes, damage cellular components, and trigger apoptosis (Neale et al., 2005). Pregnancy itself is associated with some degree of placental oxidative stress, but this is increased to pathologic levels in disorders such as preeclampsia and gestational diabetes (Marín et al., 2020).

Despite the increasing appreciation of the importance of mitochondrial function and placental metabolism, surprisingly little is known regarding the mechanisms that impact mitochondria during placental dysfunction. Elevated androgen levels are associated with several pregnancy disorders including preeclampsia, gestational diabetes and intrauterine growth restriction (Sharifzadeh F et al., 2012, Morisset AS et al., 2013), where altered mitochondrial morphology and function have also been reported (Marín et al., 2020). While androgens are

increasingly recognized as potential regulators of mitochondrial biology in a variety of cell types (Ahmad I et al., 2022), whether they directly impact placental mitochondrial function is unknown.

Given the logistical and ethical limitations of studying placental mitochondrial function during pregnancy, cell lines representing different types and phenotypes of trophoblast cells have been used for decades to investigate placental cell biology. However, each cell line has unique genetic and phenotypic characteristics that must be characterized in order to select a model best suited to the biological process and context being studied. Therefore, the primary aim of this study was to characterize the mitochondrial phenotype of three common human trophoblast cell lines to determine their suitability for evaluating placental mitochondrial responses to exogenous stimuli *in vitro*. An ovine trophoblast cell line was also investigated given the common use of sheep to study pregnancy and placental biology (Barry JS et al., 2008). These studies revealed a robust mitochondrial phenotype of the ACH-3P human trophoblast cell line, which was subsequently used to study the impact of acute and chronic androgen exposure on trophoblast mitochondrial function. Our results provide a foundation for selecting trophoblast cell lines for the investigation of placental mitochondrial function *in vitro* and suggest mitochondrial ROS production as a potential link between androgen exposure and placental oxidative stress in common pregnancy disorders.

II. Materials and Methods

II.A. Selected trophoblast cell line characteristics

For this study, ACH-3P, BeWo, and Swan-71 human trophoblast cells were used. ACH-3P cells are a first-trimester trophoblast cell line, established by the fusion of primary human first-trimester trophoblasts (gestational week 12) with a human choriocarcinoma cell line (AC1-1). ACH-3P not

only display molecular markers of extravillous trophoblast but is also capable of invading extracellular matrices, making them a useful tool for migration and invasion studies; on the other hand, their gene signature do not resemble primary trophoblast cells and do not fuse (Hiden U et al., 2007). BeWo cells are a human placental cell line that originated from choriocarcinoma and are regularly used as a model for placental villous trophoblast cell fusion and syncytialization and transplacental transport; a disadvantage of this line is that the molecular signatures resemble cytotrophoblast, syncytiotrophoblast and extravillous cytotrophoblas (Pattillo RA et al., 1968). Finally, Swan-71 cells are an hTERT-infected first trimester trophoblast cell line; expresses markers of differentiated trophoblast cells and also often used to study trophoblastic invasion in vitro (Straszewski-Chavez SL et al., 2009). In addition, an ovine iOTR cell line (immortalized ovine trophoblast cells virally infected with hTERT) (Ali A et al., 2020) was also characterized given the common use of sheep to study pregnancy and placental biology (Barry JS et al., 2008).

II.B. Cell culture and mitochondria isolation

ACH-3P and Swan-71 cells were grown in Cytiva HyClone™ Ham's Nutrient Mixture F12 medium (cat# SH30026.01) with 10% FBS and 1% penicillin-streptomycin-amphotericin (Fisher/VWR 30-005 cr). BeWo cells were cultured in ATCC F-12K medium (Kaighn's modification of Ham's F-12 medium) with 10% FBS and 1% penicillin-streptomycin-amphotericin. iOTR was cultured in DMEM/Ham’s F12 Nut Mix (1:1) base media (cat # D8062 from Sigma) with 10% FBS, 1% PSA mixture (Fisher/VWR 30-005 cr), 1% sodium pyruvate (Fisher/VWR 360-070), 1% L-glutamine (Fisher/VWR 25030-081), 1% NEAA (non-essential Amin Acids) (Fisher/VWR 40-050) and 0.5% insulin (sigma 10516-5ML). When cells reached 80

or 90% confluency in a 10 cm dish, they were harvested for respiration studies and/or mitochondrial isolation.

To isolate mitochondria, two 15 cm culture dishes were grown to 90% confluency. Mitochondria were isolated following the protocol described by Frezza for cells (Frezza et al., 2007). Cells were released from culture plates with 0.05% trypsin during a 5 min incubation at 37°C. Released cells were pooled and collected by centrifugation at 1000 g for 5 min. The cell pellet was resuspended in 2 ml of ice-cold buffer IB and homogenized using a Teflon mortar operated at 1,600 rpm. in an ice bath. The pellet was resuspended in 2 ml of fresh buffer IB containing (in mM): 200 Sucrose, mM Tris-MOPS, 1 EGTA-Tris, then centrifuged at 600 g for 10 min at 4°C. The supernatant was collected and centrifuged at 7,000g for 10 min at 4°C three times before resuspending in KME medium (100 mM KCl, 50 mM MOPS, 0.5 mM EGTA) for a final centrifugation at 7,000g for 10 min at 4°C. The final pellet (mitochondria-enriched fraction) was resuspended in 150 µl of MiR05 for fluorespirometry experiments or frozen for subsequent biochemical analyses.

II.C. Measurement of trophoblast mitochondrial function

Mitochondrial function was investigated in intact trophoblasts, digitonin-permeabilized cells, and mitochondrial-enriched fractions using a Oxygraph O2k-FluoRespirometer (Oroboros Instruments, Innsbruck, AT) consisting of two temperature-controlled chambers (37°C) containing a polarographic oxygen sensor and a custom-fitted fluorometer (O2k-Fluo LED2 module) enabling simultaneous monitoring of chamber oxygen (O_2) and H_2O_2 content as previously described in detail (Li Puma LC et al., 2019). Experiments were performed in 2 mL of MiR05 respiration medium containing (in mM) 0.5 EGTA, 3 $MgCl_2$ hexahydrate, 60 lactobionic acid, 20 taurine, 10

KH_2PO_4, 20 HEPES, 110 sucrose, and 0.1% BSA, pH 7.1 with KOH, which has been previously shown to have the highest stability and sensitivity for simultaneous measurement of O_2 and H_2O_2 flux in cells and mitochondrial isolates compared to other common mitochondrial respiration media (Komlódi T et al., 2018). Standardized instrumental and chemical calibrations were applied using Datlab software (Oroboros Instruments, Innsbruck, Austria) for accurate measurement of sample oxygen consumption rate (JO_2), which was normalized to sample protein content determined by BCA assay (Pierce, ThermoFisher). The net rate of sample H_2O_2 release (JH_2O_2) was measured during respirometry experiments by monitoring the accumulation of chamber resorufin (Ex/Em 571/585 nm), the stable fluorescent product of 1:1 oxidation of Amplex UltraRed (5 μM) by H_2O_2 in the presence of horseradish peroxidase (1 U/mL) (Starkov AA et al., 2010). Care was taken to avoid exposure of the AmR to light and to collect data within a narrow range of chamber [O2] (+/- 50 μM) during experiments to avoid confounding effects on sample JH_2O_2 measurements (Li Puma LC et al., 2019).

Fluorespirometry protocols were initiated by measurement of basal JO_2 and JH_2O_2 of intact cells, followed by the addition of digitonin (10 μg/ml) for selective permeabilization of the cell membrane, leaving mitochondrial membranes intact (Doerrier C et al., 2018). Saturating concentrations of respiratory substrates were then added to permeabilized cells (1 mM malate, 5 mM pyruvate, 10 mM glutamate, 10 mM succinate) to generate the non-phosphorylating "LEAK" JO_2 facilitated by proton leak across the inner mitochondrial membrane, followed by the addition of 2.5 mM ADP to stimulate the maximal OXPHOS-linked JO_2. The extent of respiratory control by ADP was expressed as the OXPHOS coupling control factor [1-(LEAK/OXPHOS)], where a maximal value of 1.0 represents fully coupled mitochondria (100% control of respiration by ADP), and 0 represents fully uncoupled mitochondria (0% respiratory control).

II.D. Acute action of testosterone on ACH-3P cells

To evaluate the acute effect of testosterone on ACH-3P cells, the same protocol mentioned above was performed in the Oxygraph, with the addition of 10nM Testosterone (Sigma-Aldrich, cat# T1500) after ADP treatment to one chamber and 20 µl of diluent (control) to the other (second) chamber. This concentration of testosterone corresponds to placental levels of testosterone in normal pregnancy (Shin YY et al., 2018).

II.E. Chronic Testosterone Action on ACH-3P cells

To evaluate the effect of chronic testosterone on ACH-3P, 10 nM testosterone was added to a 10 cm dish with medium containing no phenol red, and cultured for one week at 37 °C. The protocol was then performed as previously described in the Oxygraph, on treated and control (vehicle-treated) cells.

II.F. Protein extraction and Western Blot

Protein samples were extracted with a 100 ul solution of RIPA Lysis and Extraction Buffer (Thermo Scientific, cat# 89900) and the addition of 1 ul of protease and phosphatase inhibitor cocktail and 1 ul of EDTA (Thermo Scientific, cat#78440), followed by sonication and centrifugation for 10 minutes at 4 °C. Protein concentration was determined using a BCA assay (Thermo Fisher, cat# 23227). A total of 30 µg of protein sample was loaded and separated by 4-15% SdS-PAGE (Bio-Rad Mini-PROTEAN TGX Gels, Cat# 4561084) and then transferred to nitrocellulose membranes (Thermo scientific, PVDF Transfer membrane, cat# 88518). Membranes were subsequently blocked for 1 h with 5% skim milk in TBS containing 0.05%

Tween-20 (TBS-T) at room temperature. After blocking, membranes were incubated with a total OXPHOS human antibody cocktail (Abcam, ab110411; 1:1000) or a rabbit polyclonal androgen receptor antibody (Abcam, ab74272; 1:1000), overnight at 4C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:3000, Abcam ab6789 or ab6721) in 5% skim milk with TBS-T for 1 hour at room temperature. SuperSignal™ West Dura Extended Duration Substrate Reagent (Thermo Scientific™, Cat# 34076) was used to visualize antibody binding on the ChemiDoc XRS+ System (Bio Rad, cat#1708265).

II.G. Immunofluorescence staining

ACH-3P cells were cultured in a chamber slide (Millipore Sigma, cat# PZGS0416) for 24 hours. The media was discarded, and the cells were washed 3 times with PBS (Corning, cat#21-040-CV) for 5 minutes each. The cells were fixed with 1 ml of methanol for 10 minutes and washed with PBS 3 times for 5 minutes each. The slide was blocked with 1% normal goat serum (Ab7481) at 37 C for 30 minutes followed by 10 minutes at room temperature. After washing one time with PBS, a rabbit polyclonal androgen receptor antibody was added (Abcam, ab74272) and a mouse monoclonal cytochrome c antibody (Santa Cruz, sc-13156) and incubated overnight at 4°C. Thereafter, the slide was washed 3 times, 5 minutes each with PBS, and incubated with secondary antibody Alexa Fluor 555 anti-rabbit (ThermoFisher, cat# A-31572) and Alexa Fluor 647 anti-mouse (ThermFisher, cat# A-31571), for 1 hour at room temperature. Following 3 washes with PBS, the slide was covered with a mounting solution containing DAPI (Vectashiel HardSet antifade mounting medium with DAPI) for DNA staining. For negative control, the primary antibody was replaced by PBS. The pictures were obtained with a Bx63 Olympus microscope at 40x magnification and a Hammatsu Flash4.0 digital CMOS camera.

III. Results

III.A. Mitochondrial respiratory function in trophoblast cell lines

A primary goal of this study was to characterize the mitochondria functional phenotype of three well-characterized and often used human placental cell lines (ACH-3P, BeWo, Swan-71), as well as an immortalized ovine trophoblast cell line (iOTR) using an Oxygraph-2k high-resolution fluorespirometer. Basal rates of oxygen consumption (JO_2) normalized to total cellular protein content were 2-3 fold higher in ACH-3P and iOTR cells, suggesting greater mitochondrial content of these cell lines compared to Swan-71 and BeWo (**Figure 2A**). Following permeabilization of cells with digitonin to enable a direct supply of substrates to cellular mitochondria, trends of JO_2 in the LEAK state were similar to basal JO_2 across cell lines, while OXPHOS rates (following the addition of ADP) were much higher in ACH-3P compared to all cell lines (**Figure 2B**). The calculated extent of respiratory control by ADP was highest in ACH-3P and iOTR cells, indicating a greater degree of OXPHOS coupling control in these cell lines compared to Swan-71 and BeWo (**Figure 2C**). Taken together, these results demonstrate that ACH-3P cells have the most robust mitochondrial respiratory phenotype of the four cell lines tested.

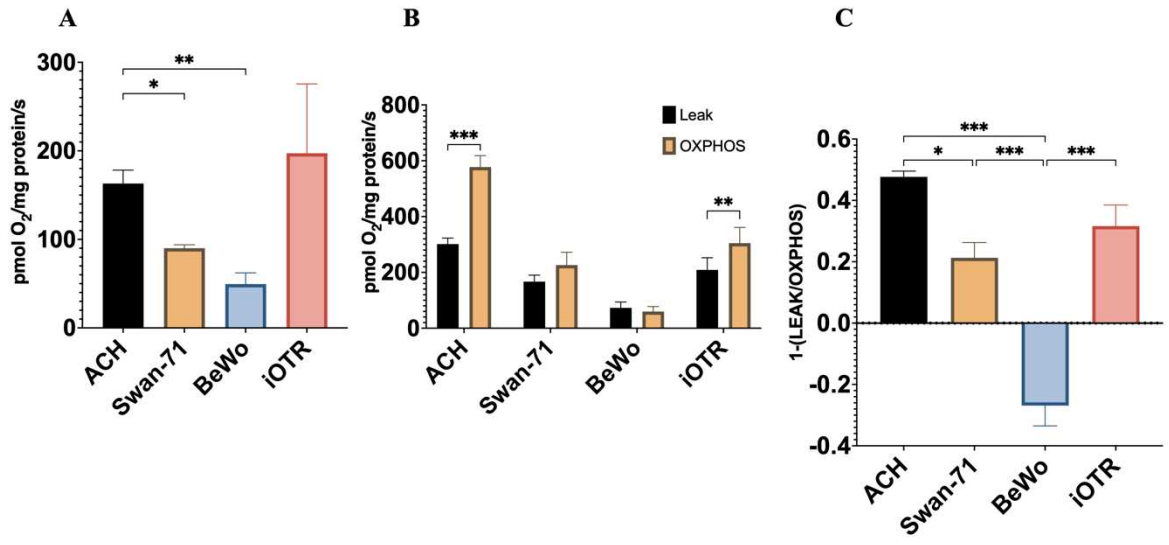

Figure 2: Mitochondrial respiratory function of common trophoblast cell lines. (A) Basal respiratory rate of intact trophoblasts prior to permeabilization. (B) Respiration rates of digitonin-permeabilized cells fueled by substrates alone (LEAK state; malate, pyruvate, glutamate, succinate), and following the addition of ADP (OXPHOS state). (C) Degree of OXPHOS coupling control for each trophoblast cell line, calculated as [1-(LEAK/OXPHOS)]. N = 6/group *P < 0.05, **P< 0.02, ***P< 0.001.

III.B. Mitochondrial H2O2 release rates in trophoblast cell lines

Basal rates of H_2O_2 release (JH_2O_2) from intact cells were similar across ACH-3P, Swan-71 and iOTR, but much lower from BeWo compared to the other cell lines (**Figure 3A**). As expected, JH_2O_2 was higher in all cell lines following digitonin-permeabilization and the addition of mitochondrial substrates in the absence of ADP (LEAK state), which declined upon the addition of ADP in all but the BeWo cells (**Figure 3B**). When expressed as a percent of JO_2, JH_2O_2 was highest in the Swan-71 cells, and exhibited the most marked decline from LEAK to OXPHOS state in the ACH-3P, Swan-71 and iOTR cells (**Figure 3C**). When considered along with JO_2 results in **Figure 2**, these findings indicate that ACH-3P cells contain robust quantities of well-coupled and highly functional mitochondria compared to the other cell lines. Therefore, we chose to

proceed with this cell line for subsequent studies investigating the impact of testosterone on mitochondria metabolism.

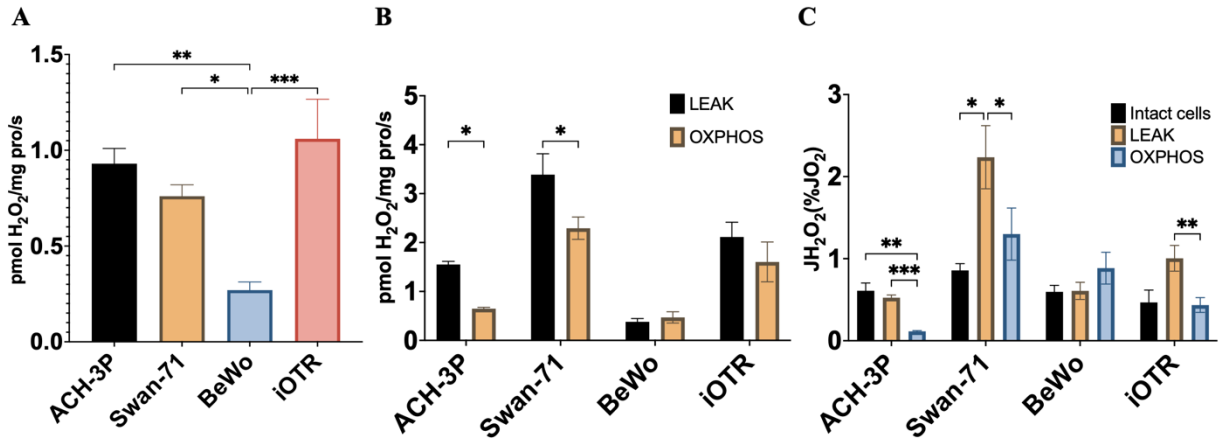

Figure 3: Hydrogen peroxide release rates from intact and permeabilized trophoblasts. (A) Basal H_2O_2 release rates from intact trophoblasts prior to permeabilization. (B) H_2O_2 release rates of digitonin-permeabilized cells fueled by substrates alone (LEAK state) and following the addition of ADP (OXPHOS state). (C) H_2O_2 release rates are expressed as a percent of oxygen consumption rate during each respiration state. N = 6/group *P < 0.05, **P< 0.02, ***P< 0.001.

III.C. Androgen receptor expression co-localizes with mitochondria in ACH-3P cells

Using Western blot, AR immunoreactive bands were observed of ~55 kDa and ~45 kDa in intact ACH-3P, Swan-71 and iOTR, and only the ~55 kDa band was observed in BeWo cells (**Figure 4A)**. Furthermore, co-localization of AR and cytochrome c was evident by immunofluorescences in cultured ACH-3P cells (**Figure 4B**). The mitochondrial isolation procedure used here allows for obtaining a mitochondrial-enriched fraction, whereby immunoblotting, the ~55 kDa band was detected (**Figure 4C**). This suggests mitochondria themselves could be a direct target of AR signaling.

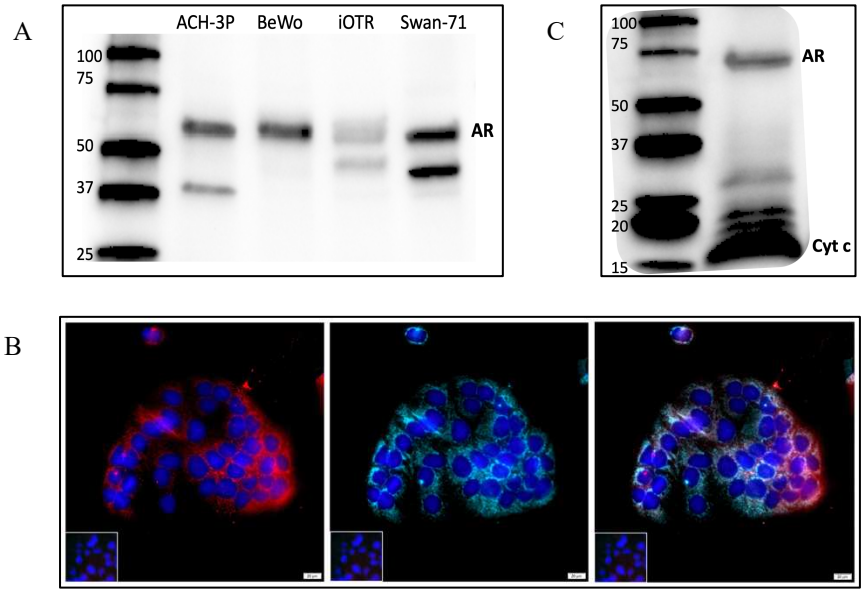

Figure 4: Androgen receptor expression in trophoblasts. (A) Androgen receptor (AR) protein expression in whole cell lysates from each of the trophoblast cell lines by immunoblot. (B) Immunofluorescence staining of AR (red), mitochondria (cytochrome *c*; cyan) and nuclei (DAPI; blue) in ACH-3P cells. The negative control stain with DAPI alone is shown in the lower left corner inset. (C) AR protein expression in ACH-3P mitochondrial isolates (enrichment confirmed by robust expression of cytochrome c) cell lysates from each of the trophoblast cell lines by immunoblot.

III.D. Acute testosterone exposure causes mitochondrial dysfunction in ACH-3P cells

To evaluate if testosterone has a direct effect on mitochondrial function, we evaluated the acute effect of testosterone treatment on JO_2 and JH_2O_2 during the OXPHOS-linked respiratory state in permeabilized ACH-3P cells **(Figure 5)**. Acute testosterone treatment tended to decrease mitochondrial JO_2 (**Figure 5A**) and increase JH_2O_2 (**Figure 5B**), but these effects were not statistically significant. However, when JH_2O_2 was expressed as a percent of JO_2, a significant increase was seen following the addition of testosterone (**Figure 5C**). These results suggest that testosterone may have deleterious effects on trophoblast mitochondria that favor an increase in ROS production during high rates of energy metabolism

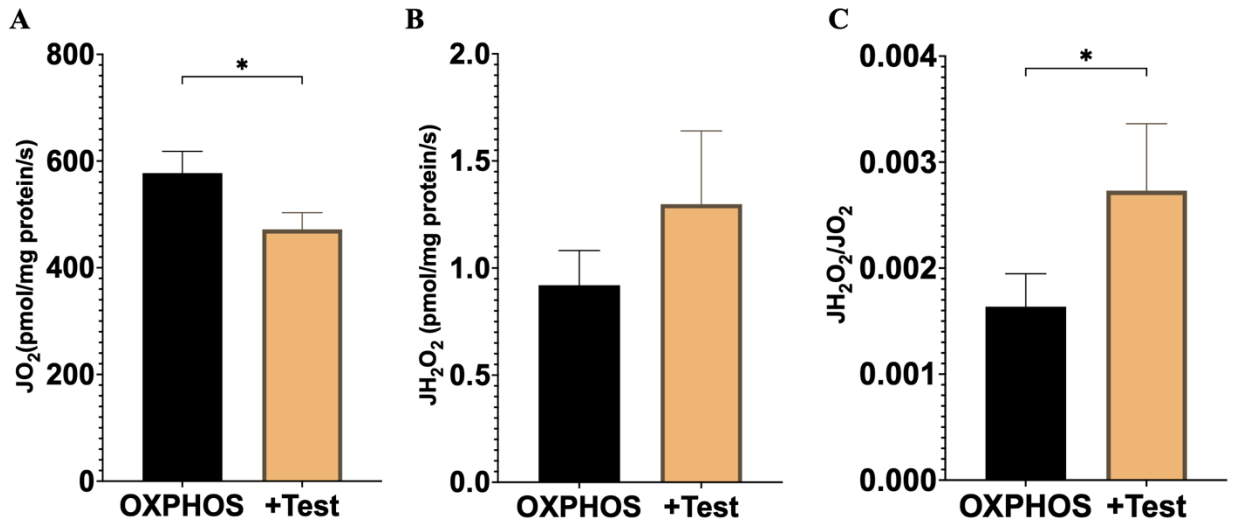

Figure 5: Impact of acute testosterone exposure on ACH-3P mitochondrial function. Testosterone (10 nM) was added to digitonin-permeabilized ACH-3P cells in the OXPHOS state to determine effects on respiration rate (A), H_2O_2 release (B), and H_2O_2 released per oxygen consumed (C), revealing a tendency of reduced oxygen consumption and increase ROS production. N = 6/group. * $P < 0.05$.

III.E. Chronic testosterone exposure increases ACH-3P mitochondrial content and activity

We next evaluated the impact of chronic (1-week) testosterone treatment on ACH-3P mitochondrial function and found that it tended to increase JO_2 in all respiratory states examined compared to untreated cells ($p < 0.05$ for main effect of testosterone by ANOVA), reaching statistical significance only in the OXPHOS state (**Figure 6A**). The calculated degree of respiratory control by ADP was higher in testosterone-treated cells (**Figure 6B**), indicating a greater degree of OXPHOS coupling control compared to untreated cells. JH_2O_2 was higher during all respiratory states after treatment with testosterone (**Figure 6C**) but was more similar when expressed as a percent of oxygen consumed, particularly during the OXPHOS state (**Figure 6D**). This suggests that chronic testosterone treatment tends to increase the overall mitochondrial content of ACH-3P cells, which was further indicated by immunoblotting analysis demonstrating

a higher expression of OXPHOS complex proteins in whole cell lysates compared to untreated controls (**Figure 6E, F**).

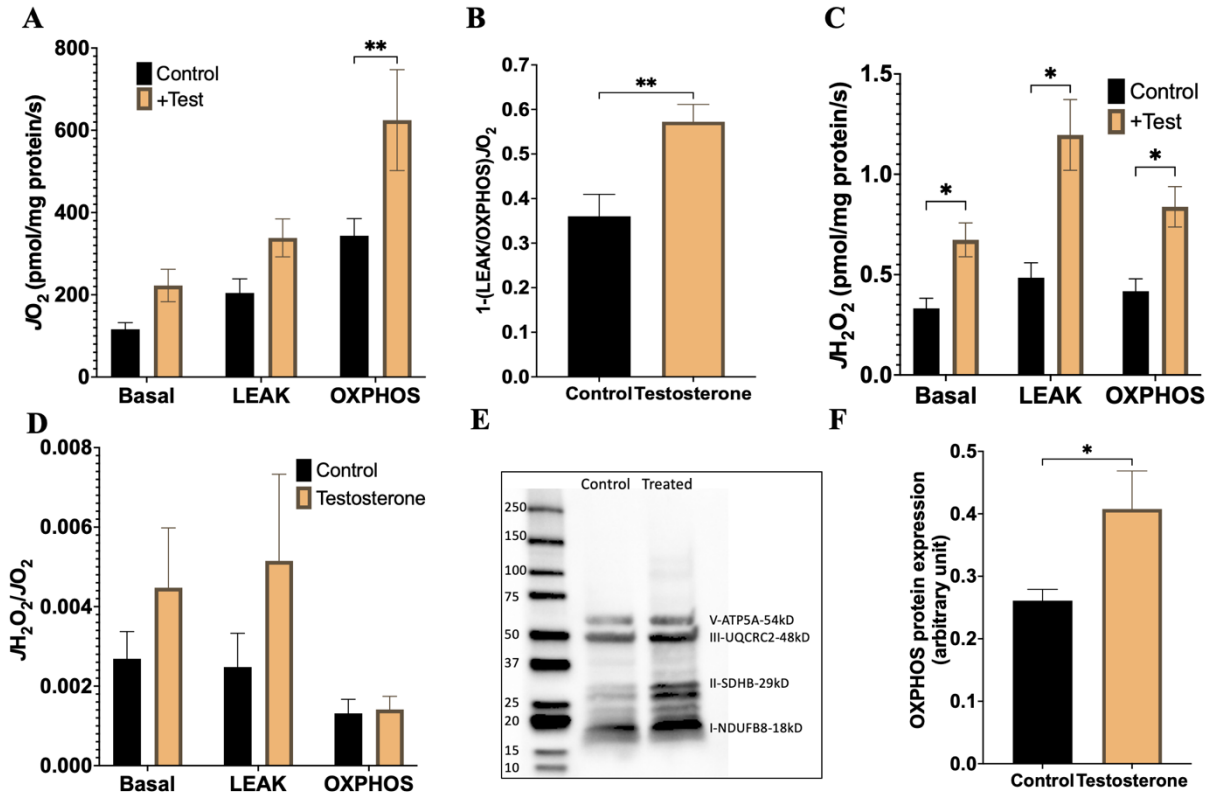

Figure 6: Chronic testosterone treatment increases ACH-3P mitochondrial content and activity. Impact of 1-week incubation of testosterone (10 nM) on oxygen consumption (A), OXPHOS coupling control (B), H_2O_2 release (C) and H_2O_2 released per oxygen consumed (D) of intact (basal) and digitonin-permeabilized ACH-3P cells in the LEAK and OXPHOS states. (E-F) Testosterone treatment increases expression of OXPHOS complexes in ACH-3P whole cell lysates, suggesting an increase in cellular mitochondrial content. N = 6/group * $P < 0.05$, **$P< 0.02$.

VI. Discussion

Mitochondrial function is critical for proper placental development and differentiation during normal pregnancy. Human placentas are inherently challenging to study *in vivo* due to inherent logistical and ethical limitations. Therefore, investigators rely on established *in vitro* model systems such as trophoblast cell lines to address scientific questions about placental biology and

health. In this study, we characterized the mitochondrial phenotypes of three well-established human and one novel sheep trophoblast cell lines and provide novel evidence for acute and chronic effects of testosterone on trophoblast mitochondrial function.

Using state-of-the-art high-resolution fluorespirometry, we found that ACH-3P, Swan-71, and iOTR cell lines all exhibit the expected responses of JO_2 and JH_2O_2 to substrates and ADP during the transitions from LEAK to OXPHOS respiratory states characteristic of functional mitochondria (Li Puma et al., 2019). However, BeWo cells exhibited much lower basal and stimulated JO_2 and JH_2O_2, and failed to increase of JO_2 in response to ADP, suggesting low quantities of poorly coupled mitochondria in this cell line. One possible explanation is that, unlike Swan-71 and ACH-3P, BeWo cells are more similar to syncytiotrophoblasts. Mitochondria in the syncytiotrophoblast layer are different from cytotrophoblast cells, being smaller and containing a more condensed matrix and vesicular cristae compared to cytotrophobast cells. Indeed, oxygen consumption rates are reportedly lower in syncytiotrophoblast than cytotrophoblast cells (Martinez F et al., 1997; see Table 1, Chapter 1), consistent with our observation in BeWo cells. Of the other cell lines characterized in this study, ACH-3P cells showed the greatest rates of coupled (OXPHOS-linked) oxygen consumption and robust basal JO_2 and JH_2O_2, consistent with a high density of functional mitochondria. As an *in vitro* model of placental biology, the ACH-3P cells are reportedly more similar to primary cells in regard to barrier integrity, glucose transport, and high metabolic activity (based on the oxidation-reduction sensitive dye Alamar Blue) compared to other cell lines, including BeWo cells (Rothbauer M et al., 2017). In addition to their robust mitochondrial phenotype, ACH-3Ps were found to express similar or higher levels of AR protein seen in the other cell lines, resembling abundant AR localization observed in first trimester placental villi (Cleys

ER et al., 2012). Therefore, ACH-3P cells are well-suited for studies investigating the effects of androgens on trophoblast mitochondrial function.

Androgens are being increasingly recognized as potential regulators of mitochondrial function, with recent studies reporting effects on mitochondrial biogenesis, fusion/fission dynamics, mitophagy, OXPHOS, and antioxidant enzyme expression, and ROS production (Chappell NR et al., 2021; Hioki T et al., 2014). Most of the evidence comes from studies demonstrating that chronic manipulation of androgen levels or AR expression/activity regulates the expression of genes relevant to mitochondrial biology through canonical binding of AR to its response element on nuclear DNA (Kobayashi A et al., 2020) To our knowledge, the present study is the first to examine the effect of acute testosterone administration on mitochondrial function in permeabilized cells, demonstrating an increase mitochondrial ROS production relative to JO_2 during maximally-stimulated OXPHOS–linked respiration. This suggests that direct exposure of mitochondria to androgens disrupts mitochondrial electron transfer, favoring a "slip" of electrons from the respiratory chain to oxygen during high rates of metabolic flux. This finding is consistent with recent evidence for mitochondrial localization of AR in a prostate cancer cell line, where it negatively impacts OXPHOS complex stability and enzymatic activity, at least in part through nongenomic activities (Bajpai P et al., 2019). We present evidence that AR may also co-localize with mitochondria in trophoblasts, which could contribute to both the acute and chronic effects of testosterone observed in ACH-3P cells herein by mechanisms that merit further investigation.

Chronic testosterone treatment increased both JO_2 and JH_2O_2 in ACH-3P cells during basal and stimulated respiration states in this study, which corresponded to a greater cellular expression of

OXPHOS complex protein expression. This suggests that testosterone treatment increased cellular mitochondrial content, which is consistent with reports that androgens positively regulate mitochondrial biogenesis and expression of OXPHOS machinery in a variety of tissue and cell types (Liu C et al., 2019, Rossetti ML et al., 2019, Usui T et al., 2014). However, the impacts of androgens and AR expression on mitochondrial phenotype appear to be somewhat tissue- and sex-dependent, with damaging effects of hyperandrogenism on ovarian follicular cells (Chappel NR et al., 2021; Song L et al., 2022) and pancreas (Mishra JS et al., 2018) of female rodents, but protective effects of androgens on prostate (Tam NN et al., 2003), heart (Apaiajai N et al., 2018) and brain (Toro-Urrego N et al., 2016) of males. This may be due to biological variations in the activity and/or expression of AR co-activators and repressors (Jafari H et al., 2022) perhaps further complicated by complex interactions between mitochondrial and nuclear AR signaling (Bajpai P et al., 2019). The *in vivo* implications of greater mitochondrial activity in trophoblasts treated with testosterone herein are unclear, but higher mitochondrial content, respiration rates and ROS production have been reported in PE and GDM placentas (Fisher JJ et al., 2021, Holland OJ etv al., 2018). Given that both PE and GDM are both associated with elevated maternal plasma levels of androgens and placental AR expression (Acromite MT et al., 1999; Uzelac PS et al., 2010), we postulate that AR-mediated increases in mitochondrial activity, particularly ROS production, could contribute to placental insufficiency and tissue damage in these conditions (Redza-Dutordoir M et al., 2016).

In summary, our studies provide a foundation for using the ACH-3P trophoblast cell line as a robust in vitro system for studying the impacts of various treatments and stimuli on placental mitochondrial function. Furthermore, we provide novel evidence that testosterone elicits acute

and chronic effects on trophoblast mitochondrial function that favors an increase in mitochondrial ROS release during high rates of energy metabolism. This could contribute to placental oxidative stress in pregnancies where androgens are elevated, establishing the need for more research on how androgen receptor signaling impacts placental mitochondria in conditions associated with hyperandrogenism such as PE and GDM.

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