DISSERTATION

ANDROGEN SIGNALING IN THE PLACENTA

Submitted by

Ellane Rachael Cleys

Department of Biomedical Sciences

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2014

Doctoral Committee:

Advisor: Gerrit Bouma
Co-Advisor: Colin Clay

Stuart Tobet
Santiago Di Pietro

ABSTRACT

ANDROGEN SIGNALING IN THE PLACENTA

Placental estrogen signaling is known to regulate placental trophoblast function and differentiation. However, the role of placental androgen signaling has never been investigated, despite the rise of maternal serum androgens throughout gestation. Recent findings have shown increased maternal serum androgen in patients with the placental induced disorder preeclampsia. Preeclampsia, a maternal hypertension and proteinuria condition instigated by insufficient trophoblast differentiation and invasion into maternal spiral arteries, is also associated with increased placental expression of androgen receptor and an increased risk of incidence in patients with polymorphisms in androgen receptor that decrease androgen signaling. These findings suggest a crucial role for placental androgen signaling. Moreover, research investigating androgen's role in cancer progression has shown that many androgen responsive genes regulate cell proliferation, differentiation to invasive phenotypes, and tissue vascularization, all processes necessary for normal placental development. Androgen signaling in tumor tissues is further regulated by androgen receptor complexes with histone lysine demethylases. These complexes are recruited to androgen response elements in DNA and dynamically regulate histone tail modifications for transcription initiation. This led us to the overall hypothesis that (1) androgen signaling in trophoblast cells is important for placental development, and (2) androgen receptor complexes with histone lysine demethylases in the placenta to regulate vascularization, growth and invasion factors in trophoblast cells. To test this hypothesis, we utilized a prenatal androgenization ewe model as well as human first trimester placental samples and immortalized

human trophoblast cell lines. Using the prenatal androgenized ewe model, we report for the first time expression of histone lysine demethylases in the placenta. Furthermore, we showed androgen receptor complexes with histone lysine demethylases and is recruited to an androgen response elements in the 5'untranslated flanking sequence of vascular endothelial growth factor in the sheep placenta. We also report that histone lysine demethylase are present in human first trimester syncytiotrophoblast and complex with androgen receptor in immortalized trophoblasts. Additionally, we demonstrated that androgen receptor complexes with histone lysine demethylases are also present in choriocarcinoma ACH-3P and BeWo cells. Dihydrotestosterone treatment in these cells led to down-regulation of androgen responsive genes, specifically *KDM3A* and *MMP2*. Inhibition of androgen receptor through flutamide treatment altered mRNA levels for genes regulating vascularization, including *HIF1α, PPARα,* and *PPARγ*. Hypoxia also decreased *CYP19* levels, however, further investigation is needed to confirm dihydrotestosterone and flutamide effect on protein expression in trophoblast cells. These data suggest that histone lysine demethylases complex with androgen receptor to regulate androgen responsive genes, including those directing placental vascularization and development. However, further experiments are needed to confirm the necessity of histone lysine demethylases for targeted androgen signaling in trophoblast cells and to determine if androgen directly regulates trophoblast differentiation and invasion. These findings suggest androgen signaling may play a critical role in placental development.

ACKNOWLEDGMENTS

I would like to thank my primary adviser, Dr. Gerrit Bouma, for generously taking me into his lab and for his support and guidance for all of my experiments, research, publications and career choices. I have truly enjoyed working with you and greatly appreciate your endless enthusiasm and support. I would also like to thank my co-adviser and committee members Dr. Colin Clay, Dr. Stuart Tobet, and Dr. Santiago Di Pietro for their encouragement, assistance, and intellectual contribution to my research and professional development. I greatly appreciate your inspiration and your drive to help me become a successful researcher and graduate student. A special thanks to Dr. Russ Anthony and Dr. Thomas Hansen for their generous mentorship and guidance throughout my graduate training as well. Your understanding and guidance has helped me develop professionally and assisted in shaping and directing my career goals. Thank you also to Dr. Quintin Winger and Dr. Jason Bruemmer for your input and time in editing publications and perfecting experiment designs. Additionally, I sincerely appreciate the endless support and guidance from the Animal Reproduction and Biotechnology Laboratory faculty, staff, and fellow graduate students. Working at the Animal Reproduction and Biotechnology Laboratory has been a wonderful experience and has granted me the opportunity to work with the most brilliant and supportive researchers and reproductive physiologists.

Thank you all so very much for contributing to my graduate research and dissertation. I hope you find the following dissertation a fair reflection of your inspiration and the generous contributions you have made to me over the past years.

DEDICATION

In dedication to my amazing family, for your endless support, encouragement and love. Thank you so much for everything Mom, Dad, and Jake.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The placenta functions as a crucial and transitory organ for the support of the developing fetus, providing an intricate network of trophoblast-lined villi interdigitated in maternal decidua and blood for nutrient and gas exchange, endocrine signaling, and fetal waste disposal. During the first trimester, trophoblast cells from the embryo rapidly proliferate and differentiate for placental development, or placentation, to occur. Trophoblast function is regulated by a vast array of transcription factors and endocrine signaling. Specifically, the hypoxic environment of the uterus regulates estrogen production from trophoblast cells, promoting cytotrophoblast differentiation to syncytiotrophoblast and invasive extravillous trophoblasts in the human placenta for continued estrogen production and uterine spiral artery remodeling, respectively. In the sheep placenta, hormone-secreting binucleate trophoblasts fuse with maternal uterine epithelium for formation of a syncytium that functions in endocrine signaling, including production and release of sex hormones into maternal circulation.

While estrogen signaling, in particular estriol, estrone and estradiol, is known to aid in trophoblast proliferation and differentiation, other factors are necessary for proper placentation to occur. Vascular endothelial growth factor (VEGF) aids in trophoblast differentiation and is necessary for placental angiogenesis. Matrix metalloproteinases (MMPs) secreted from trophoblasts break down uterine extracellular matrix for placental growth and maternal spiral artery remodeling. Growth factors such as insulin like growth factors (IGFs), epidermal growth factor (EGF), and transforming growth factors (TGFs) are also expressed in the placenta for continued proliferation and turnover of trophoblasts throughout gestation. Interestingly, many of

the proteins known to regulate angiogenesis, trophoblast function and differentiation are androgen responsive genes, including *VEGFA*, *MMP*s, *IGF*s, *EGF*, and *TGF*s.

Although androgens are known to regulate a vast array of genes that direct processes necessary for normal placentation (i.e. vascularization, growth and differentiation factors), heretofore no one has investigated androgens' role in placental development. This is of particular interest as estrogen has been shown to regulate trophoblast differentiation and invasion during the first trimester for maternal spiral artery remodel. Additionally, increased maternal serum androgens, increased placental androgen receptor (AR), and decreased placental aromatase activity have been reported in patients with preeclampsia. Preeclampsia is a placental-derived disorder where insufficient trophoblast differentiation and invasion leads to maternal hypertension, fetal growth restriction, and can result in fetal and/or maternal death. As AR protein has been immunolocalized in human trophoblast cells, these findings suggest that placental androgen signaling may have a critical function in regulating placentation through stimulating angiogenesis or trophoblast differentiation.

However, to identify the role of androgen signaling in the placenta, it is also necessary to investigate the role of histone lysine demethylases (KDMs). KDMs have been shown to form complexes with AR to initiate transcription of androgen responsive genes in cancer cells that might otherwise be epigenetically silenced. Additionally, several KDMs have been found to be androgen responsive, leading to a positive feedback on androgen signaling through KDMs in cancer cells. Although KDM presence and function has been primarily investigated in cancer, trophoblast function similarly to cancer in that they undergo an epithelial to mesenchymal

transition, express angiogenic factors to establish vascularization, and can differentiate to invade surrounding tissue. It is therefore of interest to determine if KDMs are present in the placenta and if they are capable of complexing with AR to regulate placental androgen signaling.

Identification of placental androgens' function, as well as demonstrating AR interactions with KDMs in the placenta, will aid in clarifying the complex mechanisms driving placentation. In addition, by elucidating the mechanisms of placental androgen signaling, a better understanding is achieved as to how placental disorders occur, such as preeclampsia. This could potentially lead to new opportunities for early diagnosis.

CHAPTER I: LITERATURE REVIEW

Mammalian prenatal development is supported by a multifunctional and transitory organ, the placenta (Gude et al., 2004). The placenta functions not only for nutrient transport to the fetus, but also for gas exchange, removal of waste, and endocrine functions (Knobil and Neill 1998). Through playing such a multifunctional and necessary role in neonatal development, any aberrant placentation can lead to severe complications during pregnancy (Gude et al., 2004) as well as during postnatal development in humans and agricultural animals (Resnik 2002; Wu et al., 2006). In agricultural animals, insufficient placental function can lead to low birth weights, reduced efficiency, and decreased lean muscle tissue (Wu et al., 2006). In humans, dysfunctional placentation can lead to fetal intrauterine growth restriction (IUGR), preeclampsia (PE), and adult onset of diseases such as hypertension, diabetes, and coronary heart disease (Eriksson et al., 1999; Jaquet et al., 2000; Anderson 2007). In addition to causing fetal mortality and morbidity, abnormal placentation can lead to maternal mortality, hypertension, and stroke (Redman and Sargent 2003).

One such placental induced disorder in humans is PE, which is a serious pregnancy complication characterized by maternal hypertension, proteinuria, that is typically presented with IUGR (Redman 1990; 1991) and can only be treated by delivery of the placenta (Ilekis et al., 2007). PE develops when there is insufficient trophoblast invasion and remodeling of maternal spiral arteries during the first trimester (Redman 1990; 1991; Roberts et al., 1993), possibly as a consequence of an overactive maternal immune response to placentation (Redman et al., 1999; Moffett and Hiby 2007). Many risk factors for PE have been identified, including maternal

obesity, African or Native American descent, smoking, and male fetal sex (James 1995;2008;2013). PE occurs in approximately 5-7% of pregnancies worldwide (Roberts and Cooper 2001; Redman and Sargent 2005), causing 15-20% of maternal deaths in developed countries (Roberts et al., 1993; Roberts and Redman 1993; Sibai et al., 2005). IUGR without the presentation of PE occurs in approximately 8% of pregnancies worldwide (Resnik 2002). Despite the prevalence and severity of these placental disorders, little is understood about their etiology, likely due to the multifactorial development of onset (Kaufmann et al., 2003; Ilekis et al., 2007). To better understand abnormal placental development, in the hopes of developing better diagnostics and treatment, further research into pathways regulating normal placental development is necessary.

Placental Development in the Human

The fertilized human oocyte develops into a 58-cell blastocyst by 96 hours post fertilization and enters the endometrial cavity after three days post fertilization (Knobil and Neill 1998). The developing blastocyst appositions or aligns next to the uterine endometrium and forms the junctional zone where placental cells begin to invade maternal uterine decidua approximately one week post fertilization (Knobil and Neill 1998; Kaufmann et al., 2003). The cells surrounding the outside of the blastocyst are referred to as the trophectoderm (Kaufmann et al, 2003). The trophectoderm is composed of trophoblast cells, which differentiate into cytotrophoblast cells, functioning as the proliferative bipotential progenitor cells of the placenta (Enders 1968; Kaufmann et al., 2003) (Figure 1.1). The cytotrophoblasts can further differentiate to a continuous, multinuclear layer of syncytiotrophoblast where continued cell fusion form a syncytium that will be in direct contact with maternal blood (Brosens et al., 1967; Pijnenborg et

al., 1981; Benirschkle and Kaufmann 2000; Hirano et al., 2002; Guibourdenche et al., 2009). Proliferative cytotrophoblast cell columns attach to and interdigitated the junctional zone, forming anchoring villi (Enders 1968).

The invasive extravillous trophoblasts (also referred to as interstitial extravillous cytotrophoblasts) differentiate from the cytotrophoblast at anchoring villi and invade into the maternal decidua, invading up to the first third of the myometrium by the third trimester (Brosens 1988; Blankenship, et al., 1993; Kam et al., 1999; Kaufmann et al., 2003). To aid in the invasion processes, extravillous cytotrophoblast express an array of genes, including matrix metalloproteinases 2 (MMP2) and MMP9, to break down the extracellular matrix, in particular during the first trimester when placental vascularization is primarily established (Bass et al., 1994; Cross et al., 1994). With differentiation into an invasive extravillous phenotype, there is also a decrease in expression of transforming growth factor beta-3 (TGF-β3) and tissue inhibitor of metalloproteinases-2 (TIMP2) (Lee et al., 2010).

When the extravillous cytotrophoblast invade the maternal decidua, they position themselves next to the maternal spiral arteries and form plugs during the first trimester of pregnancy (Hustin et al., 1987; Moll et al., 1988; Nanaev et al, 1995; Burton et al., 1999; Guibourdenche et al., 2009). Endovascular extravillous cytotrophoblasts differentiate from extravillous trophoblasts to invade maternal spiral artery walls and lumen (Blankenship et al., 1993; Kaufmann et al., 2003). By 10-12 weeks of pregnancy, the plugs are lost and endovascular extravillous trophoblasts replace the epithelium, leading to dilation of the arteries, increased blood flow, and reduced flow resistance to the placenta by the second trimester (Brosens et al., 1967; Pijnenborg et al., 1981;

Benirschkle and Kaufmann 2000; Hirano et al., 2002; Guibourdenche et al., 2009). Endovascular extravillous cytotrophoblast invasion and re-modeling of the spiral arteries is highest in the middle of the placental bed, forming a discoid region where extensive nutrient and gas exchange occurs (Brosens 1988; Blankenship, et al., 1993; Kam et al., 1999; Kaufmann et al., 2003).

Rapid proliferation of the placenta occurs early during pregnancy (Korgun et al., 2006; Kar et al., 2007). At about 21 days post-conception, fetal-placental vasculogenesis initiates and circulation is established by 32 days post-conception (Demir et al., 1989; Zygmunt et al., 2003; Kaufmann et al., 2004; Torry et al., 2004; Demir et al., 2004, 2007; Arroyo and Winn 2008; Burton et al., 2009; van Oppenraaij et al., 2009). Placental vascularization is in part established by villous cytotrophoblast cells expressing vascular endothelial growth factor (VEGF) and other angiogenic factors during the first trimester (Hildebrandt et al., 2001).

Although the trophoblasts continue proliferation and cell turn-over throughout pregnancy (King and Blankenship, 1993; Kaufmann and Castellucci, 1997), there remains three layers of fetal tissue (trophoblast syncytium, fetal interstitium, and fetal endothelium) (Leiser and Kaufmann 1994), that separate fetal portal blood from maternal arterial blood (Leiser and Kaufmann 1994). This direct bathing of syncytium in maternal blood gives humans a hemochorial placenta (Enders 1965; Boyd and Hamilton 1970). Additionally, as the endovascular extravillous trophoblast invasion occurs in one concentrated area, this forms a discoid region where the majority of nutrient and gas exchange occurs. Therefore, the human hemochorial placenta is also a discoidal placenta (Boud and Hamilton 1970; Leiser and Kaufmann 1994), functioning to maintain fetal-

placental growth with a highly invasive extravillous and endovascular trophoblast invasion and arterial remodeling (Kaufmann et al., 2003).

Placental Development in the Sheep

In contrast to the human placenta, endovascular invasion by trophoblast cells does not occur within the sheep placenta (Kaufmann et al., 2003; Spencer et al., 2004; Carter 2007). Instead, the maternal endothelium and connective tissue is left intact (Leiser and Kaufmann 1994). Although the method of implantation is different in the ewe compared to the human, similarities remain in the process of apposition, trophoblast differentiation, and trophoblast function.

In the ewe, the morula enters the uterus at day 4, developing into a blastocyst by day 6 (Spencer et al., 2004). By day 8, the blastocyst hatches from the zona pellucida and is located in the ipsilateral uterine horn (Rowson and Moor 1966; Spencer et al., 2004). It undergoes a period of rapid cell proliferation and elongation, moving to the contralateral horn by day 13 if a singleton is present (Rowson and Moor 1966). During this period of elongation, and in contrast to the human, the extraembryonic membranes (chorion and yolk sac) form prior to implantation (Renfree 1982; Guillomot et al., 1993; Carson et al., 2000; Spencer et al., 2004). Rapid elongation of the ovine conceptus continues until day 16, when it adheres to the uterine epithelium (Spencer et al., 2004). The ovine conceptus then increases in size approximately threefold from gestational day 20 to 30 (Spencer et al., 2004; Grazul-Bilska et al., 2011).

Attachment and implantation begins at embryonic day 16, when mononuclear trophoblast cells from the trophectoderm adhere to the endometrial luminal epithelium (Spencer et al., 2004).

Between days 14 and 16, when the ovine conceptus is rapidly elongating and beginning to adhere to the endometrial luminal epithelium, binucleate trophoblast cells begin to differentiate from the mononuclear trophoblasts through consecutive nuclear divisions without cytokinesis (Spencer et al., 2004). Mononuclear trophoblasts, comparable to the invasive extravillous trophoblast in humans, also express MMP2 and MMP9, likely for improved branching of placental villi (Riley et al., 2000). Binucleate trophoblast cells do not adhere to the endometrial luminal epithelium, but they can fuse with epithelial cells and form a syncytium or hybrid symplasm that functions similarly to the human syncytium for the synthesis and secretion of hormones (Wooding 1992; Hoffman and Wooding 1993; Spencer et al., 2004). As giant binucleate cells fuse with endometrial luminal epithelium, they form trinucleate cells that further develop into syncytial plaques as more binucleate cells migrate and fuse (Wooding 1984; Spencer et al., 2004). Also similar to the human syncytium, continued cell turn-over and binucleate cell migration and fusion maintains the syncytium in the sheep placenta (Wooding 1984; Riley et al., 2000; Spencer et al., 2004). By gestational day 22, the entire trophectoderm has adhered to the endometrial luminal epithelium at placentome sites, described below (Boshier 1969; Guillomot et al., 1981; Spencer et al., 2004).

In ruminant animals, including sheep, areas along the endometrial luminal epithelium develop plaques or caruncles, which form progressively at gestational day 14 with depression of the luminal epithelium and crypt formation (Guillomot et al., 1981; Wimsatt 1950; Spencer et al., 2004). These specialized caruncle regions are the maternal side of the developing placenta at attachment sights, called placentomes (Wimsatt 1950; Spenser et al., 2004). Placentomes are composed of both a maternal portion (caruncle) and a fetal portion (cotyledon) (Wimsatt 1950;

Spencer et al., 2004). Syncytial plaques develop specifically within the placentome, covering the surface of caruncles for nutrient and gas exchange by fusion with endometrial luminal epithelium (Cross et al., 1994; Spencer et al., 2004). Placentomes are not present in the human placenta, but they do maintain a similar structure and function compared to the discoid region of invasion and nutrient exchange in the human placenta (Leiser and Kaufmann 1994). Inter-cotyledonary regions between placentomes are characterized by smooth chorion where nutrient and gas exchange can still occur (Bjorkman 1969; Leiser and Kaufmann 1994; Spencer et al., 2004).

As the sheep placenta has limited invasion of the maternal tissue (Leiser and Kaufmann 1994; Kaufmann et al., 2003; Spenser et al., 2004; Carter 2007), there are essentially six layers of tissue separating fetal from maternal blood in areas outside of the placentomes: maternal endothelium, connective tissue, maternal epithelium, fetal trophoblast, fetal connective tissue, and fetal endothelium (Leiser and Kaufmann, 1994). This type of placenta in sheep is called synepitheliochorial placenta (Wooding 1992; Leiser and Kaufmann 1994; Carter 2007), which is capable of transferring nutrients and gas across more tissue layers than are present in the human placenta (Cross et al., 1994).

Matrix Metalloproteinases in the Placenta

Matrix metalloproteinases (MMPs) play an important role in tissue remodeling for repair, cell migration, vascularization, and placentation. In particular, extravillous cytotrophoblast cells secrete MMPs to aid trophoblast differentiation and invasion into maternal decidua, and to establish placental vascularization (Huppertz et al., 1998; Solberg et al., 2003; Isaka et al., 2003; Munaut et al., 2003; Renaud et al., 2014). There are 28 known members in the MMP family and

they all function as calcium-dependent zinc endopeptidases that cleave internal peptide bonds in polypeptides and proteins (Bode and Maskos 2003). After translation, MMP pro-protein (pro-MMP) is processed in the endoplasmic reticulum, where pro-peptide is removed to allow access to the catalytic domain. The catalytic domain of MMPs contains zinc ion (Zn^{2+}) that will interact with proteins and cleave peptide bonds, primarily through protein interactions at a glutamate residue within the MMP to initiate subsequent substrate hydrolysis. Once the pro-peptide is removed, active MMPs are secreted into the extracellular space or can be bound to the cell membrane. Substrate binding domain of MMPs functions for specificity of targeting proteins for degradation. Target proteins for MMPs are primarily extracellular matrix proteins, including collagen (types 1 through 10), gelatin, aggrecan, fibronenctin, elastin, and laminin (Bode and Maskos 2003; Manzetti et al., 2003). However, each MMP has specificity and affinity for specific extracellular matrix proteins (i.e. MMP9 targets gelatin and collagen type 4 and 5) (Manzetti et al., 2003). By this mechanism, MMPs can degrade extracellular matrix proteins, enabling cell migration or proliferation, vascularization, or other tissue remodeling processes (Munaut et al., 2003; Renaud et al., 2014).

During placentation, invading extravillous trophoblasts are known to secret MMPs to cleave extracellular matrix proteins in the decidua, aiding in the process of cell migration into maternal decidua and establishment of placental vascularization (Huppertz et al., 1998; Munaut et al., 2003; Solberg et al., 2003; Renaud et al., 2014). However, to control the function of MMPs, cells also secrete tissue inhibitors of metalloproteinases (TIMPs). TIMPs function by binding MMPs, usually at a 1:1 ration, and block MMP activity by inserting into the active site of the catalytic domain (Snoek-van Beurden and Von den Hof 2005; Stephenson et al., 2005). There are four

known TIMPs and they play a significant role in regulating tissue remodeling and cell invasion (Snoek-van Beurden and Von den Hof 2005). When extravillous trophoblasts of the placenta invade maternal decidua during the first trimester, decidual stromal cells secrete TIMPs, possibly as a mechanism to limit placental invasion (Hurskainen et al., 1996; Huppertz et al., 1998). Furthermore, research suggests that hormone secretion from trophoblasts may function in paracrine or autocrine signaling for regulation of placental MMP expression (Lee et al., 2003; Liao et al., 2003, Limaye et al., 2008; Comstock et al., 2008)

Steroidogenesis and Hormone Signaling

While placenta of all species functions for the transport of nutrients and gas for support of the developing fetal-placenta unit (Gude et al., 2004), it also has important endocrine functions necessary for fetal development and maternal signaling (Knobil and Neill 1998; Guibourdenche et al., 2009). Steroid hormone synthesis (steroidogenesis) involves the conversion of cholesterol to various steroid hormones through enzymatic processing (Miller and Auchus 2010). Free cholesterol in the cell cytoplasm, obtained either from endocytosis or *de novo* synthesis, is carried to the outer mitochondrial membrane by proteins with a StAR-related lipid transfer domain (START), such as StarD4 and StarD5 (Miller and Auchus 2010). StAR, the first-described member of the START protein family, plays the crucial role in facilitating the movement of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane (Miller and Auchus 2010). Sterol response element binding proteins (SREBPs) and 3-hydroxy-3-methylglutaryl co-enzyme A reductase regulate the biosynthesis of cholesterol (Miller and Auchus, 2010). The entire process of steroidogenesis is show in Figure 1.2, with the first and rate limiting step being P450scc (CYP11A1 or cholesterol side chain cleavage enzyme) conversion of cholesterol to pregnenolone (Miller and Auchus 2010). This occurs in the inner mitochondrial membrane (Miller and Auchus 2010). Other

steroidogenic enzymes that function in steroidogenesis belong to two family groups: cytochrome P450 enzymes (P450) or hydroxysteroid dehydrogenases (HSDs) (Miller and Auchus 2010).

Cellular regulation of steroidogenesis occurs with posttranslational modifications of P450 and HSD steroidogenic enzymes (Miller and Auchus 2010). The end result is the cell-specific production of sex steroid hormones (androgens, progesterone, and estrogens), mineralocorticoids, or glucocorticoids (Miller and Auchus 2010). The production of specific steroids is determined by the origin of the endocrine tissue or cell type as specific steroidogenic enzymes may only be expressed in a given tissue or cell, such as in the adrenal gland or ovary (Miller and Auchus 2010). The steroid hormones, once secreted by the cell, can act in a paracrine or endocrine manner (Petraglia et al., 1996). For a cell to respond to steroid hormones, however, it must express their receptors (Mangelsdorf et al., 1995). For instance, tissue specific expression of estrogen receptors 1/alpha (ESR1), estrogen receptor 2/beta (ESR2), and androgen receptor (AR) can be seen in multiple cell types and tissues, including the male and female gonads and the placenta (Leung and Steele 1992).

Estrogens encompasses a family of sex steroid hormones that have an 18 carbon structure and can refer to both naturally occurring and synthetic hormones. All estrogens bind to estrogen receptors to initiate a cellular response. The estrogen family includes estrone (E_1), estradiol (E_2), estriol (E_3), and 16-hydroxy estrone. The most common biologically active estrogen is estradiol. Similarly, androgens are a class of 19 carbon sex steroid hormones that includes the adrenal androgens, dehydroepiandrosterone (DHEA) and DHEA-S, as well as androstenedione, androstenediol, testosterone, and dihydrotestosterone (DHT). All androgens signal via binding to

the androgen receptor (AR), although, similar to estrogens, there is varying affinity of family members to the receptor (Senger 2003; Hu et al., 2010; Miller and Auchus 2010).

Steroid hormone receptors possess several domains for functional signaling, including a ligand binding domain and a DNA binding domain (Mangelsdorf et al., 1995). The traditional AR DNA binding domain or response element (ARE) is AGAACAnnnTGTTCT (Mangelsdorf and Evans 1995; Mangelsdorf et al., 1995; Comstock et al., 2008) and the traditional ESR1 DNA binding domain (ERE) is GGTCAnnnTGACC (Mangelsdorf et al., 1995). Binding of a sex hormone ligand to its receptor typically occurs within the cytoplasm or within the nucleus as steroid hormones are membrane permeable (Mangelsdorf et al., 1995). The ligand-bound receptor may then dimerize and bind directly to DNA where the DNA-response element or half-site is present (Mangelsdorf et al., 1995). Binding can also occur at other DNA sequences that are similar in sequence or shape to the traditional DNA binding domain (Comstock et al., 2008). The ligand-bound receptor further recruits other transcription cofactors to initiate transcription (Mangelsdorf et al., 1995). As this pathway of steroid hormone signaling initiates gene transcription, the effects of steroid signaling are not typically an immediate cellular response (Cooper and Hausman 2000).

However, studies have shown that steroid hormones also are capable of initiating rapid cellular responses, such as increased intracellular calcium (Berridge and Taylor 1988) that would precede gene transcription (Cooper and Hausman 2000). This suggests that steroid hormones are capable of binding cell membrane receptors to initiate a rapid cellular response via secondary messengers (Cooper and Hausman 2000). Indeed, despite traditional signaling via direct binding to DNA or

through transcription factor binding, steroid hormones appear to also signal through specific membrane receptors (Prossnitz et al., 2008; Thomas 2008). For instance, progesterone and estrogen signal through cell membrane receptors mPRα and PGMRC1, and GPR30 (GPER), respectively, possibly to initiate a different cellular response than the conventional nuclear steroid hormone receptor pathway (Prossnitz et al., 2008; Thomas 2008).

Receptor expression within a cell can also be regulated by tissue specific methylation within the promoter region, such as occurs in *AR* (Kinoshita et al., 2000; Jarrard et al., 1998), *ESR1* (Fürst et al., 2012), and *ESR2* transcription (Xue, et al., 2007). Methylation within the promoter region typically blocks gene transcription (Zhang and Meaney 2010), adding a layer of complexity to the system that ensures appropriate cellular signaling and response to steroid hormones. Additionally, tissue specific response regions may be present within the promoter region to regulate mRNA transcription, such as the placental specific promoter region for *aromatase* (*CYP19*) (Vanselow et al., 1999).

While steroid hormone signaling regulates transcription, circulating and cytoplasmic binding proteins can bind specific hormones to decrease their ability to signal while increasing their half-life (Anderson 2008). For instance, sex hormone-binding globulin (SHBG) is a glycoprotein that binds sex steroid hormones, in particular testosterone and estradiol (Bardin et al., 1981; Hammond 1990; Petra 1991). Circulating SHBG is further capable of regulating hormone signaling by transporting hormones directly to cells or tissue to initiate cellular signaling (Bordin and Petra 1980; Stanczky et al., 1986; Larriva-Sahd et al., 1991; Hryb et al., 1990).

Mature peptide hormones, in comparison, are composed of less than 50 amino acids and their signaling pathway is different from steroid hormones as they only signal via cell membrane receptors (Cooper and Hausman 2000). After a peptide hormone binds its membrane receptor, secondary messengers, such as calcium, rapidly amplify the signal of ligand binding, typically via phosphorylation of cytoplasmic proteins (Cooper and Hausman 2000). Ultimately, this leads to binding of transcriptional cofactors or repressors for regulation of gene expression (Cooper and Hausman 2000). In both situations, the products of either peptide or steroid hormone signaling can initiate gene transcription, resulting in protein products that have the potential to further regulated the transcription of other genes, referred to as secondary response genes (Cooper and Hausman 2000).

Placental Steroidogenesis and Receptor Expression

During the first trimester, at approximately the seventh week of pregnancy, sufficient syncytiotrophoblast have differentiated from cytotrophoblasts to become the primary hormone producing cells in the human placenta (Guibourdenche et al., 2009). The syncytiotrophoblast is in direct contact with maternal blood at approximately eleven weeks of gestation; it secretes hormones directly into maternal circulation (Guibourdenche et al., 2009). Syncytiotrophoblast secrete progesterone (Guibourdenche et al., 2009), human chorionic gonadotropin (hCG), placental lactogen (hPL), and placental growth hormone (hGH-v) (Jameson and Hollenberg 1993; Guibourdenche et al., 2009). While PL and GH function for mobilization of maternal nutrients for fetal-placental growth and support (Handwerger and Freemark 2000), progesterone and hCG function for uterine quiescence and pregnancy maintenance (Stoffer et al., 1977; Spencer and Bazer 2002).

As the syncytiotrophoblast is responsible for the production of progesterone in the human placenta, research has investigated the mechanisms of steroidogenesis and sex hormone signaling in this multinucleated cell layer. The steroidogenic enzymes present in the human syncytiotrophoblast include P450scc (pregnenolone production from cholesterol), 3β-HSD (progesterone and androstenedione production), 17β-HSD (testosterone production from androstenedione), and P450arom (or CYP19, estrogen production from testosterone) (Fournet-Dulguerov et al, 1987; Payne and Hales 2004; Tuckey et al., 2004; Guibourdenche et al., 2009; Sathishkumar et al., 2012). The syncytiotrophoblast in humans does not express P450 17α-hydroxylase-17:20; therefore, androgen production from pregnenolone and progesterone does not occur in the human placenta (Payne and Hales 2004; Tuckey et al., 2004; Guibourdenche et al., 2009). This, however, does not appear to be the case for ruminants, as both the goat and sheep placenta express P450c17 (Ma et al., 1999; Weng et al., 2005).

The syncytiotrophoblast in the human placenta are still capable of producing testosterone despite the lack of P450-17α (Payne and Hales 2004; Tuckey et al., 2004; Guibourdenche et al., 2009). To produce testosterone, syncytiotrophoblast endocytose maternal cholesterol via binding to lipoprotein receptor, VLDL receptor, or B1 scavenger receptor on the syncytiotrophoblast cell membrane (Guibourdenche et al., 2009). Free cytosolic cholesterol is available after lysosomal degradation of LDLs occurs (Guibourdenche et al., 2009). In the syncytiotrophoblast, the free cholesterol is selectively transported to the outer and inner mitochondrial membrane by sterol carrier protein-2 and metastatic lymph node 64 proteins, respectively (Guibourdenche et al., 2009). Within the syncytiotrophoblast's mitochondria, cholesterol can be converted into

pregnenolone through the action of P450scc, which is further converted into progesterone via enzymatic action of 3β-HSD (Guibourdenche et al., 2009).

As androgens cannot be directly produced from pregnenolone or progesterone in the human placenta, an extracellular precursor is necessary for syncytiotrophoblast androgen and estrogen biosynthesis (Guibourdenche et al., 2009). The precursor dehydroepiandrosterone sulfate (DHEA-s) is produced by fetal and maternal adrenals and reaches the placenta from fetal and maternal circulation, respectively (Calvin et al., 1963; Guibourdenche et al., 2009). Once DHEA-s is taken up by syncytiotrophoblast, it is hydrolyzed by sterol steroid sulfatases into 16α-hydroxy DHEA-s (Guibourdenche et al., 2009). 3β-HSD then metabolizes the hydrolyzed s-DHA into androstenedione, which can be further converted into testosterone by 17β-HSD (Guibourdenche et al., 2009). Placental CYP19 in the endoplasmic reticulum aromatizes androstenedione and testosterone in the syncytiotrophoblast for the production of C18-estrone and estradiol, respectively (Ryan 1959; Thompson and Siiteri 1974a,b).

While the syncytiotrophoblast is steroidogenic and produces sex steroid hormones, it also appears to be regulated by sex-steroid hormones (discussed in more detail below). To respond to paracrine and endocrine hormone signaling, trophoblasts must express sex steroid hormone receptors (reviewed in Table 1.1). Interestingly, ESR1 does not appear to be expressed in differentiated syncytiotrophoblast (Kumar et al., 2009), but is expressed in cytotrophoblasts, differentiating cytotrophoblasts, and extravillous cytotrophoblasts (Bukovsky et al., 2003a,b; Schiessl et al., 2006; Kumar et al., 2009). AR, in contrast, is immunolocalized in differentiated syncytiotrophoblast and has variable immunoreactivity in differentiating cytotrophoblasts

(Iwamura et al., 1994; Hsu et al., 2009). ESR2 has relatively low levels of nuclear immunolocalization in cytotrophoblasts and differentiating cytotrophoblasts, but can be found in the cytoplasm of the syncytiotrophoblast and extravillous cytotrophoblasts (Bukovsky et al., 2003a,b; Schiessl et al., 2006; Kumar et al., 2009). The localization of AR, ESR1, and ESR2 has not yet been determined within the sheep placenta.

Expression of sex hormone receptors and aromatase also appears to be regulated by fetal sex and pathological conditions during pregnancy. For instance, higher aromatase is present in placenta from male fetuses compared with placenta from females in normal pregnancy, while preeclampsia increases aromatase expression in placenta from female fetuses and decreases aromatase in the placenta from male fetuses (Sathishkumar et al., 2012). Additionally, ESR1 expression can also be lost in cytotrophoblast cells, possibly contributing to the reduced placental invasion noted in preeclampsia (Bukovsky et al., 2003a,b).

Syncytiotrophoblast regulation of sex hormone signaling is further complicated by the expression of sex hormone-binding globulin (SHBG) within the syncytium (Larrea et al., 1993). SHBG preferentially binds testosterone and estradiol to potentially limit immediate hormone signaling or extend sex-hormone half-life (Bardin et al., 1981; Hammond 1990; Petra 1991).

Steroidogenesis in the Maternal-Placenta-Fetal Unit

For placental steroidogenesis, maternal cholesterol is first taken up by trophoblast cells via LDL receptor-mediated endocytosis or SR-B1-mediated uptake (Guibourdenche et al., 2009; Hu et al., 2010). Once internalized within the placental cells, it can be stored as esters in lipid droplets,

incorporated as free cholesterol into cellular membranes, or, depending on the placental cell, it can be utilized for steroidogenesis. In the placenta, syncytiotrophoblast (human), binucleate cells (sheep), and giant trophoblasts and spongiotrophoblasts (mouse) are steroidogenic cells, but they lack the ability to adequately synthesize cholesterol from acetate via *de novo* synthesis. Therefore, a maternal source of cholesterol is required and, during pregnancy, higher levels of circulating cholesterol can be found in the maternal blood (Malassiné et al., 2003; Tuckey et al., 2004; Guibourdenche et al., 2009). Cholesterol that is taken up via LDL receptor-mediated endocytosis is processed in an early endosome, where decreased pH allows for the receptor to disassociate and be recycled back to the plasma membrane. The now free cytoplasmic cholesterol (including that from SR-B1 mediated uptake) is trafficked to the mitochondria in syncytiotrophoblast (human), binucleate cells (sheep), and giant trophoblasts and spongiotrophoblasts (mouse), likely following microtubule trafficking. Cholesterol is first loaded onto the outer mitochondrial membrane and then transferred to the inner mitochondrial membrane via StAR. At the inner mitochondrial membrane, side chain cleavage occurs via P450scc enzymatic activity to produce pregnenolone. Pregnenolone (in the above listed cell types) is further converted to progesterone via enzymatic activity of 3β-HSD (Guibourdenche et al., 2009; Hu et al., 2010). At this point, the progesterone is primarily released back into maternal circulation. This is of particular importance in human and sheep, where placental production of progesterone maintains pregnancy. Interestingly, the human placenta is more steroidogenic than placentas of other mammals in that it produces much greater quantities of progesterone per unit volume (Malassine et al., 2003; Senger 2003; Miller and Auchus 2010).

In the human placenta, progesterone does not undergo further processing to form estrogens or androgens, as discussed above. Instead, the human placenta required maternal and fetal contributions of dehydroepiandrosterone sulfate (DHEA-S) (Calvin et al., 1963; Mastorakos and Ilias 2003; Payne and Hales 2004; Tuckey et al., 2004; Guibourdenche et al., 2009). In contrast, the sheep and rat placenta express 17α-hydroxylase and are capable of producing androgens; this would occur in the binucleate cells (sheep and rat) and in the spongiotrophoblasts (rat). The mouse placenta is also capable of converting progesterone into androstenedione, but lacks aromatase for the production of estrogens (Rembiesa et al., 1971; Arensburg et al., 1999; Ma et al., 1999; Mastorakos and Ilias 2003). In both the human and sheep placenta, DHEA can be further processed by 3β-HSD to form androstenedione. Androstenedione is further converted to testosterone via 17β-HSD while aromatase activity (which is present in the sheep and human placenta) can produce estrone and estradiol from these precursors, respectively (Bousquet et al., 1984; Strauss et al., 1996; Wooding et al., 1996; Guibourdenche et al., 2009: Hu et al., 2010; Mondragón et al., 2012).

In humans, sheep, and rodents, the fetal gonads cannot make progesterone, estrone, estradiol, or estriol. This is because the fetus lacks the enzymes 3β-HSD and aromatase, both of which are present in the placenta (Ryan 1959; Thompson and Siiteri 1974a,b; Arensburg et al., 1999; Guibourdenche et al., 2009). However, if the fetus is a male, there will be production of testosterone and dihydrotestosterone (DHT). When this occurs, maternal LDL cholesterol is taken up by the fetal gonadal tissue via LDL receptor mediated endocytosis or SR-B1 mediated selective uptake. Once inside the cell, cholesterol will be processed as describe above. When sexual differentiation of the male testes begins to occur (at the start of the second trimester in

humans or at day 30 of gestation in sheep), the differentiating Leydig cells will convert free cholesterol into pregnenolone. Conversely, circulating fetal pregnenolone of placental origin may also be taken up for steroidogenesis. Pregnenolone in Leydig cells undergoes enzymatic processing to produce testosterone and DHT, both of which are necessary for completion of male sexual differentiation (Gehani et al., 1998; Senger 2003; Hu et al., 2010). However, fetal derived testosterone will travel through fetal circulation, back to the placenta, where aromatase activity will convert it to estradiol (Guibourdenche et al., 2009). This agrees with human studies that have shown that fetal sex does not alter maternal serum or umbilical cord blood levels of testosterone and dihydrotestosterone (Dawood and Sexena 1977; Rodeck et al., 1985).

Additionally, the maternal gonad maintains steroidogenesis throughout pregnancy, though to varying degrees in human, sheep, and rodent. To begin with, circulating LDL is also taken up by ovarian cells (granulosa, theca, small or large luteal cells) and processed as described above. In luteal cells of the sheep, human, and rat ovary, pregnenolone is converted to progesterone via 3β-HSD. The amount of progesterone produced by the corpus luteum, and the amount of luteal tissue, is dependent on the stage of pregnancy. For instance, in early sheep and human pregnancy, luteal production of progesterone is high and will maintain pregnancy. However, by the second trimester, luteal production of progesterone is supplement by the placental production (Nelson et al., 1992; Quirke et al., 2001; Senger 2003; Miller and Auchus 2010).

Placental Estrogen Signaling and Function

The major estrogens produced by the human placenta are estrone, estriol and estradiol, which begin to increase during the third to thirteenth weeks of gestation (Tulchinsky and Hobel 1953;

Siiteri and MacDonald 1963; 1966; Loriaux et al., 1972). These estrogens appears to play a very crucial and complex role during placental development in humans. Depending on the stage of gestation and hypoxic conditions, placental estrogen signals for varying cellular functions and physiological effects (Albrecht et al., 2006). Interestingly, oxygen concentration regulates aromatase activity in the placenta (Thompson and Siiteri 1974a,b; Goto and Fishman 1977; Zachariah and Juchau 1977), which can lead to impaired estrogen biosynthesis in hypoxic conditions (Aw et al., 1985; Rodesch et al., 1992). This might contribute to estradiol's varying functions between the first and second trimester, as a more hypoxic environment is present with restricted blood flow to the developing placenta during the first trimester when extensive differentiation of extravillous cytotrophoblasts and remodeling of the uterine spiral arteries occurs (Kaufmann et al., 2003). Additionally, this might lead to greater prominence of androgen signaling in syncytiotrophoblast in the first trimester as androgens up-regulates hypoxia inducible factors (HIFs) and vascular endothelial growth factor (VEGF) activity in a hypoxic environment (Shabisgh et al., 1999; Mabjeesh et al., 2003b; Cheng et al., 2004; Lissbrant et al., 2004; Boddy et al., 2005; Zhu and Kyprianou 2008).

As early as 11 weeks of gestation, HIF-1α activity is blocked by 2-methoxyestradiol (2-ME), a metabolite of estradiol that is present in the human placenta (Berg et al., 1983; Mabjeesh et al., 2003a; Ricker et al., 2004; Becker et al., 2008; Kanasaki et al., 2008). Under the hypoxic conditions that are present during the first trimester, 2-ME induces cytotrophoblast differentiation into an invasive phenotype (Lee et al., 2010). Cytotrophoblast differentiation also occurs *in vitro* as treatment of term human cytotrophoblasts with 17β-estradiol leads to differentiation into a syncytium of functionally mature syncytiotrophoblast capable of secreting

human chorionic gonadotropin (hCG) (Rama et al., 2004). Therefore, estrogen influences placental growth in the first trimester (Abdul-Karim et al., 1971) through increased syncytiotrophoblast differentiation and, thereby, increased synthesis of progesterone (Solomon 1994). It is widely accepted that the hypoxic environment during the first trimester leads to the trophoblast invasion (Zhou et al., 1993; Graham et al., 2000; Norwitz et al., 2001; Kadyrov et al., 2003; Bischof and Irminger-Finer 2005; Hayashi et al., 2005; Lyall 2006; Cohen and Bischof 2007; Lunghi et al., 2007; Robins et al., 2007; Rosario et al., 2008), likely through estradiol signaling such as with 2-ME (Lee et al., 2010).

At the start of the second trimester in primate pregnancy, there is a surge in estrogens that blocks the differentiation of cytotrophoblast cells into extravillous trophoblasts (Albrecht et al., 2006), likely facilitated through down-regulation of VEGF (Bonagura et al., 2008). This shift in estrogen function in the placenta during the second trimester, when estrogen levels surge, may be regulated by the increase in placental blood flow and oxygen at this time (Albrecht et al., 2006). However, once maternal spiral artery invasion has occurred and the high-flow, low-resistance blood flow is established to the placenta in the second trimester, high circulating estrogen levels are not required to maintain the uteroplacental blood flow that is established (Aberdeen et al., 2010), primarily because the invasion and establishment of the uteroplacental blood flow occurs in the first trimester with differentiation of the extravillous and endovascular cytotrophoblasts (Kaufmann et al., 2003).

Interestingly, reduced aromatase activity in the term placenta has been reported in preeclamptic patients, significantly reducing placental 17β-estradiol, estrone, and 2-methoxy-estradiol (Hertig

et al., 2010). This suggests that aberrant estrogen signaling during the first trimester in the preeclamptic placenta may contribute to the reduced placental invasion and pregnancy-induced hypertension presented in these patients (Redman and Sargent, 2003).

Placental Androgen Signaling and Function

Despite the research investigating the critical roles of estrogen in trophoblast cell function, there has been very limited research investigating the function of androgen signaling within the placenta. The limited research on androgen's role in placental development has primarily focused on its role in preeclampsia (PE). The earliest report from Thoumsin et al. showed that there is decreased aromatase activity in preeclamptic placentas compared to the placentas from hypertensive patients (1982). Women with PE have increase serum testosterone concentrations compared to normotensive pregnancies (Atamer et al., 2004; Ghorashi and Sheikhvatan 2008; Hsu et al., 2009), regardless of fetal gender (Lorzadeh and Kazemirad 2012). Preeclamptic patients in the third trimester have higher serum total and free testosterone levels when compared to normotensive pregnancies, although serum concentrations of DHEA-S, androstenedione, and SHBG are not different (Acromite et al., 1999; Serin et al., 2001; Salamalekis et al., 2006). An increase in androstenedione, testosterone, and free testosterone has also been reported at 17 and 33 weeks of gestation in women who eventually developed preeclampsia, while DHEAS was elevated at week 17 only (Carlsen et al., 2005). Increased serum total and free testosterone has also been reported in pregnancies with placental induced hypertension at 37 weeks of gestation (Gerulewicz-Vannini et al., 2006).

Increased placental expression of androgen receptor (AR) is also reported in the syncytiotrophoblast and stromal cells of preeclamptic placentas compared to placentas from normotensive pregnancies (Hsu et al., 2009; Sathishkumar et al., 2012). While increased serum testosterone (Atamer et al., 2004; Ghorashi and Sheikhvatan 2008; Hsu et al., 2009; Lorzadeh and Kazemirad 2012) and increase placental AR (Hsu et al., 2009; Sathishkumar et al., 2012) would suggest that androgen signaling contributes to or is a result of the pathogenesis of PE, decreased androgen signaling may also contribute to placental insufficiency (Lim et al., 2011). For instance, women are at an increased risk of developing PE if they have a polymorphism of greater than 16 GGC repeats in the transcriptional activation domain of the *AR* gene, leading to decreased AR function and expression (Lim et al., 2011).

Interestingly, by six weeks after delivery, serum testosterone and free testosterone levels from preeclamptic patients reaches circulating concentrations similar to those of normotensive, control patients (Serin et al., 2001), strongly suggesting a placental source for circulating androgen. It has been proposed that serum testosterone levels could be used as a marker for predicting late onset preeclampsia (Carlsen et al., 2005; Lorzadeh and Kazemirad 2012); however, a conflicting study has shown that serum testosterone and SHBG are not significantly different during the first and second trimester in patients that later developed preeclampsia compared to matched controls (Tuutti et al., 2011). Despite this report, these data suggest that androgen signaling may be very important for placental development and function, and that any abnormality in androgen signaling may contribute to pregnancy complications like PE. As placental androgen may play crucial roles in regulating trophoblast function and differentiation, similar to placental estrogen, future studies on androgen's role in trophoblast function and signaling are imperative.

Loss of Sex Hormone Signaling in the Placenta

Rodent knock out models of ESR1 and AR have been shown to be non-lethal (reviewed by Couse and Korach 1999; Yeh et al., 2002). ESR1 knock-out mice are infertile given the multifactorial functions of estrogen in developmental and reproductive physiology (reviewed by Couse and Korach 1999). In AR knock-out mice, only males are infertile while female mice have reduced litter sizes (Yeh et al 2002). Additionally, human cases of estrogen resistance have been reported, showing that functional loss of ESR1 is viable for fetal development (Smith et al 1994). In spite of these findings, the functional loss of ESR1 may be in part rescued with ESR2 function in some tissues (reviewed by Couse and Korach 1999). Additionally, infants born with placental aromatase deficiency (Harada et al., 1992a,b; Conte et al., 1994; Deladoëy et al., 1999) survived prenatal development (Shozu et al., 1991 Morishima et al., 1995; Carani et al., 1997), suggesting normal or at least compensatory placental development and function had occurred.

Human fetuses with androgen insensitivity also survive fetal development (Hughes and Evans 1987; Bangsboll et al., 1992; Brown 1995; Quigley et al., 1995; reviewed by Ahmed et al., 2000), suggesting that lost or perturbed sex hormone signaling may not be detrimental to placental development, despite findings of increased maternal serum androgens in compromised pregnancies (Atamer et al., 2004; Ghorashi and Sheikhvatan 2008; Hsu et al., 2009; Lorzadeh and Kazemirad 2012). However, other human case studies have suggested that functional loss of AR is correlated with multiple miscarriages (Wilson et al., 1974; Decaestecker et al., 2008). Heightened androgen signaling is also associated with recurrent spontaneous abortions and preterm labor (Karvela et al., 2008; Karjalainen et al., 2012). Given the conflicting reports on the

necessity of placental androgen signaling, further research is needed to clarify its role in normal placental development and trophoblast function.

Androgen Signaling in Cancer

To understand possible roles of androgen signaling in the placenta, insights can be obtained from studies on androgen signaling in cancer as cancer cells share many similarities to trophoblasts (Ferretti et al., 2007). For instance, cytotrophoblast cells undergo rapid cell divisions and are capable of maintaining an undifferentiated state; these features are also present in cancer (Ferretti et al., 2007). Additionally, cytotrophoblast can invade surrounding tissue, avoiding an immune response similar to cancer progression (Ferretti et al., 2007). Most importantly, trophoblasts are capable of recruiting maternal spiral arteries and remodeling them for increased vascularization of the placenta (Kaufmann et al., 2003). While cancer does not necessarily remodel arteries, tumors are capable of initiating vascularization (Ferretti et al., 2007).

Cancer cells and trophoblast cells also undergo an epithelial-mesenchymal transition (EMT). Mesenchymal cells are maintained in an undifferentiated state during gastrulation in the developing fetus (Vicovac and Aplin 1996). While mesenchymal cells are pluripotent cells capable of migration, polar epithelial cells can dedifferentiate to form mesenchymal cells in the EMT process. EMT is a normal process during gastrulation (or tissue development) in multicellular organisms. For EMT to occur, polar epithelial cells must undergo multiple changes, including loss of polarity, loss of specific cellular junctions (such as gap junctions that often separate epithelial cells and a basement membrane from underlying tissue), cytoskeletal remodeling, loss of E-cadherin expression, and redistribution of organelles. In addition, there are

molecular changes that induce epithelial cells to regain a pluripotent, mesenchymal state. Studies have shown that multiple signaling pathways are involved, but usually Wnt and Ras signaling, in cooperation with transforming growth factor beta (TGFβ) and mitogen activated protein kinase (MAPK), are necessary for EMT to occur. Once epithelial cells have returned to the de-differentiated, pluripotent state of mesenchymal cells, they are capable of migrating and proliferating via PI3K signaling, and have increased resistance to apoptosis (Theiry 2003; Kalluri and Weinberg 2009).

While EMT occurs primarily during gastrulation, it is also occurs in adult stages of multicellular organisms, likely as a mechanism to respond to physiological stress and allow for specific tissue repair mechanisms throughout the life of the organism. However, when molecular regulation of EMT goes awry, there can be uncontrolled proliferation of undifferentiated mesenchymal cells. These cells are capable of migrating to other tissue and cause disease states such as tissue fibrosis or cancer (Thiery 2003).

In addition to continuous embryonic and adult EMT, the trophoblasts in the placenta also undergoes EMT during development. The polar trophoblast cells surrounding the blastocyst undergo EMT to form cytotrophoblasts. Cytotrophoblasts, similar to mesenchymal cells, are considered pluripotent as they function as a placental stem cell, capable of migrating and differentiating into invasive extravillous cytotrophoblasts, endovascular extravillous cytotrophoblasts, and syncytiotrophoblast,. Early reports had suggested that the differentiation of cytotrophoblasts was also an EMT, although this is now debated. However, in contrasts to EMT, polar syncytiotrophoblast, which functions as an epithelial layer of the placenta, cannot revert

back to cytotrophoblast as the differentiation to the syncytiotrophoblast in the human placenta requires the loss of individual cell membranes to form the continuous, multinuclear syncytium (Vicovac and Aplin 1996).

Although placentation is similar to cancer in that there is rapid cell proliferation and migration/invasion, there are differences between the two. Primarily, in contrast to cancer, EMT in the placenta is controlled as there is no mutagenic protein or gene expression regulating the trophoblast dedifferentiation, proliferation, and invasion. Additionally, environmental signals promote EMT in the placenta, such as apposition of the blastocyst with the uterine decidua via integrin-integrin or integrin-extracellular matrix interactions (Vicovac and Aplin 1996; Senger 2003). In cancer, while environmental signals may stimulate EMT, the process is essentially uncontrolled and self-propagating (Thiery 2003). It has been suggested that placenta development and embryonic gastrulation, with their controlled EMT, are ideal models for studying mechanisms to inhibit or reverse EMT in cancer due to their overlapping molecular signaling (Kalluri and Weinberg 2009).

Therefore, since cancer cells have overlapping EMT and signaling pathways as trophoblasts cells, androgen signaling may function similarly in both the placenta and cancer. Outlined below are androgen-regulated pathways in cancer that affect cell invasion/motility, proliferation, vascularization, and immune regulation, all of which are crucial for placentation (Ferretti et al., 2007).

Androgens have been shown to regulate cell cycle progression and proliferation, either directly or through secondary response genes: *cyclin G2, cyclin-dependent kinase 8, tumor protein D52,* and *RAB4A,* a member of the RAS oncogene family (Comstock et al., 2008). Androgen signaling also increases cell proliferation via up-regulation of insulin like growth factors (IGFs) (Constáncia et al., 2002; Zhu and Kyprianou 2008). This occurs via AR-mediated up-regulation of IFG1 receptor and also through activation of *IGF1* transcription as two androgen response elements (AREs) are located on the *IGF1* promoter (Wu et al., 2007). Androgen stimulated IGF1 signaling is self-regulated as both androgens and IGF1 increase IGF binding protein 5 (*IGFBP5*) (Yoshizawa and Ogikubo, 2006). Additionally, insulin regulates AR expression and transcription through activation of the phosphatidylinositol 3-kinase transduction pathway (Manin et al., 1992; 2000; 2002). Androgens also regulate epidermal growth factors (EGF), transforming growth factors (TGFβ), and fibroblast growth factor (FGF) in prostate cancer (Zhu and Kyprianou 2008). In rat prostate epithelial cells, androgens were found to induce 66 genes that are immediate-early response genes that are known to regulate cell proliferation, cell differentiation, and cell immune/inflammatory response pathways (Asirvatham et al., 2006).

Androgen signaling also appears to regulate cell mobility by increasing expression of *fibronectin 1, fascin homolog 1, myosin heavy chain (type b), tropomyosin 1 (alpha),* and *serum/glucocorticoid regulated kinase* (Comstock et al., 2008). In addition, as androgens increase expression of matrix metalloproteinases (MMPs) MMP2, MMP9, and MMP16 (Liao et al., 2003, Limaye et al., 2008; Comstock et al., 2008), it is likely that androgen signaling may increase cell mobility and tissue invasion (Comstock et al., 2008). Androgen further regulates immune response via *T-cell receptor gamma variable 9* and *major histocompatibility complex*

(Comstock et al., 2008). Additionally, research has suggested that androgen is capable of increasing vascularization by up-regulating VEGF-A (Lissbrant et al., 2004; Cheng et al., 2004; Shabisgh et al., 1999; Zhu and Kyprianou, 2008) via hypoxia sensing HIF-1α protein (Mabjeesh et al., 2003b; Boddy et al., 2005).

Androgen Receptor Interactions with Histone Lysine Demethylases

Much of androgen signaling for cellular proliferation, motility, and vascularization was traditionally believed to occur through direct binding of ligand-bound androgen receptor (AR) to DNA for gene transcription (Mangelsdorf et al., 1995). Recent research in prostate cancer has shown that increased androgen-dependent transcription likely occurs with the aid of AR-complexed lysine demethylase 1 family/Jumonji-C domain containing histone lysine demethylases (KDMs) (Metzger et al., 2005; Yamane et al., 2006; Wissmann et al., 2007; Shin and Janknecht 2007a,b). AR complexing with KDMs appears to aid in localizing KDM activity for oxidation of methyl groups on lysines in histone N-terminal tails, leading to demethylation at mono-, di-, or tri-methylated lysines near androgen response elements (AREs) (Forneris et al., 2005; Verrier et al., 2011). While AR binding may not alter KDM conformation, alignment of lysine methyl groups to iron (Fe_{II}) in KDMs near AREs facilitates hydroxylation and demethylation in histone tails, reducing their interaction with DNA for transcription initiation (Anand and Marmorstein 2007; Couture et al., 2007; Garcia-Bassets et al., 2007). Additionally, the presence of AR-KDM complexes aids in the recruitment of transcription coactivators and RNA polymerase II (Perillo et al., 2008).

KDMs appear to form heterocomplexes with AR such that complete demethylation of histone lysine residues occurs. For instance, KDM1A and KDM3A are capable of demethylating mono- and dimethylated histone 3 lysine 9 (H3K9) when complexed with ligand bound AR (Metzger et al., 2005; Yamane et al., 2006; Wissmann et al., 2007). AR-KDM1A/KDM3A can further complex with the demethylases KDM4A, KDM4C, or KDM4D (Shin and Janknecht 2007b), which demethylate trimethylated H3K9 for increased transcription (Klose et al., 2006; Shin and Janknecht, 2007a; Wissmann et al., 2007). Interestingly, KDM1A activity has also been shown to produce hydrogen peroxide (Forneris et al., 2005), and localized hydrogen peroxide may function to amplify AR-KDM receptor signaling (Felty et al., 2005; Yang et al., 2010). Perillo suggests that the oxidation of bases with peroxide activity generates nicks that recruit 8-oxo-guanine-DNA glycosylase 1 (OGG1) and topoisomerase IIβ, relaxing the DNA strands around the histone to promote the formation of transcription initiation complexes (et al., 2008).

KDMs appear to play a crucial role in regulating androgen signaling in prostate cells. For instance, blocking KDM1A activity with the chemical pargyline in human prostate adenocarcinoma cells inhibits AR-KDM1A complex formation, decreases androgen-induced transcription, and reduces cell proliferation (Metzger et al., 2005). Additionally, up-regulation of KDM4A and KDM4C are associated with increased risk for prostate cancer recurrence (Kahl et al., 2006; Cloos et al., 2006). As KDMs regulate histone tail modifications, they also regulate localized DNA methylation and recruitment of DNA methyl-binding proteins (discussed below). Given this interaction, AR-KDM complexes provide a possible mechanism by which sex steroid hormones could regulate gene transcription and/or promoter methylation to direct trophoblast function and differentiation.

Placental Developmental Programing

Epigenetics and Methylation

Inheritance and gene expression on its most basic level follows Mendelian genetics (Cooper and Hausman 2000). However, gene expression is much more complex and follows a higher level of regulation referred to as epigenetics (Monk 1995). Epigenetic changes in gene regulation and expression are heritable, self-perpetuating, and reversible (Bonasio et al., 2010). And, because epigenetic changes are heritable and reversible, any environmental factors that change epigenetic regulation can lead to developmental programing by changing the regular pattern of gene expression (Monk 1995). Epigenetic control of gene expression occurs in multiple ways (Monk 1995), including DNA coiling, chromatin structure on nucleosomes, and methylation of cytosines on CpG islands (Holliday 1987; Monk 1995). For instance, increased gene or promoter methylation is heritable and capable of programing decreased gene expression in ensuing generations (Wigler et al., 1981; Stein et al., 1982; Doerfler 1983)

Genomic DNA is tightly coiled and bound by histones and an assortment of regulatory proteins, forming chromatin. Within the tightly wound chromatin structure lies nucleosomes, which are units of 146 base pairs of DNA wrapped around an octamer of histones. There are only four core histones, designated H2A, H2B, H3, and H4, that complex to form the octamer in the nucleosome (two dimers of H3-H4 and two dimers of H2A-H2B form the octamer). As the DNA coils around the histones, the histone N-terminal tails are directed toward the periphery of the nucleosome, making them available for modifications. Histone tails are typically modified by acetylation, phosphorylation, ubiquitination, or methylation. Histone modifications are regulated by an extensive group of enzymes, such as acetyltransferases (HAT1, FIP60, etc.), deacetylases

(SirT2), methyltransferases (SUV39H1, G9a, MLL1, etc.), lysine demethylases (KDM1A, KDM4A, KDM4D, etc.), arginine methyltransferases (CARM1, PRMT4, and PRMT5), serine/threonine kinases (Haspin, MSK1, Mst, etc.), ubiquitinases (Bmi and RNF20), and proline isomerases (ScFPR4) (Strahl and Allis 2000; Peterson and Laniel 2004; Kouzarides 2007).

Exactly where these histone modifications occur is important for determining the effects it will have on gene transcription. For instance, histone methylation can occur in promoter, insulator, and enhancer regions of genes. When methylation occurs on H3K36 (histone 3, lysine36) or H3K9 of a coding region, it can promote DNA transcription; but, when H3K36 or H3K9 methylation occurs within the reading frame of a gene, DNA transcription is silenced. Additionally, histone modifications can occur on multiple residues within the histone tails to dynamically regulate gene transcription. For instance, while acetylation has primarily been described on lysine residues, methylation has been shown to occur on lysine and arginine residues (Li 2002; Kouzarides 2007; Barski et al., 2007). As lysine residues can be monomethylated, dimethylated, or trimethylated, their methylation status can further regulate gene transcription through recruitment of transcriptional activators or repressors (Grunstein 1997; Jenuwein and Allis 2001; Rice and Allis 2001; Hake and Allis, 2006; Metzger et al., 2006).

In general, though, methylation on histone tails is considered to be repressive to gene transcription by changing the conformation of the chromatin, blocking the binding of transcription factors, and by recruiting transcriptional repressors. Acetylation, on the other hand, typically promotes gene transcription. Histone acetyltransferases (HDACs) transfer an acetyl

group onto a lysine residue in the histone tail. This loosens the binding of the DNA to the histones and increases accessibility for transcription. Histone deacetylases remove this acetyl group, functioning to retain the close DNA–histone interaction and reduce transcription (Zhang and Meaney 2010). While acetylation and phosphorylation were known to be reversible, histone methylation was traditionally considered to be a permanent silencer of gene transcription; however, it is now known that methylation is reversible and can also occasionally act as a transcription promoter (Metzger et al., 2006). Similarly, deamination, ubiquitination, and sumoylation modifications on histone tails are usually correlated with gene repression. However, ubiquitination has also been shown to increase gene expression, likely through variation in its localization or through interaction with other histone modifications and transcriptional proteins (Strahl and Allis 2000; Zhang and Reinberg 2001; Kouzarides 2002; Metzger et al., 2006).

Regulation of histone modifications is highly complex, with multiple modifications occurring on a single histone tail. These alterations can also regulate further modifications on the same and neighboring histone tails. For instance, while methylation is generally considered to be repressive to gene transcription, it is also thought to recruit acetyltransferase that can acetylate a neighboring histone tail to increase gene transcription. Given that there are multiple residues on each histone tail that can be affected, and that a complex assortment of modifications occurs on each of the histone tails, it allows for highly complex regulation of the nucleosomes' interaction with DNA, histone tail configuration, protein recruitment, and, ultimately, gene transcription. Therefore, the final effects of histone tail modifications are altered chromatin structure on the nucleosome and DNA accessibility to transcription factors (Strahl and Allis 2000; Li 2002; Kouzarides 2007).

Cellular inheritance of histone modification can be accomplished by distribution of modified histones to each of the daughter cells during replication. The modified histones are then capable of recruiting additional proteins and enzymes to renew the pattern of histone modification in both daughter cells such as by using reader-writer remodeling complexes. For example, H3K9 methylation will recruit the protein HP1. HP1 then functions to recruit methyltransferases that continue the H3K9 methylation pattern. Interestingly, HP1 has also been shown to interact with DNA methyltransferase (DNMT1) and histone deacetylase (HDAC) to block gene transcription (Burgers et al., 2002; Li 2002; Kouzarides 2007; Alberts et al., 2008).

Another form of epigenetic regulation of gene expression occurs with direct methylation on the DNA (Zhang and Meaney 2010) through the addition of methyl groups to cytosines in the DNA sequences, typically at CpG sites (Razin and Riggs 1980; Bird 1986; Holliday 1989; Bird 2002; Shilatifard 2006). These CpG sites usually occur within the promoter region, or in the 5' flanking region of a gene, where areas of heavy methylation can form to prevent genes transcription (Razin and Riggs 1980; Bird 1986; Holliday 1989; Bird 2002). Methylated DNA typically prevents transcription factor binding and is also capable of binding proteins attracted to methylated DNA, such as methyl-binding protein MECP2 and HDACs; this further blocks binding of transcription factors (Klose and Bird 2007).

DNA methyltransferases (DNMTs) regulate DNA methylation and are therefore associated with transcriptional silencing and imprinting (Hiendleder et al., 2004; Wilson et al., 2007; Beck and Rakyan 2008). Maintenance of methylation is regulated by DNMT1 and is required for embryonic development (Bird 2002; Gopalakrishnan et al., 2008; Kim et al., 2009; Ross et al.,

2010). DNMT3a and DNMT3b regulate *de novo* methylation patterns (Gopalakrishnan et al., 2008). Genome-wide reprogramming of DNA methylation and histone modifications occurs during gametogenesis and embryogenesis in various species (Feng et al., 2010), with massive demethylation occurring between the 8 cell embryo and blastocyst in mice (Monk et al., 1987). *De novo* methylation by DNMT3a and DNMT3b occurs at the time of implantation in the mouse, detectable first in the inner cell mass and occurring independently in all extraembryonic cell lineages (Monk et al., 1987; 1991). Similarly, *de novo* methylation occurs in the human embryo and developing placenta at the time of implantation (Maccani and Marsit, 2009). This also occurs with independent *de novo* methylation patterns in the extraembryonic tissues and developing inner cell mass (Monk et al., 1987; Katari et al., 2009).

In addition to histone modification and DNA methylation, a cell can inherit proteins from the cytoplasm of the progenitor cell that is self-regulating (i.e. the protein binds to DNA and promotes its own transcription). Cells can also inherit cytoplasmic small RNAs, such as microRNAs and small interfering RNAs, which regulate gene translation, are associated with chromatin-modifying complexes, and can deliver histone-modifying enzymes to chromatin. And, because miRNAs can be sequence specific, it is possible for them to deliver chromatin-modifying complexes directly to a target gene. In summary, all these mechanism of epigenetic cell memory work together to create the highly regulated and specific mechanisms for epigenetic inheritance of gene expression (Li 2002; Verdel et al., 2004; Buhler et al., 2006; Kouzarides 2007).

It is important to note, however, that genomic imprinting, a form of epigenetic gene regulation, occurs when there is differential and preferential expression of a gene from either the maternal or paternal allele (Monk 1995). Imprinting is acquired through regulation of DNA methylation and is highly regulated in the developing fetus and placenta (Monk 1995).

Imprinting in the Placenta

Imprinted genes appear to be of great importance for placental development (Georgiades et al., 2001; Tycko and Morison 2002; Angiolini et al., 2006), especially as many of the imprinted genes in the human are specifically imprinted within the placenta (Coan et al., 2005). Placental imprinted genes have a parent-of-origin imprint that led to the "Kinship theory" which suggests that the paternal imprint occurs on genes that would enable maximal nutrient allocation to the fetus and placenta and allow for maximal fetal growth (Moore and Haig 1991;Ferguson-Smith and Surani 2001; Reik et al., 2001; Reik and Walter 2002; Reik et al., 2003; Wilkins and Haig 2003; Haig 2004; Constancia et al., 2004; Angiolini et al., 2006; Myatt 2006; Wagschal and Feil 2006; Wagschal et al., 2008). For instance, the paternal imprint on long non-coding *H19* allows for maternal expression that decreases placental surface area whereas *H19* imprint loss leads to fetal and placental overgrowth (Angiolini et al., 2006; Leighton et al., 1995). Additionally, the "Kinship theory" maintains that maternal imprint occurs on genes in the placenta that would reduce excessive nutrient partitioning to the fetal-placental unit, such as *insulin-like growth factor 2* (*IGF2*), in order to ensure maternal survival of pregnancy (Moore et al., 1997; Constancia et al., 2002; Fowden 2003; Constancia et al., 2004; Sibley et al., 2004; Constancia et al., 2005; Angiolini et al., 2006). It is therefore through an equilibrium of parent-of-origin

imprinting that normal placental growth and nutrient transfer can occur (Angiolini et al., 2006; Tycko and Morison 2002; Georgiades et al., 2001; Constancia et al., 2004).

These findings suggest that any change from normal methylation status in genes integral for placental growth could lead to abnormal placentation and fetal development. Indeed, studies have suggested that this may at least be a partial cause for the development of early onset preeclampsia (PE) as increased hypomethylation (Kokalj-Volkac et al., 1998; Katari et al., 2009) is seen in thirty-four gene loci compared to control pregnancies (Yuen et al., 2010). Interestingly, essentially no changes were observed in global DNA methylation between late onset PE or intrauterine growth restriction when compared to control pregnancies at 1505 CpG sites associated with 807 genes (Yuen et al., 2010). In addition, increased genomic cytosine methylation in the placenta is associated with bovine fetal overgrowth (Hiendleder et al., 2004). Alterations in imprinting have been linked to placental pathologies (Tycko 2006; Wagschal and Feil, 2006), including increased risk for developing PE when there is maternal inheritance of imprinted gene mutations (Oudejans et al., 2004). Also, matrilineal inheritance of imprinted genes in preeclamptic patients leads to down-regulation of protein expression similar to that seen in placentas from androgenized pregnancies, such as SIRT1 deacetylates and MYST4 histone acetyltransferase (Oudejans, et al., 2004).

It now appears that epigenetic regulation of placental phenotype regulates nutrient uptake, placental growth, prenatal development, and, consequently, developmental programming (Fowden et al., 2010).

Sheep Models for Abnormal Placental and Developmental Programming

Researchers have used multiple animal models to study placental development and function; however, no animal model perfectly replicates human placentation (Carter 2007; Barry and Anthony 2008). Even the primate species macaque and baboon have less invasive placentas compared to humans (Carter 2007). While the sheep placenta has essentially no trophoblast invasion (Leiser and Kaufmann 1994), it is still a well-established model for studying maternal-fetal interactions and nutrient exchange (Carter 2007; Barry and Anthony 2008). There are many established ovine models for abnormal placental and fetal development (Anthony et al., 2003; Barry and Anthony 2008; Morrison 2008), including maternal over-nutrition, maternal under-nutrition, hyperthermia, and utero-placental embolism (Anthony et al., 2003; Morrison 2008). All of these models are effective at altering developmental programing as they drastically alter the intrauterine environment and, in turn, alter fetal-placental gene expression and development. And as the placenta regulates nutrient and oxygen supply to the fetus and regulates fetal growth, intrauterine developmental programing of the fetus is also likely strongly regulated by the placenta (Fowden et al., 2008; Vaughan et al., 2012). Therefore, any aberrant placentation, either due to genetic or environmental factors, can lead to changes in developmental programming of the fetus (Fowden et al., 2008). For instance, it has been show that perinatal exposure to a variety of chemical compounds, including estrogenic hormones, leads to developmental programing and can lead to adult onset of disease, such as obesity (Heindel and vom Saal 2009).

One such established model for developmental programing is prenatal androgenization in sheep, whereby pregnant ewes receive intramuscular injections of 100mg testosterone propionate suspended in cottonseed oil twice weekly during gestational days (GD) 30 to 90, when sexual

differentiation of the fetus occurs (Wood and Foster 1998; Robinson et al., 2002). Prenatal androgenization has been shown to cause reproductive neuroendocrine, ovarian, and metabolic dysfunction in mice, rats, sheep, and monkeys (Padmanabhan et al., 2010; Padmanabhan and Veiga-Lopez 2011).

As limited research has investigated the role of placental androgens, the model of prenatal androgenization is of particular interest in determining possible prenatal developmental programming effects of androgens. Research has shown that maternal exposure to androgen in pregnant rats impairs fetal growth due to placental insufficiency, primarily contributed to compression of spongiotrophoblast and reduced amino acid transport (Sathishkumar et al., 2009; 2011) as testosterone does not appear to cross the rat placenta to directly suppress fetal growth (Sathishkumar et al., 2011). Prenatal androgenization in pregnant ewes also leads to intrauterine growth restriction of female lambs (Manikkam et al., 2004; Steckler et al., 2005; Veiga-Lopez et al., 2011). In addition to altered programing during intrauterine growth restriction, prenatal androgenization in rats, sheep, and humans programs for increased onset of adult hypertension (King et al., 2007; Sathishkumar et al., 2009; 2011; Chinnathambi et al., 2012).

When pregnant ewes are treated with testosterone propionate from gestational day (GD) 30 to 90, maternal and fetal testosterone levels increase to that of adult male rams and GD65 control male fetuses, respectively (Veiga-Lopez et al., 2011). Using this model, prenatal androgenization in sheep programs for polycystic ovary syndrome (PCOS) as it is similar to *in utero* developmental programming for PCOS in humans with androgen excess (Recabarren et al., 2005; Padmanabhan et al., 2006; Veiga-Lopez et al., 2008). Indeed, pregnant women with PCOS

have elevated serum androgens, leading to gestational hyperandrogenism and prenatal programing (Sir-Petermann et al., 2002). Adult ewes exposed to heightened prenatal androgen develop symptoms similar to PCOS, including hyperandrogenism, phenotypic masculinization, anovulation, metabolic disruption, hyperinsulinemia, and polycystic ovarian morphology (Abbott et al., 2006; Xita and Tsatsoulis 2006; Padmanabhan et al., 2006; Manikkam et al., 2008). Prenatal programing for PCOS may be in part due to increased AR expression in granulosa cells (Ortega et al., 2009). Interestingly, testosterone treatment reduces Cyp11a1 expression in GD90 ovaries and the downstream aromatization to estrogen increases Cyp19 and 5α-reductase in GD65 lamb ovaries (Luense et al., 2011). Increased follicular recruitment and morphological changes in the ovary are seen with both prenatal testosterone and DHT treatment, while changes in follicular depletion and follicular growth are found only with prenatal testosterone treatment (Steckler et al., 2005), showing that estrogenic programming is also necessary for prenatal programming of PCOS in sheep (Smith et al., 2009; Salvetti et al., 2012).

In addition to reduced female ovarian function, prenatal androgenization also leads to reduced fertility in male lambs (Recabarren et al., 2007; Manikkam et al., 2008). Reduced fertility in males and females may in part be due to reduced estradiol negative (Wood and Foster 1998; Sarma et al., 2005; Veiga-Lopez et al., 2009) and positive feedback (Masek et al., 1999; Sharma et al., 2002; Unsworth et al., 2005; Veiga-Lopez et al., 2009), and negative progesterone feedback (Robinson et al., 1999; Veiga-Lopez et al., 2008). Prenatal conversion of testosterone excess to estrogen likely programs the timing of the luteinizing hormone (LH) surge in adult sheep, leading to the neuroendocrine disruption reported in prenatally androgenized lambs (Manikkam et al., 2008). Reduced fertility may also be due to changes in pituitary gonadotropin-

releasing hormone (GnRH) and estrogen receptor expression (Manikkam et al., 2008). Prenatal androgenization of female lambs leads to increased pituitary *GnRH*, *ESR1*, and *ESR2* mRNA, an amplified GnRH-induced LH response, LH hypersecretion, and acyclicity (Manikkam et al., 2008).

Prenatal androgenization also causes a change in placentome morphology (Astapova et al., 2004) which has also been reported in maternal nutrient restriction in sheep (Vonnahme et al., 2006). The changes in gross placentome morphology are defined as follows: type A placentome is caruncluar tissue completely surrounding the cotyledonary tissue with a cup-like structure; type B placentome is an intermediate form with cotyledonary tissue beginning to grow over the surrounding caruncle tissue; type C placentome is a flat intermediate placentome with cotyledonary tissue on one surface and caruncle tissue on the other; and type D placentome is an inverted or convex placentome with cotyledonary tissue entirely present, resembling bovine placentomes (Vatnick et al., 1991; van der Linden et al., 2012; Appendix II). Maternal nutrient restriction and prenatal androgenization increase placental placentome preference to type D, C, and B placentomes compared to control ewes that have primarily type A placentomes (Astapova et al., 2004; Vonnahme et al., 2006). It was suggested that these changes in placentome morphology toward a type D possibly compensates for maternal nutrient restriction or placental insufficiency, functioning to deliver normal nutrient and gas exchange to the developing fetus (Vonnahme et al., 2006). However, nutrient and gas exchange was not measured across placentome types and the histology has never been evaluated for changes in pathology. In a separate study where all intermediate and convex types of placentomes occurred without nutrient restriction or prenatal androgenization, it was found that type B and D placentomes had

increased capillary area density compared to type A placentomes, but placentome size, not type, may be a better indicator of placental nutrient transfer and vascularization (Vonnahme et al., 2008).

Additionally, the changes observed in gross placentome morphology in prenatal androgenization can be contributed directly to testosterone treatment or placental hormone signaling as cottonseed oil contains essentially no phytoestrogens (Grippo et al., 1999). However, it cannot be entirely ruled out that estrogenic effects in prenatal androgenization contribute to the placentome morphology as testosterone can be aromatized within the placenta (Fournet-Dulguerov et al., 1987; Payne and Hales 2004; Tuckey et al., 2004; Guibourdenche et al., 2009; Sathishkumar et al., 2012) and even DHT can be converted to 3β-diol and signal through estrogen receptor β (ESR2) (Handa et al., 2008). Abnormal fetal programing observed with prenatal androgenization may also be due to a direct effect on the developing fetus from sex hormone signaling, or it may be due to abnormal placental developmental programing (Veiga-Lopez et al., 2011). However, further research is needed to determine the effects of placental androgen signaling on programing gene expression and placenta development.

Preliminary Data and Conclusion

Using the model of prenatal androgenization in sheep for abnormal developmental programming (Padmanabhan et al., 2006), our lab has also reported a shift to type D placentomes in the placenta of androgenized ewes. We also determined that there was a decrease in global DNA methylation in androgenized placentomes compared to controls by ELISA (Halleran et al.,

2011), suggesting that aberrant sex hormone signaling disrupted normal epigenetic regulation of placental gene expression and contributed to abnormal placenta development.

As recent studies have shown that ligand bound AR is capable of complexing with demethylases for increased transcription (Metzger et al., 2005; Yamane et al., 2006; Wissmann et al., 2007; Shin and Janknecht 2007a,b), and since androgens are known to regulate cellular proliferation, migration, and vascularization pathways (Comstock et al., 2008), it is of particular importance to determine the function of androgen signaling in normal placental development and in placental developmental programming. Research into this area will fill a critical gap in our knowledge about epigenetic regulation of placental developmental programming that may underlie varying prenatal conditions (Fowden et al., 2010). Further investigation of androgen induced placental epigenetic programming should increase our understanding of placental induced pregnancy complications, in particular preeclampsia, which is associated in increased maternal serum testosterone (Acromite et al., 1999; Atamer et al, 2004; Carlsen, et al., 2005; Salamalekis et al., 2006; Gerulewicz-Vannini et al., 2006; Ghorashi and Sheikhvatan, 2008; Hsu et al., 2009), increased placental AR (Hsu et al., 2009), and reduced placental aromatase activity (Thoumsin et al., 1982; Hähnel et al., 1989; Hertig et al., 2010).

Given the known mechanisms of estrogen signaling for trophoblast differentiation, and the absence of research on androgen signaling within the placenta, this dissertation was directed to characterize the role of androgen signaling for placental development through in vivo and in vitro molecular approaches.

Justification

Abnormal placental developmental programing during the first trimester can lead to insufficient trophoblast remodeling of maternal arteries, leading to placental-induced disorders such as preeclampsia (PE) (Kaufmann et al., 2003). PE occurs in approximately 4% of live births in the United States, instigating fetal growth restriction and maternal/fetal mortality and morbidity (Ilekis et al., 2007). Preeclamptic placentas have reduced aromatase activity (Thoumsin et al., 1982; Hähnel et al., 1989; Hertig et al., 2010), increased androgen receptor (AR) (Hsu et al., 2009), and, recently, PE patients have been found to have increased serum testosterone (Acromite et al., 1999; Atamer et al, 2004; Carlsen et al., 2005; Salamalekis et al., 2006; Gerulewicz-Vannini et al., 2006; Ghorahi and Sheikhvatan, 2008). Additionally, human prenatal exposure to heightened testosterone leads to abnormal developmental programing and increased incidence of adult-onset of diseases, such as hypertension (Chinnathambi et al., 2012).

Despite these findings, there has been limited research on the physiological role of androgen on trophoblast function. In cancer cells, androgens regulate cell invasion by increasing matrix metalloproteinases (Liao et al., 2003, Limaye et al., 2008; Comstock et al., 2008). In addition, androgen also regulates vascularization and proliferation by interacting with histone lysine demethylases (KDMs) to regulate target gene transcription (Metzger et al., 2005; Yamane et al., 2006; Shin and Janknecht 2007a,b; Wissmann et al., 2007), including increased cellular vascular endothelial growth factor (VEGFA) (Lissbrant et al., 2004). This is of particular relevance as, similar to cancer cells, trophoblasts undergo an epithelial to mesenchymal transition, invade surrounding tissue, and access a blood supply (Ferretti et al., 2007), suggesting that androgens may function similarly in the placenta. The overall goal of this project is to determine

testosterone's effect on trophoblast function through its regulation of gene transcription and interaction with histone lysine demethylases.

Hypothesis

We hypothesize that placental androgen receptor interacts with histone lysine demethylases to regulate androgen responsive gene expression, in particular genes that regulate trophoblast function, differentiation, and invasion.

Aim 1: Determine the localization of androgen receptor and histone lysine demethylases in normal and prenatal androgenized ewe pregnancies, and in human first trimester trophoblasts.

Aim 2: Determine if androgen receptor complexes with histone lysine demethylases at androgen response elements in sheep and human trophoblasts to regulate gene transcription and trophoblast function.

Data from these experiments will fill a critical gap in our knowledge regarding the role of androgen in trophoblast function and differentiation. Specifically, identifying the role of androgen in regulating gene expression through interactions with KDMs provides new and critical insights on regulation of trophoblast differentiation, and will aid in understanding the pathogenesis of placental disorders, such as preeclampsia, that are associated with elevated levels of androgens. Importantly, by using both human trophoblast cells as well as sheep placentomes, insight obtained from this study will benefit and advance our understanding of placental physiology and function in humans and domestic agricultural animals. Ultimately, this

knowledge may lead to new avenues for maternal therapy in cases of placental dysfunction to improve fetal growth and postnatal health.

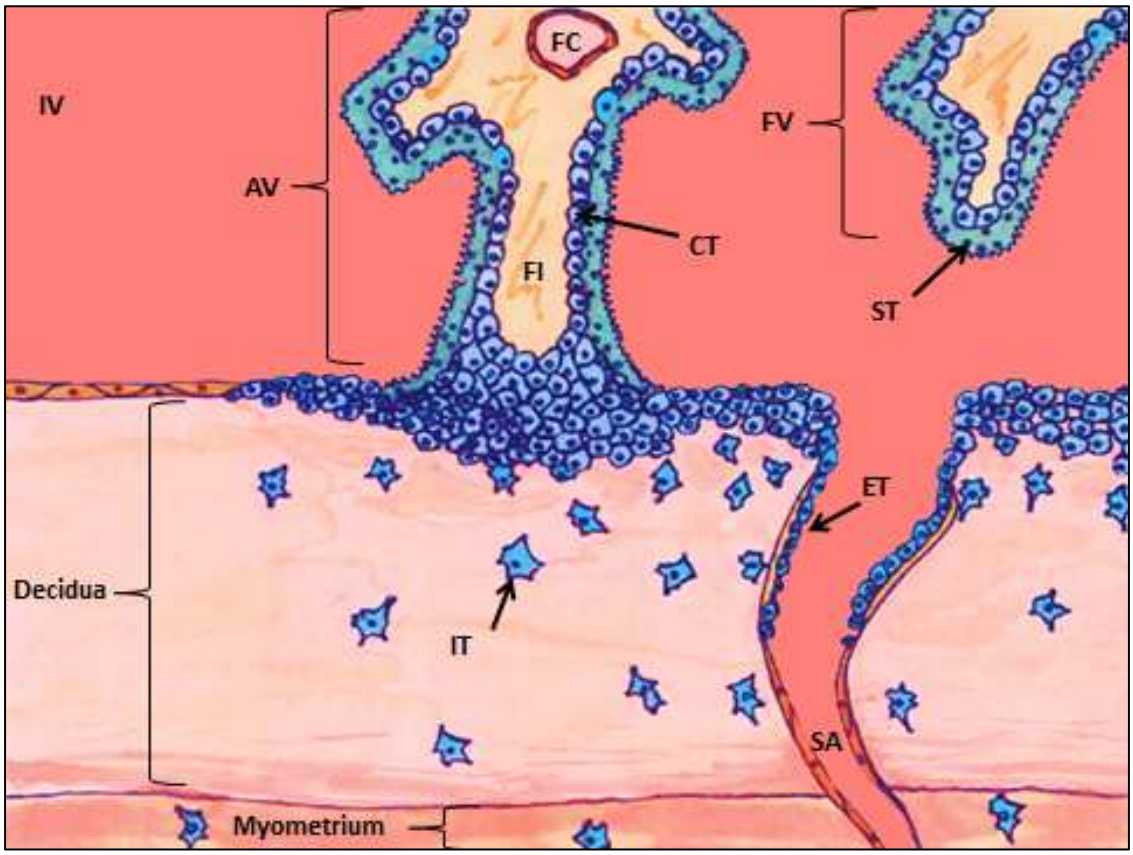


FIGURE 1.1 Structure of the Primate Placenta. AV, anchoring villi; CT, cytotrophoblasts; ET, endovascular extravillous trophoblasts; FC, fetal capillary; FI, fetal interstitium; FV, floating villi; IT, interstitial extravillous trophoblasts; IV, intervillous space; SA, spiral artery

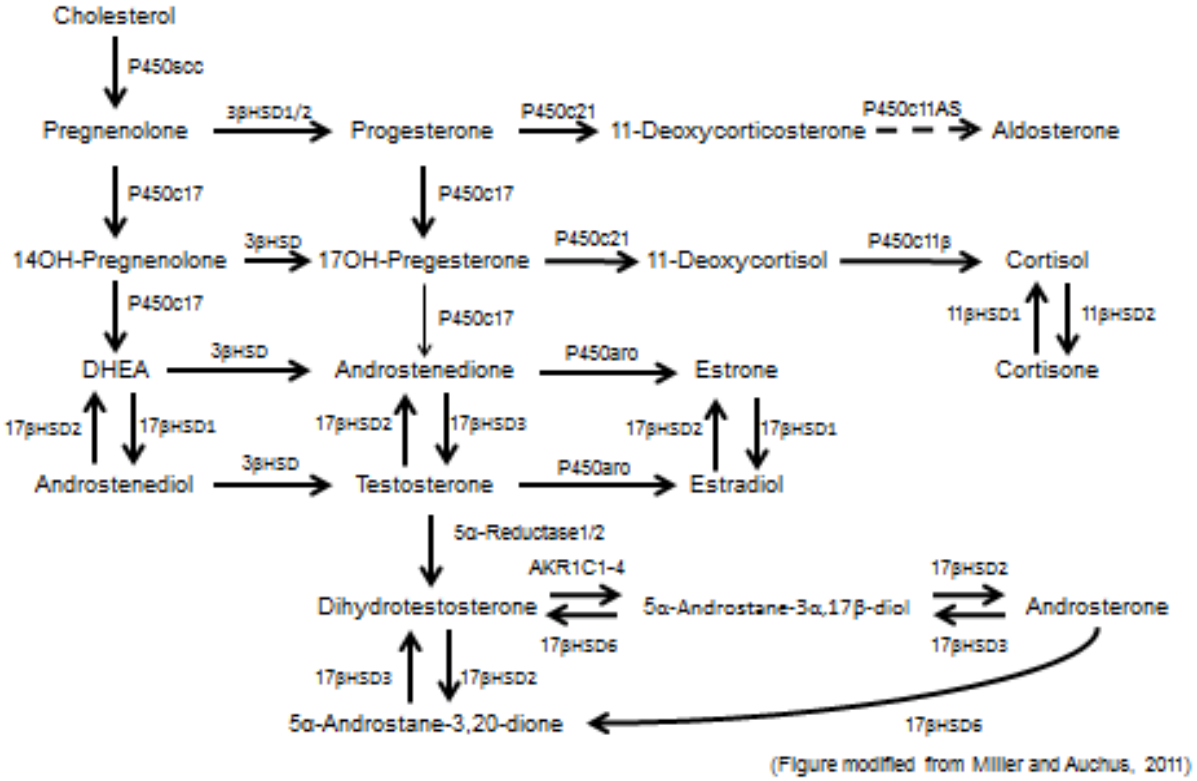


FIGURE 1.2 Steroid hormone synthesis

TABLE 1.1 Trophoblast immunolocalization of sex hormone receptors

Receptor	Syncytiotrophoblast	Differentiating Cytotrophoblast	Cytotrophoblast	Extravillous Cytotrophoblast
AR	++	−+	−	NA
ESR1	−	++	++	−+
ESR2	+	−+	−+	++

++ Positive Immunolocalization
+ Limited/Low Immunolocalization
−+ Low/Variable Immunolocalization
− Not Present
NA Not Determined

CHAPTER II: ANDROGEN RECEPOR AND HISTONE LYSINE DEMETHYLASES IN OVINE PLACENTA[1,2,3]

Summary

Sex steroid hormones regulate programing in many tissues, including programing gene expression during prenatal development. While estradiol is known to regulate placentation, little is known about the role of placental testosterone despite its rise in maternal circulation during pregnancy and placenta-induced pregnancy disorders. Using androgenized pregnant ewes, we investigated the role of testosterone in placental DNA methylation and gene expression. Placental androgenization decreased global DNA methylation in gestational day 90 placentomes and increased placental expression of genes involved in epigenetic gene regulation, angiogenesis, and growth. As androgen receptor (AR) complexes with histone lysine demethylases (KDMs) to regulate AR responsive genes in human cancers, we investigated if the same mechanism is present in the ovine placenta. AR co-immunoprecipitated with KDM1A and KDM4D in sheep placentomes, and AR-KDM1A complexes are recruited to a half-site for androgen response element (ARE) in the promoter region of *VEGFA*. Androgenized ewes also had increased cotyledon VEGFA. Finally, in human first trimester placental samples AR, KDM1A, and KDM4D immunolocalized to syncytiotrophoblast, with nuclear KDM1A and KDM4D

[1] Paper submitted to *PLoS ONE*

[2] Author list for publication: Ellane R. Cleys, Jennifer L. Halleran, Vanessa A. Enriquez, Juliano da Silveira, Quinton A. Winger, Russ V Anthony, Jason E. Bruemmer, Colin M. Clay, Gerrit J. Bouma

[3] Project supported by Agriculture and Food Research Initiative competitive grant number 2010-38420-20397 from the USDA National Institute of Food and Agriculture and by the Colorado State University College of Veterinary Medicine and Biomedical Sciences College Research Council.

immunostaining also present in the villous stroma. In conclusion, placental androgen signaling is capable of altering ovine DNA methylation and gene expression, likely through AR-KDM complex recruitment to AREs. AR and KDMs are also present in first trimester human placenta. Androgens appear to be an important regulator of trophoblast programing for placental development**,** and aberrant androgen signaling may contribute to the development of placental disorders.

Introduction

Proper placentation is required for normal fetal development and nutrient transport to the fetus. Placentation begins as trophoblast cells from the implanting embryo differentiate to form cytotrophoblasts and syncytiotrophoblast (Kaufmann et al., 2003). Trophoblast differentiation and invasion during the first trimester is essential for establishing placental vascularization for prenatal support throughout gestation (Brosens 1988, Blankenship et al., 1993; Kaufmann et al., 2003). While the placenta is widely recognized as an endocrine organ, sex steroids act on placental cells to regulate placental development and function. For example, placental estrone, estriol and estradiol signaling has been shown to regulate trophoblast differentiation and invasion in a hypoxic environment of first trimester primate placenta (Tulchinsky and Hobel 1953; Siiteri and MacDonald 1963; 1966; Loriaux et al., 1972; Albrecht et., 1999; 2004; Rama et al., 2004; Albrecht et al., 2006). Estrogen receptor alpha (ESR1) localizes primarily in cytotrophoblast and differentiating cytotrophoblast cells (Bukovsky et al., 2003a,b), and estradiol produced by the placenta regulates trophoblast invasion by increasing matrix metalloproteinase (MMP) activity and promoting angiogenesis (Niklaus et al., 2003; Albrecht et al., 2006). Although estrogen

contributes to placentation via trophoblast differentiation and uterine spiral artery remodeling (Albrecht et al., 1999; 2006), little is known about the role of placental androgens.

During human pregnancy, maternal peripheral plasma concentrations of androstenedione and testosterone increase approximately two-fold and four-fold, respectively (Mizuno et al., 1968). A similar increase in serum testosterone is seen in pregnant cows (Gaiani et al., 1984). Plasma levels of androgens return to normal, non-pregnant levels a few days postpartum, suggesting the placenta as a source of androgen production (Mizuno et al., 1968; Serin et al., 2001). Importantly, androgen receptor (AR) is present in trophoblast cells in human, cow, goat, and pig placentas (Iwamura et al., 1994; Khatri et al., 2013; Wieciech et al., 2013) and has been characterized recently in differentiating bovine invasive trophoblast giant cells (Khatri et al., 2013). In the human placenta, AR is localized to syncytiotrophoblast and vascular endothelial cells, although it also has been reported in cytotrophoblasts and invasive extra villous trophoblasts (Horie et al., 1992; Iwamura et al., 1994; Hsu et al., 2009).

Ligand bound AR functions as a dimer by binding androgen response elements (AREs: GGA/TACAnnnTGTTCT) in promoter regions of target genes for transcription initiation. Androgen signaling in non-placental tissues has been shown to regulate cell proliferation, invasion, and angiogenesis (Shabisgh et al., 1999; Mabjeesh et al., 2003; Comstock et al., 2008). Recently, AR has been shown to complex with lysine demethylase 1 family and Jumonji-C domain containing histone demethylases (KDMs) to regulate AR responsive genes (Yamane et al., 2006; Shi and Janknecht 2007; Wissmann et al., 2007). KDMs function to regulate gene expression by demethylating mono-, di-, and tri-methylated lysines on histone N-terminal tails

(Verrier et al., 2011), increasing DNA access to sex hormone receptors and coactivators for transcription initiation (Perillo et al., 2008). Additionally, KDMs have been shown to be co-activators of *AR* and *ESR1* transcription (Shi and Janknecht 2007; Perillo et al., 2008; Kawazu et al., 2011; Garcia-Bassets et al., 2007), and to increase expression of sex hormone responsive genes (Metzger et al., 2005; Yamane et al., 2006; Shin and Janknecht 2007; Wissmann et al., 2007; Coffey et al., 2013). While KDMs appear to play a prominent role in regulating gene expression, they have not been identified in placental tissue.

The overall goal of this study was to identify a role for androgen signaling in placental cells. To this end, we used a well-established model of prenatal androgenization in ewes that were treated with testosterone propionate from gestational day GD30 to GD90 (Robinson et al., 2002). Previous studies clearly demonstrate this model leads to abnormal fetal programming and intrauterine growth restriction (Manikkam et al., 2004), abnormal placental development (Beckett et al., 2011), and offspring with reduced fertility, hyperinsulinemia, and polycystic ovarian syndrome (Recabarren et al., 2005; Dumesic et al., 2007; Veiga-Lopez et al., 2008). The presence and role of placental AR has not been investigated in this model, and its contribution to abnormal placental development and gene expression has not been previously examined.

Materials and Methods

Prenatal Androgenization and Placentome Collection

All experiments were approved by the Colorado State University Institutional Animal Care and Use Committee. Fourteen crossbred ewes were bred and randomly assigned to one of two groups, control or testosterone propionate (TP) treated. Control ewes received intramuscular

injections of 2 mL vehicle (cottonseed oil) biweekly from gestational day GD30 to GD90 (n=7). TP treated ewes received biweekly intramuscular injections of 100 mg of testosterone propionate resuspended in 2 mL of cottonseed oil as previously described (n=7) (Robinson et al., 2002). On GD90, ewes were anesthetized, uteri were surgically removed, fetal lambs were weighed, and the placentas were collected. Placentomes from each placenta were isolated and classified as either type A, B, C, or D based on gross morphology (van der Linden et al., 2012). The total number of placentomes, placentome types, and total weight of placentomes were recorded. For placentome types A, B, and C, the cotyledon was separated from the caruncle and processed separately; for type D placentomes this was not possible. The 5 placentomes closest to the umbilicus were used for analysis.

Of the five placentomes closest to the umbilicus, two were randomly selected for fixation in ice cold 4% PFA overnight at 4°C. The following day, PFA was removed and the placentomes were stored in 70% ethanol at 4°C until embedded in paraffin blocks. Remaining cotyledon and caruncle tissue was snap frozen in liquid nitrogen and stored at -80°C until processed. Prior to RNA, DNA, and protein isolation, samples were pulverized in liquid nitrogen. Pulverized tissue was stored at -80°C until placed directly in appropriate lysis buffer for each isolation protocol. To avoid any possible prejudices in data due to fetal sex, placentomes from both male and female fetuses from each treatment group were used for analysis. For all experiments, unless otherwise noted, there were 4 type A placentomes from controls, 3 type A placentomes from testosterone treated ewes.

First Trimester Human Placenta Samples

Human first trimester placental samples were obtained from elective terminations from anonymous, non-smoking, non-drug using patients, in accordance with the Colorado State University Institutional Biosafety Committee. Samples were stored in sterile PBS upon collection and were transferred to ice cold 4% PFA upon receipt. Samples were stored overnight at 4°C in PFA, then transferred to 70% ethanol at 4°C until embedded in paraffin blocks. For immunohistolocalization, one 8.6 weeks of gestation sample and one 11.5 weeks of gestation sample were repeated in triplicate. Tissue sections of 5µm thickness were taken from the center of paraffin blocks for immunohistolocalization.

DNA Isolation and ELISA of Global DNA Methylation

Genomic DNA was isolated using approximately 10 mg of pulverized ovine cotyledon and caruncle tissue using Wizard Genomic DNA Isolation Kit (Promega, #A1125) per manufacturer's protocol. Concentration and purity of DNA was determined using a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). DNA was stored at -80°C until used for analysis. MethylFlash Methylated DNA Quantification Kit (Epigentek, P1034) was used for detection of 5-methylcytosine as per the manufacturer's protocol. 100 ng of DNA from each sample was used in duplicate. Standards were used in duplicate from 0.5 ng/µL to 10 ng/µL to quantify 5-methylcytosine in samples. Colorimetric readings at 450 nm were recorded on a model 680 microplate reader (BioRad). Average absorbance values for the negative control were subtracted from each sample to obtain normalized values. A linear equation was calculated from standard curve dilutions. Normalized values were divided by the slope of the linear equation multiplied by two to obtain the amount of 5-mehtylcytosine present in each sample (ng of 5-mC).

Statistical analysis was performed using ANOVA followed by Tukey pairwise comparison (Minitab 16). P-values less than 0.05 were considered statistically significant.

Protein Isolation and Western Blot

Approximately 2 mg of pulverized ovine placentome tissues were added directly to 2mL of ice cold lysis buffer containing 0.48M Tris pH7.5, 10mM EGTA pH8.6, 10mM EDTA pH8.0, and 0.1% (w/v) PMSF and protease inhibitor. Samples were sonicated on ice for 5 minutes and centrifuged at 10,000 rpm for 10 minutes at 4°C. Supernatant was collected and frozen prior to protein concentration analysis using a Bradford standard curve (BioRad). Supernatant was diluted in 6x SDS-DTT loading dye with 0.375M Tris pH6.8, 4M glycerol, 0.21M SDS, 0.6M DTT, and 0.06% (w/v) bromophenol blue. β-mercaptoethanol (1.75 μL) and water was added to reach a final concentration of 4.29 μg/μL of protein in 35 μL (150 μg total protein). Samples were boiled for 5 minutes after addition of β-mercaptoethanol, then electrophoresed at 95 volts in 10% Tris-HCL polyacrylamide gels (BioRad). For ESR1 blots, 10% Tris polyacrylamide gels were made using 10.2% acrylamide/bis solution (BioRad), 3.5mM SDS, 4.5mM TEMED (BioRad), and 43.3mM APS. Protein was electrophoresed in an ice cold running buffer containing 50mM Tris, 384mM glycine, and 7mM SDS. Protein was transferred onto 0.2 μm nitrocellulose membranes (Protran) for 1 hour at 200 milliamps at 4°C in transfer buffer containing 2mM Tris, 150 mM glycine, 5M methanol, and 3.5mM SDS. After protein transfer, blots were blocked for non-specific binding with 2% milk-TBST for 1 hour at room temperature. After blocking, blots were washed with TBST and left overnight at 4°C with primary antibody diluted in 2% milk-TBST. Antibodies used for Western blot analysis are listed in Table 2.1 with their respective dilutions. After incubation with primary antibody, blots were washed in TBST

and incubated for 1 hour at room temperature with secondary antibodies, goat-anti-rabbit-HRP (Abcam ab6721, 1:1000) or goat-anti-mouse-HRP (Abcam ab6789, 1:1000). Blots were subsequently washed in TBST and ECL Prime Western Blotting Detection System (Amersham Biosciences) was applied to detect immunoreactivity. Chemiluminescent bands were detected using the Storm Scanner 860 (Amersham Biosciences). AR, ESR2, and KDM1A immunoreactive bands were confirmed by preabsorption of antibody with available blocking peptides for one hour at room temperature prior to overnight incubation.

Immunoreactive bands were quantified using ImageJ software (NIH). Pixelation for the protein of interest was divided by that of β-actin for each sample to normalize for protein loading. The average amount of normalized protein was then compared between control and TP treated ewe cotyledon and caruncle tissue using an ANOVA followed by Tukey pairwise comparison (Minitab 16).

Immunohistolocalization

To determine cellular localization of selected proteins in ovine type A placentomes from control ewes (n=4) and first trimester human placenta (n=2), 5μm paraffin sections were taken from the center of the tissue blocks. Slides were deparaffinized with Citra-solve (Fisherbrand) and rehydrated with washes of decreasing percentages of ethanol. Slides were boiled for 15 minutes in 10mM sodium citrate pH6 for antigen retrieval using 5 minute intervals with 2 minute breaks. Non-specific peroxidase activity was inhibited by a 30 minute incubation in 3% hydrogen peroxide solution in PBS. Slides were incubated for 1 hour in 2% Superblock Blocking Buffer (Thermo Scientific, Waltham, MA) in PBS prior to incubation with KDM1A and KDM4D

antibodies, and in 2% goat serum in PBS preceding all other antibodies. After blocking, slides were washed in PBS. Antibody dilutions are listed in Table 2.1. AR, ESR1, and ESR2 antibodies were diluted in 2% goat serum in PBS. KDM1A and KDM4D antibodies were diluted in 2% Superblock Blocking Buffer in PBS. Primary antibodies were incubated overnight at 4°C. Control slides were incubated with 2% Superblock or 2% goat serum in PBS with exclusion of primary antibody. AR and ESR2 blocking peptides (Santa Cruz sc816-P and Abcam ab3564, respectively) were used at a 3:1 and 1:1 ratio with antibody to confirm specific immunolocalization. After incubation with primary antibody, slides were washed in PBS and incubated with secondary antibody for 30 minutes at room temperature. A goat-anti-rabbit polyclonal secondary antibody tagged with HRP (Abcam ab6721, 1:1000) was used for AR, ESR2, KDM1A, and KDM4D detection. A goat-anti-mouse polyclonal secondary antibody tagged with HRP (Abcam ab6789, 1:2000) was used for ESR1 detection. Slides were washed in PBS and stained using avidin-biotin staining with diaminobenzidine (DAB) peroxidase substrate kit (Vector Labs, Burlingame, CA) to detect HRP immunoreactivity. Slides were washed again in PBS and dehydrated prior to mounting.

Placentome RNA Isolation and Real Time PCR

Prior to real time PCR amplification, primer specificity was confirmed using DNA sequencing of PCR amplicons. Briefly, PCR analysis was performed using a placentome cDNA pool and GoTaq (Promega) per the manufacturer's recommendations. PCR product was electrophoresed on a 1% agarose gel with a 100bp ladder (New England BioLabs) to determine the presence of expected amplicon sizes. The amplicon band was excised from the agarose gel and DNA was isolated using QIAquick Gel Extraction Kit (Qiagen). The resulting DNA was sequenced at the

Colorado State University Proteomics and Genomics Laboratory. The sequences were blasted using NCBI blast to confirm specificity. Primer efficiency was assessed using serial dilutions of a cDNA pool from placentome samples. Standard curves from serial dilutions were analyzed on the Roche LightCycler 480 (Roche, Basel, Switzerland), and primer efficiencies used for real time PCR assay were calculated and are listed in Supplemental Table 2.1.

Total RNA was isolated from pulverized samples using the RNeasy Mini kit (Qiagen) and were treated with RNase-Free DNase (Qiagen) to eliminate any genomic DNA contamination. RNA quality and purity was determined with a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies) and RNA aliquots were stored at -80° C. Total RNA was processed for reverse transcription using qScript (BioRad). 1µg of total RNA was added to each reverse transcription reaction with 4µL reverse transcriptase QScript Supermix (Quanta) and nuclease free water up to 20µL total reaction volume. Reverse transcription of total RNA was performed according to manufacturer's specifications with 5 minutes 25°C, 30 minutes 42°C, 5 minutes 85°C, and holding at 4°C for use the same day. Resulting cDNA was used as a template for real time PCR quantification. cDNA was diluted 1:4 in nuclease free water prior to loading into real time PCR reactions. For real time PCR analysis, 2.5 µl of nanopure water was added to every 5µl of LightCycler 480 SYBR Green I Master (Roche, Basel Switzerland). Primer sets were added to reach a final concentration of 0.5µM in each reaction and 1µl of diluted cDNA was added for a final cDNA concentration of 1.25 ng/µL in a total volume of 10µL. Samples were loaded into 384 well LightCycler 480 plates (Roche) and analyzed using a LightCycler 480 PCR system (Roche Applied Science) in duplicate. Real time PCR cycle conditions were 95°C for 5 minutes, proceeded by 45 cycles of denaturing at 95°C for 30 seconds, annealing at 60°C for 15 seconds,

and extension at 72°C for 10 seconds. Following real time PCR amplification, melt peaks were generated with an incubation at 95°C for 30 seconds to confirm a single amplicon was present. Cp values were normalized using the geometric mean of *RN18s* and *GAPDH*. Statistical analysis between TP treated and control samples used an ANOVA followed by Tukey pairwise comparison (Minitab 16). Results reported for real time PCR data use $2^{-\Delta Cp}$ values for statistical analysis and graphs, and fold changes between $2^{-\Delta Cp}$ values in tables for comparison (Schmittgen et al., 2008).

Coimmunoprecipitation of AR with KDM1A and KDM4D from Placentomes

Protein was isolated from pulverized snap frozen cotyledon and caruncle tissue from control and TP treated ewes in RIPA buffer containing 10mM Tris-HCl pH 7.5, 140mM NaCl, 1mM EDTA, 0.5mM EGTA, 1% Triton X-100, 0.1% SDS, 0.1% proteinase inhibitor, and 0.1% 100mM PMSF. Protein concentration was determined by a BCA assay (Pierce). 250 μg of cotyledon and 250 μg caruncle protein were diluted in RIPA buffer to reach a final concentration of 1 μg/μL in 500 μL RIPA buffer. Antibodies were combined with 500 μL of diluted placentome protein and incubated for an hour at 4° C on a rotational mixer. 2 μg of AR antibody (Santa Cruz, sc816), 2 μg of KDM1A antibody (Abcam, ab17721), 2 μg KDM4D antibody (Abcam, ab93694), or 1 μg secondary antibody as a negative control (Abcam, anti-rabbit ab6721) were added to each sample. Preabsorption of AR and KDM1A antibodies with their blocking peptides (4 μg) occurred at room temperature 1 hour prior to incubation with placentome protein. Following antibody incubation, 20 μL of A/G PLUS agarose beads (Santa Cruz, sc2003) were added for an hour at 4° C on a rotational mixer. Samples were briefly spun at 2500 rpm for 5 minutes at 4° C. Agarose beads were centrifuged and washed two times with RIPA buffer followed by three

washes with PBS. Protein was resuspended in 6.67 μL 6x Western blot loading dye (described above) diluted with 31.33 μL RIPA buffer, then stored at -80° C overnight. The following day, samples were thawed and 2 μL of β-mercaptoethanol was added to reach a final volume of 40 μL. Samples were immediately incubated at 95° C for 12 minutes and centrifuged at 10,000 x g for 1 minute at room temperature. 20 μL of immunoprecipitated protein was loaded per lane on 10% Tris-HCl gels (BioRad) and electrophoresed. Western blot protocol and antibody dilutions followed those described above and in Table 2.1.

Chromatin Immunoprecipitation with AR and KDM1A from Placentomes

ChIP was performed using ChIP-It Express High Throughput (Actif Motif) following the manufacturer's protocol. Approximately 2 mg of pulverized cotyledon and caruncle tissue from control and TP treated ewes was added to 500 μL of ice cold PBS. Cotyledon and caruncle tissue was mixed for each ewe and mechanically homogenized by pestle on ice. 14 μL of 37% formaldehyde (VWR International) was added, samples were vortexed, then incubated on ice for 8 minutes. 57 μL of 125 mM glycine was added to quench crosslinking. Samples were vortexed and incubated on ice for 5 minutes. Samples were centrifuged for 2 minutes at 10,000 x g at 4° C. Supernatant was aspirated off and samples were resuspended in 500 μL ice cold PBS. Samples were centrifuged again, supernatant was removed, and samples were resuspended in 500 μL PBS. 130 μL of lysis buffer containing 50mM Tris HCl pH8.0, 10mM EDTA, 1% SDS, and 1:100 proteinase inhibitor was added. Samples were homogenized by pestle, vortexed, and incubated on ice for 30 minutes. Samples were homogenized again then sonicated using a Biorupter for 30 minutes with cycles of 5 seconds on, 5 seconds off. 400 μL of RIPA buffer was added, then samples were centrifuged for 10 minutes at 12,000 x g at 4° C. Supernatant was

placed in a new tube and remaining sample was resuspended again in 400 μL RIPA buffer and centrifuged. Supernatant was combined and DNA concentration was determined by Nanodrop 1000 Spectrophotometer (NanoDrop Technologies). 6.3 μg of DNA was placed in a new tube for immunoprecipitation.

25 μL of magnetic G-protein beads (Active Motif) were added with 10 μL of ChIP buffer 1 (Actif Motif) and 1 μL of proteinase inhibitor. 2 μg of AR antibody (Santa Cruz, sc816), 2 μg of KDM1A antibody (Abcam, ab17721), or 2 μg anti-rabbit secondary antibody (Abcam ab6721, negative control) were added to each sample. RIPA buffer was added to bring the total immunoprecipitation reaction volume up to 200 μL. Samples were incubated overnight at 4° C on a rotational mixer, and briefly spun at 2500 rpm for 1 minute at 4° C. Magnetic G-protein beads were pulled down and washed two times with 200 uL of ChIP buffer 1, then washed 3 times with 100 μL of ChIP buffer 2 (Actif Motif). Magnetic beads were then resuspended in elution buffer AM2 and incubated at room temperature for 15 minutes on a rotational rotor. Samples were centrifuged at 2500 rpm for 1 minute at room temperature. 50 μL of reverse crosslink buffer was added and samples were vortexed. Magnetic beads were pulled down and supernatant was removed and placed in a new tube. 10 μL of input cross-linked DNA was added to a tube containing 88 μL of ChIP buffer 2 and 2 μL of 5 M NaCl as a PCR positive control (ChIP input DNA). All samples were incubated overnight at 64° C. Samples were cooled to room temperature and 2 μL of proteinase K was added. Samples were incubated for 1 hour at 37° C, then 2 μL of Proteinase Stop Solution was added.

1 μL of ChIP sample was added per each PCR reaction in technical triplicates. Sonicated DNA samples and ChIP input DNA were used as positive controls for PCR. PCR and amplicon sequencing was performed as described above. Androgen response element (ARE) half-sites were identified in a -5000bp region of the 5' flanking sequence of bovine and human *VEGFA* as the promoter region of ovine *VEGFA* has not been published. Primers were designed to amplify an ARE region of genomic DNA situated between two ARE half-site in bovine *VEGFA*, 181 nucleotides away from the upstream ARE and 44 nucleotides away from the downstream ARE, and was located 3,118 nucleotides away from the coding region. Primers also were designed to span a region 1,774 nucleotides from an ARE half-sites (non-ARE) in the -5,000 5' flanking sequence in the human *VEGFA,* located 4,786 nucleotides away the coding region (primers listed in Table 2.2).

Results

Placentome Morphology and Fetal Growth

The total number and weight of placentomes collected from control and TP treated ewes did not differ at GD 90 (Figure 2.1A and 2.1B). Placentas from TP treated ewes had a reduced number of type A placentomes (P=0.001) and an increased number of type C (P=0.002) and type D (P=0.06) placentomes compared to control ewes (Figure 2.1A). There was no difference between weight of male and female fetuses in control pregnancies or between male and female fetuses from TP treated ewes. However, female fetuses from TP treated ewes weighed less (P=0.022) compared to female fetuses from control ewes (Figure 2.1C).

Global DNA Methylation in Placentomes

Colorimetric ELISA revealed a decrease in 5-methylcytosine in type A placentomes from TP treated ewes compared to type A placentomes from control ewes at GD90 (P=0.025, Figure 2.2). No difference was found in 5-methylcytosine levels between control or TP treated ewe type A to type D placentomes.

Changes in Epigenetic Factors and Steroid Hormone Receptors

Transcript levels for several genes involved in epigenetics (*KDM*'s, *DNA methyltransferases* (*DNMT*s), and imprinted long non-coding *H19*) were assessed in GD90 placentomes from control and TP treated ewes (Figure 2.3, Figure 2.4, and Supplemental Figure 2.1). While no difference was observed in *KDM3A* or *KDM4A* mRNA levels (Supplemental Figure 2.1), *KDM4C* was lower in type A cotyledons from TP treated ewes compared to controls (P<0.001, Figure 2.3). *H19* mRNA levels increased in cotyledon tissue from type A placentomes from TP treated ewes compared to controls (P=0.005, Figure 2.3). *DNMT1* levels were increased in type A cotyledon tissue from TP treated ewes compared to controls (P<0.001, Figure 2.3). Increased DNMT1 protein also was observed in TP treated ewe type A cotyledon and placentome tissue (P<0.001, Figure 2.3). No difference was observed in *DNMT3a* or *DNMT3b* with TP treatment (Supplemental Figure 2.1). TP treatment did not alter placentome *AR*, *KDM1A*, and *KDM4D* mRNA levels (Figure 2.4).

Further characterization of AR, KDM1A and KDM4D in GD90 ovine placentomes was completed to determine their response to TP treatment (Figure 2.4). AR protein increased in caruncle tissue from type A placentomes in TP treated ewes compared to controls (P=0.014)

(Figure 2.4). Preabsorption with the complementary AR peptide resulted in loss of immunoreactive band (Supplemental Figure 2.2A). No significant difference was observed in KDM1A protein. Although TP treatment did not alter *KDM4D* mRNA levels, KDM4D protein levels increased in type A placentome tissue from TP treated ewes when compared to controls ($P<0.001$), but was not different in type D placentomes from TP treated ewes (Figure 2.4).

Localization of AR, KDM1A and KDM4D in GD90 Placentomes

In type A placentomes from control ewes, nuclear AR immunolocalized to the trophoblast and syncytium (Figure 2.5). KDM1A coimmunolocalized with AR to the nucleus of the trophoblast. AR and KDM4D coimmunolocalized to the syncytium. Predominate immunostaining for KDM1A was localized to the trophoblast nuclei while KDM4D localized to the apical surface of the syncytium (Figure 2.5).

mRNA Levels of Androgen Responsive Genes in Placentomes

Real time PCR of androgen responsive genes (Androgen Responsive Gene Database; http://argdb.fudan.edu.cn/) known to regulate placentation was undertaken to determine if TP treatment altered relative mRNA levels (Figure 2.6 and Supplemental Figure 2.3). *Insulin-like growth factor 2* (*IGF2*) was decreased in cotyledon tissue from type A placentomes from TP treated ewes compared to controls ($P<0.001$, Figure 2.6). *IGF2* was increased in type D placentomes from TP treated ewes compared to type A placentomes from controls ($P<0.001$). *IGF binding protein* (*IGFBP*) *1* and *2 (IGFBP2)* both decreased in caruncle tissue from type A placentomes from TP treated ewes compared to controls ($P<0.001$). *IGFBP2* also was observed to decrease in placentomes from TP treated ewes compared to type A placentomes from control

ewes (P<0.001). *Aromatase (CYP19)* mRNA levels increased in type D placentomes from TP treated ewes compared to type A placentomes from controls (P<0.001, Figure 2.6). *Vascular endothelial growth factor* (*VEGFA*) increased in TP treated ewe type D placentomes compared to type A placentomes from control (P<0.001, Figure 2.6). VEGFA monomer increased in cotyledon tissue in type A placentomes from TP treated ewes compared to controls (P=0.005, Figure 2.6).

AR Complexes with Histone Demethylases and Binds to VEGFA

Coimmunoprecipitation and chromatin immunoprecipitation were used to determine if AR interacts with KDM1A and/or KDM4D in ovine placenta to regulate androgen responsive *VEGFA*. Co-immunoprecipitation and Western blot revealed AR binds to KDM1A and KDM4D in GD90 placentomes (Figure 2.7A), and preabsorption with AR or KDM1A blocking peptide decreased or inhibited immunoreactive bands, respectively (Supplemental Figure 2.2B). Furthermore, both AR and KDM1A protein bind the same DNA region in the 5' flanking sequencing of *VEGFA* containing an ARE half-site (Figure 2.7B) according to PCR amplification. PCR with primers designed for a non-ARE region in the 5' flanking sequencing of *VEGFA* did not amplify in AR or KDM1A chromatin immunoprecipitation samples, but was present in genomic DNA (data not shown).

Immunolocalization of AR, KDM1A, and KDM4D in First Trimester Human Placenta

In both the 8.6 and 11.5 weeks of gestation samples, AR, KDM1A, and KDM4D immunolocalized to nuclei in syncytiotrophoblast (Figure 2.8). Additional KDM1A immunostaining was observed in cells in the villous stroma at 8.6 weeks of gestation, but not at

11.5 weeks of gestation. KDM4D immunostaining was also observed in villous stroma at both 8.6 and 11.5 weeks of gestation (Figure 2.8).

Discussion

Prenatal androgenization models previously have been employed to study abnormal developmental programing (Robinson et al., 2002; Manikkam et al., 2004; Recabarren et al., 2005; Dumesic et al., 2007; Veiga-Lopez et al., 2008; Beckett et al., 2011). In our study, prenatal androgenization from GD30 to 90 led to reduced fetal weight of female lambs. Androgen induced intrauterine growth restriction has been described in sheep (Manikkam et al., 2004; Recabarren et al., 2005) and prenatal androgenized rats (Sathishkumar et al., 2011) along with reduced placental weights (Sun et al., 2012). These data strongly suggest androgen as a potent regulator of developmental programing for fetal growth and placental function. We also report that prenatal androgenization either directly or indirectly leads to abnormal placentome morphology. While the increase in type C and D placentomes has been associated previously with prenatal androgenization (Vonnahme et al., 2006), increased presence of type C and D placentomes in pregnancies complicated by maternal nutrient restriction corresponds to increased interdigitated fetal villi, heightened cotyledon proliferation, and capillary density (Hoet and Hanson 1999; Osgerby et al., 2004; Ford et al., 2004; Ford et al., 2006; Vonnahme et al., 2006). However, it still is unclear how prenatal androgenization contributes to intrauterine growth restriction.

In addition to altered ovine placentome morphology, we observed decreased global DNA methylation in placentomes from TP treated ewes, suggesting abnormal epigenetic regulation of

trophoblast gene expression. DNA methylation is regulated by DNA methyltransferases (DNMTs), with DNMT1 functioning to maintain cytosine methylation while DNMT3A and DNMT3B function for de novo cytosine methylation patterns, such as those required during reprograming for embryonic development and imprinting (Klose and Bird 2006). Proper regulation of DNA methylation in trophoblasts is necessary for normal placenta developmental programing. Reduction of DNA methyltransferases (DNMT1, DNMT3a and DNMT3b) in choriocarcinoma (BeWo) cells prevents cell migration (Rahnama et al., 2006) and reduced placental DNMT1 expression in in vitro produced ovine embryos leads to early embryonic loss (Ptak et al., 2013). Loss of DNA methylation imprinting in long non-coding *H19* and neighboring *IGF2* in the placenta also has been associated with Beckwith-Wiedemann Syndrome and increased prenatal and placental growth (Angiolini et al., 2006; Weksberg et al., 2010). In the prenatal androgenization ewe model, we report up-regulated *H19* and *IGF2* in placentomes from TP treated ewes, suggesting that excess prenatal androgen exposure may lead to dysregulated imprinting in the placenta via decreased global DNA methylation, contributing to abnormal fetal growth observed with prenatal androgenization (Manikkam et al., 2004; Sathishkumar et al., 2011).

DNMTs dynamically regulate gene expression by working in concert with site-specific adaptations of amino acids in histone N-terminal tails, including methylation of lysine residues (Johnson et al., 2002; Klose and Bird 2006; Ooi et al., 2007; Cedar and Bergman 2009). KDMs demethylate lysines in histones to typically increase DNA accessibility and promote transcription in the presence of transcription activators, such as ligand-bound nuclear receptors (Johnson et al., 2002; Klose and Bird 2006;Ooi et al., 2007; Perillo et al., 2008). KDMs interact with sex

hormone receptors and regulate androgen and estrogen signaling (Kawazu et al., 2011; Coffey et al., 2013). Knock-out of *KDM1A* in mice results in early embryonic lethality (Wang et al., 2007; 2009). We report that not only are AR and histone demethylases KDM1A and KDM4D present in ovine placenta and first trimester human placenta, but AR co-localizes and complexes with KDM1A and KDM4D in ovine placental tissue. This agrees with previous studies that reported AR, ESR1, and ESR2 heterocomplexes with KDMs to regulate target genes (Metzger et al., 2005; Yamane et al., 2006; Garcia-Bassets et al., 2007; Shin and Janknecht 2007; Wissmann et al., 2007; Perillo et al., 2008; Kawazu et al., 2011; Verrier et al., 2011; Coffey et al., 2013), though this is the first study to our knowledge to show this interaction also occurs in placental tissue.

With the decreased DNA methylation observed in placental tissue from TP treated ewes, the shift in placentome morphology may be a direct result of altered gene expression, more specifically from increased growth and angiogenic factors induced by androgen signaling in placental tissues. In GD90 placentomes from TP treated ewes, we observed increased mRNA levels of AR responsive genes *insulin-like growth factor 2 (IGF2)* and *vascular endothelial growth factor (VEGF),* and increased protein levels of AR and VEGFA. During prenatal development, IGFs and VEGFA regulate placentation and fetal growth. IGFs are expressed throughout the placenta of various species, including maternal decidualized cells, cytotrophoblasts, and chorionic mesoderm (Hill et al., 1993; Han and Carter 2000) and function as a key regulator of prenatal growth (Randawa and Cohen 2005). Decreased *IGFBP1* and *IGFBP2* in caruncle tissue from TP treated ewes may be an additional mechanism to heighten local IGF signaling in androgenized

placenta (Hill et al., 1993), explaining the rise in placentome overgrowth observed with the increase in type C and D placentomes (Hoet and Hanson 1999; Osgerby et al., 2004).

VEGFA also stimulates fetal and placental growth through increased angiogenesis (Zhou et al., 2002; 2003). While androgen responsive VEGFA stimulates placental and prenatal angiogenesis, it also stimulates differentiation of extravillous trophoblasts that remodel maternal spiral arteries in the human decidua for enhanced blood flow to the placenta (Zhou et al., 2002; 2003; Bdolah et al., 2004). Insufficient extravillous trophoblast differentiation and invasion has been associated with placental insufficiency and the development of pregnancy induced pathologies, including fetal growth restriction and preeclampsia (Bdolah et al., 2004). Multiple studies have also shown that maternal plasma testosterone is increased in cases of severe preeclampsia (Acromite et al., 1999; Serin et al., 2001; Salamalekis et al., 2006), with placentas from preeclamptic pregnancies expressing increased syncytiotrophoblast and stromal cell expression of AR (Hsu et al., 2009; Sathishkumar et al., 2011). Mutations in *AR* are correlated with increased risk for the development of preeclampsia (Lim et al., 2011). As we demonstrated AR and KDM1A binding to an ARE in *VEGFA*'s promoter region in ovine placental tissue, placental androgens may have a critical function in regulating trophoblast function in sheep, possibly through VEGFA signaling, with increased placental androgen production occurring in an effort to correct reduced trophoblast differentiation and invasion.

This proposed mechanism for placental androgen signaling is further supported by evidence that hypoxia induces AR activity and expression of androgen-regulated genes, including VEGFA (Shabisgh et al., 1999; Mabjeesh et al., 2003; Comstock et al., 2008), while androgen withdrawal

leads to hypoxia (Milosevic et al., 2007). Additionally, KDMs also are regulated by hypoxia to promote vascularization, invasion, migration, and cell proliferation in cancerous tissues (Metzger et al., 2005; Shin and Janknecht 2007; Yang et al., 2010; Coffey et al., 2013). This is of particular interest as hypoxia-regulated trophoblast differentiation and invasion in the first trimester is required to establish proper placentation (Zhou et al., 1993; Kaufmann et al., 2003; Bdolah et al., 2004).

Prenatal androgenization and increased ligand could promote AR-KDM1A complex recruitment to AR-target genes in the placenta to stimulate gene transcription by promoting reduced methylation signatures near androgen responsive genes (Johnson et al., 2002; Perillo et al., 2008). Therefore, ligand-dependent recruitment of AR-KDM1A to androgen responsive genes could regulate growth, invasion, and angiogenesis and, as such, placental development and function. However, despite the observed up-regulation in mRNA levels for androgen responsive genes regulating growth, invasion, and angiogenesis, and the advancement in differentiated placentome morphology, GD90 prenatal androgenized ewe lambs had reduced fetal weight, suggesting that increased androgen signaling, either directly or indirectly, dysregulated fetal developmental programing through other factors, such as placental nutrient transport, that were not investigated.

However, in this model of prenatal androgenization we cannot rule out the effects of placental estradiol as the ovine placenta expresses active aromatase (CYP19) (France et al., 1987) and increased *CYP19* was observed in type A placentomes from TP treated ewes. Further investigation into the interaction of ESRs and KDMs in the placenta also is necessary given the

known role of estradiol in regulating trophoblast differentiation (Niklaus et al., 2003; Rama et al., 2004; Albrecht et al., 2004; 2006) and the ability of ESR1 to complex with KDMs (Garcia-Bassets et al., 2007; Perillo et al., 2008; Yang et al., 2010; Kawazu et al., 2011). Furthermore, our data indicates ESR1 colocalizes with KDM1A in the nucleus of trophoblasts in the villous epithelium in GD90 ovine placentomes.

In conclusion, the present study revealed that prenatal androgenization alters ovine placentome development through decreased global DNA methylation and altered gene transcription of angiogenic and growth factor pathways. Furthermore, we demonstrated that AR complexes to KDM's in ovine placenta, and that AR and KDM1A bind to the same promoter region of androgen responsive gene *VEGFA* in trophoblast cells. These results indicate androgen signaling functions jointly with KDMs to play a key regulatory role in placentation and function. These findings also have important implications as to androgens' regulation of human trophoblast differentiation and function as KDM1A and KDM4D immunolocalized in syncytiotrophoblast and in villous stromal cells in first trimester human placenta. Current studies are underway to determine the role of histone demethylases in human placentation and to determine androgen's effects on trophoblast differentiation.

TABLE 2.1. List of antibodies and their dilutions used for Western blot or IHC protocols

Protein	Antibody for Western Blot and Dilution Utilized	Blocking Peptide Ratio to Antibody	Antibody for IHC and Dilution Utilized
AR	Santa Cruz sc816 **1:500**	Santa Cruz sc816-P **3:1**	ThermoScientific PA1-110 **1:10**
KDM1A	Abcam ab17721 **1:500**	Abcam ab17763 **2:1**	Abcam ab17721 **1:100**
KDM4D	Abcam ab93694 **1:300**		Abcam ab93694 **1:2000**
VEGFA	Santa Cruz sc152 **1:500**		
ESR1	ThermoScientific MA1-310 **1:50**		Abcam ab2746 **1:10**
ESR2	Abcam ab3577 **1:500**	Abcam ab3564 **1:1**	Abcam 3577 **1:50**
DNMT1	Abcam ab92453 **1:1000**		
β-actin	Santa Cruz sc47778 **1:1000**		

TABLE 2.2. List of primer sequences used for PCR of ChIP samples spanning an ARE and non-ARE region in the -5000bp 5' flanking sequence of *VEGFA*.

Gene	ARE/non-ARE Region	Primer Sequence	Amplicon Size
VEGFA	**ARE**	**F-5' CTCTGTCTGGGCTGCTCTCT** **R-5' TCCTCCCATGGACGTAACTC**	**179**
VEGFA	**Non-ARE**	**F-5' GCCTGTAATCCCAGCACTCT** **R-5' GAGCAATTCTCCTGCCTCAG**	**181**

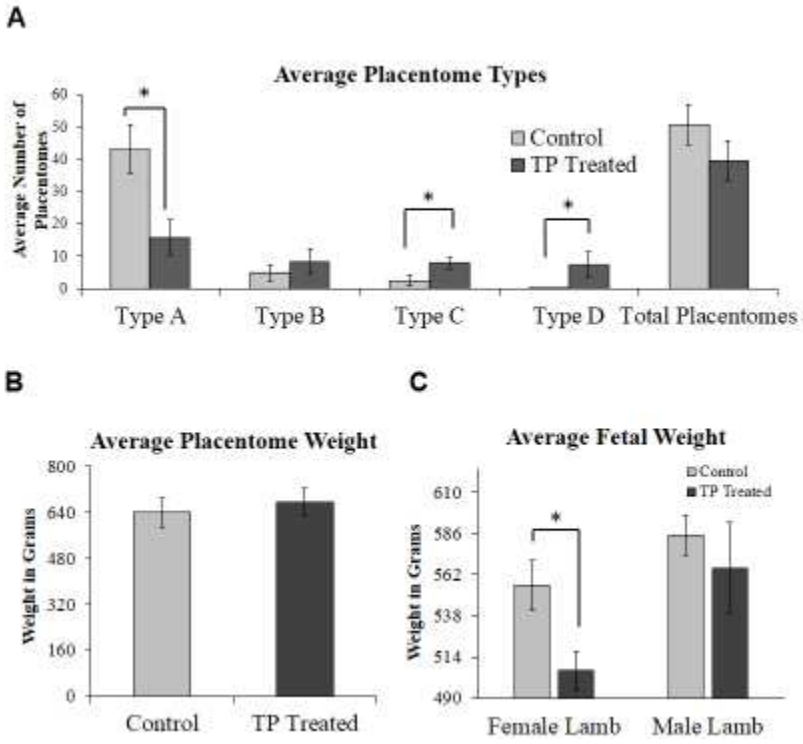


FIGURE 2.1. Placentome morphology and fetal weight. **A**) Testosterone propionate treatment (TP) decreased the number of type A placentomes, and increased type C and type D placentomes collected at gestational day 90. **B**) TP treatment did not affect placental weight while **C**) female fetuses from TP ewes had significantly reduced body weight at gestational day 90 compared to female fetuses from controls. * Indicates $P \leq 0.06$

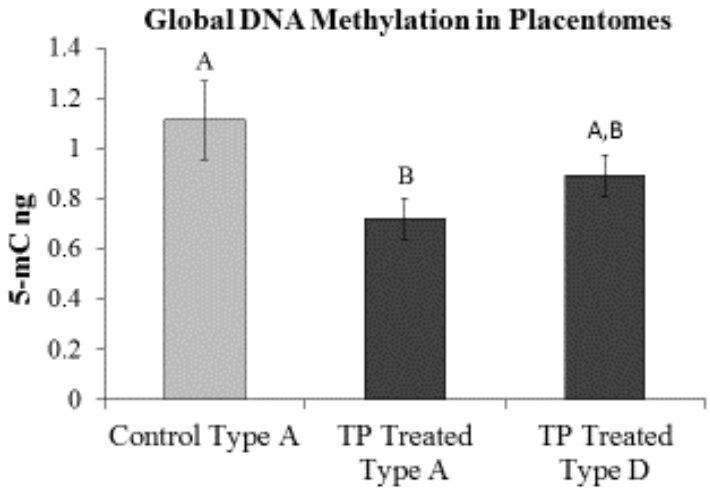


FIGURE 2.2. Global methylation in ovine GD90 placentomes. Global DNA methylation decreased in type A placentomes from TP treated ewes when compared to type A placentomes from controls. No difference was observed between control type A placentomes compared with type D placentomes from TP treated ewes or between type A and D placentomes from TP treated ewes. Different letters indicate statistical difference of $P<0.05$.

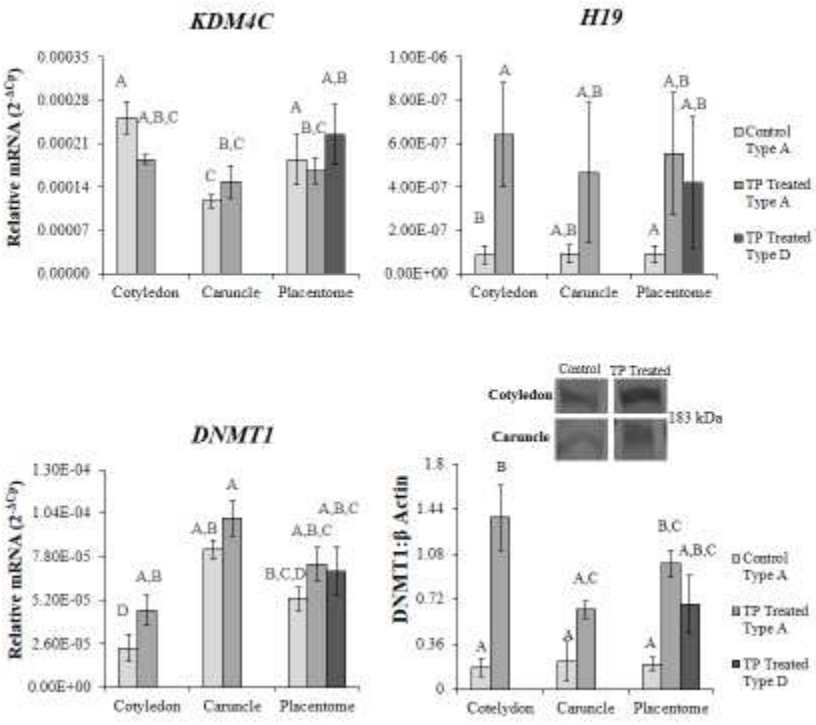


FIGURE 2.3. Differential levels of epigenetic regulators with TP treatement. Analysis of relative mRNA levels for genes regulating histone and DNA methylation and imprinting in ovine placentome tissue from real time PCR. *KDM4C* was decreased in type A placentomes from TP treated ewes compared to controls. *H19* was increased in cotyledon tissue from type A placentomes in TP treated ewes compared to controls. *DNMT1* increased in TP treated ewe type A cotyledon tissue compared to controls. DNMT1 increased in TP treated ewe type A placentomes ($P<0.001$). Different letters indicate statistical difference of $P<0.05$.

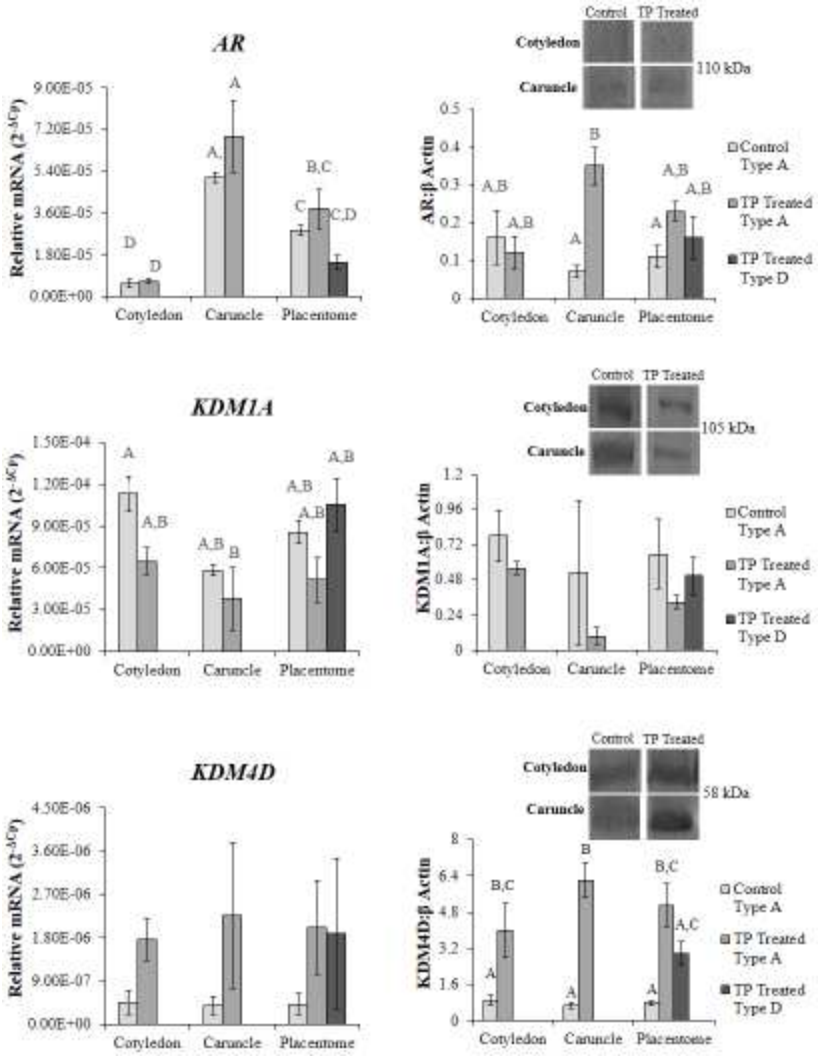


FIGURE 2.4. Real time PCR and representative Western blot depicting placentome mRNA and protein levels in control and TP treated ewes. TP treatment did not alter mRNA for *AR*, *KDM1A*, or *KDM4D*. AR was increased in type A caruncle tissue from TP treated ewes compared to controls. No difference was found in KDM1A, though KDM4D increased in type A placentomes from TP treated ewes compared to controls. Placentome values represent average of cotyledon and caruncle quantified mRNA levels in type A cotyledons. Different letters indicate statistical difference of $P<0.05$.

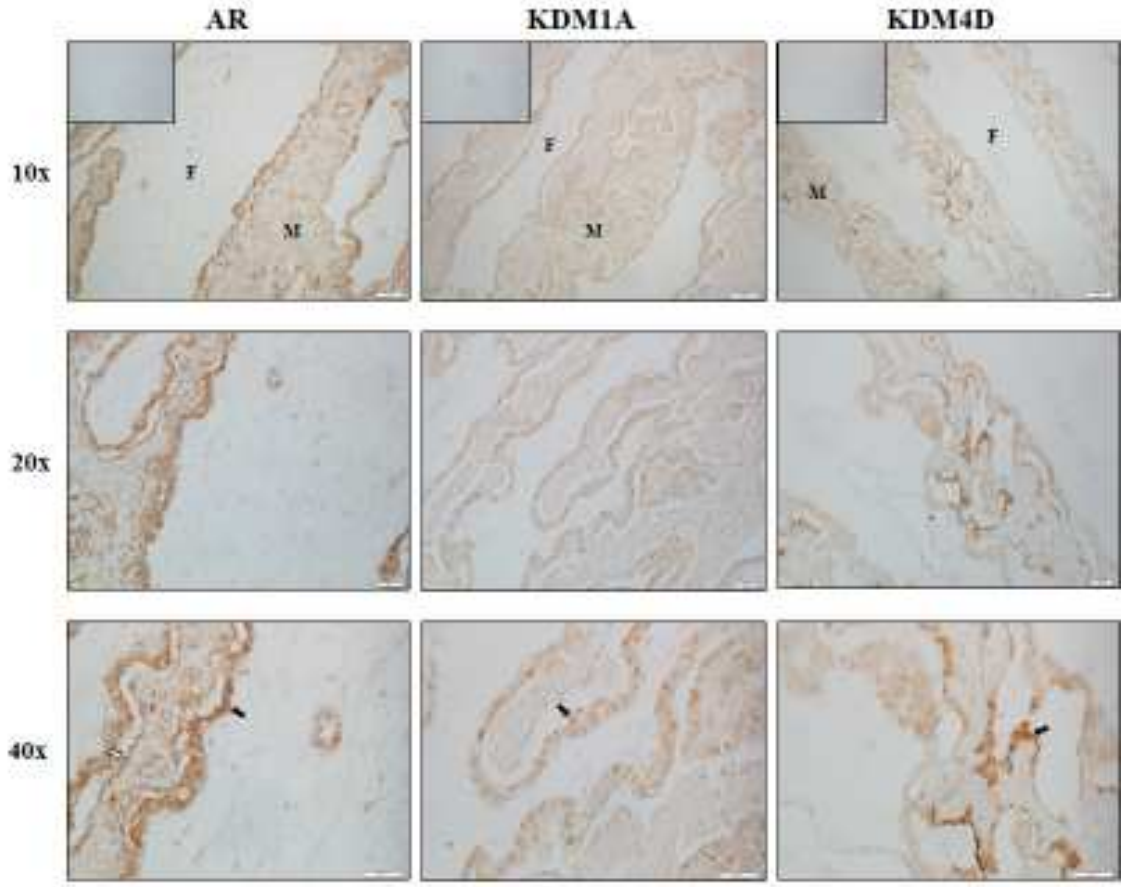


FIGURE 2.5. Serial images of immunolocalized AR, KDM1A, and KDM4D in type A placentomes from control ewes at GD90. Inserts represent control IHC straining. Immunolocalization of AR was present in the trophoblasts of the villous epithelium with primarily nuclear staining (arrow). AR immunostaining also was present in the surface of the syncytium (white arrow). KDM1A immunolocalized to the nucleus of the trophoblasts in the villous epithelium (arrow), while KDM4D immunostaining was prominent in the apical surface of the syncytium (arrow). F, fetal cotyledon; M, maternal caruncle

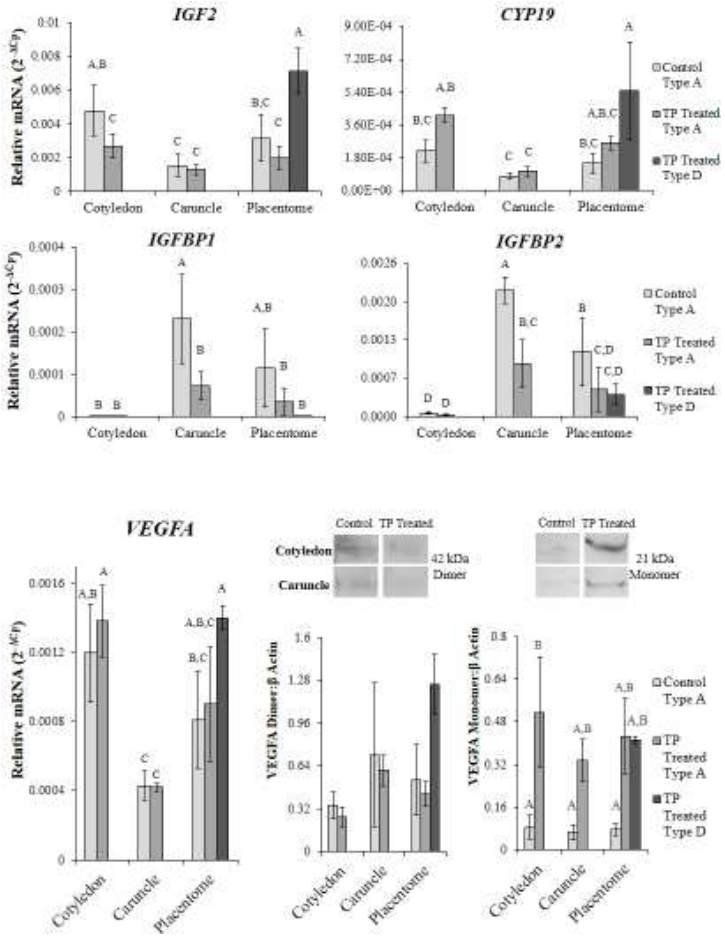


FIGURE 2.6. Real time PCR results of androgen responsive genes known to regulate trophoblast differentiation and proliferation. *IGF2* increased in type D placentomes from TP treated ewes compared to type A placentomes from control or TP treated ewes. *IGFBP1* and *IGFBP2* both decreased in caruncle tissue from type A placentomes from TP treated ewes compared to controls. *IGFBP2* also decreased in type A and D placentomes from TP treated ewes compared to control type A placentomes. *CYP19* increased in type D placentomes form TP treated ewes compared to type A placentomes from controls. TP treated ewes had increased *VEGFA* in type D placentomes compared to type A placentomes from control ewe. TP treated ewes had increased VEGFA monomer in type A placentome cotyledon tissue. Different letters indicate statistical difference of $P<0.05$.

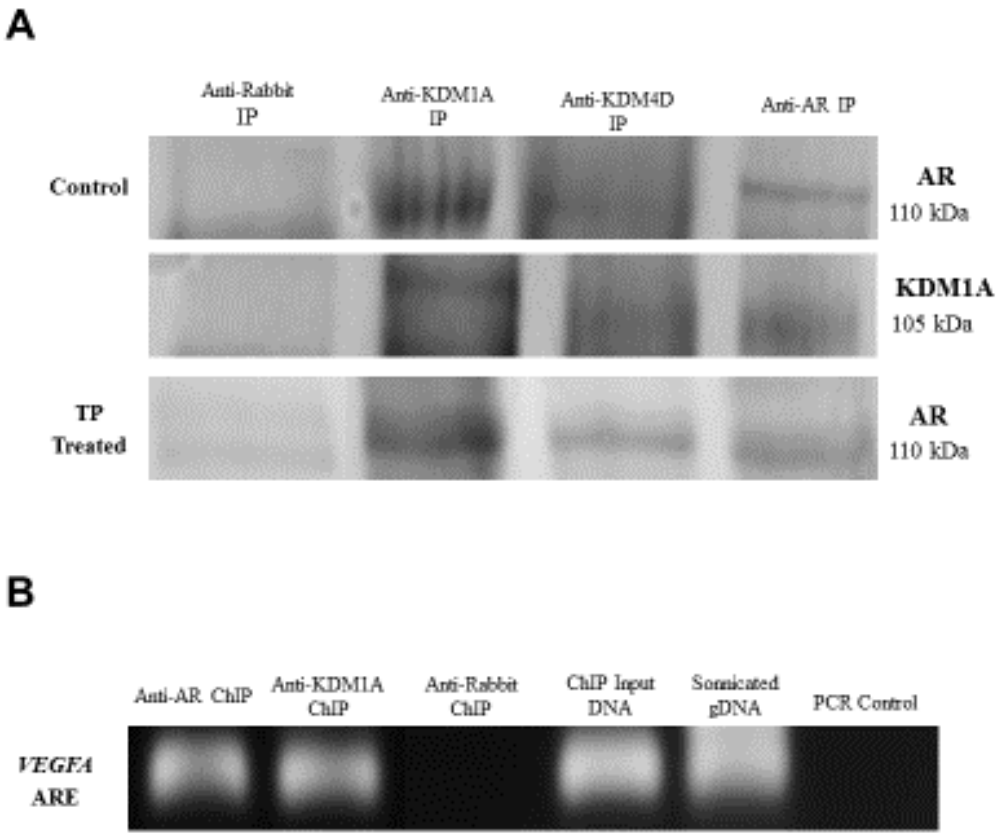


FIGURE 2.7. Interaction of KDMs with AR. **A**) Immunoprecipitation of AR, KDM1A, and KDM4D from placentome protein isolated from control and TP treated ewes followed by Western blot detection of co-immunoprecipitated protein. Anti-rabbit IP represents pull down by secondary antibody used for Western blot immunolabeling. Antibody preabsorption with blocking peptide was used as a negative control. **B**) PCR of an ARE promoter region of *VEGFA* from ChIP samples. IP, immunoprecipitate; ChIP, chromatin immunoprecipitation; ARE, primers spanning androgen response element in promoter region

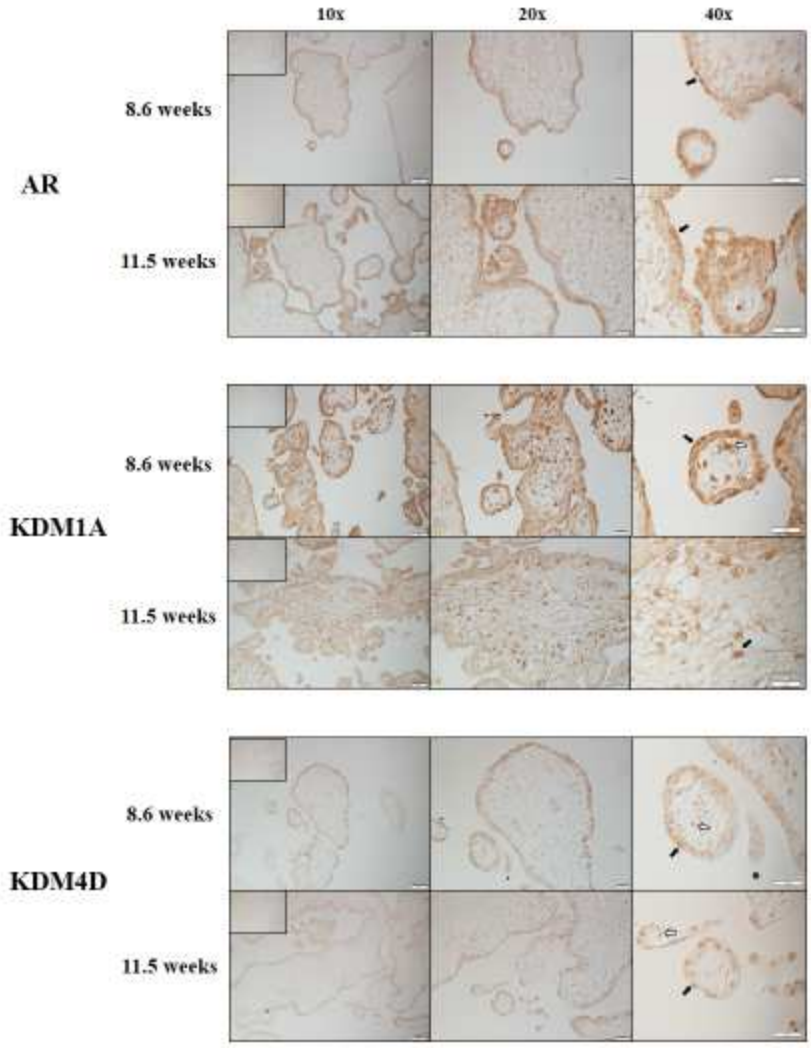
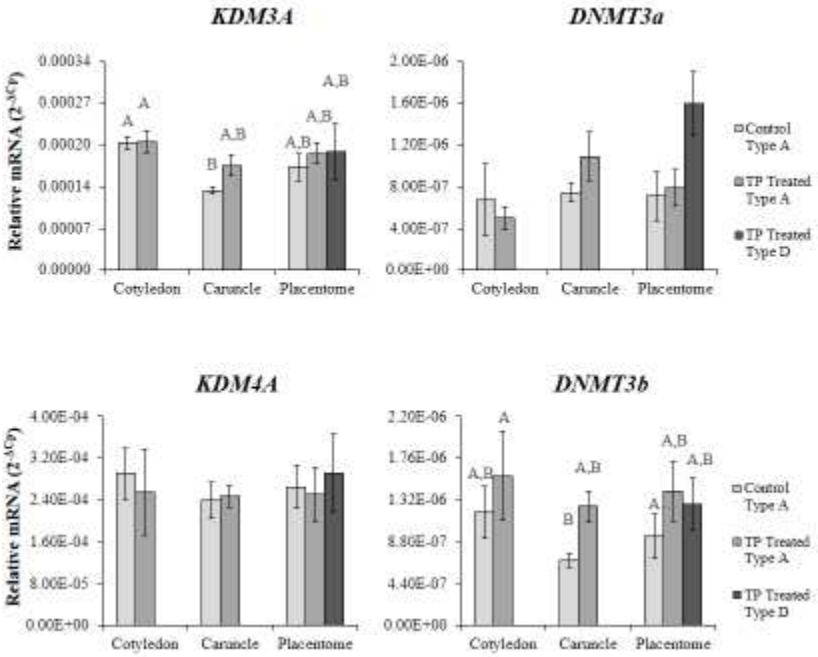


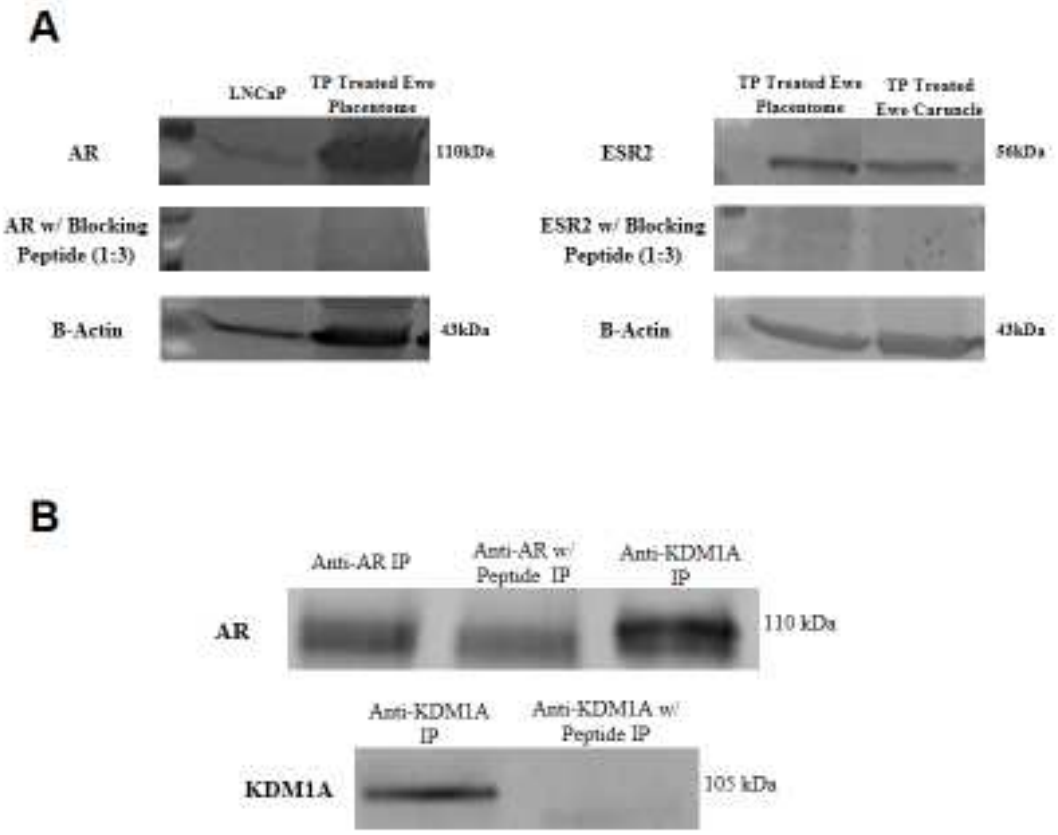
FIGURE 2.8. Immunolocalization of AR, KDM1A, and KDM4D in first trimester human placenta samples. Inserts are representative images from control slides. AR immunostaining was present in the nuclei in the syncytium at 8.6 and 11.5 weeks of gestation (arrow). KDM1A immunostaining also localized to the nuclei in the syncytium at 8.6 and 11.5 weeks of gestation. KDM1A nuclear immunostaining also was localized in cells in the villous stroma (white arrow) at 8.6 weeks of gestation. KDM4D Immunolocalization was similar to KDM1A, with nuclear immunostaining present in the syncytium (arrow) and in the villous stroma (white arrow) at both 8.6 and 11.5 weeks of gestation.

SUPPLEMENTAL TABLE 2.1. List of primer sequences used for real time PCR of sheep placentomes.

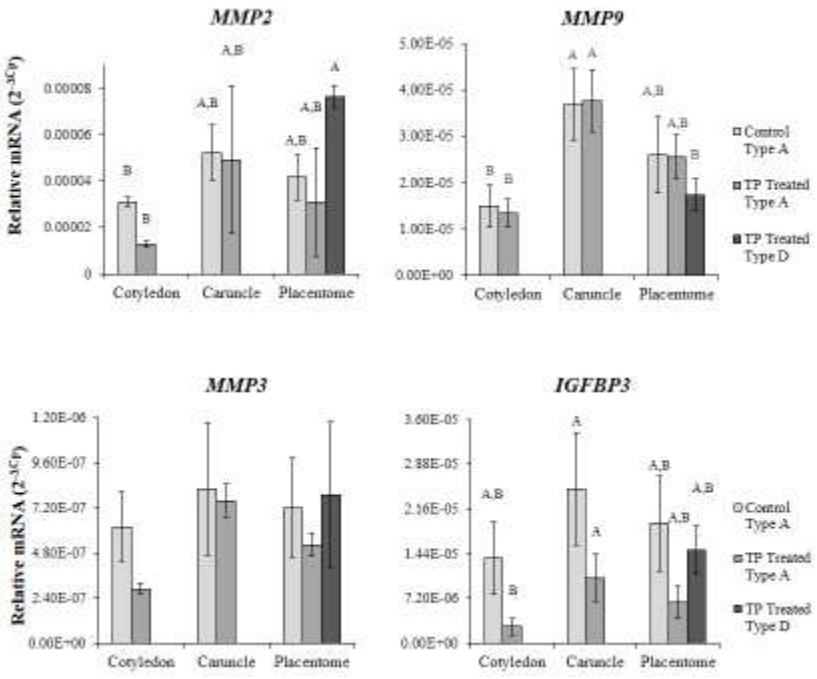
Gene	Primer Sequence	Amplicon Size	Primer Efficiency
AR	**F-5' TCCTGGATGGGGCTTATGGT** **R-5' GCCTCATTCGGACACACTGG**	**150**	**99.8%**
ESR1	**F-5' CGCGTCCTGGACAAGATCAC** **R-5' TGCTCCATGCCTTTGTTGCT**	**142**	**92.4%**
ESR2	**F-5' CTGTCGACTGCGGAAGTGCT** **R-5' ATGGGTGCACCGTTCCTCTT**	**146**	**87.6%**
CYP19	**F-5' GTTGTGCCTATTGCCAGCAT** **R-5' AACCTGCAGTGGGAAATGAG**	**137**	**88.7%**
DNMT1	**F-5' TTCTGCAGCAAGAAGAGCAA** **R-5' AGAAGTCCTGGAGGCACTCA**	**229**	**99.2%**
DNMT3A	**F-5' AGCACAACGGAGAAGCCTAA** **R-5' GTTCTTGCAGTTTTGGCACA**	**180**	**96.3%**
DNMT3B	**F-5' TGCAGACAGCACCGAGTATC** **R-5' CTGCTGGAATCTCGGAGAAC**	**190**	**90.1%**
H19	**F-5' AATAGAAGCCCCCTGGGTGT** **R-5' CCCCATCAGATCCCTGTCAT**	**152**	**83.1%**
KDM1A	**F-5' ACATTGCAGTTGTGGTTGGA** **R-5' GACCCCAGAGCCTATGATGA**	**216**	**99.1%**
KDM3A	**F-5' GCCAACATTGGAGACCACTT** **R-5' GCACCTTGTTGGCAGTTTTT**	**230**	**95.6%**
KDM4A	**F-5' TGGATCGAGTATGGCAAACA** **R-5' TCTCAGGGCCAGTTCACTCT**	**201**	**86.0%**
KDM4C	**F-5' TGCACTTGCAGGAAAGAC** **R-5' ACACTGGAAGCTCCTGGATG**	**206**	**98.1%**
KDM4D	**F-5' GGCAGAGTACCGCCACTTAG** **R-5' GGTTCCACTGCTTCGTGTTT**	**167**	**86.3%**
MMP2	**F-5' ACCAGAGCACCATTGAGACC** **R-5' TGGATCCGAGAAAACCGTAG**	**220**	**86.5%**
MMP3	**F-5' GCAAGCAGGTTACCCAAGAG** **R-5' GGCTCCATGGATTGTGTCTT**	**156**	**99.5%**
MMP9	**F-5' CTCCTACTCCTCCTGCACCA** **R-5' GCGTCCATCGGAGGTACA**	**205**	**99.3%**
VEGF	**F-5' TCACCAAAGCCAGCACATAG** **R-5' GCGAGTCTGTGTTTTTGCAG**	**179**	**97.9%**
IGF2	**F-5' GACCGCGGCTTCTACTTCAG** **R-5' AAGAACTTGCCCACGGGGTAT**	**202**	**98.6%**
IGFBP1	**F-5' TGATGACCGAGTCCAGTGAG** **R-5' GTCCAGCGAAGTCTCACAC**	**248**	**94.5%**
IGFBP2	**F-5' CAATGGCGAGGAGCACTCTG** **R-5' TGGGGATGTGTAGGGAATAG**	**330**	**88.3%**
IGFBP3	**F-5' CTCAGAGCACAGACACCCA** **R-5' GGCATATTTGAGCTCCAC**	**335**	**95.7%**
GAPDH	**F-5' GATTGTCAGCAATGCCTCCT** **R-5'GGTCATAAGTCCCTCCACGA**	**94**	**94.3%**
RN18s	**F-5' GAGGCCCTGTAATTGGAATGAG** **R-5' GCAGCAACTTTAATATACGCTATTGG**	**119**	**97.2%**



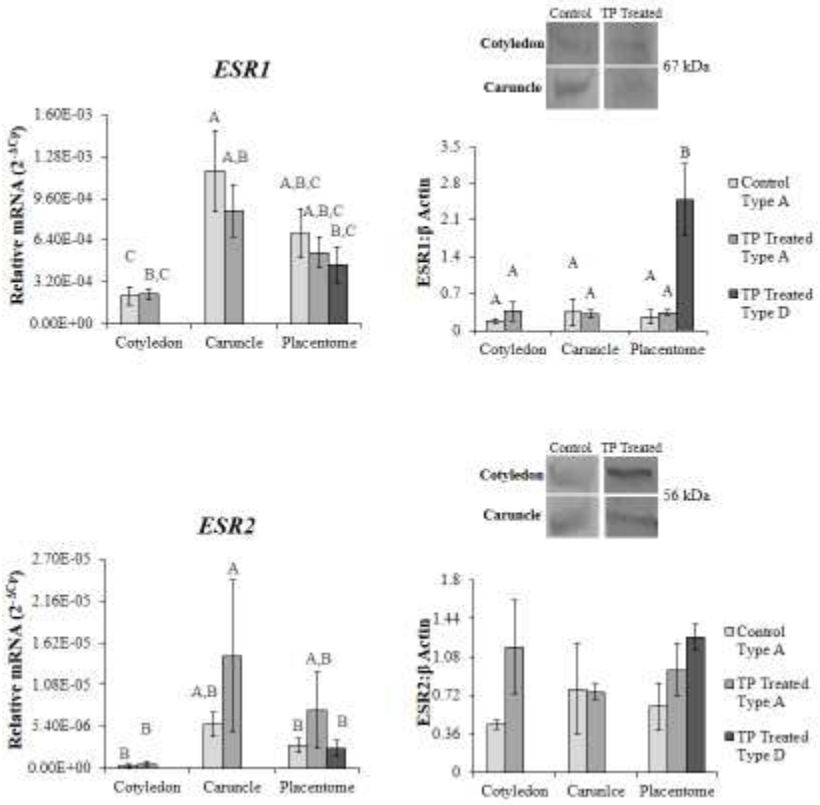
SUPPLEMENTAL FIGURE 2.1. Real time PCR results for other epigenetic regulators. Difference in letter indicates significant difference of $P<0.05$.



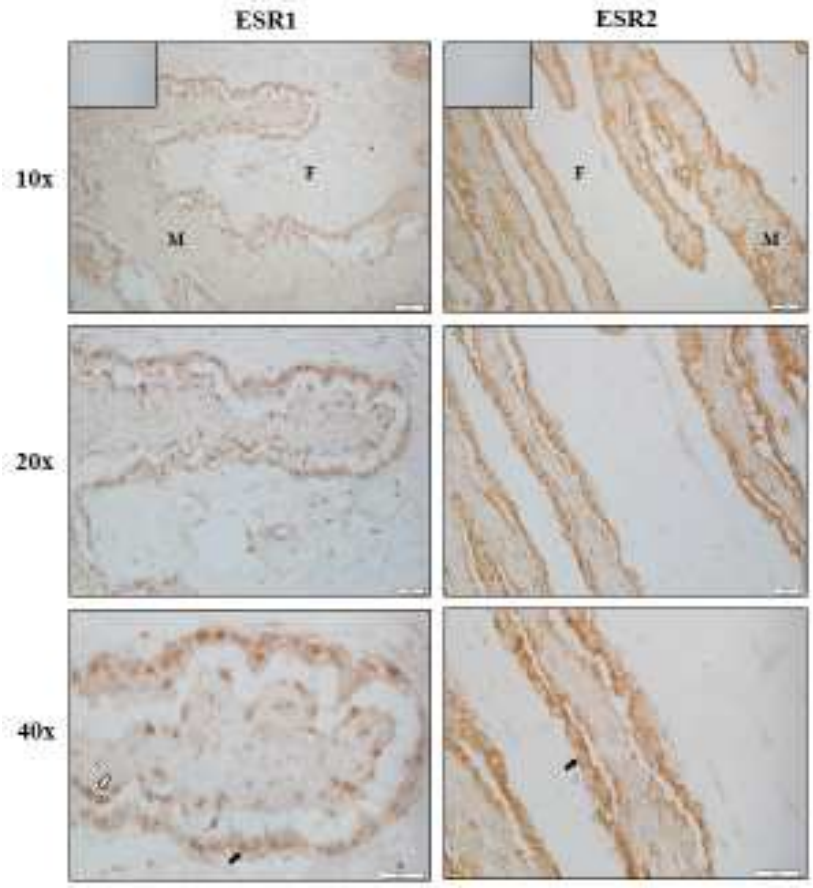
SUPPLEMENTAL FIGURE 2.2. Representative Western blot blocking peptide controls. **A**) Loss of immunoreactive band for AR when antibody is preabsorbed with AR blocking peptide at a 1:3 ratio. Loss of ESR2 immunoreactive band when antibody is preabsorbed with ESR2 blocking peptide at a 1:1 ratio. LNCaP, human prostate adenocarcinoma cells. **B**) Reduced AR immunoreactive band in immunoprecipitation of AR when antibody is preabsorbed with blocking peptide (1:1 dilution). Loss of KDM1A immunoreactive band in immunoprecipitation when antibody is preabsorbed with blocking peptide (1:1 dilution). IP, immunoprecipitation



SUPPLEMENTAL FIGURE 2.3. Real time PCR results for other androgen responsive genes known to regulate trophoblast differentiation and proliferation. Placentome values represent average of cotyledon and caruncle quantified mRNA levels in type A cotyledons. Difference in letter indicates significant difference of $P<0.05$.



SUPPLEMENTAL FIGURE 2.4. ESR1 and ESR2 placentome mRNA and protein levels in control and TP treated ewes. ESR1 increased in type D placentomes from TP treated ewes. No difference was observed in ESR2 mRNA or protein. Difference in letter indicates significant difference of $P<0.05$.



SUPPLEMENTAL FIGURE 2.5. Immunolocalization of ESR1 and ESR2 in type A placentomes from control ewes at GD90. Inserts are representative images from control IHC slides. ESR1 immunolocalization was present in the nucleus of the trophoblast layer (arrow), although nuclear immunostaining was also present in the syncytium (white arrow). In contrast, ESR2 immunolocalization was present primarily within the cytoplasm of the trophoblasts (arrow). F, fetal cotyledon; M, maternal caruncle

REFERENCES

Acromite MT, Mantzoros CS, Leach RE, Hurwitz J, Dorey LG 1999 Androgens in preeclampsia. Am J Obstet Gynecol 180(1):60-63

Albrecht ED, Pepe GJ 1999 Central integrative role of oestrogen in modulating the communication between the placenta and fetus that results in primate fetal-placental development. Placenta 20:129–139

Albrecht ED, Robb VA, Pepe GJ 2004 Regulation of placental vascular endothelial growth/permeability factor expression and angiogenesis by estrogen during early baboon pregnancy. J Clin Endocrinol Metab 89(11):5803-5809

Albrecht ED, Bonagura TW, Burleight DW, Enders AC, Aberdeen GW, Pepe GJ 2006 Suppression of extravillous trophoblast invasion of uterine spiral arteries by estrogen during early baboon pregnancy. Placenta 27:483-490

Angiolini E, Fowden A, Coan P, Sandovici I, Smith P, Dean W, Burton G, Tycko B, Reik W, Sibley C, Constância M 2006 Regulation of placental efficiency for nutrient transport by imprinted genes. Placenta 27(Supplement A):Vol. 20

Bdolah Y, Sukhatme VP, Karumanchi SA 2004 Angiogenic Imbalance in the Pathophysiology of Preeclampsia: Newer Insights. Semin Nephrol 24:548-556

Beckett EM, Veiga-Lopez A, DeHaan AM, Padmanabhan V 2011 Developmental programming: role of androgen and insulin in advancing placentome differentiation in testosterone treated sheep. Abstract of the 44th Annual Meeting of the Society for the Study of Reproduction, July 31-Auguat 4, 2011, Portland, Oregon. Biol Reprod 85(suppl):Abstract 301

Blankenship TN, Enders AC, King BF 1993 Trophoblastic invasion and the development of uteroplacental arteries in the macaque: immunohistochemical localization of cytokeratins, desmin, type IV collagen, laminin, and fibronectin. Cell Tissue Res 272:227-236

Brosens IA 1988 The uteroplacental vessels at term: the distribution and extent of physiological changes. Trophoblast Res 3:61-68

Bukovsky A, Caudle MR, Cekanova M, Fernando RI, Wimalasena, J, Foster JS, Henley DC, Elder RF 2003a Placental expression of estrogen receptor beta and its hormone binding variant-comparison with estrogen receptor alpha and a role for estrogen receptors in asymmetric division and differentiation of estrogen-dependent cells. Reprod Biol Endocrinol 1:36-57

Bukovsky A, Cekanova M, Caudle MR, Wimalasena J, Foster JS, Henley DC, Elder RF 2003b Expression and localization of estrogen receptor-alpha protein in normal and abnormal term

placentae and stimulation of trophoblast differentiation by estradiol. Reprod Biol Endocrinol 1:13-31

Cedar H, Bergman Y 2009 Linking DNA methylation and histone modification: patterns and paradigms. Nature Genet 10:295-304

Coffey K, Rogerson L, Ryan-Munden C, Alkharaif D, Stockley J, Heer R, Sahadevan K, O'Neill D, Jones D, Darby S, Staller P, Mantilla A, Gaughan L, Robson CN 2013 The lysin demethylase, KDM4B, is a key molecule in androgen receptor signaling and turnover. Nucleic Acids Res 1-14

Comstock CE, Burd CJ, Jessen WJ, Knudsen KE 2008 Gene profiling analysis of androgen receptor mediated function. Genomics in endocrinology: DNA microarray analysis in endocrine health and disease. Eds Handweger S, and Aronow B. Contemp Endocrinol. Humana Press:Totowa. 2:83-113

Dumesic DA, Abbott DH, Padmanabhan V 2007 Polycystic ovary syndrome and its developmental origins. Endocrin Metab Disorders 8(2):127-141

Ford SP, Vonnahme KA, Drumhiller MC, Reynolds LP, Nijland MJ, Nathanielsz PW 2004 Arteriolar density and capillary volume increase as placentomes advance from type A through type D developmental stages. Biol Reprod 71(Suppl): 509 Abstr

Ford SP, Nijland MJ, Miller MM, Hess BW, Nathanielsz PW 2006 Maternal undernutrition advanced placentomal type, in association with increase placentomal size and cotyledonary (cot) blood flow. J Soc Gynecol Investig 13(Suppl): 212 Abstr

France JT, Mason JI, Magness RR, Murry BA, Rosenfeld CR 1987 Ovine placental aromatase: studies of activity levels, kinetic characteristics and effects of aromatase inhibitors. J Steroid Biochm 28(2):155-160

Gaiani R, Chiesa F, Mattili M, Nannetti G, Galeati F 1984 Androstenedione and testosterone concentrations in plasma and milk of the cow throughout pregnancy. J Reprod Fertil 70:55-59

Garcia-Bassets I, Kwon Y-S, Telese F, Prefontaine GG, Hutt KR, Cheng CS, Ju B-G, Ohgi KA, Wang J, Escoubet-Lozach L, Rose DW, Glass CK, Fu X-D, Rosenfeld MG 2007 Histone methylation-dependent mechanisms impose ligand dependency for gene activation by nuclear receptors. Cell 128(3):505-518

Han VKM, Carter AM 2000 Spatial and temporal patterns of expression of messenger mRNA for insulin-like growth factors and their binding proteins in the placenta of man and laboratory animals. Placenta 21(4):289-305

Hill DJ, Clemmons DR, Riley SC, Bassett N, Challis JRG 1993 Immunohistochemical localization of insulin-like growth factors (IGFs) and IGF binding proteins -1, -2, and -3 in human placenta and fetal membranes. Placenta 14(1):1-12

Hoet JJ, Hanson MA 1999 Intrauterine nutrition: its importance during critical periods for cardiovascular and endocrine development. J Physiol 514:617-627

Horie K, Takakura K, Imai K, Liao S, Mori T 1992 Immunohistochemical localization of androgen receptor in the human endometrium, decidua, placenta, and pathological conditions of the endometrium. Hum Reprod 7:1461-1466

Hsu T-Y, Lan K-C, Tsai C-C, Ou C-Y, Cheng B-H, Tsai M-Y, Kang Y-Y, Tung Y-H, Wong Y-H, Huang K-E 2009 Expression of androgen receptor in human placentas from normal and preeclamptic pregnancies. Taiwanese J Obstet Gynecol 48(3):262-267

Iwamura M, Abrahamsson PA, Benning CM, Cockett AT, di Sant'Agnese PA 1994 Androgen receptor immunostaining and its tissue distribution in formalin-fixed, paraffin-embedded sections after microwave treatment. J Histochem Cytochem 42:783-788

Johnson LM, Cao X, Jacobsen SE 2002 Interplay between two epigenetic marks: DNA methylation and histone H3 lysine 9 methylation. Curr Biol 12(6):1360-1367

Kaufmann P, Black S, Huppertz B 2003 Endovascular Trophoblast Invasion: Implications for the pathogenesis of intrauterine growth retardation and preeclampsia. Biol Reprod 69:1-7

Kawazu M, Saso K, Tong KI, McQuire T, Goto K, Son D-O, Wakeham A, Miyagishi M, Mak TW, Okada H 2011 Histone demethylase JMJD3B functions as a co-factor of estrogen receptor in breast cancer proliferation and mammary gland development. PLoS ONE 6(3):e17830

Khatri P, Hoffmann B, Schuler G 2013 Androgen receptor is widely expressed in bovine placentomes and up-regulated during differentiation of bovine trophoblast giant cells. Placenta 34:416-423

Klose RJ, Bird AP 2006 Genomic DNA methylation: the mark and its mediators. Trends Biochem Sci 31(2):89-97

Lim JH, Kim SY, Lee SW, Park SY, Han JY, Chung JH, Kim MY, Yang JH, Ryu HM 2011 Association between genetic polymorphisms in androgen receptor gene and risk of preeclampsia in Korean women. J Assist Reprod Genet 28:85-90

Loriaux DL, Ruder HJ, Knab DR, Lipsett MB 1972 Estrone sulfate, estrone, estradiol and estriol plasma levels in human pregnancy. J Clin Endocrinol Metab 35(6):887-891

Mabjeesh NJ, Willard MT, Fredrickson CE, Zhong H, Simons JW 2003 Androgens stimulate hypoxia-inducible factor 1 activation via autocrine loop of tyrosine kinase receptor/phosphatidylinositol 3'-kinase/protein kinase B in prostate cancer cells. Clin Cancer Res 9:2416-2425

Manikkam M, Crespi EJ, Doop DD, Herkimer C, Lee JS, Yu S, Brown MB, Foster DL, Padmanabhan V 2004 Fetal programming: prenatal testosterone excess leads to fetal growth retardation and postnatal catch-up growth in sheep. Endocrinology 145(2):790-798

Metzger E, Wissmann M, Yin N, Muller JM, Schneider R, Peters AH, Gunther T, Buettner R, Schule R 2005 LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. Nature 437:436-439

Milosevic M, Chung P, Parker C, Bristow R, Toi A, Panzarella T, Warde P, Catton C, Menard C, Bayley A, Gospodarowicz M, Hill R 2007 Androgen withdrawal in patients reduces prostate cancer hypoxia: implications for disease progression and radiation response. Cancer Res 67:6022-6025

Mizuno M, Lobotsky J, Lloyd CW, Kobayashi T, Murasawa Y 1968 Plasma androstenedione and testosterone during pregnancy and in the newborn. J Clin Endocrinol Metab 28(8):1133-1142

Niklaus AL, Aberdeen GW, Babischkin JS, Pepe GJ, Albrecht ED 2003 Effect of estrogen on vascular endothelial growth/permeability factor expression by glandular epithelial and stromal cells in the baboon endometrium. Biol Reprod 68:1997–2004

Ooi SKT, Qiu C, Berstein E, Li K, Jia D, Yang Z, Erdjument-Bromage H, Tempst P, Lin S-P, Allis CD, Cheng X, Bestor TH 2007 DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA. Nature 448(7154):714-717

Osgerby JC, Wathes DC, Howard D, Gadd TS 2004 The effect of maternal undernutrition on the placental growth trajectory and the uterine insulin-like growth factor axis in the pregnant ewe. J Endocrinol 182:89-103

Perillo B, Ombra MN, Bertoni A, Cuozzo C, Sacchetti S, Sasso, A, Chiariotti L, Malorni A, Abbondanza C, Accedimento EV 2008 DNA Oxidation as triggered by H3K9me2 demethylation drives estrogen-induced gene expression. Science 319:202-206

Ptak GE, D'Agostino A, Toschi P, Fidanza A, Zacchini F, Czernik M, Monaco F, Loi P 2013 Post-implantation mortality of in vitro produced embryos is associated with DNA methyltransferase I dysfunction in sheep placenta. Hum Reprod 28(2):298-305

Rahnama F, Shafiei F, Cluckman PD, Mitchell MD, Lobie PE 2006 Epigenetic regulation of human trophoblastic cell migration and invasion. Endocrinology 147(11):5275-5283

Rama S, Petrusz P, Rao AJ 2004 Hormonal regulation of human trophoblast differentiation: a possible role for 17β-estradiol and GnRH. Mol Cellular Endocrinol 218:79-94

Randawa R, Cohen P 2005 The role of the insulin-like growth factor system in prenatal growth. Mol Genet Metab 86(1-2):84-90

Recabarren SE, Padmanabhan V, Codner E, Lobos A, Durán C, Vidal M, Foster DL, Sir-Petermann T 2005 Postnatal developmental consequences of altered insulin sensitivity in female sheep treated prenatally with testosterone. Am J Physiol Endocrinol Metab 289:E801-E806

Robinson JE, Birch RA, Taylor JA, Foster DL, Padmanabhan V 2002 In utero programming of sexually differentiated gonadotrophin releasing hormone (GnRH) secretion. Domest Anim Endocrinol 23:43-53

Salamalekis E, Bakas P, Vitoratos N, Eleptheriadis M, Creatsas G 2006 Androgen levels in the third trimester of pregnancy in patients with preeclampsia. Eur J Obstet Gynecol Reprod Biol 126:16-19

Sathishkumar K, Elkins R, Chinnathambi V, Gao H, Hankins GDV, Yallampalli C. 2011 Prenatal testosterone-induced fetal growth restriction is associated with down-regulation of rat placental amino acid transport. Reprod Biol Endocrinol 9:110-122

Schmittgen TD, Livak KJ 2008 Analyzing real-time PCR data by the comparative CT method. Nature Protocols 3(6): 1101-1108

Serin IS, Kula M, Basbug M, Unlühizarci K, Güçer S, Tayyar M 2001 Androgen levels of preeclamptic patients in the third trimester of pregnancy and six weeks after delivery. Acta Ostet Gynecol Scand 80:1009-1013

Shabisgh A, Tanji N, D'Agati V, Burchardt M, Rubin M, Goluboff ET, Heitjan D, Kiss A, Buttyan R 1999 Early effects of castration on the vascular system of the rat ventral prostate gland. Endocrinology 140:1920-1926

Shin S, Janknecht R 2007 Activation of androgen receptor by histone demethylases JMJD2A and JMJD2D. Biochem Bioph Res Commun 359:742-746

Siiteri PK, MacDonald PC 1963 The utilization of circulating dehydroisoandrosterone sulfate for estrogen synthesis during human pregnancy. Steroids 2(6):713-730

Siiteri PK, MacDonal PC 1966 Placental estrogen biosynthesis during human pregnancy. J Clin Endocrinol Metab 26(7):751-761

Sun M, Maliqueo M, Benrick A, Hohansson J, Shao R, Hou L, Jansson T, Wu X, Stener-Victorin E 2012 Maternal androgen excess reduces placental and fetal weights, increases placental steroidogenesis, and leads to long-term health effects in their female offspring. Am J Physiol Endocrinol Metab 303:E1373-E1385

Tulchinsky D, Hobel CJ 1953 Plasma human chorionic gonadotropin, estrone, estradiol, estriol, progesterone, and 17-alpha-hydroxyprogesterone in human pregnancy. 3. Early normal pregnancy. Am J Obstet Gynecol 117(7):884-893

van der Linden DS, Sciascia Q, Sales F, McCord SA 2012 Placental nutrient transport is affected by pregnancy rank in sheep. J Ani Sci 91(2):644-653

Veiga-Lopez A, Ye W, Phillips DJ, Herkimer C, Knight PG, Padmanabhan V 2008 Developmental programming: deficits in reproductive hormone dynamics and ovulatory outcomes in prenatal, testosterone-treated sheep. Biol Reprod 78:636-647

Verrier L, Vandromme M, Trouche D 2011 Histone demethylases in chromatin cross-talks. Biol Cell 103(8):381-401

Vonnahme KA, Hess BW, Nijland MJ, Nathanielsz PW, Ford SP 2006 Placentomal differentiation may compensate for maternal nutrient restriction in ewes adapted to harsh range conditions. J Anim Sci 84(12):3451-3459

Wang J, Scully K, Zhu X, Cai L, Zhang J, Prefontain GG, Krones A, Ohgi KA, Zhu P, Garcia-Bassets I, Liu F, Taylor H, Lozach J, Jayes FL, Korach KS, Flass CK, Fu X-D, Rosenfeld 2007 Opposing LSD1 complexes function in developmental gene activation and repression programs. Nature 466:882-887

Wang J, Hevi S, Kurash JK, Lei H, Gay F, Bajko J, Su H, Sun W, Chang H, Xu G, Gaudet F, Li E, Chen T 2009 The lysine demethylase LSD1 (KDM1) is required for maintenance of global DNA methylation. Nature Genet 41(1):125-129

Weksberg R, Shuman C, Beckwith JB 2010 Beckwith-Wiedemann Syndrome. J Hum Genet 18:8-14

Wieciech I, Durlej-Grzesiak M, Stomczyńska M 2013 Influence of the antiandrogen flutamide on the androgen receptor gene expression in the placenta and umbilical cord during pregnancy in the pig. Acta Histochem 115(3):290-295

Wissmann M, Yin N, Müller JM, Greschik H, Fodor BD, Jenuwein T, Vogler C, Schneider R, Günther T, Buettner R, Metzger E, Schüle R 2007 Cooperative demethylation by JMJD2C and LSD1 promotes androgen receptor-dependent gene expression. Nature Cell Biol 9(3):347-353

Yamane K, Toumazou C, Tsukada Y-I, Erdjument-Bromage H, Tempst P, Wong J, Zhang Y 2006 JHDM2A, a JmjC-contain H3K9 demethylase, facilitates transcription activation by androgen receptor. Cell 125:483-495

Yang J, Jubb AM, Pike L, Buffa FM, Turley H, Baban D, Leek R, Gatter KC, Ragoussis J, Harris AL 2010 The histone demethylase JMJD2B is regulated by estrogen receptor α and hypoxia, and is a key mediator of estrogen induced growth. Cancer Res 70(16):6456-6466

Zhou Y, Chiu K, Brescia RJ, Combs CA, Katz MA, Kitzmiller JL, Heilbron DC, Fisher SJ 1993 Increased depth of trophoblast invasion after chronic constriction of the lower aorta in rhesus monkeys. Am J Obstet Gynecol 169:224-229

Zhou Y, McMaster M, Woo K, Janatpour M, Perry J, Karpanen T, Alitalo K, Damsky C, Fisher SJ 2002 Vascular endothelial growth factor ligands and receptors that regulate human cytotrophoblast survival are dysregulated in severe preeclampsia and hemolysis, elevated liver enzymes, and low platelets syndrome. Am J Pathol 160:1405–1423

Zhou Y, Bellingard V, Feng KT, McMaster M, Fisher SJ 2003 Human cytotrophoblasts promote endothelial survival and vascular remodeling through secretion of Ang 2, PIGF, and VEGF-C. Dev Biol 263:114–125

CHAPTER III: ANDROGEN RECEPTOR INTERACTS WITH HISTONE LYSINE DEMETHYLASES AND REGULATES TRANSCRIPTION IN CHORIOCARCINOMA CELLS[4,5]

Summary

Recently, we reported that androgen receptor (AR) complexes with histone lysine demethylases (KDMs) in the ovine placenta and that KDMs immunolocalized to the syncytiotrophoblast and nuclei of cells in the villous stroma of the human first trimester placenta. While KDMs complex with AR and are regulated by androgen signaling, their expression and activity is increased under hypoxic conditions. Hypoxia also drives trophoblast differentiation in the first trimester, in part through regulation of estrogen signaling in trophoblast cells. Increased AR activity has also been reported in hypoxic conditions for other cell types, though hypoxia-mediated regulation of androgen signaling in the placenta has not been previously investigated. We therefore investigated the response of choriocarcinoma cell lines ACH-3P and BeWo to dihydrotestosterone (DHT) treatment with and without androgen inhibition through flutamide treatment. Additionally, we investigated if ACH-3P response to DHT treatment was altered under hypoxic conditions. We report that DHT treatment increased *AR* mRNA levels in ACH-3P at 48 hours, but down-regulated known androgen responsive genes, such as *KDM3A*, *KDM4D*, and *MMP2* in ACH-3P and BeWo cells. Flutamide treatment affected genes regulating

[4] Paper in progress for submission

[5] Project supported by Agriculture and Food Research Initiative competitive grant number 2010-38420-20397 from the USDA National Institute of Food and Agriculture and by the Colorado State University College of Veterinary Medicine and Biomedical Sciences College Research Council.

vascularization, including increased *HIF1α* in hypoxic conditions and decreased *PPARy* in normoxic conditions. *CYP19* decreased in hypoxic conditions compared to normoxic cells. While androgen responsive genes did not appear to respond to DHT as would be predicted, protein expression levels in response to treatment have not yet been investigated. Additionally, identification of AR and KDMs in the immortalized trophoblast cell line Swan71 suggests an alternative, better model than choriocarcinoma cells for future experiments in characterizing androgen signaling and interaction with KDMs and HIF1α in human trophoblast cells.

Introduction

During the first trimester, cytotrophoblast cells in human placental villi differentiate to form syncytiotrophoblast and invasive extravillous trophoblasts for placental growth, endocrine function, invasion and remodeling of the uterine spiral arteries (Brosens et al., 1967; Enders 1968; Blankenship et al., 1993; Brosens 1988; Kaufmann et al., 2003). Inadequate trophoblast differentiation and invasion leads to placental insufficiency and the development of pregnancy induced disorders, including fetal growth restriction and preeclampsia (Knobil and Neill 1998; Eriksson et al., 1999; Jaquet et al., 2000; Anderson 2007). Trophoblast differentiation is regulated by the hypoxic uterine environment of the first trimester, in particular through activation of hypoxia inducible factor 1 (HIF1), increased placental vascularization through HIF1 target vascular endothelial factor (VEGF), and regulation of estrogen signaling (Brogi et al., 1996; Kingdom and Kaufmann 1997; Salceda and Cara 1997; Ehleben et al., 1998; Huang et al., 1998; Shweiki et al., 1992; Adelman et al., 2000; Chandel et al., 2000; Semenza 2000; Tissot van Patot et al., 2004; Rama et al., 2004; Albrecht et al., 2004). Estradiol produced by the placenta in the hypoxic first trimester is suggested to regulate trophoblast invasion by increasing matrix

metalloproteinases (MMPs) and promoting angiogenesis (Kaufmann et al., 2003; Albrecht et al., 2006). However, the increased uterine-placental blood flow in the second trimester, coupled with the rapid rise in estrone, estriol, and estradiol production by the growing placenta, is suggested to block further trophoblast differentiation into the invasive phenotype (Tulchinsky and Hobel 1953; Siiteri and MacDonald 1963; 1966; Loriaux et al., 1972; Aw et al., 1985; Rodesch et al., 1992; Zhou et al., 1993; Rama et al., 2004; Albrecht et al., 2006; Lee et al., 2010). Although these estrogens appear to regulate trophoblast function in the first trimester, no research has investigated the role of androgen signaling in trophoblast cells. As hypoxia has been shown to increase androgen receptor (AR) activity in other cell lines, there is potential for the hypoxic environment during the first trimester to also regulate placental androgen signaling (Shabisgh et al., 1999; Mabjeesh et al., 2003; Cheng et al., 2004; Lissbrant et al., 2004; Boddy et al., 2005; Zhu and Kyprianou 2008).

Normal trophoblast function and placental health may be controlled by placental androgen signaling as maternal serum androgen levels rise rapidly throughout gestation through placental production and secretion into maternal vasculature (Mizuno et al., 1968; Serin et al., 2001). By the second trimester, maternal plasma concentrations of androstenedione and testosterone reach levels two-fold and four-fold greater than non-pregnant women, respectively, (Mizuno et al., 1968). Additionally, multiple studies have shown that maternal plasma testosterone is increased in cases of severe preeclampsia and placenta-induced hypertension (Acromite et al., 1999; Serin et al., 2001; Atamer et al., 2004; Salamalekis et al., 2006; Gerulewicz-Vannini et al., 2006; Ghorashi and Sheikhvatan 2008; Hsu et al., 2009; Lorzadeh and Kazemirad 2012). In preeclamptic patients, serum testosterone raises two to three times higher than normal levels

during pregnancy, with increased testosterone detectably higher at 17 weeks of gestation (Atamer et al., 2004; Carlsen et al., 2005). As AR is localized in cytotrophoblast, syncytiotrophoblast, invasive extravillous trophoblasts and vascular endothelial cells, the placental production of androgens has the potential for autocrine or paracrine regulation of trophoblast function (Horie et al., 1992; Iwamura et al., 1994; Hsu et al., 2009; Khatri et al., 2013; Wieciech et al., 2013).

Hypoxia has been shown to increase expression of androgen responsive genes, such as hypoxia inducible factor 1α (HIF1α) and vascular endothelial growth factor (VEGF) which function to promote angiogenesis and vascularization of the placenta (Shabisgh et al., 1999; Mabjeesh et al., 2003; Cheng et al., 2004; Lissbrant et al., 2004; Boddy et al., 2005; Zhu and Kyprianou 2008). Additionally, androgen withdrawal in prostate cancer has been shown to reduce tissue hypoxia (Milosevic et al., 2007). While androgen signaling in trophoblasts may in part be regulated by hypoxia, lysine demethylase 1 family and Jumonji-C domain containing histone demethylases (KDMs) have been shown to complex with AR to regulate androgen signaling (Metzger et al., 2005; Yamane et al., 2006; Shin and Janknecht 2007; Wissmann et al., 2007; Cloos et al., 2006; Coffey et al., 2013). In addition, KDMs are also regulated by hypoxia to promote vascularization, invasion, migration, anti-apoptosis, and cell proliferation (Metzger et al., 2005; Shin and Janknecht 2007; Beyer et al., 2008; Yang et al., 2010; Kawazu et al., 2011; Shi et al., 2011; Coffey et al., 2013).

Recently, we described nuclear immunolocalization of KDM1A and KDM4D in syncytiotrophoblast and villous stroma of first trimester human placenta. It was therefore the

goal of this study to determine if KDMs were present in choriocarcinoma cells and to investigate the response of choriocarcinoma cells to androgen treatment. In particular, we aimed to determine the changes in androgen responsive genes that are known to regulate trophoblast differentiation and placental angiogenesis: MMPs, insulin-like growth factors (IGFs), HIF1α, VEGFA, and VEGFA regulator PPARα/*y* (Charnock-Jones et al., 1994; Huppertz et al., 1998; Lee et al., 2001; Constância et al., 2002; Kudo and Boyd 2002; Solberg et al., 2003 Wieser et al., 2008; Renaud et al., 2014). Additionally, we tested whether expression of syncytin, syncytin receptor (ASCT2), and kisspeptin (KiSS1), genes known to regulate trophoblast fusion and invasion, respectively (Pötgens et al., 2004; Dhillo et al., 2006; Hiden et al., 2007a; Armstrong et al., 2009; Gauster et al., 2009; Vargas et al., 2009), were increased in choriocarcinoma cells following androgen treatment.

Materials and Methods

Cell Culture Treatment with DHT, Flutamide, and Hypoxia

Human first trimester choriocarcinoma ACH-3P (Hiden et al., 2007b) and BeWo cells (Trowsdale et al., 1980) between passage 13 and 17 were plated on 10cm^2 plastic dishes. Cells were maintained in phenol red-free Ham's F12 medium (Caisson) supplemented with 10% charcoal stripped FBS (Cellgro), 1% PSA (Cellgro) and cultured at 37°C with 5% CO_2 and 95% air (normoxic 20% O_2) or with 2% O_2, 5% CO_2, and 92.8% N_2 (hypoxic). Immortalized human first trimester trophoblast Swan71 cells (Straszewski-Chavez et al., 2009) were maintained in normoxic conditions with phenol red-free DMEM/F12 1:1 media (Hyclone) containing 2.5mM L-glutamine supplemented with 10% charcoal stripped FBS (Cellgro), 1% PSA (Cellgro), 1mM sodium pyruvate (Cellgro), 0.1mM non-essential amino acids (Hyclone). Prior to treatment, 1.5 x

10^6 and 1 x 10^6 ACH-3P and BeWo cells, respectively, were seeded per dish and allowed to plate overnight. Dihydrotestosterone (DHT) and the antiandrogen drug flutamide (Sigma Aldrich) were dissolved in 100% ethanol and further diluted in PBS to obtain working stock solutions (Nheu et al., 2011).

DHT was added at concentrations of 0.01nM, 0.1nM, and 1nM to mimic a physiological range (Dawood and Saxen 1977). Control cells received equal volume and concentration of diluted ethanol in PBS (vehicle). When treated with flutamide, 10 μM flutamide in complete phenol red-free media supplemented with charcoal stripped serum was added to cell culture plates 3 hours prior to DHT administration. Cells were maintained up to 48 hours and observed to ensure treatments did not result in cell death. With the preliminary treatment of ACH-3P cells, cell pellets were collected at 24 and 48 hours post-treatment. In all subsequent cell culture experiments, cell pellets were collected at 48 hours post-treatment.

To collect cell pellets, culture plates were first treated with trypsin (Cellgro) and cells were collected in complete phenol-red free media containing charcoal-stripped serum in conical tubes. Tubes were centrifuged for 3 minutes at 1000 x g and media was removed from cell pellets. Cell pellets were then snap frozen and stored at -80°C prior to RNA and protein isolation. Each treatment contained at least four biological replicates (n=4 per treatment). For statistical analysis, ACH-3P cells collected at 24 hours were analyzed separately from cell pellets collected at 48 hours. ACH-3P cells kept under hypoxic conditions were compared to cells under normoxic culture conditions using an ANOVA with Tukey's pairwise comparison (Minitab 16) with statistical significance at $P \leq 0.05$.

Cell Pellet RNA Isolation and Real Time PCR

Primers were designed using Primer3Plus online tool when a previously published primer sequence was not readily identified (Table 3.1). Primer specificity was confirmed using DNA sequencing of PCR amplicons prior to real time PCR. A pool of cDNA was used for PCR amplification with GoTaq (Promega) as per the manufacturer's specifications. PCR product was electrophoresed and the resulting amplicon band was excised from the agarose gel. DNA was extracted from the agarose using QIAquick Gel Extraction Kit (Qiagen) and was sequenced at the Colorado State University Genomics and Proteomics Laboratory. Sequence alignment with androgen responsive genes was confirmed with NCBI blast to verify specificity. Serial dilutions of a cellular cDNA pool were used to created standard curves. Standard curves were analyzed on the Roche Light Cycler 480 (Roche, Basel, Switzerland) to assess primer efficiency, including primer sequences obtained from publications.

The protocol for RNA isolation, cDNA synthesis, and real time PCR follows that described in Chapter II. Total RNA was isolated from snap frozen cell pellets using the RNeasy Mini kit (Qiagen) followed by treatment with RNase-Free DNase (Qiagen) to eliminate any genomic DNA contamination. RNA concentration and purity was determined with a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and RNA aliquots were stored at -80° C. Reverse transcription of total RNA utilized qScript cDNA synthesis (Bio-Rad, Hercules, CA) according to manufacturer's specifications. 1μg of total RNA was added to each reverse transcription reaction with 4μL reverse transcriptase Supermix (Quanta QScript) and nuclease free water up to 20μL total reaction volume. The resulting cDNA was used the same day as a template for real time PCR analysis.

For real time PCR analysis, cDNA was diluted 1:4 in nuclease free water prior to loading into real time PCR reactions. 5µl of Light Cycler 480 SYBR Green I Master (Roche, Basel Switzerland) and 2.5 µl of nanopure water per reaction was added to an Eppendorf tube to create a master mix. Primer sets of forward and reverse primer were added to the master mix to reach a final concentration of 0.5µM in each reaction. 1µl of diluted cDNA was added per well with 9 µL master mix containing primer, water, and SYBR Green I, resulting in a final cDNA concentration of 1.25 ng/µL in a total volume of 10µL. Samples were loaded into 384 well Light Cycler 480 plates (Roche) and analyzed using a Roche Light Cycler 480 (Roche) in duplicate. Real time PCR cycle conditions followed manufacturer's specifications with primer annealing at 60°C for 15 seconds. Following real time PCR amplification, melt peaks were generated with an initial incubation at 95°C for 30 seconds to confirm the presence of a single amplicon. Cp values were normalized to *RN18s*. Statistical analysis used an ANOVA and Tukey's pairwise comparison (Minitab 16) with statistical significance at $P<0.05$. Results reported for real time PCR data use $2^{-\Delta Cp}$ values (Schmittgen and Livak 2008) for statistical analysis and graphs.

Protein Isolation and Western Blot

Protein isolation and Western blot detection follows the protocol outlined in Chapter II. Protein was isolated from cell pellets suspended in 600µL of M-PER (Pierce) containing 0.1% PMSF and protease inhibitor. Samples were sonicated on ice for 2 minutes and centrifuged at 10,000 rpm for 10 minutes at 4°C. Supernatant was collected and protein concentration was determined using a Bradford standard curve (BioRad). 50µg total protein was diluted in 6x SDS-DTT loading dye with 0.375M Tris pH6.8, 60% glycerol, 12% SDS, 0.6M DTT, and 0.06% bromophenol blue with 1.75 µL β-mercaptoethanol and water to reach a final concentration of

1.43 μg/μL of protein in 35 μL. After addition of β-mercaptoethanol, samples were boiled for 5 minutes then electrophoresed at 95 volts in 10% Tris-HCL polyacrylamide gels (BioRad). Protein was electrophoresed in an ice cold running buffer containing 1.2% Tris, 5.76% glycine, and 0.4% SDS. Protein was then transferred onto 0.2 μm nitrocellulose membranes (Protran) for 1 hour at 200 milliamps at 4°C in transfer buffer containing 2mM Tris, 150 mM glycine, 20% methanol, and 0.1% SDS. After protein transfer, nitrocellulose blots were blocked with 2% milk-TBST for 1 hour at room temperature to prevent non-specific antibody binding.

After blocking in milk-TBST, blots were washed with TBST. Primary antibody diluted in 2% milk-TBST and left overnight at 4°C. The next morning, primary antibody was removed and blots were washed in TBST. Goat-anti-rabbit-HRP (Abcam ab6721, 1:1000) was applied to nitrocellulose blots and incubated for 1 hour at room temperature. Blots were subsequently washed in TBST and ECL Prime Western Blotting Detection System (Amersham Biosciences) was applied to detect immunoreactivity. Chemiluminescent bands were detected using the Chemidoc MP System (BioRad). Primary antibodies that were used for Western blot analysis are listed in Table 3.2 with their respective dilutions.

Coimmunoprecipitation of AR with KDM1A

Protein was isolated from snap frozen ACH-3P and BeWo cell pellets as described above. Coimmunoprecipitation follows the protocol listed in Chapter II with minor alterations. 250 μg of cellular protein was diluted in RIPA buffer to reach a final concentration of 0.5 μg/μL in 500 μL RIPA buffer containing 5μL PIC and PMSF. Antibodies were combined with the 500 μL of diluted cellular protein and incubated for an hour at 4° C on a rotational mixer. 2 μg of AR

antibody (Santa Cruz, sc816), 2 μg of KDM1A antibody (Abcam, ab17721), or 1 μg secondary antibody as a negative control (Abcam, anti-rabbit ab6721) were added to each sample. Following antibody incubation, 20 μL of A/G PLUS agarose beads (Santa Cruz, sc2003) were added for an hour at 4° C on a rotational mixer. Samples were briefly centrifuged at 2500 rpm for 5 minutes at 4° C. Agarose beads were centrifuged down and washed two times with RIPA buffer followed by three washes with PBS. Protein was resuspended in 6.67 μL 6x Western blot loading dye (described above) diluted with 31.33 μL RIPA buffer, then stored at -80° C overnight. The following day, samples were thawed and 2 μL of β-mercaptoethanol was added to reach a final volume of 40 μL. Samples were immediately incubated at 95° C for 8 minutes and centrifuged at 10,000 x g for 1 minute at room temperature. 20 μL of immunoprecipitated protein was loaded per lane on 10% Tris-HCl gels (BioRad) and electrophoresed. Western blot protocol and antibody dilutions followed those described above and in Table 3.2.

Results

AR and KDM1A Protein Expression and Interaction in Choriocarcinoma Cells

Immunoreactive bands of 110kDa and 105kDA corresponding to AR and KDM1A, respectively, were detected in protein isolated from ACH-3P and BeWo control cells (Figure 3.1A). Furthermore, immunoprecipitation with KDM1A antibody in control ACH-3P and BeWo cellular protein immunoprecipitated AR (Figure 3.1B).

Gene Expression Levels in Choriocarcinoma Cells

DHT Response: DHT treatment of ACH-3P (Supplemental Figure 3.1) and BeWo cells (data not shown) did not appear to lead to cell death. ACH-3P treatment with 1nM DHT decreased *AR*

compared to 0.01nM DHT at 24 hours (P=0.027, Figure 3.2). 0.1nM DHT increased *AR* levels at 48 hours compared to control and 1nM DHT (P=0.024, Figure 3.2). ACH-3P treatment with 1nM DHT decreased *KDM3A* at 24 hours compared to all other treatments (P<0.001), but had no effect at 48 hours post-treatment (Figure 3.2). 1nM DHT also resulted in decreased *KDM4A* (P=0.049) and *KDM4D* (P=0.004) levels compared to 0.01nM DHT in ACH-3P cells at 24 hours (Figure 3.2). 1nM DHT decreased *MMP2* compared to control and 0.01nM DHT at 24 hours (P<0.001, Figure 3.2). *PPARy* also decreased with 1nM DHT treatment at 24 hours compared to 0.01nM DHT treatment (P=0.046, Figure 3.2). Treatment with DHT in ACH-3P cells at 24 and 48 hours did not affect relative mRNA levels of other androgen responsive genes (Figure 3.2) or genes regulating trophoblast differentiation that were investigated (Figure 3.3).

In BeWo cells, *KDM3A* decreased at 48 hours post-treatment with all doses of DHT (P=0.003, Figure 3.4). *KDM4D* decreased in BeWo cells with 0.1nM DHT treatment compared to control (P=0.05, Figure 3.4). 0.01nM DHT decreased *MMP2* compared to control cells (P=0.03, Figure 3.4). Similar to ACH-3P cells, DHT treatment did not affect the relative mRNA levels of other androgen responsive genes that were analyzed in BeWo cells at 48 hours post-treatment (Figure 3.4).

Hypoxia and Flutamide Treatment: 10µM flutamide treatment increased *HIF1α* compared to ACH-3P control cells in hypoxic conditions (P=0.023, Figure 3.5). In hypoxic conditions, addition of 10µM flutamide to 1nM DHT increased *VEGFA* compared to 1nM DHT alone (P=0.001, Figure 3.5). *VEGFA* levels were also higher hypoxic conditions compared to normoxic when 1nM DHT with 10µM flutamide was present (P=0.001, Figure 3.5). In normoxic

conditions, treatment with 10µM flutamide, with or without 1nM DHT, decreased *PPARy* levels (P=0.017, Figure 3.5). All ACH-3P treatments in hypoxic conditions decreased *CYP19* levels compared to control normoxic levels (P=0.002, Figure 3.5). Additionally, treatment of normoxic cells with 10µM flutamide, with or without 1nM DHT, also decreased *CYP19* compared to normoxic control (P=0.002, Figure 3.5).

In BeWo cells, 10µM flutamide treatment decreased *KDM1A* (P=0.013), *KDM3A* (P=0.023), and *KDM4A* (P=0.001) compared to control and/or 1nM DHT treated cells under normoxic conditions (Figure 3.6A). 10µM flutamide decreased *MMP2* (P=0.004) and *VEGFA* (P=0.016) compared to control and 1nM DHT treated BeWo cells (Figure 3.6B). Treatment with either 1nM DHT or 10µM flutamide decreased *HIF1α* compared to control (P=0.005, Figure 3.6B). 1nM DHT decreased *PPARα* in BeWo cells compared to control; 10µM flutamide decreased *PPARα* further compared to control and 1nM DHT treatment (P=0.001, Figure 3.6B). In comparison, *PPARy* decreased with just 10µM flutamide treatment (P=0.013, Figure 3.6B).

AR and KDMs in Telomerase Immortalized Swan71 Cells

In the human first trimester telomerase-immortalized trophoblast cell line Swan71, *AR* and *KDM*s transcripts were present as determined by end-point PCR analysis (Figure 3.7A). *KDM1A*, *KDM3A*, *KDM4A*, *KDM4C*, and *KDM4D* were all amplified via PCR. Western blot immunodetection showed the presence of AR, KDM1A, and KDM4D protein in Swan71 cells (Figure 3.7B).

Discussion

Previously, we reported that prenatal androgenization altered mRNA and protein levels of vascular endothelial growth factor A (VEGFA), a recognized androgen responsive gene that regulates placental vascularization and trophoblast differentiation (Chapter II). Here, we report that dihydrotestosterone (DHT) treatment in choriocarcinoma cells led to decreased *KDM3A*, *KDM4D*, *MMP2*, and *PPARy* in normoxic conditions and increased *HIF1α* with flutamide treatment in hypoxic conditions. Many of the other androgen responsive genes investigated did not show a response to DHT treatment at the mRNA level, including *VEGFA*, the imprinted non-coding *H19*, and *insulin-like growth factors* (*IGF*s). The decreased or apparent lack of response in androgen responsive genes' relative mRNA levels contradicts expected results of increased transcription in response to DHT treatment. However, due to time constraints, protein expression in response to DHT treatment was not investigated, and it is possible effects are observed post-translation.

This is of particular relevance as androgens' effect on gene expression may be observed only at the protein level, meaning that an androgen-induced response in the expression or activity of trophoblast differentiation regulators syncytin, syncytin receptor (ASCT2), or kisspeptin (KiSS1) cannot yet be ruled out (Charnock-Jones et al., 1994; Lee et al., 2001; Kudo and Boyd 2002; Bilban et al., 2004; Pötgens et al., 2004; Hiden et al., 2007a; Wieser et al., 2008). Similarly, while hypoxia did not increase *HIF1α* or *VEGFA* relative mRNA levels, hypoxic conditions are reported to increase functional protein activity, but not necessarily mRNA levels, of HIF1α and HIF1 target genes VEGFA and matrix metalloproteinases (MMPs) (Salceda and Cara 1997; Huang et al., 1998; Yu et al., 1998; Chandel et al., 2000; Haddad et al., 2000; Rajakumar and

Conrad 2000; Pringle et al., 2010). Therefore, to clarify androgens' effect on placental gene expression and function, detection of altered protein expression in response to DHT treatment needs to be completed.

Increased interest is being placed on the role of androgens in placentation, specifically its role in the etiology of preeclampsia. Preeclampsia develops with insufficient trophoblast differentiation, invasion, and uterine vascular remodeling, leading to a potentially life-threatening disorder of maternal hypertension and fetal nutrient and growth restriction (Redman 1990; 1991; Ilekis et al., 2007). Preeclamptic placentas are reported to have increased syncytiotrophoblast and stromal cell expression of androgen receptor (AR) compared to placentas from normotensive pregnancies, suggesting that placental AR is dynamically regulated by placental health (Ghidini and Salafia 2005; Hsu et al., 2009; Sathishkumar et al., 2011). Additionally, increased maternal serum androgens have been characterized in preeclamptic patients (Acromite et al., 1999; Serin et al., 2001; Atamer et al., 2004; Carlsen et al., 2005; Salamalekis et al., 2006; Ghorashi and Sheikhvatan 2008; Hsu et al., 2009; Lorzadeh and Kazemirad 2012). Although the gestational timing of increased placental testosterone is debated, it is suggest that increased maternal serum androgens could serve as an early diagnostic for detecting preeclampsia (Dawood and Saxena 1977; Carlsen et al., 2005; Tuutti et al., 2011).

In light of these reports, placental androgens may crucial regulate trophoblast differentiation or placental function. This speculation is further supported by the fact that hypoxia regulates trophoblast differentiation and also increases androgen signaling by inducing HIF1α, AR, and β-catenin complex recruitment to androgen response elements (AREs) for transcription initiation

(Adelman et al., 2000; Semenza 2000). In hypoxic conditions, androgens also increase VEGF by increasing translation and stability of HIF1α, stimulating angiogenesis (Mabjeesh et al., 2003). Placental hypoxia has also been shown to reduce aromatase activity (Thompson and Siiteri 1974a,b; Goto and Fishman 1977; Zachariah and Juchau 1977). This agrees with our finding of reduced *aromatase* (*CYP19*) in ACH-3P cells in hypoxic verses normoxic conditions. As androgen deprivation reduces hypoxia in prostate cancer (Shabisgh et al., 1999), the increased placental androgen production in preeclampsia may be a compensatory mechanism for insufficient trophoblast differentiation and sustained placental hypoxia, leading to the heightened circulating androgens that cause maternal vascular endothelial cell damage, vasoconstriction, and hypertension (Song and Martin 2006; Reckelhoff 2001). However, the absence of androgens is suggested to have similar effects on cardiovascular health, signifying a dynamic role of androgens in regulating systemic vascularization and vasodilation (Park et al., 2006; Nheu et al., 2011).

While hypoxia is shown to regulate androgen signaling, it also induces HIF1 complexes with histone lysine demethylases (KDMs) (Beyer et al., 2008; Wellmann et al., 2008). KDMs regulate gene expression by demethylating mono-, di-, or tri-methylated lysines residues on histone N-terminal tails (Shi 2007; Verrier et al., 2011). Hypoxia increases KDM expression and increases site specific histone lysine demethylation in promoter regions of hypoxia-inducible genes (Beyer et al., 2008; Wellmann et al., 2008; Yang et al., 2010). Additionally, HIF1α forms transcription initiation complexes with KDMs and sex hormone receptors (Yang et al., 2010). Multiple KDMs, including KDM1A and KDM4D, have been shown to be co-activators of AR and function to increase expression of androgen responsive genes (Shin and Janknecht 2007, Metzger

et al., 2005; Yamane et al 2006; Wissmann et al., 2007; Coffey et al., 2013). By this mechanism, KDMs and AR can function dynamically in hypoxic conditions to regulate cellular proliferation or differentiation (Cloos et al., 2006).

In additional to regulation by hypoxia and control of androgen signaling, KDM3A has been shown to down regulate peroxisome proliferator-activated receptor α (PPARα) (Metzger et al., 2005; Shin and Janknecht 2007; Beyer et al., 2008; Yang et al., 2010; Kawazu et al., 2011; Coffey et al., 2013). PPARs, specifically PPARγ, regulates trophoblast differentiation, metabolism, and angiogenesis, and increases placental production of human chorionic gonadotropin and placental lactogen (Barak et al., 1999; Kubota et al., 1999; Tarrade 2001a-c; Barak et al., 2002; Schild et al., 2002; Pavan et al., 2003; Fournier et al., 2007a-b; Wieser et al., 2008). Increased placental PPARγ and PPARα has been characterized in pregnancy disorders, including preeclampsia with intrauterine growth restriction, gestational diabetes mellitus, and placentas from miscarriages (Wieser et al., 2008; Toth et al., 2009; Holdsworth-Carson et al., 2010). In choriocarcinoma cells, inhibition of AR and treatment with DHT altered *PPARγ* in normoxic choriocarcinoma cells. Together, these findings suggest that androgens may regulate PPARs through KDM activity, suggesting another mechanism for androgen regulation of trophoblast function and differentiation.

Regulation of gene expression and trophoblast differentiation through AR-KDM-HIF1α complexes is of particular interest in the placenta, where hypoxia-regulated trophoblast differentiation and invasion in the first trimester is required to establish proper placentation (Zhou et al., 1993; Adelman et al., 2000; Semenza 2000; Soares et al., 2012). However,

trophoblast response to hypoxia in some *in vitro* experiments is atypical. Hypoxia was shown to inhibit choriocarcinoma cells from differentiating to syncytium and to maintain cytotrophoblast proliferation over differentiation into an invasive phenotype (Alsat et al., 1996; Genbacev et al., 1996; Nelson et al., 1999; Kudo et al., 2003; Hu et al., 2007). In hypoxic BeWo cells, *syncytin* was reduced as opposed to the increase seen *in vivo* (Kudo and Boyd 2002; Kudo et al., 2003). Additionally, the BeWo cell line is heterogeneous, being composed of cells with epithelial and fibroblast phenotypes, as well as a minor percentage of multinuclear giant cells; this heterogeneous compilation results in altered gene expression and function in a portion of BeWo cells, likely contributing the lack of response observed with DHT treatment (Aplin 1991; Martell and Ruddon 1990; Alpin et al., 1992).

ACH-3P cells are also composed of a heterogeneous cell population, though the origins are suggested to arise from villous and extravillous trophoblasts as the cell line was created from fusion of a choriocarcinoma cell line (ACI-I) with trophoblasts isolated from placental villi at 12 weeks of gestation (Hiden et al., 2007b). Although ACH-3P cells may provide a model for studying paracrine signaling in first trimester placental villi (Hiden et al., 2007b), the cell-specific localization of AR and KDMs in first trimester trophoblasts (Figure 2.8) may result in an uneven or inconsistent cellular response to DHT treatment *in vitro*. As we report the expression of KDMs in the immortalized human first trimester trophoblast cell line Swan71, this cell line may provide a more suitable model than choriocarcinoma cells for further characterizing hypoxia's effect on AR-KDM signaling in the placenta. Swan71 cells originated from 7-week of gestation primary trophoblasts, immortalize with human telomerase reverse transcriptase (Straszewski-Chavez et al., 2009). Swan71 cells appear to have a more homogenous phenotype

with limited human chorionic gonadotrophin production, suggesting their maintained undifferentiated state (Straszewski-Chavez et al., 2009). Therefore, as Swan71 cells expressing AR and KDMs, they may offer a more applicable model for AR-KDM placental signaling for trophoblast function and differentiation, albeit an imperfect replacement for primary trophoblasts from first trimester placental explants.

In conclusion, these experiments report limited changes in choriocarcinoma mRNA levels in response to DHT treatment. Further research is necessary to investigate altered trophoblast protein levels and function in response to androgen signaling. As Swan71 cells express AR and KDMs, and are derived from first trimester trophoblasts (Straszewski-Chavez et al., 2009), they are a potential model for future studies characterizing placental androgen signaling mechanisms. By further elucidating the effects of placental androgen signaling, a better understanding of the etiology and diagnosis of placental disorders, such as preeclampsia, may be achieved.

TABLE 3.1. List of primer sequences used for real time PCR.

Gene	Primer Sequence	Amplicon Size	Citation
AR	**F-5' TCCTGGATGGGGCTTATGGT** **R-5' GCCTCATTCGGACACACTGG**	**150**	
KDM1A	**F-5' ATCTGCAGTCCAAAGGATGG** **R-5' GCCAACAATCACATCGTCAC**	**232**	
KDM3A	**F-5' CAGCCAGCACATCTCCTCTAAAC** **R-5' TGGATTTGCTTAAAGGTGGGAGG**	**144**	**Cho et al., 2011**
KDM4A	**F-5' GGAAGCCACGAGCATCCTATG** **R-5' GGAACTCTCGAACAGTCATGG**	**134**	**Kawazu et al., 2011**
KDM4C	**F-5' CTGTCACCTAGTGCGGAACAA** **R-5' ATGATGGTTAGGGCAGTGTCT**	**133**	**Kawazu et al., 2011**
KDM4D	**F-5' TTTCCCTATGGCTACCATGC** **R-5' TCATAGCGTTCAGGTTGCAG**	**179**	
MMP2	**F-5' AGATCTTCTTCTTCAAGGACCGGTT** **R-5' GGCTGGTCAGTGGCTTGGGGTA**	**225**	**Munaut et al., 2003**
MMP12	**F-5' ACACCTGACATGAACCGTGA** **R-5' AGCAGAGAGGCGAAATGTGT**	**392**	
IGF1	**F-5' CAGACAAGCCCACAGGGT** **R-5' GGTGGGCTTACCTTCTG**	**520**	**Bonapace et al., 2012**
IGF2	**F-5' GACCGCGGCTTCTACTTCAG** **R-5' AAGAACTTGCCCACGGGGTAT**	**202**	
VEGFA	**F-5' TCACCAAAGCCAGCACATAG** **R-5' GCGAGTCTGTGTTTTTGCAG**	**179**	
HIF1α	**F-5' CCCAATGGATGATGACTTCC** **R-5' TGGGTAGGAGATGGAGATGC**	**204**	
PPARy	**F-5' GCTGTGCAGGAGATCACAGA** **R-5' GGGCTCCATAAAGTCACCAA**	**225**	
CYP19	**F-5' CAAACCCACCTGCTAGTGTG** **R-5' TTGTAGCACAGGCAAGTGGC**	**237**	**Watzka et al., 1996**
SYNCYTIN1	**F-5' GAAGGCCCTTCATAACCAATGA** **R-5' GATATTTGGCTAAGGAGGTGATGTC**	**82**	**Chen et al., 2006**
ASCT2	**F-5' CCGCTTCTTCAACTCCTTCAA** **R-5' ACCCACATCCTCCATCTCCA**	**122**	**Chen et al., 2006**
KiSS1	**F-5' GCCATTAGAAAAGGTGGCCTC** **R-5' TTGTAGTTCGGCAGGTCCTTC**	**278**	**Bilban et al., 2004**
H19	**F-5' ACCCCCTGCGGTGGACGGTT** **R-5' TGGAATGCTTGAAGGCTGCT**	**427**	**Takai et al., 2001**
18s	**F-5' GAGGCCCTGTAATTGGAATGAG** **R-5' GCAGCAACTTTAATATACGCTATTGG**	**119**	

TABLE 3.2. List of antibodies and their dilutions used for Western blot protein detection.

Protein	Antibody and Dilution Utilized
AR	Santa Cruz sc816 **1:500**
KDM1A	Abcam ab17721 **1:500**
KDM4D	Abcam ab93694 **1:300**

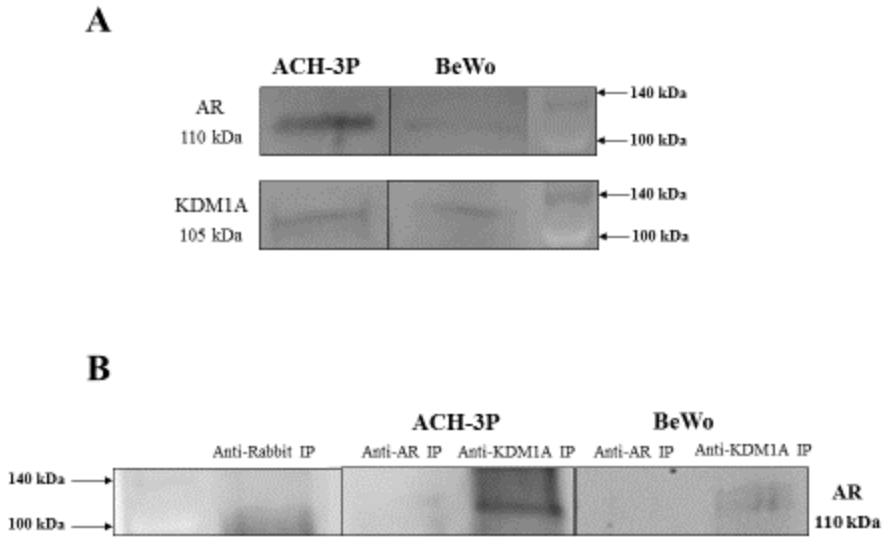


FIGURE 3.1. Detection and interaction of AR and KDM1A in choriocarcinoma cells. **A)** Western blot detection of AR and KDM1A in ACH-3P and BeWo cells. **B)** Coimmunoprecipitation of AR with KDM1A in ACH-3P and BeWo cells. Immunoprecipitation with rabbit-IgG antibody was used as negative control.

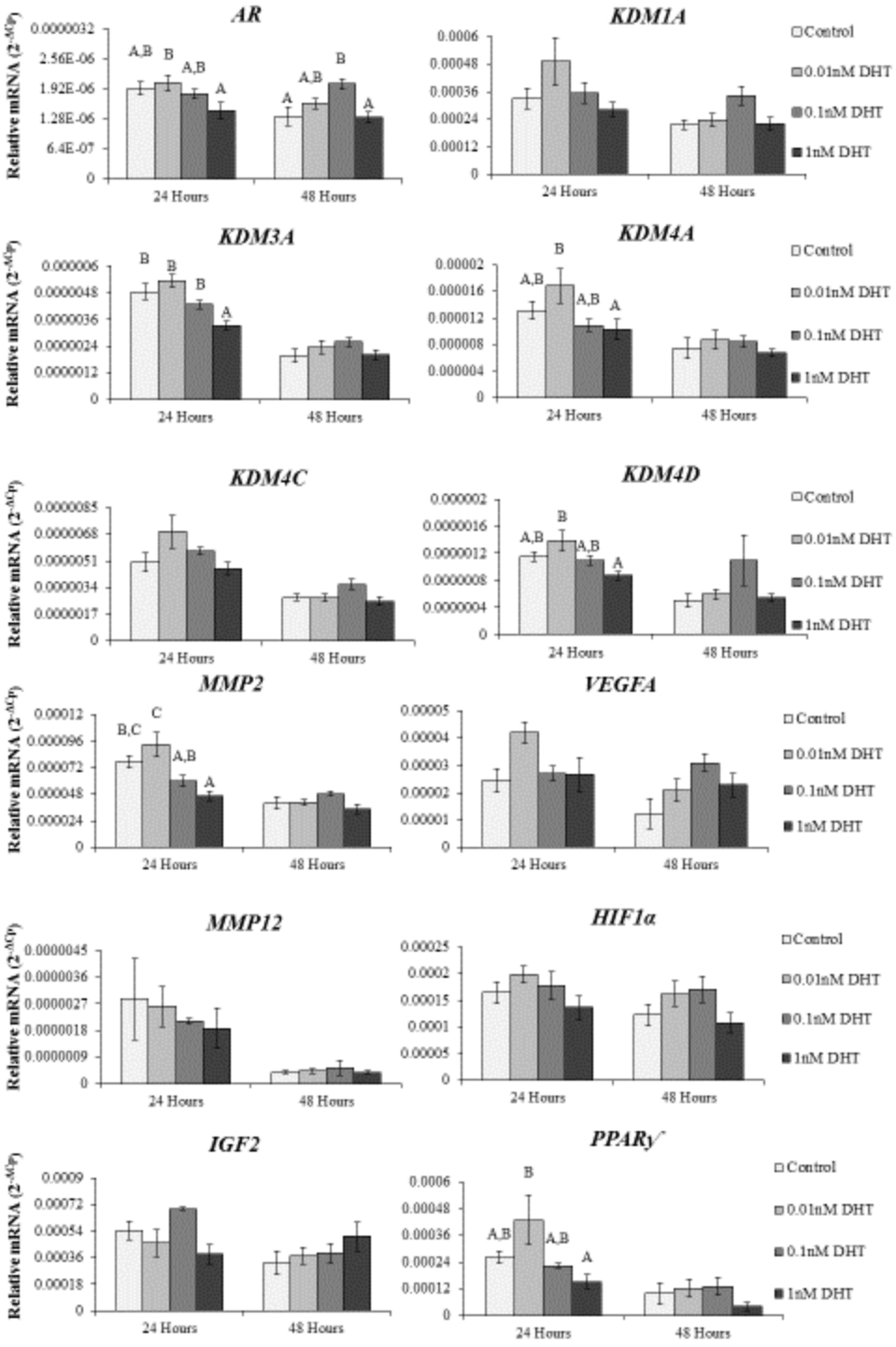


FIGURE 3.2. Changes in relative mRNA levels of androgen responsive genes with DHT treatment in ACH-3P cells.

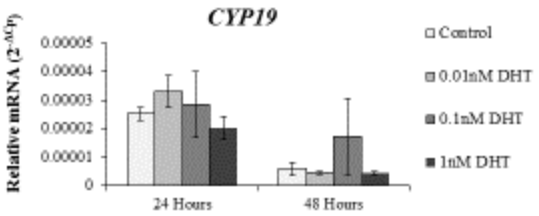


FIGURE 3.2 Continued. Changes in relative mRNA levels of androgen responsive genes with DHT treatment in ACH-3P cells.

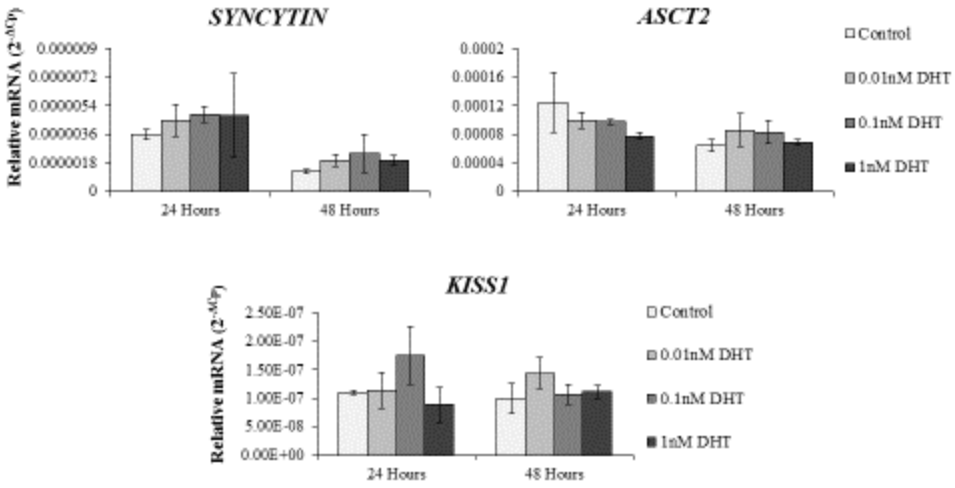


FIGURE 3.3. Relative changes in mRNA in ACH-3P cells for genes regulating trophoblast differentiation with DHT treatment.

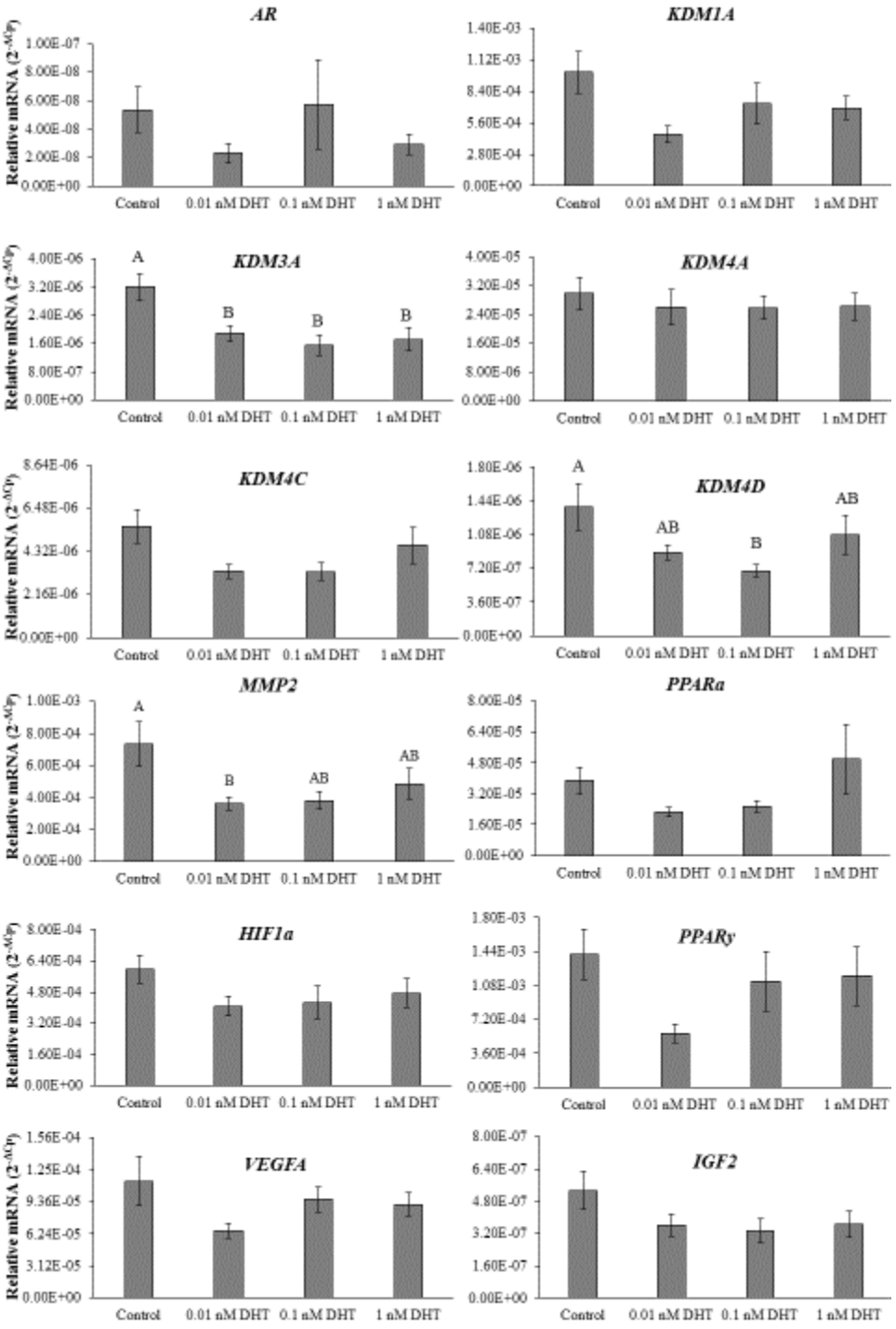


FIGURE 3.4. Changes in relative mRNA levels of androgen responsive genes with DHT treatment in BeWo cells.

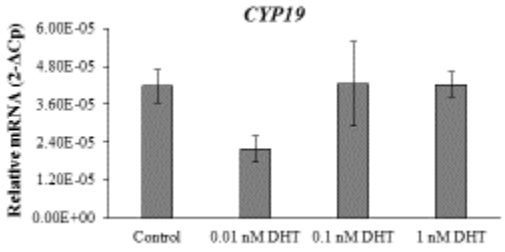


FIGURE 3.4 Continued. Changes in relative mRNA levels of androgen responsive genes with DHT treatment in BeWo cells.

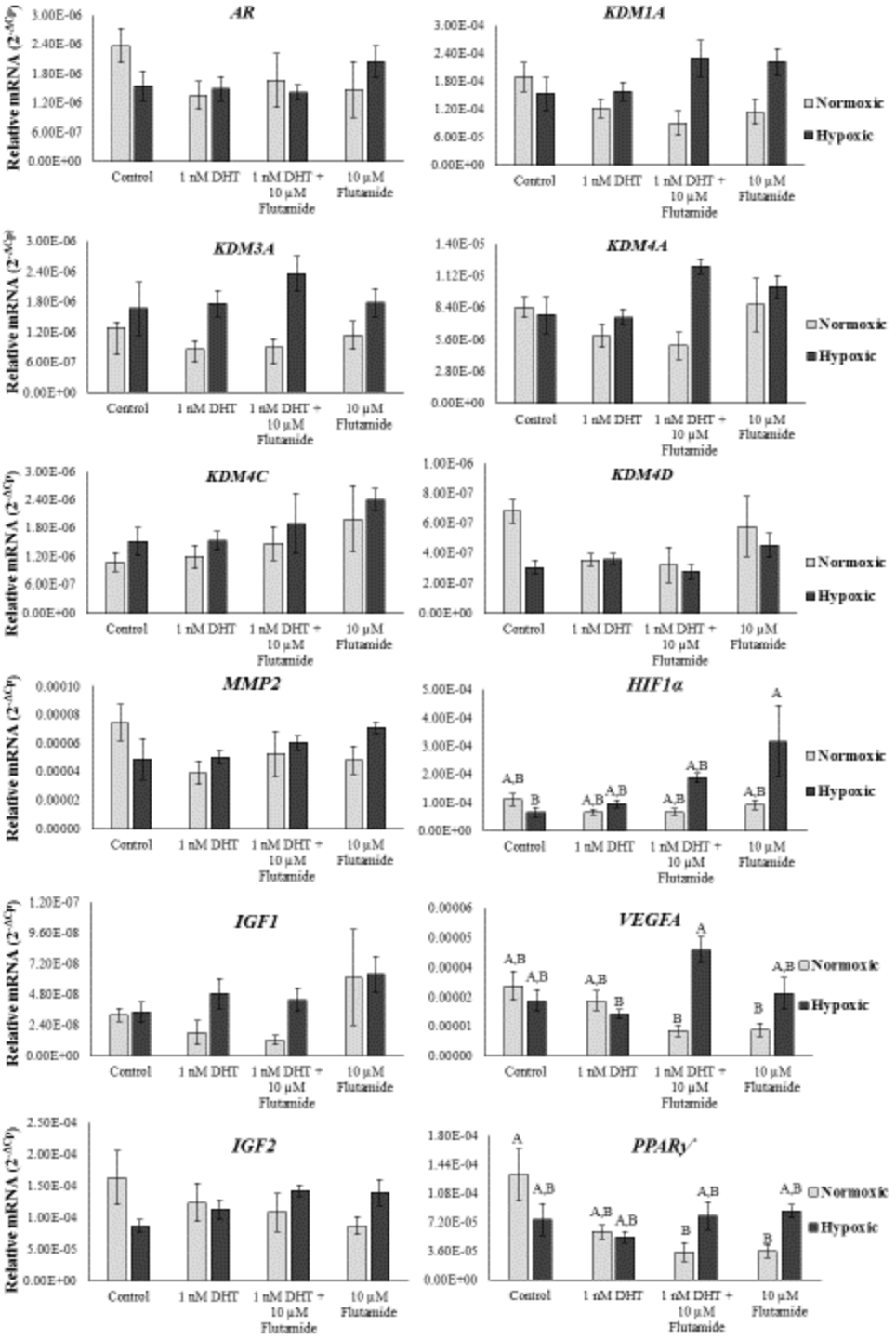


FIGURE 3.5. Changes in relative mRNA levels with DHT treatment, hypoxia, and AR inhibition 48 hours after treatment in ACH-3P cells.

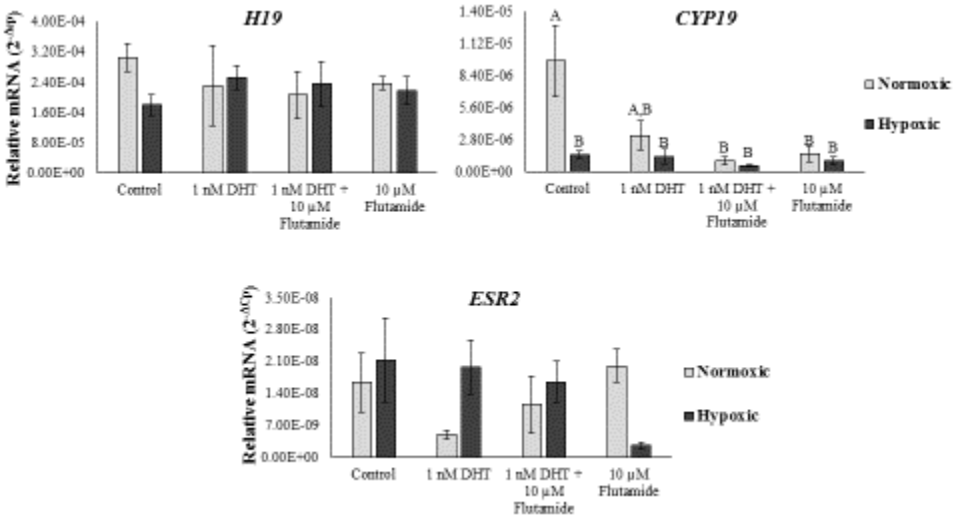


FIGURE 3.5 Conintued. Changes in relative mRNA levels with DHT treatment, hypoxia, and AR inhibition 48 hours after treatment in ACH-3P cells.

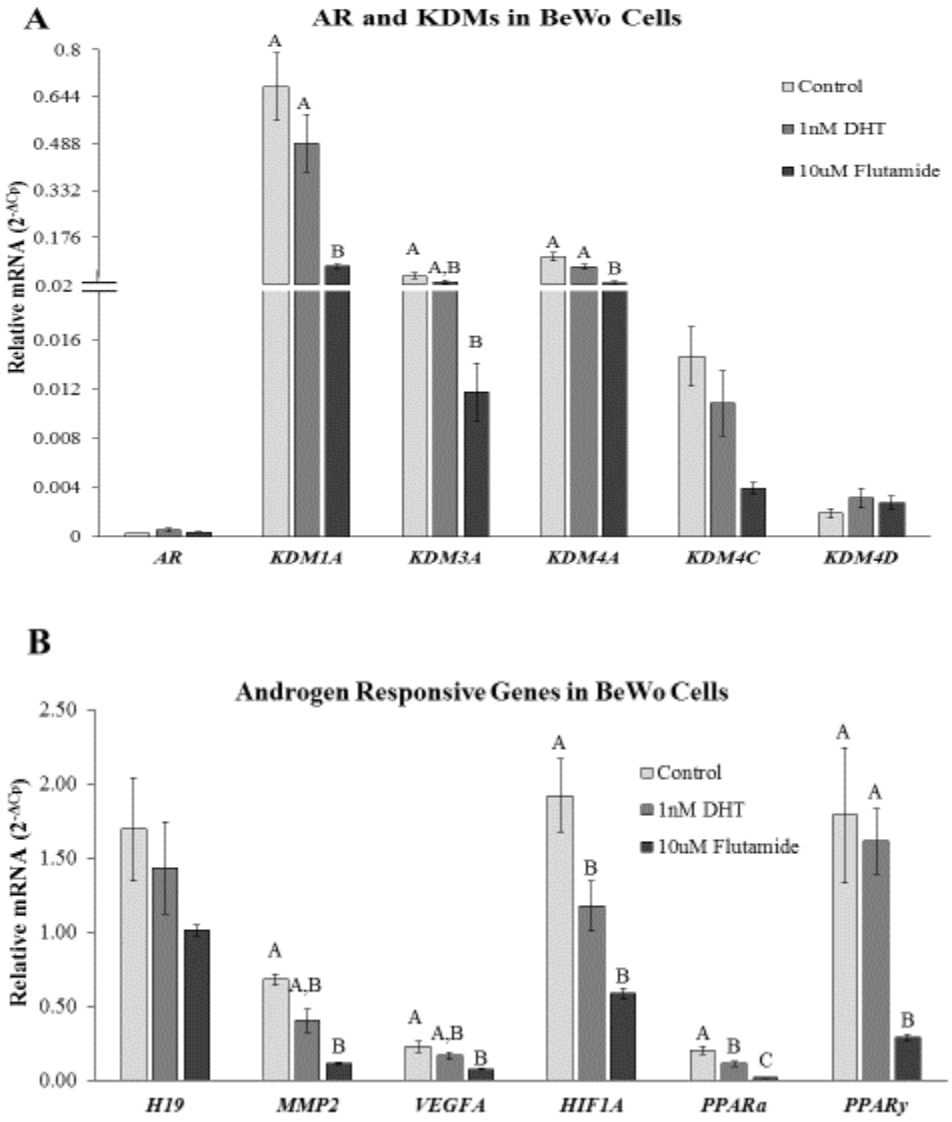


FIGURE 3.6. Changes in relative mRNA associated with DHT treatment and AR inhibition in BeWo cells. **A)** *AR* and *KDM* response to DHT and flutamide treatment. **B)** Response to DHT and flutamide treatment for androgen responsive genes.

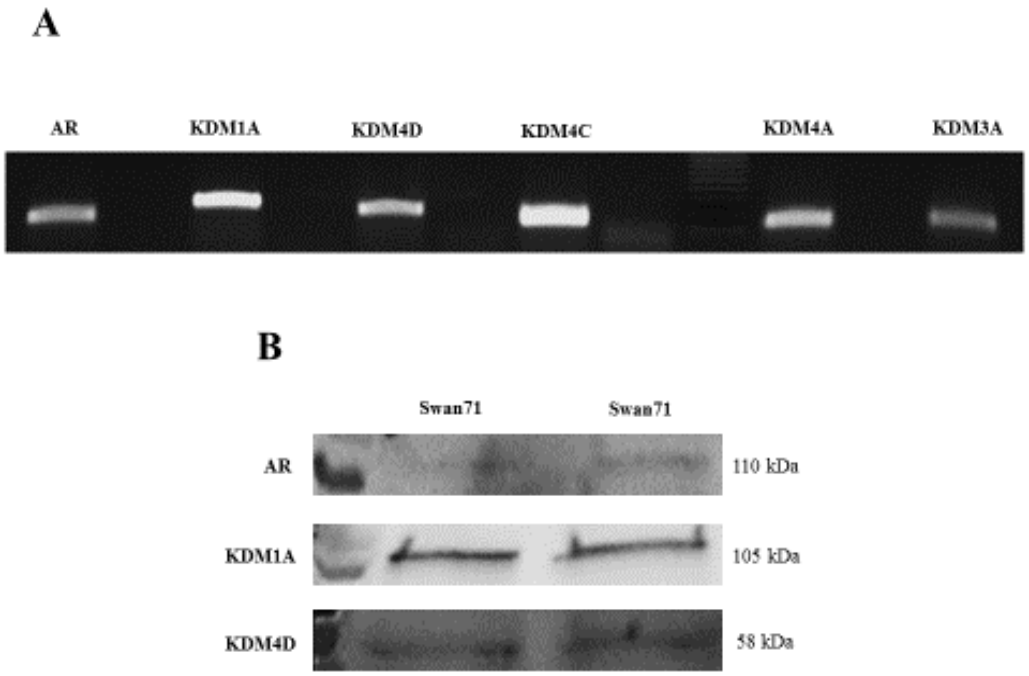
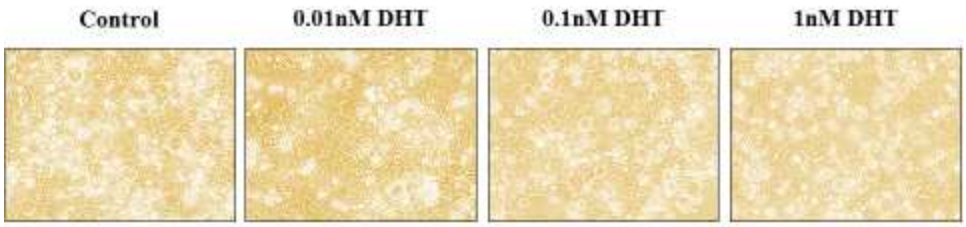


FIGURE 3.7. Expression of AR and KDMs in Swan71 Cells. **A)** Endpoint PCR for *AR* and *KDM*s **B)** Western blot detection of AR, KDM1A, and KDM4D.



SUPPLEMENTAL FIGURE 3.1. ACH-3P cells at 48 hours post-DHT treatment. Images were taken to confirm that noticable cell death did not occur with DHT treatment.

REFERENCES

Acromite MT, Mantzoros CS, Leach RE, Hurwitz J, Dorey LG 1999 Androgens in preeclampsia. Am J Obstet Gynecol 180(1):60-63

Adelman DM, Gertsenstein M, Nagy A, Simon MC, Maltepe 2000 Placental cell fates are regulated in vivo b HIF-mediated hypoxia responses. Genes Dev 14:3191-3203

Albrecht ED, Robb VA, Pepe GJ 2004 Regulation of placental vascular endothelial growth/permeability factor expression and angiogenesis by estrogen during early baboon pregnancy. J Clin Endocrinol Metab 89(11):5803-5809

Albrecht ED, Bonagura TW, Burleight DW, Enders AC, Aberdeen GW, Pepe GJ 2006 Suppression of extravillous trophoblast invasion of uterine spiral arteries by estrogen during early baboon pregnancy. Placenta 27:483-490

Alsat E, Wyplosz P, Malassin A, Guibourdenche J, Porquet D, Nessmann C, Evain-Brion D 1996 Hypoxia impairs cell fusion and differentiation process in human cytotrophoblast, in vivo. J Cell Physiol 168: 346-353

Anderson CM 2007 Preeclampsia: exposing future cardiovascular risk in mother and their children. J Obstet Gynecol Neonat Nursing 36(1):3-8

Aplin JD 1991 Implantation, trophoblast differentiation and haemochorial placentation: mechanistic evidence in vivo and in vitro. J Cell Sci 99:681-692

Aplin JD, Sattar A, Mould AP 1992 Variant choriocarcinoma (BeWo) cells that differ in adhesion and migration of fibronectin display conserved patterns of integrin expression. J Cell Science 103:435-444

Armstrong RA, Reynolds RM, Leask R, Shearing CH, Calder AA, Riley SC 2009 Decreased serum levels of kisspeptin in early pregnancy are associated with intra-uterine growth restriction and pre-eclampsia. Prenatal Diagnosis 29(10):982-985

Atamer Y, Erden AC, Demir B, Koçygit Y, Atamer A 2004 The relationship between plasma levels of leptin an androgen in healthy and preeclamptic pregnant women. Acta Obstet Gynecol Scand 83:425-430

Aw TY, Jones DP, O'Shannessy DJ, Priest JH, Priest RE 1985 Oxygen dependence of oestrogen production by human placental microsomes and cultured choriocarcinoma cells. J Steroid Biochem 22(6):753-758

Barak Y, Nelson MC, Ong ES, Jones YZ, Ruiz-Lozano P, Chien KR, Koder A, Evans RM 1999 PPAR gamma is required for placental, cardiac, and adipose issue development. Mol Cell 4:585-595

Barak Y, Liao D, He W, Ong ES, Nelson MC, Olefsky JM, Boland R, Evans RM 2002. Effects of peroxisome proliferator-activated receptor delta on placentation, adiposity, and colorectal cancer. Proc Natl Acad Sci USA 99(1):303-308

Beyer S, Kristensen MM, Jensen KS, Johansen JV, Staller P 2008 The histone demethylases JMJD1A and JMJD2B are transcriptional targets of hypoxia-inducible factor HIF. J Biological Chem 283:36542-36552

Bilban M, Ghaffari-Tabrizi N, Hintermann E, Bauer S, Molzer S, Zoratti C, Malli R, Sharabi A, Hiden U, Braier W, Knöfler M, Andreae F, Wagner O, Quaranta V, Desoye G 2004 Kisspeptin-10, a KiSS-1/metastin-derived decapeptide is a physiological invasion inhibitor of primary human trophoblasts. J Cell Science 117:119-1328

Blankenship TN, Enders AC, King BF 1993 Trophoblastic invasion and the development of uteroplacental arteries in the macaque: immunohistochemical localization of cytokeratins, desmin, type IV collagen, laminin, and fibronectin. Cell Tissue Res 272:227-236

Boddy JL, Fox SB, Han C, Campo L, Turley H, Kanga S, Malone PR, Harris AL 2005 The androgen receptor is significantly associated with vascular endothelial growth factor and hypoxia sensing via hypoxia-inducible factors HIF-1a, HIF-2a, and the prolyl hydroxylases in human prostate cancer. Hum Cancer Biol 11(21)7658-7663

Bonapace G, Concolino D, Formicola S, Strisciuglio P 2012 A novel mutation in a patient with insulin-like growth factor 1 (IGF1) deficiency. J Med Genet 40:913-917

Brogi E, Schatteman G, Wu T, Kim EA, Varticovski L, Keyt B, Isner JM 1996 Hypoxia-induced paracrine regulation of vascular endothelial growth factor receptor expression. J Clin Invest 97(2):469-476

Brosens IA, Robertson WB, Dixon HG 1967 The Physiological response of the vessels of the placental bed to normal pregnancy. J Pathol Bacteriol 93:569-579

Brosens IA 1988 The uteroplacental vessels at term: the distribution and extent of physiological changes. Trophoblast Res 3:61-68

Carlsen SM, Romundstad P, Jacobsen G 2005 Early second-trimester maternal hyperandrogenemia and subsequent preeclampsia: a prospective study. Acta Obstet Gynecol Scand 84:117-121

Chandel NS, McClintock DS, Feliciano CE, Wood TM, Melendez JA, Rodriguez AM, Schumacher PT 2000 Relative oxygen species generated at mitochondrial complex III stabilize HIF-1α during hypoxia: a mechanism of O_2 sensing. J Biol Chem 275:25130-25138

Charnock-Jones DS, Sharkey AM, Boocock CA, Ahmed A, Plevin R, Ferrara N, Smith SK 1994 Vascular endothelial growth factor receptor localization and activation in human trophoblast and choriocarcinoma cells. Biol Reprod 51:524-530

Chen C-P, Wang K-G, Chen C-Y, Yu C, Chuang H-C, Chen H 2006 Altered placental syncytin and its receptor ASCT2 expression in placental development and preeclampsia. BJOG 113:152-158

Cheng L, Zhang S, Sweeney CJ, Kao C, Gardener TA, Eble JN 2004 Androgen withdrawal inhibits tumor growth and is associated with decrease in angiogenesis and VEGF expression in androgen-independent CWR22Rv1 human prostate cancer model. Anticancer Res 24:2135-2140

Cho H-S, Toyokawa G, Daigo Y, Hayami S, Masuda K, Ikawa N, Yamane Y, Maejima K, Tsunoda T, Field HI, Kelly JD, Neal DE, Ponder BAJ, Maehara Y, Nakamura Y, Hamamoto R 2011 The JmjC domain-containing histone demethylase KDM3A is a positive regulator of the G_1/S transition in cancer cells via transcriptional regulation of the HOXA1 gene. Int J Cancer 131:E179-E189

Cloos PA, Christensen J, Agger K, Maiolica A, Rappsilber J, Antal T, Hansen KH, Helin K 2006 The putative oncogene GASC1 demethylates tri- and dimethylated lysine 9 on histone H3. Nature 442:307-311

Coffey K, Rogerson L, Ryan-Munden C, Alkharaif D, Stockley J, Heer R, Sahadevan K, O'Neill D, Jones D, Darby S, Staller P, Mantilla A, Gaughan L, Robson CN 2013 The lysin demethylase, KDM4B, is a key molecule in androgen receptor signaling and turnover. Nucleic Acids Res 1-14

Constância M, Hemberger M, Hughes J, Dean W, Ferguson-Smith A, Fundele R, Stewart F, Kelsey G, Fowden A, Sibley C, Reik W 2002 Placental-specific IGF-II is a major modulator of placental and fetal growth. Nature 417:945-948

Dawood MY, Saxen BB 1977 Testosterone and dihydrotestosterone in maternal and cord blood and in amniotic fluid. Am J Obstet Gynecol 129(1):37-42

Dhillo WS, Savage P, Murphy KG, Chaudhri OB, Patterson M, Nijher GM, Foggo VM, Dancey GS, Mitchell H, Seckl MJ, Ghatei MA, Bloom SR 2006 Plasma kisspeptin is raised in patients with gestational trophoblastic neoplasia and falls during treatment. Am J Physiology Endocr Metab 291:E878-E884

Ehleben W, Bolling B, Merten E, Parwol T, Strohmaier AR, Acker H 1998 Cytochromes and oxygen radicals as putative members of oxygen sensing pathway. Pespir Physiol 114:25-36

Enders AC 1968 Fine structure of anchoring villi of human placenta. Am J Anat 122(3):419-451

Eriksson JG, Forsén T, Tuomilehto J, Winter PD, Osmond C, Barker DJP 1999 Catch-up growth in childhood and death from coronary heart disease: longitudinal study. BMH 318(7181):427-431

Fournier T, Handschuh K, Tsatsaris V, Evian-Brian D 2007a Involvement of PPARy in human trophoblast invasion. Placenta 28:S76-S81

Fournier T, Tsatsaris V, Handschuh K, Evain-Brion D 2007b PPARs and the placenta. Placenta 28:65-76

Gauster M, Moser G, Orendi K, Huppertz B 2009 Factors involved in regulating trophoblast fusion: potential role in the development of preeclampsia. Placenta 30:S49-S54

Genbacev O, Joslin R,Damsky CH, Polliotti BM, Fisher SJ 1996 Hypoxia alters early gestation human cytotrophoblast differentiation/invasion in vitro and models the placental defects that occur in preeclampsia. J Clin Invest 97(2):540-550

Gerulewicz-Vannini D, Camero Y, Salas J, Hernández-Andrade E 2006 High plasmatic androgen levels in women affected with pregnancy-induced hypertension. Rev Invest Clin Abstract 58(3):228-233

Ghorashi V, Sheikhvata M 2008 The relationship between serum concentration of free testosterone and pre-eclampsia. Polish J Endocrinol 59(5):390-392

Goto J, Fishman J 1977 Participation of a non-enzymatic transformation in the biosynthesis of estrogens from androgens. Science 195:80-81

Haddad JJ, Olver RE, Land SC 2000 Antioxidant/prooxidant equilibrium regulates HIF1α and NF-κB redox sensitivity: evidence for inhibition by glutathione oxidation in alveolar epithelial cells. J Biol Chem 275:21130-21139

Hiden U, Bilban M, Knöfler M, Desoye G 2007a Kisspeptins and the placenta: regulation of trophoblast invasion. Rev Endocr Metab Disord 8:31-39

Hiden U, Wadsck C, Prutsch N, Gauster M, Weiss U, Frank H-G, Schmitz U, Fast-Hirsh C, Hengstschläger M, Pötgens A, Rüben A, Knöfler M, Haslinger P, Huppertz B, Bilban M, Kaugmann P, Desoye G 2007b The first trimester human trophoblast cell line ACH-3P: a novel tool to study autocrine/paracrine regulatory loops of human trophoblast subpopulations- TNF-α stimulates MMP15 expression. BMC Dev Biol 7:137-150

Holdsworth-Carson SJ, Lim R, Mitton A, Whitehead C, Rice GE, Permezel M, Lappas M 2010 Peroxisome proliferater-activated receptors are altered in pathologies of the human placenta: gestational diabetes mellitus, intrauterine growth restriction and preeclampsia. Placenta 31:222-229

Horie K, Takakura K, Imai K, Liao S, Mori T 1992 Immunohistochemical localization of androgen receptor in the human endometrium, decidua, placenta, and pathological conditions of the endometrium. Hum Reprod 7:1461-1466

Hsu T-Y, Lan K-C, Tsai C-C, Ou C-Y, Cheng B-H, Tsai M-Y, Kang Y-Y, Tung Y-H, Wong Y-H, Huang K-E 2009 Expression of androgen receptor in human placentas from normal and preeclamptic pregnancies. Taiwanese J Obstet Gynecol 48(3):262-267

Hu R, Jin H, Zhou S, Yang P, Li X 2007 Proteomic analysis of hypoxia-induced responses in the syncytialization of human placental cell line BeWo. Placenta 28:399-407

Huang EC, Hively WP, DePinho RA, Varmus HE 1998 Regulation of hypoxia-inducible factor 1α is mediated by an O_2-dependent degredation domain via ubiquitin-protesome pathway. Pro Natl Acad Sci 95:7987-7992

Huppertz B, Kertshanska S, Demir AY, Frank H-G, Kaufmann P 1998 Immunohistochemistry of matrix metalloproteinases (MMP), their substrates, and their inhibitors (TIMP) during trophoblast invasion in the human placenta. Cell Tissue Res 291:133-148

Ilekis J, Reddy UM, Roberts JM 2007 Preeclampsia—a pressing problem: an executive summary of a national institute of child health and human development workshop. Reproductive Sciences 14:508-525

Iwamura M, Abrahamsson PA, Benning CM, Cockett AT, di Sant'Agnese PA 1994 Androgen receptor immunostaining and its tissue distribution in formalin-fixed, paraffin-embedded sections after microwave treatment. J Histochem Cytochem 42:783-788

Jaquet D, Gaboriau A, Czernichow P, Levy-Marchal C 2000 Insulin resistence early in adulthood in subjects born with intrauterine growth retartdation. J Clin Endocrinol Metab 84(4):1401-1406

Kaufmann P, Black S, Huppertz B 2003 Endovascular Trophoblast Invasion: Implications for the pathogenesis of intrauterine growth retardation and preeclampsia. Biol Reprod 69:1-7

Kawazu M, Saso K, Tong KI, McQuire T, Goto K, Son D-O, Wakeham A, Miyagishi M, Mak TW, Okada H 2011 Histone demethylase JMJD3B functions as a co-factor of estrogen receptor in breast cancer proliferation and mammary gland development. PLoS ONE 6(3):e17830

Khatri P, Hoffmann B, Schuler G 2013 Androgen receptor is widely expressed in bovine placentomes and up-regulated during differentiation of bovine trophoblast giant cells. Placenta 34:416-423

Kingdom JCR, Kaufmann P 1997 Oxygen and placental villous development origins of fetal hypoxia. Placenta 18:613-621

Knobil E, Neill JD Editors. 1998. Placenta: Implantation and Developmental. Enc Reprod Acad Press: San Diego. Vol 3:848-855

Kubota N, Terauchi Y, Miki H, Tamemoto H, Yamauchi T, Komeda K, Satoh S, Nakano R, Ishii C, Sugiyama T, Eto K, Tsubamoto Y, Okuno A, Murakami K, Sekihara H, Hasegawa G, Naito M, Toyoshima Y, Tanaka S, Shiota K, Kitamura T, Fujita T, Ezaki O, Aizawa S, Nagai R, Tobe

K, Kimura S, Kadowaki T 1999 PPARy mediates high-fat diest-induced adipocyte hypertrophy and insulin resistance. Mol Cell 4(4):597-609

Kudo Y, Boyd CAR 2002 Changes in expression and function of syncytin and its receptor, amino acid transport system B^0 (ASCT2), in human placental choriocarcinoma BeWo cells during syncytialization. Placenta 23:536-541

Kudo Y, Boyd CAR, Sargent IL, Redman CWG 2003 Hypoxia alters expression and function of syncytin and its receptor during trophoblast cell fusion of human placental BeWo cells: implications for impaired trophoblast syncytialisation in pre-eclampsia. Biochem Bioph Acta 1638:63-71

Kudo Y, Boyd CAR, Sargent IL, Redman CWG, Lee JM, Freeman TC 2004 An analysis using DNA microarray of the time course of gene expression during syncytialization of a human placental cell line (BeWo). Placenta 25:479-788

Lee X, Keith JC Jr, Stumm N, Moutsatsos I, McCoy JM, Crum CP, Genest D, Chin D, Ehrenfels C, Pijnenborg R, Van Assche FA, Mi S 2001 Downregulation of placental syncytin expression and abnormal protein localization in pre-eclampsia. Placenta 22:808-812

Lee SB, Wong AP, Kanasaki K, Xu Y, Shenoy VK, McElrath TF, Whitesides GM, Kalluri R 2010 2-Methoxyestradiol induces cytotrophoblast invasion and vascular development specifically under hypoxic conditions. Epithelial and Mesenchymal Cell Biology-Preeclampsia. Am J Pathol 176(2):710-720

Lissbrant IF, Hammarsten P, Lissbrant E, Ferrara N, Rudolfsson SH, Bergh A 2004 Neutralizing VEGF bioactivity with a soluble chimeric VEGF-receptor protein flt(1-3) IgG inhibits testosterone-stimulated prostate growth in castrated mice. Prostate 58:57-65

Loriaux DL, Ruder HJ, Knab DR, Lipsett MB 1972 Estrone sulfate, estrone, estradiol and estriol plasma levels in human pregnancy. J Clin Endocrinol Metab 35(6):887-891

Lorzadeh N, Kazemirad S 2012 The effects of fetal gender on serum human chorionic gonadotropin and testosterone in normotensive and preeclamptic placentas. J Pregnancy ID874290:1-6

Mabjeesh NJ, Willard MT, Fredrickson CE, Zhong H, Simons JW 2003 Androgens stimulate hypoxia-inducible factor 1 activation via autocrine loop of tyrosine kinase receptor/phosphatidylinositol 3'-kinase/protein kinase B in prostate cancer cells. Clin Cancer Res 9:2416-2425

Metzger E, Wissmann M, Yin N, Muller JM, Schneider R, Peters AH, Gunther T, Buettner R, Schule R 2005 LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. Nature 437:436-439

Milosevic M, Chung P, Parker C, Bristow R, Toi A, Panzarella T, Warde P, Catton C, Menard C, Bayley A, Gospodarowicz M, Hill R 2007 Androgen withdrawal in patients reduces prostate cancer hypoxia: implications for disease progression and radiation response. Cancer Res 67:6022-6025

Mizuno M, Lobotsky J, Lloyd CW, Kobayashi T, Murasawa Y 1968 Plasma androstenedione and testosterone during pregnancy and in the newborn. J Clin Endocrinol Metab 28(8):1133-1142

Munaut C, Noel A, Hougrand O, Foidart J-M, Boniver J, Deprez M 2003 Vascular endothelial growth factor expression correlates with matrix metalloproteinases MT1-MMP, MMP-2 and MMP-9 in human glioblastomas. Int J Cancer 106:848-855

Nelson DM, Johnson RD, Smith SD, Anteby EY, Sadovsky Y 1999 Hypoxia limits differentiation and up-regulates expression and activity of prostaglandin H synthase2 in cultured trophoblast from term human placenta. Am J Obstet Gynecol 180:896-902

Nheu L, Nazareth L, Xu G-Y, Xiao F-Y, Luo R-Z, Komesaroff P, Ling S 2011 Physiological effects of androgens on human vascular endothelial and smooth muscle cells in culture. Steroids 76(14):1590-1596

Park S-Y, Kim Y-J, Gao AC, Mohler JL, Onate SA, Hidalgo AA, Ip C, Park E-M, Yoon SY, Park Y-M 2006 Hypoxia increases androgen receptor activity in prostate cancer cells. Cancer Res 66:5121-5129

Pavan L, Tarrade A, Hermonet A, Delouis C, Titeux M, Vidaud M, Thérond P, Evain-Brion D, Fournier T 2003 Human invasive trophoblasts transformed with simian virus 40 provide a new tool to study the role of PPARy in cell invasion process. Carcinogenesis 24(8):1325-1336

Pötgens AJG, Drewlo S, Kokozidou M, Kaufmann P 2004 Syncytin: the major regulator of trophoblast fusion? Recent development and hypotheses on its action. Hum Reprod Update 10(6):487-496

Pringle KG, Kind KL, Sferrazzi-Perri AN, Thompson JG, Roberts CT 2010 Beyond oxygen: complex regulation and activity of hypoxia inducible factors in pregnancy. Hum Reprod Update 16(4):415-431

Rama S, Petrusz P, Rao AJ 2004 Hormonal regulation of human trophoblast differentiation: a possible role for 17β-estradiol and GnRH. Mol Cell Endocrinol 218:79-94

Rajakumar A, Conrad KP 2000 Expression, ontogeny, and regulatioin of hypoxia-inducible transcription factors in the human placenta. Biol Reprod 63:559-569

Reckelhoff JF 2001 Gender differences in regulation of blood pressure. Hypertension 37:1199-1208

Redman CWG 1990 Platelets and the beginnings of pre-eclampsia. N England J Med 323:478-480

Redman CW, 1991 Current topic: pre-eclampsia and the placenta. Placenta 12(4):301-308

Renaud SJ, Kubota K, Rumi MAK, Soares MJ 2014 The FOS transcription factor family differentially controls trophoblast migration and invasion. J Biological Chemistry 289(8):5025-5039

Rodesch F, Simon P, Donner C, Jauniaux E 1992 Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. Obstetrics and Gynecology 80:283-285

Salamalekis E, Bakas P, Vitoratos N, Eleptheriadis M, Creatsas G 2006 Androgen levels in the third trimester of pregnancy in patients with preeclampsia. Eur J Obstet Gynecol Reprod Biol 126:16-19

Salceda S, Cara J 1997 Hypoxia-inducible factor 1α (HIF1α) protein is rapidly degraded by ubiquitin-proteasome system under normoxic conditions: its stabilization by hypoxia depends upon redox-induced changes. J Biol Chem 272:22643-22647

Sathishkumar K, Elkins R, Chinnathambi V, Gao H, Hankins GDV, Yallampalli C 2011 Prenatal testosterone-induced fetal growth restriction is associated with down-regulation of rat placental amino acid transport. Reprod Biol Endocrinol 9:110-122

Schild RL, Schaiff WT, Carlson MG, Carlson MG, Cronbach EJ, Melson DM, Sadovsky Y 2002 The activity of PPAR gamma in primary human trophoblasts in enhanced by oxidized lipids. J Clin Endocrinol Metab 87:1105-1110

Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nature Protocols 2008;3(6): 1101-1108

Semenza GL 2000 HIF-1 and human disease: one highly involved factor. Genes Dev 14:1983-1991

Serin IS, Kula M, Başbug M, Ünlühizarci K, Güçer Ş, Tayyar M 2001 Androgen levels of preeclamptic patients in the third trimester of pregnancy and six weeks after delivery. Acta Obstet Gynecol Scand 80:1009-1013

Shabisgh A, Tanji N, D'Agati V, Burchardt M, Rubin M, Goluboff ET, Heitjan D, Kiss A, Buttyan R 1999 Early effects of castration on the vascular system of the rat ventral prostate gland. Endocrinology 140:1920-1926

Shin S, Janknecht R 2007 Activation of androgen receptor by histone demethylases JMJD2A and JMJD2D. Biochem Bioph Res Commun 359:742-746

Shweiki D, Itin A, Soffer D, Keshet E 1992 Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359:843-845

Soares MJ, Chakraborty D, Renaud SJ, Kubota K, Bu P, Konnq T, Rum MAK 2012 Regulatory pathways controlling the endovascular invasive trophoblast cell lineage. J Reprod Dev 58(3):283-287

Siiteri PK, MacDonald PC 1963 The utilization of circulating dehydroisoandrosterone sulfate for estrogen synthesis during human pregnancy. Steroids 2(6):713-730

Siiteri PK, MacDonal PC 1966 Placental estrogen biosynthesis during human pregnancy. J Clin Endocrinol Metab 26(7):751-761

Solberg H, Rinkenberger J, Danø K, Lund LR 2003 A functional overlap of plasminogen and MMPs regulates vascularization during placental development. Development 130: 4439-4450

Song J, Martin DS 2006 Rho kinase contributes to androgen amplification of renal vasoconstrictor responses in spontaneously hypertensive rat. J Cardiovascular Pharmacol 48(3):103-109

Strszewski-Chavez SL, Abrahams VM, Alvero AB, Aldo PB, Ma Y, Guller S, Romero R, Mor G 2009 The isolation and characterization of a novel telomerase immortalized first trimester trophoblast cell line, Swan 71. Placenta 30:939-948

Takai D, Gonzales FA, Tsai YC, Thayer MJ, Jones PA 2001 Large scale mapping of methylcytosines in CTCF-binding sites in the human *H19* promoter and aberrant hypomethylation in human bladder cancer. Human Mol Genet 10(23):2619-2626

Tarrade A, Lai Kuen R, Malassine A, Ricottet V, Blain P, Vidaud M Evain-Brion D 2001a Characterization of human villious and extravillous trophoblasts isolated from first trimester placenta. Lab Invest 81:1199-1211

Tarrade A, Schoonjans K, Guibourdenche J, Bidart JM, Vidaud M, Auwerx J, Rochette-Egly C, Evain-Brion D 2001b PPARγ/RXRα heterodimers are involved in human CGβ synthesis and human trophoblast differentiation. Endocrinology 142(10):4504-4514

Tarrade A, Schoonjans K, Paran L, Auwerx J, Rochette-Egly C, Evain-Brion D, Fournier T 2001c PPARγ/RXRα heterodimers control human trophoblast invasion. J Clin Endocrinol Metab 86(10):5017-5024

Thompson EA, Siiteri PK 1974a The involvement of human placental microsomal cytochrome P-450 in aromatization. J Biol Chem 249:5373-5378

Thompson EA, Siiteri PK 1974b Utilization of oxygen and reduced nicotinamide adenine dinucleotide phosphate by human placental microsomes during aromatization of androstenedione. J Biol Chem 249:5364-5372

Tissot van Patot MC, Bendrick-Peart J, Beckey VE, Serkova N, Zwerdlinger L 2004 Greater vascularity, lowered HIF-1/DNA binding, and elevated GSH as markers of adaptation to in vivo chronic hypoxia. Am J Physiol Lung Cell Mol Physiol 287:L525-L532

Toth B, Bastag M, Mylonas I, Scholz C, Makovitzky J, Krize S, Thaler C, Friese K, Jeschke 2009 Peroxisome proliferator-activated receptor-gamma in normal hyman pregnancy and miscarriage. Acta Histochemica 111:372-378

Trowsdale J, Travass P, Bodmer WF, Patillo RA 1980 Expression of HLA-A, -B and -C and fl2m antigens in human choriocarcinoma cell lines J Exp Med 152: 115

Tulchinsky D, Hobel CJ 1953 Plasma human chorionic gonadotropin, estrone, estradiol, estriol, progesterone, and 17-alpha-hydroxyprogesterone in human pregnancy. 3. Early normal pregnancy. Am J Obstet Gynecol 117(7):884-893

Tuutti EK, Hämäläinen EK, Sainio SM, Hiilesmaa VK, Turpeinen UL, Alfthan HV, Stenman U-HE 2011 Serum testosterone levels during early pregnancy in patients developing preeclampsia. Scand J Clin Lab Invest 71:413-418

Vargas A, Moreau J, Landry S, LeBellego F, Tougaily C, Rassart É, Lafond J, Barbeau B 2009 Syncytin-2 plays an important role in the fusion of human trophoblast cells. J Mol Biol 392(2):301-318

Verrier L, Vandromme M, Trouche D 2011 Histone demethylases in chromatin cross-talks. Biol Cell 103(8):381-401

Watzka M, Waha A, Koch A, Schmutzler RK, Bidlingmaier F, von Deimling A, Klingmuller D, Stoffel-Wagner B 1996 An optimized protocol for mRNA Quantification using nested competitive RT-PCR. Biochem Bioph Res Comm 231:813-817

Wellmann S, Bettkober M, Zelmer A, Seeger K, Faigle M, Eltzschig HK, Buhrer C 2008 Hypoxia upregulates the histone demethylase JMJD1A via HIF-1. Biochem Bioph Res Comm 372(4):892-897

Wieciech I, Durlej-Grzesiak M, Stomczyńska M 2013 Influence of the antiandrogen flutamide on the androgen receptor gene expression in the placenta and umbilical cord during pregnancy in the pig. Acta Histochem 115(3):290-295

Wieser F, Waite L, Depoix C, Taylor RN 2008 PPAR action in human placental development and pregnancy and its complications. PPAR Res:527048

Wissmann M, Yin N, Muller JM, Greschik H, Fodor BD, Jenuwein T, Vogler C, Schneider R, Gunther T, Buettner R, Metzger E, Schule R 2007 Cooperative demethylation by JMJD2C and LSD1 promotes androgen receptor-dependent gene expression. National Cell Biol 9:347-353

Yamane K, Toumazou C, Tsukada Y-I, Erdjument-Bromage H, Tempst P, Wong J, Zhang Y 2006 JHDM2A, a JmjC-contain H3K9 demethylase, facilitates transcription activation by androgen receptor. Cell 125:483-495

Yang J, Jubb AM, Pike L, Buffa FM, Turley H, Baban D, Leek R, Gatter KC, Ragoussis J, Harris AL 2010 The histone demethylase JMJD2B is regulated by estrogen receptor α and hypoxia, and is a key mediator of estrogen induced growth. Cancer Res 70(16):6456-6466

Yu AY, Frid MG, Shimada LA, Wiener CM, Stenmark K, Semenza GL 1998 Temporal, spatial and oxygen regulated expression of hypoxia-inducible factor 1 in the lung. Am J Physiol 275:L818-L826

Zachariah PK, Juchau MR 1977 Inhibition of human placental mixed-function oxidation with carbon monoxide: reversal with monochromatic light. J Steroid Biochem 8:221-228

Zhou Y, Chiu K, Brescia RJ, Combs CA, Katz MA, Kitzmiller JL, Heilbron DC, Fisher SJ 1993 Increased depth of trophoblast invasion after chronic constriction of the lower aorta in rhesus monkeys. Am J Obstet Gynecol 169:224-229

Zhu M-L, Kyprianou N 2008 Androgen receptor and growth factor signaling cross-talk in prostate cancer cells. Endocrine-Related Cancer 15:841-849

CHAPTER IV: DISCUSSION AND CONCLUSIONS

From the experiments conducted, it is apparent that androgen receptor (AR) and histone lysine demethylases (KDMs) are present in ovine and human placenta, as well as choriocarcinoma and immortalized human trophoblast cell lines. Additionally, these are the first studies to demonstrate AR-KDM complexes are present in placental tissue and choriocarcinoma cells. These findings suggest that, similar to previous reports in cancer cell lines (Metzger et al., 2005; Yamane et al., 2006; Wissmann et al., 2007; Shin and Janknecht 2007a,b), AR-KDM complexes in the placenta may function to regulate transcription of androgen responsive genes, including those known to regulate cellular proliferation, invasion, and tissue angiogenesis (Liao et al., 2003; Limaye et al., 2008; Shi 2007; Comstock et al., 2008). However, as of yet, we have not directly addressed the functionality of AR-KDM complexes or androgen signaling in the placenta. For instance, the altered gene expression observed in the placentas from prenatal androgenized ewes could partially be due to placental aromatization of testosterone propionate and it therefore cannot be implied solely as the results of placental androgen signaling. Additionally, while dihydrotestosterone (DHT) treatment altered relative mRNA levels of *KDM3A*, *KDM4D*, *PPARγ* and *MMP2* in choriocarcinoma cells, the gene expression change was not as predicted and functional protein levels in response to DHT treatment have not yet been investigated. Furthermore, androgens' effect on trophoblast differentiation into an invasive or syncytial phenotype should be tested in future experiments.

Data from these experiments put forward additional areas for future investigation. For example, while only KDM1A and KDM4D interactions with placental AR were tested, the presence and

functionality of other KDMs in the placenta may prove to be of great importance. When trophoblast stem cells differentiate, they lose tri-methylation on histone 3 lysine 27 (H3K27me3) on the X-chromosome (Plath et al., 2003; Silva et al., 2003), suggesting that other histone demethylases also are involved in regulating placentation than the demethylases KDM1A, a H3K4me1/me2 and H3K9me1/me2 demethylase (Metzger et al., 2005; Shi and Whetstine 2007; Shi 2007), and KDM4D, a H3K9me2/me3 demethylase (Shin and Janknecht 2007b). KDMs may also act as corepressors of gene transcription (Wang et al., 2007; Verrier et al., 2011), and could function to decrease the expression of key regulatory genes necessary for placentation and placental function. Additionally, as estrogen signaling is known to regulate trophoblast differentiation (Albrecht and Pepe 1999; Albrecht et al., 2006), characterization of estrogen receptor (ESR)-KDM complexes and function in placental tissue could further elucidate the mechanisms controlling trophoblast differentiation.

To date, limited research on the function of placental androgen signaling has been directly investigated. An experiment using daily injections of flutamide, an AR inhibitor, during porcine pregnancy has been completed; however, flutamide was only injected for seven days at two time points (GD 83-89 and GD 101-107) near term, after the critical window of placenta formation (Spencer et al., 2004; Wieciech et al., 2013). No difference was noted in fetal growth or placental pathology, but this is likely due to the brief window of flutamide treatment after placental establishment, making it unlikely to affect placental development or fetal growth. Ideally, direct inhibition of AR in the placenta prior to implantation and throughout pregnancy would be ideal to determine the role of androgen signaling for placentation and fetal growth. Treatment with flutamide is unlikely to be with best option to accomplish this as it requires daily injections of

doses near 50mg/kg of body weight and would act systemically. Instead, trophoblast specific lentiviral knockdown of AR *in vivo* is ideal to characterize placental androgens' function at different points of gestation in domestic agricultural species (Purcell et al., 2009). Recent acquisition of the dominant-negative mutant ARΔ142-337 (ARΔTR) provides an excellent opportunity for targeted silencing of androgen signaling in future *in vitro* and *in vivo* experiments as ARΔTR binds ligand, dimerizes with endogenous AR, and prevents transcription of androgen responsive genes (Zhou et al., 1995; Palvimo et al., 1993; Titus et al., 2012).

Further characterization of placental androgen signaling is necessary to clearly identify its role in regulating trophoblast function and differentiation. There are currently conflicting reports as to the likely requirement of androgen signaling for normal placentation and prenatal development. For instance, human infants with placental aromatase deficiency have normal fetal growth (Shozu et al., 1991; Harada et al., 1992a,b; Conte et al., 1994; Morishima et al., 1995; Carani et al., 1997; Deladoëy et al., 1999), as do fetuses with mutations in AR that lead to partial androgen insensitivity (Hughes and Evans 1987; Bangsboll et al., 1992; Brown 1995; Quigley et al., 1995; reviewed by Ahmed et al., 2000), suggesting normal or at least compensatory placental development and function had occurred. Additionally, genetic males with AR mutations leading to complete androgen insensitivity (with lack of ligand binding to AR) have normal fetal growth; however, bioassays on AR functionality and DNA binding in cases of complete or partial androgen insensitivity has not been completed (Ahmed et al., 2000). In contrast, case studies on familial androgen insensitivity found that females who are carriers for partial or complete androgen insensitivity report multiple miscarriages (Wilson et al., 1974; Decaestecker et al., 2008) and that heightened androgen signaling through an AR-polymorphism is associated with

recurrent spontaneous abortions and preterm labor (Karvela et al., 2008; Karjalainen et al., 2012).

As androgen production and signaling may be crucial for placental development, redundancy in sex-hormone signaling may be present to protect normal placentation, possibly explaining the conflicting reports on the requirement of placental androgens. For instance, sex hormones have been shown to have similar overlapping functions, including regulation of genes promoting cellular proliferation and vascularization (Mueller et al., 2000; Inoue et al., 2002; Marin-Castaño et al., 2003; Comstock et al., 2008). Therefore, if there is loss of function in either ESRs or AR, the alternate sex hormone may compensate to some extent to rescue placentation and trophoblast function. This sex hormone cross-talk may in part be accomplished through androgen binding to ESRs to regulate estrogen-responsive genes while estrogens can bind and act through AR to initiate physiological effects, although the relative binding affinity is low when sex steroid hormones are at physiologic levels (Yeh et al., 1998; 2002; Panet-Raymond et al., 2000). Additionally, heterodimerization of AR with ESRs led to decreased transcriptional activity (Panet-Raymond et al., 2000). It is therefore unlikely that this mechanism of sex hormone cross-talk would be capable of rescuing expression of all androgen responsive genes in the case abnormal placental androgen levels. Several nuclear receptors, namely glucocorticoid, progesterone, and mineralocorticoid receptors (GR, PR, and MR), may also function to partially regulate androgen responsive genes as they have a similar response element as AR (Claessens et al., 2008). However, while sharing a similar response element in DNA, AR initiation of transcription also requires enhancer regions with binding sites for additional transcription activators, such as Sp1, with multiple response element repeats, suggesting that sequences

flanking DNA response elements regulate AR binding over alternate nuclear receptors to maintain specificity of gene response (Claessens et al., 2008). As KDMs have been shown to regulate both estrogen and androgen responsive gene transcription, and to recruit sex hormone receptors to DNA in the absence of ligand (Metzger et al., 2005; Yamane et al., 2006; Wissmann et al., 2007; Shin and Janknecht 2007a,b; Perillo et al., 2008; Kawazu et al., 2011), they may offer another level of regulation and/or protection of normal trophoblast function and differentiation. However, further research is necessary to clarify this proposed mechanism of placental sex hormone signaling.

In conclusion, this dissertation it is the first report of KDMs and AR-KDM complexes in placental tissue. Additionally, changes in choriocarcinoma relative mRNA levels in response to DHT treatment is reported. These findings, along with the increased maternal serum androgen levels throughout gestation (Mizuno et al., 1968; Serin et al., 2001) and increased androgen levels in preeclampsia (Acromite et al., 1999; Serin et al., 2001; Atamer et al., 2004; Salamalekis et al., 2006; Gerulewicz-Vannini et al., 2006; Ghorashi and Sheikhvatan 2008; Lorzadeh and Kazemirad 2012), suggest placental androgen signaling may crucially regulate trophoblast function or differentiation. It is therefore of great interest to determine if androgen signaling directly regulates human cytotrophoblast differentiation into a syncytial or invasive phenotype. Further studies are needed to determine if the presence of KDMs and their interaction with AR are required for androgen signaling in trophoblast cells. These findings indicate the necessity of continued research in clarifying androgen's role in regulating trophoblast function.

REFERENCES

Abbott DH, Padmanabhan V, Dumesic DA 2006 Contributions of androgen and estrogen to fetal programming of ovarian dysfunction. Reprod Biol Endocrin 4:17-25

Abdul-Karim RW, Nesbitt REL, Drucker M, Rizk PT 1971 The regulatory effect of estrogens on fetal growth. I Placental and Fetal body weights. Am J Obstet Gynecol 109:656-661

Aberdeen GW, Baschat AA, Harman CR, Weiner CP, Langenberg PW, Pepe GJ, Albrecht ED 2010 Uterine and fetal blood flow indexes and fetal growth assessment after chronic estrogen suppression in the second half of baboon pregnancy. Am J Physiol-Heart C 298:H881-H889

Acromite MT, Mantzoros CS, Leach RE, Hurwitz J, Dorey LG 1999 Androgens in preeclampsia. Am J Obstet Gynecol 180(1):60-63

Ahmed SF, Cheng A, Kovey L, Hawkins JR, Martin H, Rowland J, Shimura N, Tait AD, Hughes IA 2000 Phenotypic features, androgen receptor binding, and mutational analysis in 278 clinical cases reported as androgen insensitivity syndrome. J Clin Endocrinol Metab 85(2):658-665

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P 2008 Molecular Biology of the Cell. 5th Ed. Garland Science, Taylor & Francis: Madison, NY. 211-233. 1468

Albrecht ED, Pepe GJ 1999 Central integrative role of oestrogen in modulating the communication between the placenta and fetus that results in primate fetal-placental development. Placenta 20:129–139

Albrecht ED, Bonagura TW, Burleight DW, Enders AC, Aberdeen GW, Pepe GJ 2006 Suppression of extravillous trophoblast invasion of uterine spiral arteries by estrogen during early baboon pregnancy. Placenta 27:483-490

Anand R, Marmorstein R 2007 Structure and mechanism of lysine-specific demethylase enzymes. J Biol Chem 282:35425-35429

Anderson CM 2007 Preeclampsia: exposing future cardiovascular risk in mother and their children. J Obstet Gynecol Neonatal Nursing 36(1):3-8

Anderson DC 2008 Sex-Hormone-Binding Globulin. Clin Endocrinol 3(1):69-96

Angiolini E, Fowden A, Coan P, Sandovici I, Smith P, Dean W, Burton G, Tycko B, Reik W, Sibley C, Constância M 2006 Regulation of placental efficiency for nutrient transport by imprinted genes. Placenta 27:S98-S102

Anthony RV, Scheaffer AN, Wright CD, Regnault TRH 2003 Ruminant models of prenatal growth restrictions. Reproduction in domestic ruminants V. Proc Sixth Int Sym Reprod Domest Rumin. Crieff, Scotland, UK, August 2002 pp. 183-194

Arensburg J, Payne AH, Orly J 1999 Expression of steroidogenic genes in maternal and extramebryonic cells during early pregnancy in mice. Endocrinology 140(11):5220-5232

Arroyo JA, Winn VD 2008 Vasculogenesis and angiogenesis in the IUGR placenta. Sem Perinatol 32: 172-177

Astapova O, Steckler TL, Jackson LM, MohanKumar PS, Ford SP, Fosteur DL, Padmanabhan V 2004 Fetal programming: testosterone treatment during pregnancy advances placentome differentiation. 15th Annual Pediatric Research Symposium Department of Pediatrics and Communicable Diseases. CS Mott Children's Hospital, MI. Abstract 27

Atamer Y, Erden AC, Demir B, Koçygit Y, Atamer A 2004 The relationship between plasma levels of leptin an androgen in healthy and preeclamptic pregnant women. Acta Obstet Gynecol Scand 83:425-430

Aw TY, Jones DP, O'Shannessy DJ, Priest JH, Priest RE 1985 Oxygen dependence of oestrogen production by human placental microsomes and cultured choriocarcinoma cells. J Steroid Biochem 22(6):753-758

Bangsboll S, Qvist I, Lebech PE, Lewinsky M 1992 Testicular feminization syndrome and associated gonadal tumors in Denmark. Acta Obstet Gynecol Scand 71:63-66

Bardin CW, Musto NA, Gunsalus GL, Kotite N, Cheng S-L, Larrea F, Becker R 1981 Extracellular androgen binding protein. A Rev Physiol 43:189-198

Barry JS, Anthony RV 2008 The pregnant sheep as a model for human pregnancy. Theriogenology 69:22-67

Barski A, Cuddapah S, Cui K, Roh T-Y, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K 2007 High-resolution profiling of histone methylations in the human genome. Cell 129:823-837

Bass KE, Morrish D, Roth I, Bhardwaj D, Taylor R, Zhou Y, Fisher SJ 1994 Human cytotrophoblast invasion is up-regulated by epidermal growth factor: evidence that paracrine factors modify this process. Dev Biol 164(2):550-561

Beck S, Rakya VK 2008 The methylome: approaches for global DNA methylation profiling. Trends Genet 24:231-237

Becker CM, Rohwer N, Funakoshi T, Cramer T, Bernhardt W, Bisner A, Folkman J, D'Amato RJ 2008 2-Methoxyestradiol inhibits hypoxia-inducible factor-1(alpha) and suppresses growth of lesions in mouse model endometriosis. Am J Pathol 172:534-544

Benirschke K, Kaufmann P 2000 Pathology of the human placenta, 4th edition. Springer-Verlag:New York

Berg D, Sonsalia R, Kuss E 1983 Concentrations of 2-methoxyoestrogens in human serum measured by heterologous immunoassay with an 125I-labelled ligand. Acta Endocrinol 103:282-288

Berridge MJ, Taylor CW 1988 Inositol triphosphate and calcium signaling. Cold Spring Harbor Sym Quant Biol 53:927-933

Bird AP 1986 CpG-rich islands and the function of DNA methylation. Nature 321:209-213

Bird AP 2002 DNA methylation patterns and epigenetic memory. Genes Dev 16:6-21

Bischof P, Irminger-Finger I 2005 The human cytotrophoblastic cell, a mononuclear chameleon. Int J Biochem Cell Biol 37:1-16

Bjorkman N 1969 Light and electron microscopic studies on cellular alterations in the normal bovine placentome. Anat Rec 163:17-30

Blankenship TN, Enders AC, King BF 1993 Trophoblastic invasion and the development of uteroplacental arteries in the macaque: immunohistochemical localization of cytokeratins, desmin, type IV collagen, laminin, and fibronectin. Cell Tissue Res 272:227-236

Boddy JL, Fox SB, Han C, Campo L, Turley H, Kanga S, Malone PR, Harris AL 2005 The androgen receptor is significantly associated with vascular endothelial growth factor and hypoxia sensing via hypoxia-inducible factors HIF-1a, HIF-2a, and the prolyl hydroxylases in human prostate cancer. Hum Cancer Biol 11(21):7658-7663

Bode W, Maskos K 2003 Structural basis of the matrix metalloproteinases and their physiological inhibitors, the tissue inhibitors of metalloproteinase. Biol Chem 384:863-872

Bonagura TW, Pepe GJ, Enders AC, Albrecht ED 2008 Suppression of extravillous trophoblast vascular endothelial growth factor expression and uterine spiral artery invasion by estrogen during early baboon pregnancy. Endocrinology 149(10):5078-5087

Bonasio R, Tu S, Reinberg D 2010 Molecular signals of epigenetic states. Science 330:612-616

Bordin S, Petra PH 1980 Immunocytochemical localization of the plasma sex steroid-binding protein (SBP) in tissues of the adult male monkey, Macaca nemestrina. Proc Natl Acad Sci USA 77:5678-5682

Boshier DP 1969 A histological and histochemical examination of implantation and early placentome formation in sheep. J Reprod Fert 19:51-61

Bousquet J, Lye SJ, Challis JRG 1984 Comparison of leucine enkephalin and adrenocorticotrophin effects on adrenal function in fetal and adult sheep. J Reprod Fert 70:499-506

Boyd JD, Hamilton WJ 1970 The Human Placenta. Heffer & Sons:Cambridge 212

Brosens IA, Robertson WB, Dixon HG 1967 The Physiological response of the vessels of the placental bed to normal pregnancy. J Pathol Bacteriol 93:569-579

Brosens IA 1988 The uteroplacental vessels at term: the distribution and extent of physiological changes. Trophoblast Res 3:61-68

Brown TR 1995 Human androgen insensitivity syndrome. J Androl 16:299-303

Buhler M, Verdel A, Moazed D 2006 Tehtering RITS to a nascent transcript initiates RNAi- and heterochromatin-dependent gene silencing. Cell 125:873-886

Bukovsky A, Cekanova M, Caudle MR, Wimalasena J, Foster JS, Henley DC, Elder RF 2003a Expression and localization of estrogen receptor-alpha protein in normal and abnormal term placentae and stimulation of trophoblast differentiation by estradiol. Reprod Biol Endocrinol 1:13-31

Bukovsky A, Caudle MR, Cekanova M, Fernando RI, Wimalasena, J, Foster JS, Henley DC, Elder RF 2003b Placental expression of estrogen receptor beta and its hormone binding variant-comparison with estrogen receptor alpha and a role for estrogen receptors in asymmetric division and differentiation of estrogen-dependent cells. Reprod Biol Endocrinol 1:36-57

Burgers WA, Fuks F, Kouzarides T 2002 DNA methyltransferases get connected to chromatin. Trends Genet 18(6):275-277

Burton B, Jauniaux E, Watson A 1999 Maternal arterial connections to the placental intervillous space during the first trimester of human pregnancy: the Boyd collection revisited. Am J Obstet Gynecol 181:718-724

Burton GJ, Charnock-Jones DS, Jauniaux E 2009 Regulation of vascular growth and function in human placenta. Reproduction 138:895-902

Calvin HI, VandeWiele RL, Liberman S 1963 Evidence that steroid sulfates serve as biosynthetic intermediates in vivo conversion of pregnenolone-sulfate-S^{35} to dehydroisoandrosterone sulfate-S^{35}. Biochem 2(4):648-653

Carani C, Qin K, Simoni M, Faustini-Fustini M, Serpente S, Boyd J, Korach KS, Simpson ER 1997 Effect of testosterone and estradiol in a man with aromatase deficiency. N Engl J Med 337:91-95

Carlsen SM, Romundstad P, Jacobsen G 2005 Early second-trimester maternal hyperandrogenemia and subsequent preeclampsia: a prospective study. Acta Obstet Gynecol Scand 84:117-121

Carson DD, Bagchi I, Dey SK, Enders AC, Fazleabas AT, Lessey BA, Yoshinaga K 2000 Embryo implantation. Dev Biol 223:217-237

Carter AM 2007 Animal Models of Human Placentation- a review. Placenta 21:S41-S47

Cheng L, Zhang S, Sweeney CJ, Kao C, Gardener TA, Eble JN 2004 Androgen withdrawal inhibits tumor growth and is associated with decrease in angiogenesis and VEGF expression in androgen-independent CWR22Rv1 human prostate cancer model. Anticancer Res 24:2135-2140

Chinnathambi V, Balakrishnan M, Yallampalli C, Sathishkumar K 2012 Prenatal testosterone exposure leads to hypertension that is gonadal hormone-dependent in adult rat male and female offspring. Biol Reprod 86(5):137, 1-7

Claessens F, Denayer S, Tilborgh NV, Kerkhofs S, Helsen C, Haelens A 2008 Diverse roles of androgen receptor (AR) Domains in AR-mediated signaling. Nucl Recept Signal 6:e008

Cloos PA, Christensen J, Agger K, Maiolica A, Rappsilber J, Antal T, Hansen KH, Helin K 2006 The putative oncogene GASC1 demethylates tri- and dimethylated lysine 9 on histone H3. Nature 442:307-311

Coan PM, Burton GJ, Ferguson-Smith AC 2005 Imprinted genes in the placenta- a review. Placenta 26:S10-S20

Cohen M, Bischof P 2007 Factors regulating trophoblast invasion. Gynecol Obstet Inv 64:126-130

Comstock CE, Burd CJ, Jessen WJ, Knudsen KE 2008 Gene Profiling Analysis of Androgen Receptor Mediated Function. Genomics in Endocrinology: DNA Microarray Analysis in Endocrine Health and Disease. Contemporary Endocrinology. Ed Handweger, S., and Aronow, B. Humana Press. Totowa, NJ. II:83-113

Constancia M, Hemberger M, Hughes J, Dean W, Ferguson-Smith P, Fundele R, Stweart F, Kelsey G, Fowden A, Sibley C, Reik W 2002 Placental-specific IGF-II is a major modulator of placental and fetal growth. Nature 417:945-948

Constancia M, Kelsey G, Reik W 2004 Resourceful imprinting. Nature 432:53-57

Constancia M, Angiolini W, Sandovici I, Smith P, Smith R, Kelsey G, Dean W, Ferguson-Smith A, Sibley CP, Reik W, Fowden A 2005 Adaptation of nutrient supply to fetal demand in the mouse involves interaction between the Igf2 gene and placental transport systems. Proc Natl Acad Sci USA 102:19219-19224

Conte FA, Grumbach MM, Ito Y, Fisher CR, Simpson ER 1994 A syndrome of female pseudohermaphrodism, hypergonadotropic hypogonadism, and multicystic ovaries associated with missense mutations in the gene encoding aromatase (P450). J Clin Endocrinol Metab 78(6):1287-1292

Cooper GM, Hausman RE 2000 The Cell- a molecular approach. 5th Ed. ASM Press:Washington DC:603-644

Couse JF, Korach KS 1999 Estrogen receptor null mice: what have we learned and where will they lead us? Endocrine Rev 20(3):358-417

Couture J-F, Collazo E, Ortiz-Tello PA, Brunzelle JS, Trievel RC 2007 Specificity and mechanism of JMJD2A, a trimethyllysine-specific histone demethylase. Nat Struct Mol Biol 14:689-695

Cross JC, Werb Z, Fisher S 1994 Implantation and the placenta: key pieces of the development puzzle. Science 266(5190):1508-1518

Dawood MY, Saxen BB 1977 Testosterone and dihydrotestosterone in maternal and cord blood and in amniotic fluid. Am J Obstet Gynecol 129(1):37-42

Decaestecker K, Philiber P, de Baete E, Hoebeke P, Kaufman J-M, Sultan C, T'Sjoen G 2008 A novel mutation c.118deIA in exon 1 of the androgen receptor gene resulting in complete androgen insensitivity syndrome within a large family. Am Society Reprod Med 89(5): 1260.e3

Deladoëy J, Flück C, Bex, M, Yoshimura N, Harada N, Mullis PE 1999 Aromatase deficiency caused by a novel $P450_{AROM}$ gene mutation: impact of absent estrogen production on serum gonadotropin concentration in a boy. J Clin Endocrinol Metab 84(11):4050-4053

Demir R, Kaufmann P, Castellucci M, Erbengi T, Kotowski A 1989 Fetal vasculogenesis and angiogenesis in human placental villi. Acta Anatomica 136:192-203

Demir R, Kayisli UA, Seval Y, Celik-Ozenci C, Korgun ET, Demir-Weusten AY, Huppertz B 2004 Sequential expression of VEGF and its receptors in human placental villi during very early pregnancy: differences between placental vasculogenesis and angiogenesis. Placenta 25:560-572

Demir R, Seval Y, Huppertz B 2007 Vasculogenesis and angiogenesis in the early human placenta. Acta Hitochem 109:257-265

Doerfler W 1983 DNA methylation and gene activity. Ann Rev Biochem 52:93-124.

Enders AC 1965 A comparative study of the fine structure of the trophoblast in several hemochorial placentas. Am J Anat 116:29-67

Enders AC 1968 Fine structure of anchoring villi of human placenta. Am J Anat 122(3):419-451

Eriksson JG, Forsén T, Tuomilehto J, Winter PD, Osmond C, Barker DJP 1999 Catch-up growth in childhood and death from coronary heart disease: longitudinal study. BMJ 318(7181):427-431

Felty Q, Xiong W-C, Sun D, Sarkar S, Singh KP, Parkash J, Roy D 2005 Estrogen-induced mitrochondrial reactive oxygen species as signal transducing messengers. Biochem 44(18):6900-6909

Feng S, Jacobsen SE, Reik W 2010 Epigenetic reprogramming in plant and animal development. Science 330:622-627

Ferguson-Smith AC, Surani MA 2001 Imprinting and the epigenetic asymmetry between parental genomes. Science 293:1086-1089

Ferretti C, Bruni L, Dangles-Marie V, Pecking AP, Bellet D 2007 Molecular circuits shared by placental and cancer cells, and their implications in the proliferative, invasive and migratory capacities of trophoblasts. Hum Reprod Update 13(2):121-141

Forneris F, Binda C, Vanoni MA, Mattevi A, Battaglioli E 2005 Histone demethylation catalyzed by LSD1 is a flavin-dependent oxidative process. FEBS Lett 579:2203-2207

Fournet-Dulguerov N, MacLusky NJ, Leranth CZ, Todd R, Mendelson CR, Simpson ER, Naftolin F 1987 Immunocytochemical localization of aromatase cytochrome P-450 and estradiol dehydrogenase in the syncytiotrophoblast of the human placenta. J Clin Endocrinol Metab 65:757–763

Fowden AL 2003 The insulin-like growth factors and feto-placental growth. Placenta. 24:803-812

Fowden AL, Forhead AJ, Coan PM, Burton GJ 2008 The Placenta and Intrauterine Programming. J Neuroendocrinol 20:439-450

Fowden AL, Coan PM, Angiolini E, Burton GJ, Constancia M 2010 Imprinted genes and the epigenetic regulation of placental phenotype. Prog Bioph Mol Biol 106:281-288

Fürst RW, Meyer HHD, Schweizer G, Ulbrich SE 2012 Is DNA methylation an epigenetic contribution to transcriptional regulation of the bovine endometrium during the estrous cycle and early pregnancy? Mol Cell Endocrinol 348:67-77

Garcia-Bassets I, Kwon Y-S, Telese F, Prefontaine GG, Hutt KR, Cheng CS, Ju B-G, Ohgi KA, Wang J, Escoubet-Lozach L, Rose DW, Glass CK, Fu X-D, Rosenfeld MG 2007 Histone methylation-dependent mechanisms impose ligand dependency for gene activation by nuclear receptors. Cell 128(3):505-518

Gehani FE, Zhang FP, Pakarinen P, Rannikko A, Huhtaniemi I 1998 Gonadotropin-independent regulation of steroidogenesis in the fetal rat testis. Biol Reprod 58(1):116-123

Georgiades P, Watkins M, Burton GJ, Gerguson-Smith AC 2001 Roles for genomic imprinting and the zygotic genome in placental development. Proc Natl Acad Sci USA 98:4522-4527

Gerulewicz-Vannini D, Camero Y, Salas J, Hernández-Andrade E 2006 High plasmatic androgen levels in women affected with pregnancy-induced hypertension. Rev Invest Clin 58(3):228-233

Ghidini A, Salafia CM 2005 Gender differences of placental dysfunction in severe prematurity. British J Obstet Gynecol 112:140-144

Ghorashi V, Sheikhvata M 2008 The relationship between serum concentration of free testosterone and pre-eclampsia. Polish J Endocrinol 59(5):390-392

Gopalakrishnan S, Van Emburgh BO, Robertson KD 2008 DNA methylation in development and human disease. Epigenet Dev Hum Disease 647:30-38

Goto J, Fishman J 1977 Participation of a non-enzymatic transformation in the biosynthesis of estrogens from androgens. Science 195:80-81

Graham CH, Postovit LM, Park H, Canning MT, Fitzpatrick TE 2000 Role of oxygen in regulation of trophoblast gene expression and invasion. Placeta 21:443-450

Grazul-Bilska AT, Johnson ML, Borowicz PP, Minten M, Bilski JJ, Wroblewski R, Velimirovish M, Coupe LR, Redmer DA, Reynolds LP 2011 Placental development during early pregnancy in sheep: cell proliferation, global methylation, and angiogenesis in the fetal placenta. Reproduction 141:529-540

Grippo AA, Xie Y, Rougeau BL, Wyatt WV 1999 Analysis of phytoestrogens by high performance liquid chromatography. J Arkansas Acad Sci 53:61-66

Grunstein M 1997 Histone acetylation in chromatin structure and transcription. Nature 389:349-352

Gude NM, Roberts CT, Kalionis B, King RG 2004 Growth and Function of the normal human placenta. Thrombosis Res 114(5-6):397-407

Guibourdenche J, Fournier T, Malassiné A, Evain-Brion D 2009 Development and hormonal functions of the human placents. Folia Histochem Cytobiol 47(5):s35-s42.

Guillomot M, Flechon JE, Wintenberger-Torres S 1981 Conceptus attachment in the ewe: an ultrastructural study. Placenta 2:169-182

Guillomot M, Flechon JE, Leroy F 1993 Blastocyst development and implantation. Reproduction in Mammals and Man. Eds Thibault C, Levasseur MC, Hunter RHF. Ellipses:Paris. 387-411

Hähnel ME, Martin JD, Michael CA, Hähnel R 1989 Metabolism of androstenedione by placental microsomes in pregnancy hypertension. Clin Chimica Acta 181(1):103-108

Haig D 2004 Genomic imprinting and kinship: how good is the evidence? Annual Rev Genet 38:553-585.

Hake SB, Allis CD 2006 Histone H3 variants and their potential role in indexing mammalian genomes: the "H3 barcode hypothesis". Proc Natl Acad Sci USA 103:6428-6435

Halleran J, da Silveira JC, Winger Q, Bouma, GJ 2011 Sheep Placentome and Developmental Programming. College of Veterinary Medicine and Biomedical Sciences Research Day, Colorado State University.

Hammond GL 1990 Molecular properties of corticosteroid binding globulin and the sex-steroid binding proteins. Endocrin Rev 11:65-79

Handa RJ, Pak TR, Kudwa AE, Lund TD, Hinds L 2008 An alternate pathway for androgen regulation of brain function: activation of estrogen receptor β by the metabolite of dihydrotestosterone, 5α-androstane-3β, 17β-diol. Horm Behav 53:741-752

Handwerger S, Freemark M 2000 The roles of placental growth hormone and placental lactogen in the regulation of human fetal growth and development. J Pediatr Endocrinol Metab 13(4):506-512

Harada N, Ogawa H, Shozu M, Yamada K 1992a Genetic studies to characterize the origin of the mutation in placental aromatase deficiency. Am J Hum Gene 51:666-672

Harada N, Ogawa H, Shozu M, Yamada K, Suhara K, Nishida E, Takagi Y 1992b Biochemical and molecular genetic analyses on placental aromatase ($P\text{-}450_{AROM}$) deficiency. J Biol Chem 267:4781-4785

Hayashi M, Sakata M, Takeda T, Tahara M, Yamamoto T, Minekawa R, Isobe A, Tasaka K, Murata Y 2005 Hypoxia up-regulates hypoxia-inducible factor-1alpha expression through RhoA actiation in trophoblast cells. J Clin Endocrinol Metab 90:1712-1719

Heindel HH, vom Saal FS 2009 Role of nutrition and environmental endocrine disrupting chemicals during the perinatal period on the aetiology of obesity. Mol Cell Endocrinol 304:90-96

Henderson BE, Bernstein L, Ross RK, Depue RH, Judd HL 1988 The early in utero oestrogen and testosterone environment of Blacks and Whites. British J Cancer 57:216-218

Hertig A, Liere P, Chabbert-Buffet N, Fort J, Pianos A, Eychenne B, Cambourg A, Schumacher M, Berkane N, Lefevre G, Uzan S, Rondeau E, Rozenberg P, Rafestin-Oblin M-E 2010 Steroid profiling in preeclamptic women: evidence for aromatase deficieny. Am J Obstet Gynecol 203:477.e1-9

Hiendleder S, Mund C, Reichenbach HD, Wenigerkind H, Brem G, Zakhartchenko V, Lyko F, Wolf E 2004 Tissue-specific elevated genomic cytosine methylation levels are associated with an overgrowth phenotype of bovine fetuses derived by in vitro techniques. Biol Reprod 71:217-223

Hildebrandt VA, Babischkin JS, Koos RD, Pepe GJ, Albrecht ED 2001 Developmental regulation of vascular endothelial growth/permeability factor messenger ribonucleic acid levels in and vascularization of the villous placenta during baboon pregnancy. Endocrinology 142(5):2050-2057

Hirano H, Imai Y, Ito H 2002 Spiral artery of placenta: development and pathology: immunohistochemical, microscopical, and electron-microscopic study. Kobe J Medical Sci 48:13-23.

Hoffman LH, Wooding FB 1993 Giant and binucleate trophoblast cells of mammals. J Exp Zoology 266:559-577

Holliday R 1987 The inheritance of epigenetic defects. Science 238:163-170.

Holliday R 1989 DNA methylation and epigenetic mechanisms. Cell Bioph 15:15-20

Hryb PJ, Khan MS, Romas NA, Rosner W 1990 The control of the interaction of sex hormone-binding globulin with its receptor by steroid hormones. J Biol Chem 265:6048-6054

Hsu T-Y, Lan K-C, Tsai C-C, Ou C-Y, Cheng B-H, Tsai M-Y, Kang Y-Y, Tung Y-H, Wong Y-H, Huang K-E 2009 Expression of androgen receptor in human placentas from normal and preeclamptic pregnancies. Taiwanese J Obstet Gynecol 48(3):262-267

Hu J, Zhang Z, Shen W-J, Azhar S 2010 Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid hormones. Nutrition Metab 7:47-72

Hughes IA, Evans BAJ 1987 Androgen insensitivity in forty-nine patients: classification based on clinical and androgen receptor phenotypes. Horm Res 28:25-29

Huppertz B, Kertshanska S, Demir AY, Frank H-G, Kaufmann P 1998 Immunohistochemistry of matrix metalloproteinases (MMP), their substrates, and their inhibitors (TIMP) during trophoblast invasion in the human placenta. Cell Tissue Res 291:133-148

Hurskainen T, Höyhtyä M, Tuuttila A, Oilarinen A, Autio-Harmainen H 1996 mRNA expressions of TIMP-1, -2, and -3 and 92-KD type IV collagenase in early human placenta and decidual membranes as studied by in situ hybridization. J Histochem Cytochem 44(12):1379-1388

Hustin J, Jauniaux E, Schapps J 1987 Histological study of the materno-embryonic interface in spontaneous abortion. Placenta 11:477-480

Ilekis J, Reddy UM, Roberts JM 2007 Preeclampsia—a pressing problem: an executive summary of a national institute of child health and human development workshop. Reprod Sci 14:508-525.

Inoue A, Yoshida N, Omoto Y, Oguchi S, Yamori T, Kiyama R, Hayashi S 2002 Development of cDNA microarray for expression profiling of estrogen-responsive genes. J Mol Endocrinol 29:175-192

Isaka K, Usuda S, Ito H, Sagawa Y, Nakamura H, Nishi H, Suzuki Y, Li YF, Takayama M 2003 Expression and activity of matrix metalloproteinase 2 and 9 in human trophoblasts. Placenta 24(1):53-64

Iwamura M, Abrahamsson PA, Benning CM, Cockett AT, di Sant'Agnese PA 1994 Androgen receptor immunostaining and its tissue distribution in formalin-fixed, paraffin-embedded sections after microwave treatment. J Histochem Cytochem 42:783-788

James WH 1995 Sex ratios of offspring and the causes of placental pathology. Hum Reprod 10:1403-1406

James WH 2008 Evidence that mammalian sex ratios at birth are partially controlled by parental hormone levels around the time of conception. J Endocrinol 198:3-15

James WH 2013 Letter to the editor: Change in paternity, pre-eclampsia, fetal growth retardation and maternal testosterone concentration. J Obstet Gynecol 33:217

Jamesone J, Hollenberg A 1993 Regulation of chorionic gonadotropin gene expression. Endocrinol Rev 14:203-221

Jaquet D, Gaboriau A, Czernichow P, Levy-Marchal C 2000 Insulin resistence early in adulthood in subjects born with intrauterine growth retartdation. J Clin Endocrinol Metab 84(4):1401-1406

Jarrard DF, Kinoshita H, Shi Y, Sanderfur C, Hoff D, Meisner LF, Chang C, Herman JG, Isaacs WB, Nassif N 1998 Methylation of the androgen receptor promoter CpG island is associated with loss of androgen receptor expression in prostate cancer cells. Cancer Res 58:5310-5314

Jenuwein T, Allis CD 2001 Translating the histone code. Science 293:1074-1080

Kadyrov M, Schitz V, Black S, Kaufmann P, Huppertz B 2003 Preeclampsia and maternal anaemia display reduced apoptosis and opposite invasive phyenotypes of extravillous trophoblast. Placenta 24:540-548

Kahl P, Gullotti L, Heukamp LC, Wolf S, Friedrichs N, Vorreuther R, Solleder G, Bastian PJ, Ellinger J, Metzger E, Schule R, Buettner R 2006 Androgen receptor coactivators lysine-specific histome demethylases 1 and four and a half LIM domain protein 2 predict risk of prostate cancer recurrence. Cancer Res 66:11341-11347

Kalluri, R, Weinberg, RA 2009 The basics of epithelial-mesenchymal transition. J Clin Inv 119(6):1420-1428

Kam EPY, Gardner L, Loke YW, King A 1999 The role of trophoblast in the physiological change in decidual spiral arteries. Hum Reprod 14:2131-2138.

Kanasaki K, Palmsten K, Sugimoto H, Ahad S, Hamano Y, Xie L, Parry S, Augustin HG, Gattone VH, Folkman J, Strauss JF, Kalluri F 2008 Deficiency in catechol-O-methyltransferase and 2-methoxyoestradiol is associated with pre-eclampsia. Nature 453:1117-1121

Kar M, Ghosh D, Sengupta J 2007 Histochemical and morphological examination of proliferation and apoptosis in human first trimester villous trophoblast. Hum Reprod 22:2814-2823

Karjalainen MK, Huusko JM, Ulvila J, Sotkasiira J, Luukkoren A, Teramo K, Plunkett J, Anttila V, Palotie A, Haataja R, Muglia LJ, Hallma M 2012 A potential novel spontaneous preterm birth gene, *AR*, identified by linkage and association analysis of X chromosomal markers. PLoS ONE 7(17):e51378

Karvela M, Stenfanakis N, Papadopoulou S, Tsitilou SG, Tsilivakos V, Lamnissou K 2008 Evidence for association of the G1733A polymorphism of the androgen receptor gene with recurrent spontaneous abortions. Fert Sterility 90(5):210.e9-.e12

Katari S, Turan N, Bibikova M, Erinle O, Chalian R, Foster M, Gaughan JP, Coutifaris C, Sapienza C 2009 DNA methylation and gene expression differeces in children conceived in vitro or in vivo. Hum Mol Genet 18:3769-3778

Kaufmann P, Castellucci M 1997 Extravillous trophoblast in the human placenta. Trophoblast Res 10:21-65

Kaufmann P, Black S, Huppertz B 2003 Endovascular Trophoblast Invasion: Implications for the Pathogenesis of Intrauterine Growth Retardation and Preeclampsia. Biol Reprod 69:1-7

Kaufmann P, Mayhew TM, Charnock-Jones DS 2004 Aspects of human fetoplacental vasculogenesis and angiogenesis. II. Changes during normal pregnancy. Placenta 25:114-126

Kawazu M, Saso K, Tong KI, McQuire T, Goto K, Son D-O, Wakeham A, Miyagishi M, Mak TW, Okada H 2011 Histone demethylase JMJD3B functions as a co-factor of estrogen receptor in breast cancer proliferation and mammary gland development. PLoS ONE 6(3):e17830

Keelan JA, Mattes E, Tan HW, Dinan A, Newnham JP, Whitehouse AJO, Jacoby P, Hickey M 2013 Androgen concentrations in umbilical cord blood and their association with maternal, fetal and obstetric factors. PLoS ONE 7(8):e42827 1-8

Kiiveri, S, Liu, J, Westerholm-ormio, M, Narita, N, Wilson, DB, Voutilinen, R, Heikinheimo, M 2002 Differential expression of GATA-4 and GATA-6 in fetal and adult mouse and human adrenal tissue. Endocrinology 143(8):3136-3143

Kim JK, Samaranayake M, Pradhan S 2009 Epigenetic mechanisms in mammals. Cell Mol Life Sci 66:596-659

King BF, Blankenship TN 1993 Expression of proliferating cell nuclear antigen (PCNA) in developing macaque placentas. Placenta 14:A36

King AJ, Olivier NB, Mohankumar PS, Lee JS, Padmanabhan V, Fink GD 2007 Hypertensioncaused by prenatal testosterone excess in female sheep. Am J Physiol Endocrinol Metab 292:E1837-E1841

Kinoshita H, Shi Y, Sandefur C, Meisner LF, Chang C, Choon A, Reznikoff CR, Bova GS, Friedl A, Jarrard DF 2000 Methylation of the androgen receptor minimal promoter silences transcription in human prostate cancer. Cancer Res 60:3623-3630

Klose RJ, Yamane K,Bae Y, Zhang D, Erdjument-Bromage H, Tempst P, Wong J, Zhang Y 2006 The transcriptional repressor JHDM3A demethylates trimethyl histone H3 lysine 9 and lysine 36. Nature 442:312-316

Klose RJ, Bird AP 2007 Genomic DNA methylation: the mark and its mediators. Trends Biochem Sci 31:89-97

Knobil E, Neill JD 1998 Placenta: Implantation and Development. Enc Reprod. Academic Press: San Diego. 3:848-855

Korgun ET, Celik-Ozenci C, Acar N, Cayli S, Desoye G, Demir R 2006 Location of cell cycle regulators cyclin B1, cyclin A, PCNA, Ki67, and cell cycle inhibitors p21, p27 and p57 in human first trimester placenta and deciduas. Histochem Cell Biol 125:615-624

Kouzarides T 2002 Histone methylation in transcriptional control. Curr Op Genet Dev 12:198-209

Kouzarides T 2007 Chromatin modifications and their function. Cell 128:693-705

Kumar P, Kamat A, Mendelson CR 2009 Estrogen receptor alpha (ERα) mediates stimulatory effects of estrogen on *aromatase* (CYP19) gene expression in human placenta. Mol Endocrinol 23(6):784-793

Larrea F, DÍaz L, Cariño C, Larriva-Sahd J, Carrillo L, Orozoco H, Ulloa-Aguirre A 1993 Evidence that human placenta is a site of sex hormone-binding globulin gene expression. J Steroid Biochem Mol Biol 46(4):497-505

Larriva-Sahd J, Orozco H, Hernández-Pando R, Oliart RM, Musto NA, Larrea F 1991 Immunohistochemical demonstration of androgen-binding protein in the rat prostatic gland. Biol Reprod 45:417-423

Lee YJ, Lee EB, Kwon YE, Lee JJ, Cho WS, Kim HA, Song YM 2003 Effect of estrogen on the expression of matrix metalloproteinase (MMP)-1, MMP-3, and MMP-13 and tissue inhibitor of metalloproternase-1 in osteoarthritis chondrocytes. Rheumatol Int 23:282-288

Lee SB, Wong AP, Kanasaki K, Xu Y, Shenoy VK, McElrath TF, Whitesides GM, Kalluri R 2010 2-Methoxyestradiol induces cytotrophoblast invasion and vascular development specifically under hypoxic conditions. Epithelial and Mesenchymal Cell Biology-Preeclampsia. Am J Pathol 176(2):710-720

Leighton PA, Ingram RS, Eggenschwiler J, Efstratiadis A, Tilgham SM 1995 Disruption of imprinting caused by deletion of the H19 gene region in mice. Nature 375:34-39

Leiser R, Kaufmann P 1994 Placental structure: in a comparative aspect. Exp Clin Endocrinol 102:122-134

Leung PCK, Steele GL 1992 Intracellular signaling in the gonad. Endocrine Rev 13(3):476-498

Li E 2002 Chromatin modification and epigenetic reprogramming in mammalian development. Nature Rev 3:662-673

Liao X, Thrasher B, Pelling J, Holzbeierlein J, Sang Q-XA, Li B 2003 Androgen stimulates matrix metalloproteinase-2 expression in human prostate cancer. Cancer 144(5):1656-1663

Lim JH, Kim SY, Lee SW, Park SY, Han JY, Chung JH, Kim MY, Yang JH, Ryu HM 2011 Association between genetic polymorphisms in androgen receptor gene and risk of preeclampsia in Korean women. J Assisted Reprod Genet 28:85-90

Limaye AM, Desai KV, Chavalmane AK, Kondaiah P 2008 Regulation of mRNA encoding MMP-9 and MMP-2, and their inhibitros TIMP-1 and TIMP-2 by androgens in the rat ventral prostate. Mol Cell Endocrinol 294(1-2):10-18

Lissbrant IF, Hammarsten P, Lissbrant E, Ferrara N, Rudolfsson SH, Bergh A 2004 Neutralizing VEGF bioactivity with a soluble chimeric VEGF-receptor protein flt(1-3) IgG inhibits testosterone-stimulated prostate growth in castrated mice. Prostate 58:57-65

Loriaux DL, Ruder HJ, Knab DR, Lipsett MB 1972 Estrone sulfate, estrone, estradiol and estriol plasma levels in human pregnancy. J Clin Endocrinol Metab 35(6):887-891

Lorzadeh N, Kazemirad S 2012 The effects of fetal gender on serum human chorionic gonadotropin and testosterone in normotensive and preeclamptic placentas. J Pregnancy ID874290:1-6

Luense LJ, Veiga-Lopez A, Padmanabhan V, Christenson LK 2011 Developmental programming: gestational testosterone treatment alters fetal ovarian gene expression. Endocrinology 152(12):4974-4983

Lunghi L, Ferretti ME, Medici S, Biondi C, Vesce F 2007 Control of human trophoblast function. Reprod Biol Endocrinol 5:6

Lyall F 2006 Mechanisms regulating cytotrophoblast invasion in normal pregnancy and pre-eclampsia. Aust NZ J Obstet Gynaecol 46:266-273

Ma XH, Wu WX, Nathanielsz PW 1999 Differential effects of natural and synthetic glucocorticoids on cytochrome 17α-hydroxylase ($P\text{-}450_{17\alpha}$) and cytochrome P-450 side-chain cleavage (P_450_{scc}) messenger ribonucleic acid in the sheep placenta. Am J Obstet Gynecol 180(5):1215-1221

Mabjeesh NJ, Escuin D, LaVallee TM, Pribluda VS, Swrtz GM, Johson MS, Willard MT, Zhong H, Simons JW, Giannakakou P 2003a 2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and disregulating HIF. Cancer Cell 3:363-375

Mabjeesh NJ, Willard MT, Fredrickson CE, Zhong H, Simons JW 2003b Androgens stimulate hypoxia-inducible factor 1 activation via autocrine loop of tyrosine kinase receptor/phosphatidylinositol 3'-kinase/protein kinase B in prostate cancer cells. Clin Cancer Res 9:2416-2425

Maccani MA, Marsit CJ 2009 Epigenetics in the placenta. Am J Reprod Immunol 62:78-89

Malassine A, Frendo JL, Evan-Brion D 2003 A comparison of placental development and endocrine functions between the human and mouse model. Hum Reprod Update 9(6):531-539

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM 1995 The nuclear receptor superfamily: the second decade. Cell 83:835-839

Manin M, Veyssiere G, Cheyvialle D, Chevalier M, Lecher P, Jean C 1992 In vitro androgenic induction of a major protein in epithelial cell subcultures from mouse vas deferens. Endocrinology 131:2378-2386

Manin M, Martinez A, Van Der Schueren B, Reynaert I, Jean C 2000 Acquisition of androgen-mediated expression of mouse vas deferens protein (MVDP) gene in cultured epithelial cells and in vas deferens during postnatal development. J Andrology 21:641-650

Manin M, Baron S, Goossens K, Beaudoin C, Jean C, Veyssiere G, Verhoeven G, Morel L 2002 Androgen receptor expression is regulated by the phophoinositide 3-kinase/Akt pathway in normal and tumoral epithelial cells. Biochem J 366:729-736

Manikkam M, Crespi EJ, Doop DD, Herkimer C, Lee JS, Yu S, Brown MB, Foster DL, Padmanabhan V 2004 Fetal programming: prenatal testosterone excess leads to fetal growth retardation and postnatal catch-up growth in sheep. Endocrinology 145(2):790-798

Manikkam M, Thompson RC, Hrkimer C, Welch KB, Flak J, Karsch FJ, Padmanabhan V 2008 Developmental programming: impact of prenatal testosterone excess on pre- and postnatal gonadotropin regulation in sheep. Biol Reprod 78:648-660

Manzetti S, McCulloch DR, Herignton AC, van der Spoel D 2003 Modeling of enzyme-substrate complexes for the metalloprotease MMP-3, ADAM-9, and ADAM-10. J Computer-Aided Mol Design 17:551-565

Marin-Castaño ME, Elliot SJ, Potier M, Karl M, Striker LJ, Stiker GE, Csaky KG, Cousins SW 2003 Regulation of estrogen receptors and MMP-2 expression by estrogen in human retinal pigment epithelium. Invest Ophthalmol Visual Sci 44(1):50-59

Masek KS, Wood RI, Foster DL 1999 Prenatal dihydrotestosterone differentially masculinizes tonic and surge modes of luteinizing hormone secretion in sheep. Endocrinology 140:3459-3466

Mastorakos G, Ilias I 2003 Maternal and fetal hypothalamic-pituitary-adrenal axes during pregnancy and postpartum. Ann NY Acad Sci 997(1):136-149

Metzger E, Wissmann M, Yin N, Müller JM, Schneider R, Peters AH, Günther T, Buettner R, Schüle R 2005 LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. Nature 437:436-439

Metzger E, Wissmann M, Schüle R 2006 Histone demethylation an androgen-dependent transcription. Curr Op Genet Dev 16:513-517

Miller WL, Auchus RJ 2010 The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. Endocrine Rev 32(1):81-151

Mizuno M, Lobotsky J, Lloyd CW, Kobayashi T, Murasawa Y 1968 Plasma androstenedione and testosterone during pregnancy and in the newborn. J Clin Endocrinol Metab 28(8):1133-1142

Moffett A, Hiby SE 2007 How does the maternal immune system contribute to the development of pre-eclampsia. Placenta 28:S51-S56

Moll W, Nienartowicz A, Hees H, Wrobel K-H, Lenz A 1988 Blood flow regulation in the uteroplacental arteries. Trophoblast Res 3:83-96

Mondragón JA, Valdez RA, Gómez, Rosales AM, Romano MC 2012 Study of the steroidogenic pathways involved in goat placental androgen and estrogen synthesis. Small Rumin Res 106:173-177

Monk M, Boubelik M, Lehnert S 1987 Temporal and regional changes in DNA methylation in the embryonic, extra-embryonic, and germ cell lineages during mouse embryo development. Development 99:371-382

Monk M, Adams RLP, Rinaldi A 1991 Decrease in DNA methylase activity during preimplantation development in the mouse. Development 112:189-192

Monk M 1995 Epigenetic programming of differential gene expression in development and evolution. Dev Genet 17:188-197

Moore T, Haig D 1991 Genomic imprinting in mammalian development: a parental tug-of-war. Trends Genet 7:45-49

Moore T, Constancia M, Zubair M, Bailleul B, Feil R, Sasaki H, Reik W 1997 Multiple imprinted sense and antisense transcripts, differentialy methylation and tandem repeats in a putative imprinting control region upstream of mouse *Igf2*. Proc Natl Acad Sci USA 94(23):12509-12514

Morishima A, Grumbach MM, Simpson ER, Fisher C, Qin K 1995 Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. J Clin Endocrinol Metab 80:3689-3698

Morrison JL 2008 Brief review: sheep models of intrauterine growth restriction: fetal adaptations and consequences. Clin Exp Pharmacol Physiol 35:730-743

Mueller MD, Vigne J-L, Minchenko A, Leboric DI, Leitman DC, Taylor RN 2000 Regulation of vascular endothelial growth factor (VEGF) gene transcription by estrogen receptors α and β. Pro Natl Acad Sci 97(20):10972-10977

Munaut C, Noel A, Hougrand O, Foidart J-M, Boniver J, Deprez M 2003 Vascular endothelial growth factor expression correlates with matrix metalloproteinases MT1-MMP, MMP-2 and MMP-9 in human glioblastomas. Int J Cancer 106:848-855

Nanaev AK, Chwalisz K, Frank HG, Kohnen G, Hegele-Hartung C, Kaufmann P 1995 Physiological dilation of uteroplacental arteries in the guinea pig depends on nitric oxide synthase activity of extracillous trophoblast. Cell Tissue Res 282:407-421

Nelson SE, McLean MP, Jayatilak PG, Gibori G 1992 Isolation, characterization, and culture of cell subpopulations forming the pregnant rat corpus luteum. Endocinology 130(2):954-966

Norwitz ER, Schust DJ, Fisher SJ 2001 Implantation and the survival of early pregnancy. N Engl J Med 345:400-1408

Ortega HH, Salvetti NR, Padmanabhan V 2009 Developmental programming: prenatal androgen excess disrupts ovarian steroid receptor balance. Reproduction 137:865-877

Oudejans CBM, Mulders J, Lachmeijer AMA, van Dijk M, Könst AAM, Westerman BA, van Wijk IJ, Leegwater PAJ, Kato HD, Matsuda T, Wake N, Dekker GA, Pals G, ten Kate LP, Blankenstein MA 2004 The parent-of-origin effect of 10q22 in pre-eclamptic famales coincides with two regions clustered for genes with down-regulated expression in androgenetic placentas. Mol Hum Reprod 10(8):589-598

Padmanabhan V, Manikkam M, Recabarren S, Foster D 2006 Prenatal testosterone excess programs reproductive metabolic dysfunction in the female. Mol Cell Endocrinol 246:165-174

Padmanabhan V, Sarma HN, Savabieasfahani M, Steckler TL, Veiga-Lopez A 2010 Developmental reprogramming of reproductive and metabolic dysfuction in sheep: native steroids vs. environmental steroid receptor modulators. Int J Andro 33:394-404

Padmanabhan V, Veiga-Lopez A 2011 Developmental origin of reproductive and metabolic dysfunctions: androgenic vs. estrogenic reprogramming. Semin Reprod Med 29:173-186

Palvimo JJ, Kallio PJ, Ikonen T, Mehto M, Janne OA 1993 Dominant negative regulation of trans-activation by the rat androgen receptor: roles of the N-terminal domain and heterodimer formation. Mol Endocrinol 7:1399-1407

Panet-Raymond V, Gottlieb B, Beitel LK, Pinsky L, Trifiro MA 2000 Interactions between androgen and estrogen receptors and the effects on their transactivational properties. Mol Cell Endo 167:139-150

Payne AH, Hales DB 2004 Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocrine Rev 25:947-970

Perillo B, Ombra MN, Bertoni A, Cuozzo C, Sacchetti S, Sasso, A, Chiariotti L, Malorni A, Abbondanza C, Accedimento EV 2008 DNA Oxidation as triggered by H3K9me2 demethylation drives estrogen-induced gene expression. Science 319:202-206

Peterson CL, Laniel M-A 2004 Histones and histone modifications. Curr Biol 14(14):R546-R551

Petra PH 1991 The plasma sex steroid binding protein (SBP or SHBG). A critical review of recent developments on the structure, molecular biology and function. J Steroid Biochem Mol Biol 40:735-753

Petraglia F, Florio P, Nappi C, Genazzni AR 1996 Peptide signaling in human placenta and membranes: autocrine, paracrine, and endocrine mechanisms. Endocrine Rev 17:156-186

Pijnenborg R, Robertson W, Brosens I, Dixon G 1981 Trophoblast invasion and the establishment of haemochorial placentation in man and laboratory animals. Placenta 2:71-91

Plath K, Fang J, Mynarczyk-Evans SK, Cao R, Worringer KA, Wang H, de la Cruz CC, Otte AP, Panning B, Zhang Y 2003 Role of histone H3 lysine 27 methylation in X inactivation. Science 300(5616):131-135

Prossnitz ER, Oprea TI, Sklar LA, Arterburn JB 2008 The ins and outs of GPR30: a transmembrane estrogen receptor. J Steroid Biochem Mol Biol 109:350-353

Purcell SH, Cantlon JD, Wright CD, Henkes LE, Seidel GE, Anthony RV 2009 The involvement of proline-rich 15 in early conceptus development in sheep. Biol Reprod 81(6):1112-1121

Quigley CA, De Bellis A, Marschke KB, El-Awady MK, Wilson EM, French FS 1995 Androgen receptor defects: historical, clinical, and molecular perspectives. Endocr Rev 16:271-321

Quirke LD, Juengel JL, Tisdall DJ, Lun W, Heath DA, McNatty KP 2001 Ontogeny of steroidogenesis in the fetal sheep gonad. Biol Reprod 65(1):216-228

Rama S, Petrusz P, Rao AJ 2004 Hormonal regulation of human trophoblast differentiation: a possible role for 17β-estradiol and GnRH. Mol Cell Endocrinol 218:79-94

Razin A, Riggs AD 1980 DNA methylation and gene function. Science 210:604-610

Recabarren SE, Padmanabhan V, Codner E, Lobos A, Durán C, Vidal M, Foster DL, Sir-Petermann T 2005 Postnatal developmental consequences of altered insulin sensitivity in female sheep treated prenatally with testosterone. Am J Physiol Endocrinol Metab 289(5):E801-E806

Recabarren SE, Lobos A, Figueroa Y, Padmanabhan V, Foster DL, Sir-Peterman T 2007 Prenatal testosterone treatment alters LH and testosterone responsiveness to GnRH agonist in male sheep. Biol Res 40:329-338

Redman CWG 1990 Platelets and the beginnings of pre-eclampsia. N England J Med 323:478-480

Redman CW 1991 Current topic: pre-eclampsia and the placenta. Placenta 12(4):301-308

Redman CWG, Saks GP, Sargent IL 1999 Preeclampsia: an excessive maternal inflammatory response to pregnancy. Am J Obstet Gynecol 180(2):499-506

Redman CWG, Sargent IL 2003 Pre-eclampsia, the placenta and the maternal systemic inflammatory response. Placenta 24(A):521-527

Redman CW, Sargent IL 2005 Latest advances in understanding preeclampsia. Science 308:1592-1594

Reik W, Dean W, Walter J 2001 Epigenetic reprogramming in mammalian development. Science 293:1089-1093

Reik W, Walter J 2002 Genomic imprinting: parental influence on the genome. Nat Rev Cenet 2:21-32

Reik W, Constancia M, Fowden A, Anderson N, Dean W, Ferguson-Smith A, Tycko B, Sibley C 2003 Regulation of supply and demand for maternal nutrients in mammals by imprinted genes. J Physiol 547:35-44

Rembiesa R, Marchut M, Warchol A 1971 Formation and metabolism of progesterone by the mouse placenta in vivo. J Steroid Biochem 2:111-119

Renaud SJ, Kubota K, Rumi MAK, Soares MJ 2014 The FOS transcription factor family differentially controls trophoblast migration and invasion. J Biol Chem 289(8):5025-5039

Renfree MB 1982 Implantation and placentation. Reproduction in Mammals. Eds Austin CR, Short RV. Cabridge University Press:Cambridge. 26-69

Resnik R 2002 Intrauterine growth restriction. Obstet Gynecol 99(3):490-496

Rice JC, Allis CD 2001 Histone methylation versus histone acetylation: new insights into epigenetic regulation. Curr Opin Genet Dev 13:263-273

Ricker JL, Chen Z, Yang XP, Pribluda VS, Swartz GM, Van Waes C 2004 2-Methoxyestradiol inhibits hypoxia-inducable factor 1alpha, tubor growth, and angiogenesis and augments paclitaxel efficacy in head and neck squamous cell carcinoma. Clin Cancer Res 10:8665-8673

Riley SC, Webb CJ, Leask R, McCaig FM, Howe DC 2000 Involvement of matrix metalloproteinases 2 and 9, tissue inhibitor of metalloproteinases and apoptosis in tissue remodeling in the sheep placenta. Reproduction 118:19-27

Roberts JM, Taylor RN, Friedman SA, Goldfien A 1993 Fetal Medical Review. Ed. W. Dunlop. Arnold: London

Roberts JM, Redman CWG 1993 Pre-eclampsia: more than pregnancy induced hypertension. Lancet 341(8858):1447-1451

Roberts JM, Cooper DW 2001 Pathogenesis and genetics of pre-eclampsia. Lancet 357:53-56

Robins JC, Jeizer A, Hardiman A, Hubert M, Handwefer S 2007 Oxygen tension directs the differentiation pathway of human cytotrophoblast cells. Placenta 28:1141-1146

Robinson JE, Forsdike RA, Taylor JA 1999 In utero exposure of female lambs to testosterone reduces the sensitivity of the gonadotropin-releasing hormone neuronal network to inhibition by progesterone. Endocrinology 140:5797-5805

Robinson JE, Birch RA, Taylor JA, Foster DL, Padmanabhan V 2002 In utero programming of sexually differentiated gonadotrophin releasing hormone (GnRH) secretion. Dom Anim Endocrinol 23:43-53

Rodeck CH, gill D, Rosenberg DA, Collins WP 1985 Testosterone levels in midtrimester maternal and fetal plasma and amniotic fluid. Prenatal Diagn 5(3):175-181

Rodesch F, Simon P, Donner C, Jauniaux E 1992 Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. Obstet Gynecol 80:283-285

Rosario GX, Konno T, Soares MJ 2008 Maternal hypoxia activates endovascular trophoblast cell invasion. Dev Biol 314:362-375

Ross JP, Suetake I, Tajima S, Molloy PL 2010 Recombinant mammalian DNA methyltransferase activity on model transcriptional gene silencing short RNA-DNA heteroduplex substrates. Biochem J 432:323-332

Rowson LE, Moor RM 1966 Development of the sheep conceptus during the first fourteen days. J Anat 100:777-785

Ryan KJ 1959 Biological aromatization of steroids. J Biol Chem 234:268-272

Salamalekis E, Bakas P, Vitoratos N, Eleptheriadis M, Creatsas G 2006 Androgen levels in the third trimester of pregnancy in patients with preeclampsia. Eur J Obstet Gynecol Reprod Biol 126:16-19

Salvetti NR, Ortega HH, Veiga-Lopez A, Padmanabhan V 2012 Developmental programming: impact of prenatal testosterone excess on ovarian cell proliferation and apoptotic factors in sheep. Biol Reprod 87(1):22,1-10

Sarma HN, Manikkam M, Herkimer C, Dell'Orco J, Welch KB, Foster DL, Padmanabhan V 2005 Fetal programming: excess prenatal testosterone reduces postnatal luteinizing hormone, but not follicle-stimulating hormone responsiveness, to estradiol negative feedback in the female. Endocrinology 146:4281-4291

Sathishkumar K, Elkins R, Yallampalli U, Yallampalli C 2009 Elevated androgen levels during pregnancy impair fetal growth due to placental insufficiency and programs for adult hypertension in rats. Society for the Study of Reproduction Abstract. Biol Reprod 81:250

Sathishkumar K, Elkins R, Chinnathambi V, Gao H, Hankins GDV, Yallampalli C 2011 Prenatal testosterone-induced fetal growth restriction is associated with down-regulation of rat placental amino acid transport. Reprod Biol Endocrin 9:110-122

Sathishkumar K, Balakrishnan M, Chinnathambi V, Chauhan M, Hankins GDV, Yallampalli C 2012 Fetal sex-related dysregulation in testosterone production and their receptor expression in the human placenta with preeclampsia. J Perinat 32:328-335

Schiessl B, Mylonas I, Kuhn C, Kunze S, Schulze S, Friese K, Jeschke U 2006 Expression of estrogen receptor-alpha, estrogen receptor-beta and placental endothelial and inducible NO synthase in intrauterine growth restricted and normal placentals. Arch Med Res 37:967-975

Senger PL 2003 Pathways to pregnancy and parturition. 2nd Ed. Current Conceptions, Inc:Pullman

Serin IS, Kula M, Başbug M, Ünlühizarci K, Güçer Ş, Tayyar M 2001 Androgen levels of preeclamptic patients in the third trimester of pregnancy and six weeks after delivery. Acta Obstet Gynecol Scand 80:1009-1013

Shabisgh A, Tanji N, D'Agati V, Burchardt M, Rubin M, Goluboff ET, Heitjan D, Kiss A, Buttyan R 1999 Early effects of castration on the vascular system of the rat ventral prostate gland. Endocrinology 140:1920-1926

Sharma TP, Herkimer C, West C, Ye W, Birch R, Robinson JE, Foster DL, Padmanabhan V 2002 Fetal programming: prenatal androgen disrupts positive feedback actions of estradiol but does not affect timing of puberty in female sheep. Biol Reprod 66:924-933

Shi Y 2007 Histone lysine demethylases emerging roles in development physiology and disease. Nature Rev Genet 8:829-833

Shi Y, Whetshine JR 2007 Dynamic regulation of histone lysine methylation by demethylases. Mol Cell 25(1):1-14

Shilatifard A 2006 Chromatin modifications by methylation and ubiquitination: implications in the regulation of gene expression. Ann Rev Biochem 75:243-269

Shin S, Janknecht R 2007a Activation of androgen receptor by histone demethylases JMJD2A and JMJD2D. Biochem Bioph Res Co 359:742-746

Shin S, Janknecht R 2007b Diversity within the JMJD2 histone demethylases family. Biochem Bioph Rsearch Co 353:973-977

Shozu M, Akasofu K, Harada T, Kubota Y 1991 A new casue of female pseudohermaphroditism: placental aromatase deficiency. J Clin Endocrinol Metab 72:560-566

Sibai B, Dekker G, Kupferminc M 2005 Pre-eclampsia. Lancet 365(9461):785-799

Sibley CP, Coan PM, Ferguson-Smith AC, Dean W, Hughes J, Smith P, Reik W, Burton GJ, Fowden AL, Constância M 2004 Placental-specific insulin-like growth factor 2 (IGF2) regulates the diffusional exchange characteristic of the mouse placenta. Proc Natl Acad Sci USA 101:8204-8208

Siiteri PK, MacDonald PC 1963 The utilization of circulating dehydroisoandrosterone sulfate for estrogen synthesis during human pregnancy. Steroids 2(6):713-730

Siiteri PK, MacDonald PC 1966 Placental estrogen biosynthesis during human pregnancy. J Clin Endocrinol Metab 26(7):751-761

Silva J, Mak W, Zvetkova I, Appanah R, Nesterova TB, Webster Z, Peters AHFM, Jenuwein T, Otte AP, Brockdorff N 2003 Establishment of histone H3 methylation on the inactive X chromosome requires transient recruitment of Eed-Enx1 polycomb group complexes. Develop Cell 4(4):481-495

Sir-Petermann T, Maliqueo M, Angel B, Lara HE, Pérez-Bravo F, Recabarren SE 2002 Maternal serum androgens in pregnant women with polysistic ovarian syndrome: possible implications in prenatal androgenization. Hum Reprod 17(10):2573-2579

Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med 331(16):1056-1061

Snoek-van Beurden RAM, Von den Hof JW 2005 Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors. BioTechniques 38:73-83

Solberg H, Rinkenberger J, Danø K, Lund LR 2003 A functional overlap of plasminogen and MMPs regulates vascularization during placental development. Development 130: 4439-4450

Solomon S 1994 The placenta as an endocrine organ: steroids. Physiology of Reproduction. 2nd Ed. Eds Knobil E, Neill JD. Raven Press:New York. 2:863-873

Spencer TE, Bazer FW 2002 Biology of progesterone action during pregnancy recognition and maintenance of pregnancy. Front Biosci 7:1879-1898

Spencer TE, Johnson GA, Bazer FW, Burghardt RC 2004 Implantation mechanisms: insights from the sheep. Reprod Rev 128:657-668

Stanczky FZ, Namkung PL, Fritz MA, Novy MJ, Petra PH 1986 The influence of sex steroid binding protein on the metabolic clearance rate of testosterone. In Binding Proteins of Steroid Hormones. Eds Forest MG, Pugeat M. Colloque/INSERM. John Libbey:London. 149:555-563

Steckler T, Wang J, Bartol FF, Roy SK, Padmanabhan V 2005 Fetal programming: prenatal testosterone treatment causes intrauterine growth retardation, reduces ovarian reserve and increases ovarian follicular recruitment. Endocrinol 146(7):3185-3193

Stein R, Gruenbaum Y, Pollack Y, Razin A, Cedar H 1982 Clonal inheritance of the pattern of DNA methylation in mouse cells. Proc Natl Acad Sci USA 79:61-65

Stephenson CD, Lockwood CJ, Ma Y, Guller S 2005 Throbin-dependent regulation of matrix metalloproteinase (MMP)-9 levels in human fetal membranes. J Maternal-Fetal Neonat Med 18(1):17-22

Stoffer RL, Nixon WE, Gulyas BJ, Hodgen GD 1977 Gonadotropin-sensitive progesterone production by Rhesus monkey luteal cells in vitro: a function of age of the corpus luteuim during the menstrual cycle. Endocrinology 100(2):506-512

Strahl BD, Allis CD 2000 The language of covalent histone modification. Nature 403:41-45

Strauss JF, Martinez F, Kiriakidou M 1996 Placental steroid hormone synthesis: unique features and unanswered questions. Biol Reprod 54:303-311

Thiery JP 2003 Epithelial-mesenchymal transitions in development and pathologies. Curr Op Cell Biol 15:740-746

Thomas P 2008 Characteristics of membrane progestin receptor alpha (mPRα) and progesterone membrane receptor component one (PGMRC1) and their roles in mediating rapid progestin actions. Front Neuroendocrinol 29(2):292-312

Thompson EA, Siiteri PK 1974a The involvement of human placental microsomal cytochrome P-450 in aromatization. J Biol Chem 249:5373-5378

Thompson EA, Siiteri PK 1974b Utilization of oxygen and reduced nicotinamide adenine dinucleotide phosphate by human placental microsomes during aromatization of androstenedione. J Biol Chem 249:5364-5372

Thoumsin HJ, Alsat E, Cedard L 1982 In vitro aromatization of androgens into estrogens in placental insufficiency. Gynecol Obstet Invest 13(1):37-43

Titus MA, Zeithaml B, Kantor B, Li X, Haack K, Moore DT, Wilson EM, Mohler JL, Kafri T 2012 Dominant-negative androgen receptor inhibition of intracrine androgen-dependent growth of castration-recurrent prostate cancer. PLoS ONE 7(1):e30192

Torry DS, Hinrich N, Torry RJ 2004 Determinants of placental vascularity. Am J Reprod Immunol 51:257-268

Tuckey RC, Bose HS, Czerwionka I, Miller WL 2004 Molten globule structure and steroidogenic activity of N-218 MLN64 in human placenta mitochondria. Endrocrinology. 145:1700-1707

Tulchinsky D, Hobel CJ 1953 Plasma human chorionic gonadotropin, estrone, estradiol, estiol, progesterone, and 17-alpha-hydroxyprogesterone in human pregnancy. 3. Early Normal Pregnancy. Am J Obstet Gynecol 117(7):884-893

Tuutti EK, Hämäläinen EK, Sainio SM, Hiilesmaa VK, Turpeinen UL, Alfthan HV, Stenman U-HE 2011 Serum testosterone levels during early pregnancy in patients developing preeclampsia. Scand J Clin Lab Inv 71:413-418

Tycko B, Morison IM 2002 Physiological functions of imprinted genes J Cell Physiol 192:245-258

Tycko B 2006 Imprinted genes in placental growth and obstetric disorders. Cytogenetic Genome Res 113:271-278

Unsworth WP, Taylor JA, Robinson JE 2005 Prenatal programming of reproductive neuroendocrine function: the effect of prenatal androgens on the development of estrogen positive feedback and ovarian cycles in the ewe. Biol Reprod 72:619-627

van der Linden DS, Sciascia Q, Sales F, McCord SA 2012 Placental nutrient transport is affected by pregnancy rank in sheep. J Ani Sci 91(2):644-653

van Oppenraaij RH, Koning AH, Lisman BA, Boer K, van den Hoff JJ, van der Spek PJ, Steegers EA, Exalto N 2009 Vasculogenesis and angiogenesis in the first trimester human placenta: an innovative 3D study using an immersive virtual reality system. Placenta 30:220-222

Vanselow J, Zsolnai A, Fésüs L, Fürbass R, Schwerin M 1999 Placenta-specific transcripts of the aromatase encoding gene include different untranslated first exons in sheep and cattle. Eur J Biochem 265(1)318-324

Vatnick I, Scholknecht R, Darrigrand R, Bell AW 1991 Growth and metabolism of theplacenta after unilateral fetectomy in twin pregnant ewes. J Dev Physiol 15:351-356

Vaughan OR, Sferruzzi-Perri AN, Coan PM, Fowden AL 2012 Environmental regulation of placental phenotype: implications for fetal growth. Reprod Fert Dev 24:80-96

Veiga-Lopez A, Ye W, Phillips DJ, Herkimer C, Knight PG, Padmanaghan V 2008 Developmental programming: deficits in reproductive hormone dynamics and ovulatory outcomes in prenatal, testosterone-treated sheep. Biol Reprod 78:636-647

Veiga-Lopez A, Astapova OI, Aizenberg EF, Lee JS, Padmanabhan V 2009 Developmental programming: contribution of prenatal androgen and estrogen to estradiol feedback systems and periovulatory hormonal dynamics in sheep. Biol Reprod 80:718-725

Veiga-Lopez A, Steckler TL, Abbott D, Welch KB, MohanKumar PS, Phillips DJ, Refsal K, Padmanabhan V 2011 Developmental programming: impact of excess prenatal testosterone on intrauterine fetal endocrine milieu and growth in sheep. Biol Reprod 84:87-96

Verdel A, Jia S, Gerber S, Sugiyama T, Gygi S, Grewal SI, Moazed D 2004 RNAi-mediated targeting of heterochromatin by the RITS complex. Science 303:672-676

Verrier L, Vandromme M, Trouche D 2011 Histone demethylases in chromatin cross-talks. Biol Cell 103(8):381-401

Vicovac L, Aplin JD 1996 Epithelial-mesenchymal transition during trophoblast differentiation. Cells Tissues Organs 156(3):202-216

Vonnahme KA, Hess BW, Nijland MJ, Nathanielsz PW, Ford SP 2006 Placentomal differentiation may compensate for maternal nutrient restriction in ewes adapted to harsh range conditions. J Anim Sci 84:3451-3459

Vonnahme KA, Arndt WJ, Johnson ML, Borowicz PP, Reynolds LP 2008 Effect of morphology on placentome size, vascularity, and vasoreactivity in late pregnant sheep. Biol Reprod 79:976-982

Wagschal A, Feil R 2006 Genomic imprinting in the placenta. Cytogenet Genomic Res 113:90-98

Wagschal A, Sutherland HG, Woodfine K, Henckel A, Chebli K, Schulz R, Oakey RJ, Bickmore WA, Feil R 2008 G9a histone methyltransferase contributes to imprinting in the ouse placenta. Mol Cell Biol 28: 1104-1113

Wang J, Scully K, Zhu X, Cai L, Zhang J, Prefontain GG, Krones A, Ohgi KA, Zhu P, Garcia-Bassets I, Liu F, Taylor H, Lozach J, Jayes FL, Korach KS, Flass CK, Fu X-D, Rosenfeld 2007 Opposing LSD1 complexes function in developmental gene activation and repression programs. Nature 466:882-887

Weng Q, Medan MS, Ren LQ, Watanabe G, Tsubota T, Taya K 2005 Immunolocalization of steroidogenic enzymes in the corpus luteum and placenta of the Japanese Shiba goat. J Reprod Dev 51(2):247-252

Wieciech I, Durlej-Grzesiak M, Stomczyńska M 2013 Influence of the antiandrogen flutamide on the androgen receptor gene expression in the placenta and umbilical cord during pregnancy in the pig. Acta Histochem 115:290-295

Wigler M, Levy D, Perucho M 1981 The somatic replication of DNA methylation. Cell 24:33-40

Wilkins JF, Haig D 2003 What good is genomic imprinting: the function of parent-specific gene expression. Nat Rev Genet 4:359-368

Wilson JD, Harrod MJ, Goldstein JL, Hemsell DL, MacDonald PC 1974 Familial incomplete male pseudohermaphroditism, Type 1: evidence for androgen resistance and variable clinical manifiestations in a family with the Reifenstein syndrome. N Engl J Med 290:1097-1103

Wilson AS, Power BE, Molloy PL 2007 DNA hypomethylation and human diseases. Biochem Bioph Acta 1775:138-162

Wimsatt WA 1950 New histological observation of the placenta of sheep. Am J Anat 87:391-436

Wissmann M, Yin N, Muller JM, Greschik H, Fodor BD, Jenuwein T, Vogler C, Schneider R, Gunther T, Buettner R, Metzger E, Schule R 2007 Cooperative demethylation by JMJD2C and LSD1 promotes androgen receptor-dependent gene expression. Nat Cell Biol 9:347-353

Wood RI, Foster DL 1998 Sexual differentiation of reproductive neuroendocrine function in sheep. Rev Reprod 3:130-140

Wooding FB 1984 Role of binucleate cells in fetomaternal cell fusion at implantation in the sheep. Am J Anat 170:233-250

Wooding FBP 1992 The synepitheliochorial placenta of ruminants: binucleate cell fusions and hormone production. Placenta 13:101-113

Wooding FBP, Morgan G, Monaghan S, Hamon M, Heap RB 1996 Functional specialization in the ruminant placenta: evidence for two populations of fetal binucleate cells of different selective synthetic capacity. Placenta 17(1):75-86

Wu G, Bazer FW, Wallace JM, Spencer TE 2006 Board-invited review: Intrauterine growth retardation: implications for the animal sciences. J Anim Sci 84:2316-2337

Wu Y, Zhao W, Zhao J, Pan J, Wu Q, Zhang Y, Bauman WA, Cardozo CP 2007 Identification of androgen response elements in the insulin-like growth factor I upstream promoter. Endocrinology 148:2984-2993

Xita N, Tsatsoulis A 2006 Fetal programming of polycystic ovary syndrome by androgen excess: evidence from experimental, clinical, and genetic association studies. J Clin Endocrinol Metab 91(5):1660-1666

Xue Q, Lin Z, Cheng YH, Huang CC, March E, Yin P, Milad MP, Confino E, Reierstad S, Innes J, Bulun SE 2007 Promoter methylation regulates estrogen receptor 2 in human endometrium and endometriosis. Biol Reprod 77:681-687

Yamane K, Toumazou C, Tsukada Y, Erdjument-Bromage H, Tempst P, Wong J, Zhang Y 2006 JHDM2A, a JmjC-containing H3K9 demthylase, facilitates transcription activation by androgen receptor. Cell 125:483-495

Yang J, Jubb AM, Pike L, Buffa FM, Turley H, Baban D, Leek R, Gatter KC, Ragoussis J, Harris AL 2010 The histone demethylase JMJD2B is regulated by estrogen receptor α and hypoxia, and is a key mediator of estrogen induced growth. Cancer Res 70(16):6456-6466

Yeh S, Miyamoto H, Shima H, Chang C 1998 From estrogen to androgen receptor: a new pathway for sex hormones in prostate. Proc Natl Acad Sci USA 95:5527-5532

Yeh S, Tsai M-Y, Xu Q, Mu X-M, Lardy H, Huang K-E, Lin H, Yeh S-D, Altuwaijri S, Zhou X, Xing L, Boyce BF, Hung M-C, Zhange S, Gan L, Chang C 2002 Generation and characterization

of androgen receptor knockout (ARKO) mice: an *in vivo* model for the study of androgen functions in selective tissues. Proc Natl Acad Sci 99(21):13498-13503

Yoshizawa A, Ogikubo S 2006 IGF binding protein-5 synthesis is regulated by testosterone through transcriptional mechanisms in androgen responsive cells. Endocrine J 53:811-818

Yuen RKC, Peñaherrera MS, von Dadelsen P, McFadden DE, Robinson WP 2010 DNA methylation profiling of human placentas reveals promoter hypomethylation of multiple genes in early-onset preeclampsia. Eur J Hum Genet 18:1006-1012

Zachariah PK, Juchau MR 1977 Inhibition of human placental mixed-function oxidation with carbon monoxide: reversal with monochromatic light. J Steroid Biochem 8:221-228.

Zhang Y, Reinberg D 2001 Transcription regulation by histone methylation: interplay between different covalent modification of the core histone tails. Genes Dev 15:2343-2360

Zhang T-Y, Meaney MJ 2010 Epigenetic and the environmental regulation of the genome and its function. Annual Rev Psychol 61:439-466

Zhou Y, Chiu K, Brescia RJ, Combs CA, Katz MA, Kitzmiller JL, Heilbron DC, Fisher SJ 1993 Increased depth of trophoblast invasion after chronic constriction of the lower aorta in rhesus monkeys. Am J Obstet Gynecol. 169:224-229

Zhou ZX, Lane MV, Kemppainen JA, French FS, Wilson EM 1995 Specificity of ligand-dependent androgen receptor stabilization: receptor domain interactions influence ligand dissociation and receptor stability. Mol Endocrinol 9(2):208-218

Zhu M-L, Kyprianou N 2008 Androgen receptor and growth factor signaling cross-talk in prostate cancer cells. Endocrine-Related Cancer 15:841-849

Zygmunt M, Herr F, Münstedt K, Lang U, Liang OD 2003 Angiogenesis and vasculogenesis in pregnancy. Eur J Obstret Gynecol Reprod Biol 110:S10-S18

APPENDIX

APPENDIX I: MIRNA PROFILE IN SERUM EXOSOMES DURING THE FIRST HALF OF PREGNANCY AND MID-GESTATION IN SHEEP[6,7,8]

Summary

While circulating levels of maternal serum exosomes increase during pregnancy, and while placental specific microRNAs (miRNAs) have been identified in humans, little is known about exosomes and miRNAs during pregnancy in agriculture animals. In this study, we characterized the expression of 94 miRNAs in ovine placentomes at gestation day (GD) 90 by real time PCR. We also investigated the presence of these 94 miRNAs in exosome samples isolated from maternal jugular blood in non-pregnant ewes and at GD30 and GD90, and in umbilical blood collected at GD90. In maternal jugular exosome samples, 13 miRNAs were present in lower and 12 miRNAs were present in higher amounts at GD90 compared to non-pregnant (GD0) or GD30. Additionally, 12 miRNAs were present in higher amounts in umbilical venous exosomes compared to umbilical arterial exosomes, with only miR-132 amounts being lower in exosomes isolated from umbilical venous blood than umbilical arterial blood. In placentome samples, miR-34c and miR135a amounts were higher in the cotyledon tissue than caruncle, while miR-183 and miR-379 amounts were higher in caruncle than cotyledon tissue. Only miR-379 was differentially expressed in all serum exosomes and placentome samples. Pathway analysis

[6] Paper submitted to *Molecular Reproduction and Development*

[7] Author list for publication: Ellane R. Cleys, Jennifer L. Halleran, Joanna Hergenreder, Vanessa A. Enriquez, Juliano da Silveira, Jason E. Bruemmer, Quinton A. Winger, Gerrit J. Bouma

[8] Project supported by Agriculture and Food Research Initiative competitive grant number 2010-38420-20397 from the USDA National Institute of Food and Agriculture and by the Colorado State University College of Veterinary Medicine and Biomedical Sciences College Research Council.

predicted that differentially expressed maternal serum exosomal miRNAs target Cellular Growth and Proliferation and Organ Development pathways, while umbilical serum exosomal and placentomes miRNAs were predicted to target Cellular Development and Organismal/Embryonic Development. This is the first report characterizing serum exosomal miRNAs as well as placentome miRNAs in sheep during pregnancy.

Introduction

MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate gene expression and function by binding complementary sequences in target transcripts to either promote mRNA degradation or to inhibit translation (Cai et al 2009). MiRNA target binding to mRNAs occurs most commonly in the 3'UTR, but can also occur in the coding region itself or the 5'UTR. It has been estimated that miRNAs regulate 30-80% of all genes in the human genome. While a single miRNA can regulate hundreds of transcripts, a single transcript can be regulated by many miRNAs (reviewed in Lu and Clark, 2012). miRNAs are therefore considered as transcription factors responsible for regulating and fine-tuning signaling pathways, with overall effects on cell differentiation, proliferation, apoptosis, metabolism, and cellular secretion. MiRNAs also play important roles in regulation of gene function in various aspects of reproductive biology, including development and function of the reproductive tracts, germ cell development and maturation, fertilization, and placental function (Wilfred et al 2007; Sonkoly et al 2008; Morales Prieto and Markert, 2011; Laddha et al 2013).

Successful establishment of pregnancy requires placental development and altered maternal immune response to fetal-placental cells. While pregnancy involves extensive communication

and signaling between cells and tissues, hormones and secreted molecules, such as matrix metalloproteinases, are known to regulate cellular signaling for placentation to occur. However, one mechanism not often thought of in context of cell communication during pregnancy is the release and uptake of extracellular vesicles. Cells secrete a wide variety of membranous vesicles, often characterized by size, shape or content. Although their nomenclature still is obscure, with vesicles being referred to as microparticles, ectosomes, shedding vesicles, microvesicles, or exosomes (Burger et al., 2013), interest in exosomes and microvesicles has expanded greatly with recent discoveries that they function also to transfer mRNAs and miRNAs between cells or tissues (Valadi et al., 2007).

Exosomes are small, ~40-160nm sized vesicles that are derived through formation of intraluminal vesicles inside late endosomes or multivesicular bodies (Keller et al., 2006; Camussi et al., 2010). Exosomes are released into extracellular space following fusion of multivesicular bodies with the plasma membrane. Due to their origin as intraluminal vesicles in multivesicular bodies, exosomes are surrounded by a phospholipid bilayer containing transmembrane proteins; additionally, exosomes carry RNAs and proteins, including tetraspanins, heat shock proteins, and adhesion molecules (Fevrier and Raposo, 2004; Gyorgy et al., 2011). Studies have shown that exosomes aid in regulating immune modulation, and that pregnancy in women is associated with increased levels of maternal circulating exosomes (Taylor et al., 2006; reviewed in Théry et al., 2002; Southcombe et al., 2011). Moreover, small-RNA library sequencing approaches have identified numerous miRNAs in human placental tissue, as well as their secreted exosomes (Luo et al., 2009). These include placental-specific miRNAs and a large cluster of primate specific miRNAs on chromosome 19 called C19MC (Bentwich et al., 2005; Zhang et al., 2008; reviewed

in Ouyang et al., 2014). Therefore, exosome-mediated delivery of biological material (RNAs and protein) during pregnancy is thought to play important roles in trophoblast physiology, immune modulation, and maternal-fetal communication.

To date, little is known about exosomes and miRNAs during pregnancy in agriculture animals. In this study, we examined the presence of miRNAs in placentomes at gestation day (GD) 90 as well as their presence in circulating exosomes during mid-gestation in sheep. More specifically, presence of miRNAs was assessed using real time PCR of isolated maternal jugular serum exosomes, umbilical artery and vein serum exosomes, and caruncle and cotyledon GD90 placentome tissue. Differentially expressed miRNAs were further analyzed for their potential involvement in regulating cellular pathways using Ingenuity Pathway Analysis.

Results

Previously we used real time PCR profiling of miRNAs in fetal sheep gonads, taking advantage of the fact that many mature miRNA sequences are highly conserved across mammalian species (Torley et al., 2011). In this study, we used the same approach and determined the presence of 94 mature miRNAs in exosomes isolated from maternal serum, as well as umbilical cord serum and placental tissues (Appendix Table I.III). Following real time PCR, amplification and melt curve analysis was conducted to ensure exponential amplification of single cDNA products. A miRNA was considered detected if amplification occurred at crossing-point value (Cp) less than 37 cycles. Initial profile analysis revealed the presence of 6 mature miRNAs (miR-92b, miR-138, miR-206, miR-182, miR-302d, and miR-320) that were detected in all samples at levels that were not statistically different between samples with a standard deviation less than 1.4. These

miRNAs were selected as internal references and their geometric mean was used to normalize Cp values in each sample and experiment (Vandesompele et al., 2002). Statistical difference is reported with $p \leq 0.05$ (Appendix Table I.II).

Exosomes in Serum of Ewes

Exosomes were isolated using a combination of differential centrifugation and ExoQuick precipitation as described previously (da Silveira et al., 2012; 2014). Western blot analysis revealed the presence of distinct 70kDa band for HSP70, a positive marker for exosomes, and absence of the predicted 15kDa band for Cytochrome C (Appendix Figure I.I), a negative marker for exosomes (Lancaster and Febbraio 2005).

miRNAs in Exosomes Isolated from Maternal Circulation of Non-Pregnant (GD0), GD30, and GD90 Ewes

In this study we used real time PCR to determine the presence of mature miRNAs in exosomes isolated from maternal circulation at GD30 and GD90. As indicated in Appendix Figure I.II and I.III, a total of 25 miRNAs were differentially present in exosomes isolated from serum of non-pregnant, GD30, and GD90 ewes. Of these miRNAs, miR-27a, miR-30c, miR-34c, miR-92b miR-125-3p, miR-132, miR-142-5p, miR-143, miR-181a, miR-210, and miR-323-5p were significantly ($P < 0.05$) lower in exosomes isolated from maternal serum at GD90 compared to GD30 (Appendix Figure I.II). In addition, exosomal miR-19a and miR379 were significantly lower at GD90 compared to GD0 (Appendix Figure I.II). Alternatively, miR-183, miR-196b, miR204, miR-206, miR-216a, miR-216b, miR-320, and miR-328 were significantly ($P < 0.05$)

higher in exosomes isolated from maternal serum at GD90 compared to GD30, and miR-181c, miR-199a-3p, miR-200b, and miR-302d compared to GD0 (Appendix Figure I.III).

Ingenuity Pathway Analysis (IPA) analysis revealed that several pathways are predicted to be regulated by the 13 exosomal miRNAs that are significantly lower in serum at GD90, including cell cycle, cellular development, cellular growth and proliferation, and cellular death and survival (Appendix Table I.I). Additionally, the miRNAs that decreased at GD90 are associated with physiological system development of digestive, hepatic and organ systems and is correlated with cancer, organismal injury and abnormalities, reproductive system disease, endocrine system disorders, and gastrointestinal disease (Appendix Table I.I).

Conversely, the 12 exosomal miRNAs that were significantly increased at GD90 compared to GD30 and/or GD0 are predicted to target pathways related to cell-to-cell signaling and interaction, cellular death and survival, and cellular development (Appendix Table I.I). Similar to the miRNAs that were decreased at GD90, miRNAs that increased were also associated with organ development, though other physiological systems included tissue development, cardiovascular, skeletal and muscular system development and function (Appendix Table I.I). Diseases that were associated with the 12 miRNAs that increased at GD90 are similar to those correlated with the decreased miRNAs, including organismal injury and abnormalities, reproductive system disease, cancer, and gastrointestinal disease.

miRNAs in Exosomes Isolated from Umbilical Arterial and Venous Serum at GD90

Real time PCR analysis revealed significant differences in 13 miRNAs in exosomes isolated from umbilical arterial compared to umbilical venous serum (Appendix Figure I.IV). Interestingly, of the 13 miRNAs that were differentially expressed ($P < 0.05$), 12 were increased in umbilical venous serum exosomes compared to umbilical arterial serum exosomes; miR-23a, miR-23b, miR-24, miR-26a, miR-27a, miR-29c, miR-30a, miR-30d, miR-148b, miR-199a-3p, miR-223, and miR-379 were present at higher levels in exosomes isolated from umbilical vein compared to arterial serum. Only miR-132 was increased ($P < 0.05$) in exosomes isolated from umbilical arterial serum.

Pathway analysis revealed that the differentially expressed umbilical exosomal miRNAs are predicted to target pathways related to cell development, cellular growth and proliferation, cell cycle and cell-to-cell signaling and function (Appendix Table I.I). Differentially expressed umbilical exosomal miRNAs were also associated with organismal, organ and tissue development. Similar to the differentially expressed maternal serum exosomal miRNAs, umbilical serum exosomal miRNAs are also associated with cancer and gastrointestinal disease. Other disorders that are correlated with umbilical exosomal miRNAs include endocrine system disorders and metabolic disease (Appendix Table I.I).

miRNA Levels in Cotyledon and Caruncle Tissue from GD90 Placentomes

Real time PCR analysis revealed that 4 miRNAs were differentially ($P<0.05$) expressed between cotyledon and caruncle tissue at GD90. MiR-34c and miR-135a were lower in GD90 caruncle, whereas miR-183 and miR-379 were significantly lower in GD90 cotyledon tissues (Appendix

Figure I.V). Pathway analysis of all 4 differentially expressed placentome miRNAs predicted cellular development as the primary molecular pathway affect (Appendix Table I.I). Additionally, differentially expressed placentome miRNAs are associated with embryonic development and connective tissue development and function. All 4 miRNAs were associated with organismal injury and abnormalities, endocrine system disease, and gastrointestinal disease (Appendix Table I.I).

Discussion

In this study we report on the presence of exosomes and their miRNA in maternal circulation during pregnancy in sheep. The identification of exosomes containing miRNAs during pregnancy itself is not new and has been extensively investigated in women during normal pregnancy as well as in pregnancies effected by the placental induced disorder preeclampsia. In cattle, cultured uterine epithelial cells secrete exosomes containing the antiviral protein myxovirus resistance 1, and sheep exosomes have been isolated from uterine luminal fluid and their RNA (including miRNA) and protein content has been characterized (Racicot et al., 2012; Burns et al., 2014). Moreover, it has been postulated that these exosomes could play a role in conceptus-maternal communication during early pregnancy (Burns et al., 2014). However, to our knowledge, this is the first report characterizing exosomal miRNAs from maternal circulation during pregnancy (GD30 and GD90), as well as from umbilical artery and vein.

Taylor (et al., 2006) reported that exosomes quantities are greatly increased in serum at term, and, based on presence of T-cell activation markers, proposed a role for these “pregnancy exosomes” in modulating the immune system. Luo and colleagues (2009) further suggest a

possible function of secreted placental exosome miR-517A, based on bioinformatics analysis, in regulating tumor necrosis factor signaling. Moreover, the human placenta is enriched in a number of specific miRNAs; these miRNAs also are detected in exosomes in circulation, and the level of these miRNAs in maternal blood decrease after delivery, suggesting a placental source (Luo et al., 2009). Unique to the primate placenta is the presence of a miRNA cluster C19MC on chromosome 19, which contains 46 miRNAs. MiRNAs from this cluster have been described in placental-secreted exosomes (Donker et al., 2012). Moreover, recent studies indicate that C19MC secreted in trophoblast exosomes inhibits viral replication by inducing autophagy in recipient target cells (Delorme-Axford et al., 2013). Although the function of placental derived exosomes and their biological content in pregnancy is still largely unknown, there remains considerable interest in the possible use of extracellular placental miRNAs and protein as diagnostic markers of placental disorders (e.g., reviewed in Ouyang et al., 2014; Morales and Markert 2011; Redman and Sargent, 2007).

Using a previously published approach, we assessed the presence of miRNAs using real time PCR profiling, taking advantage of the conserved nature of mature miRNA sequences between mammalian species. Differential expression of miR181a and miR-196 in maternal serum exosomes at GD90 could suggest a possible mechanism for regulating maternal immune response to the fetal-placental unit, as both miRNAs control immune reaction and inflammation signaling pathways (Sonkoly et al 2008). Additionally, miR-143, which was also differentially expressed in GD90 maternal serum exosomes, is reported to regulate adipocyte differentiation, a process that is highly regulated during pregnancy for proper fetal growth and development (Wilfred et al 2007). Furthermore, of the 94 miRNAs examined, 12 miRNAs were lower in

exosomes collected from umbilical arterial compared to venous blood. These findings suggest a maternal origin for delivery to fetal-placental circulation, or placental filtration. In contrast, miR-132, which was higher in umbilical arterial exosomes than venous exosomes, may be released from the placentomes and carried towards the maternal circulation. Of the 12 miRNAs that were lower in umbilical arterial exosomes, several miRNAs, including miR-23a, miR-27a, 26a, and miR-199a-3p, have been detected in term placenta (Luo et al., 2009). Interestingly, miR-135a was previously reported to be up-regulated in circulation in preterm birth (Montenegro et al., 2009), and this miRNA was detected in GD90 caruncle tissue. The role of these miRNAs in placental function is unknown, however it may be of interest to further characterize these miRNA in exosomes as it relates to comprised pregnancies.

miR-379 was significantly lower in exosomes isolated from maternal circulation as well as umbilical arterial serum at GD90. Moreover level of this miRNA was increased in caruncle compared to cotyledon tissues. miR-379 belongs to the chromosome 14 miRNA cluster (C14MC); C14MC is the largest known miRNA cluster in humans, consisting of 54 miRNAs, and is widely conserved in placental mammals (Morales Prieto et al., 2014). MiRNAs belonging to this cluster are highly expressed in first trimester placenta and decrease by the third trimester, similar to our findings of decreased miR-379 in maternal circulation between non-pregnant ewes and GD90. Abnormal expression of C14MC contributes to cancer development (Laddha et al., 2013), and down-regulation of miR-379 has been reported in placentas from women with severe preeclampsia (Xu et al., 2014). While these observations suggest a role for miR-379 in trophoblast function and pregnancy, our findings of differential miR-379 expression in the

pregnant ewe may offer a potential *in vivo* model for further characterization of its role and function.

Serum exosomes are actively being pursued as new diagnostic markers, including in pregnancy and diseases. However, it is important to note that in most cases (including this study) the source of exosomes in maternal circulation is unknown. In addition, although exosomes and other cell-secreted vesicles, such as microvesicles, are implicated in tissue and cell-communication processes, data demonstrating selective packaging and/or transfer of exosome content has been lacking. Although uncovering a functional role of exosomal miRNAs was beyond the scope of this study, IPA analysis was conducted to reveal potential signaling pathways for further insight on the function of pregnancy-related exosomal miRNAs. As maternal serum exosomal miRNAs were predicted to target "Cellular Growth and Proliferation" and "Organ Development", and as umbilical serum exosomal and placentomes miRNAs were predicted to target "Cellular Development" and "Organismal/Embryonic Development", the differentially expressed miRNAs at GD90 appear to have dynamic functions in possibly regulating fetal/placental growth and development.

Finally, it is important to note that all exosomal miRNAs reported in this study were detected in the placentome tissue. Because the approach used herein was to profile the presence of 94 known, conserved miRNAs sequences, it is likely many more miRNAs remain to be identified. In future studies, the use of NextGen Sequencing approaches will provide much needed insight into the characterization of all placental miRNAs as well as their presence in placental-secreted exosomes.

In summary, this study presents new data on the presence of miRNAs in exosomes isolated from maternal circulation during the approximate first half of pregnancy in the ewe, as well as characterizing the presence of miRNAs in exosomes isolated from umbilical cord serum. Although the function and possible role of these miRNAs is unknown, exosomal miRNAs serve as novel markers that could be used to monitor pregnancy status and placental function, and may serve a critical, though still uncharacterized, role in modulating maternal-fetal communication for successful pregnancy maintenance.

Materials and Methods

Animal Care and Serum Collection

All experiments were approved by the Colorado State University Institutional Animal Care and Use Committee. Tissue samples were collected from 4 crossbred ewes that had received intramuscular injections of 2 mL vehicle (cottonseed oil) biweekly from gestational day GD30 to GD90 as part of a separate study (Chapter 2). Five to seven days after observed estrus, ewes were synchronized using 2 intramuscular injections of 1 mL Lutalyse® given four hours apart. The day of Lutalyse® injection was designated as gestational day GD0. Forty-eight hours after injection, ewes were bred with 2 different intact, fertile rams and checked daily throughout the course of the study to ensure they did not return to estrus. Estrus was observed as mounting behavior from surgically vasectomized rams. For analysis, each time point of serum or tissue collection utilized the same 4 ewes.

Serum Exosome Collection and miRNA Isolation

Maternal jugular blood samples were collected at GD0, 30, and 90. Samples were allowed to clot for 1 hour at room temperature and were centrifuged at 2,000 x g for 10 minutes. Serum was isolated and differentially centrifuged to remove cell debris, followed by sequential centrifugation at 300 x g for 10 minutes, 2,000 x g for 10 minutes, and 10,000 x g for 30 minutes (Théry et al 2006). Exosomes were isolated by adding 400 μL of final supernatant to 100 μL of Exoquick™ (System Biosciences). Samples were stored overnight at 4 °C. The following morning, serum exosomes were pelleted by centrifugation at 1,500 x g for 30 minutes. Supernatant was removed and serum exosome pellets were resuspended in 250 μL PBS. After resuspending in PBS, 750 μL TRI Reagent BD (Sigma-Aldrich) was added, samples were snap frozen and stored at -80 °C until miRNA isolation.

Exosomal miRNAs were isolated using TRI Reagent (Sigma-Aldrich) per manufacturer's instructions with the following adjustments. Samples were thawed at room temperature, homogenized, and incubated at room temperature for 5 minutes. Next, 200 μL of chloroform was added and samples were vortexed for 15 seconds. Samples were incubated for 5 minutes at room temperature, and centrifuged at 12000 x g for 15 minutes. The aqueous phase was removed and stored for protein isolation. To each remaining sample, 500 μL isopropanol was added and tubes were inverted and placed at room temp for 10 minutes. Samples were centrifuged at 16,100 x g for 8 minutes, supernatant was removed, 1 mL of 75% EtOH was added, and samples were centrifuged at 16,100 x g for 5 minutes. Supernatant was removed, 1 mL of 75% EtOH was added, and samples were centrifuged for another 5 minutes. Supernatant was removed, tubes were inverted, and RNA pellets were air dried for 5 minutes at room temperature. The RNA

pellet was resuspended by adding 20 μL of 55°C nuclease-free water and incubating at 55°C for 10-15 minutes. Genomic DNA was removed by adding 2 μL of 10X DNase1 Buffer and 1 μL of DNAse from DNA-*free* DNase Treatment (Life Technologies) to each sample. Samples were incubated for 30 minutes at 37°C, and the reaction was stopped by adding 3.5 μL of inactivation reagent and incubating samples at room temperature for 30 minutes. Total RNA purity and concentration was determined with a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies). RNA aliquots were stored at -80° C until further use.

Placentome Collection and miRNA Isolation

Ewes were anesthetized at GD90 and five placentomes closest to the umbilicus were collected. Caruncle and cotyledon sections of the placentomes were separated, snap frozen in liquid nitrogen, and stored at -80°C. Prior to miRNA isolation, samples were pulverized in liquid nitrogen. miRNA was isolated using mirVana™ (Ambion) isolation kit according to manufacturer's protocol.

Real Time PCR Analysis

From 128 mature miRNA sequences that are conserved between human, mouse, and/or sheep (Torley et al 2011), 94 mature miRNAs were selected for analysis due to published reports of their expression in (human, murine) placenta or maternal serum or correlation with pregnancy disorders (Bentwich et al., 2005; Zhang et al., 2008; 2012; Luo et al., 2009; Seabrook et al., 2013; reviewed in Ouyang et al., 2014). The relative level of 94 mature miRNAs (Appendix Table I.III) was assessed in maternal serum exosomes, umbilical serum exosomes, and placentome tissue. Quantifiable, reverse transcribed miRNAs were generated using the miScript

II Reverse Transcriptase Kit (Qiagen) with 100 ng RNA, 4 μL 5x miScript HiFlex solution, 2 μL 10x Nucleic solution, 2 μL of miScript Reverse Transcriptase, and RNase-free water to reach a final volume of 20μL. Reverse transcribed miRNAs was loaded into each real time PCR reaction containing 0.6 μL of 10x Universal Reverse Primer (Qiagen), 1.5 μL specific forward primer, 3 μL miScript SYBR Green (Qiagen), and 1.37 μL nuclease-free water for a final reaction volume of 6.5 μL per reaction. Real time PCR cycling conditions consisted of an initial incubation at 95°C for 15 minutes, followed by 40 cycles at 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, followed by melt curve analysis to confirm amplification of single cDNA products.

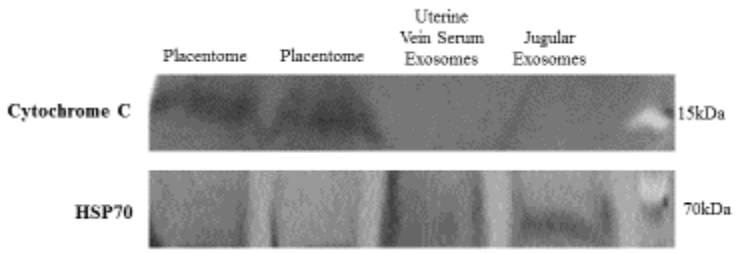
Differences in the presence of miRNAs levels were determined by normalizing the raw Cp values to the geometric mean of 6 miRNAs (miR-92b, miR-138, miR-206, miR-182, miR-302d, miR-320) that were chosen as internal reference as they were present in all samples, showed little variability (standard deviation less than 1.4 and standard error of the mean less than 0.255) across all samples, and were not statistically different between samples. Statistical analysis was performed on normalized data using an ANOVA with Tukey's pair-wise comparison (Minitab16) on maternal serum exosome samples and a two-tailed student's t-test (Excel) on umbilical serum exosome and placentome miRNA samples with statistical significance at p-values less than or equal to 0.05. To identify possible pathways predicted to be targeted by miRNAs, Ingenuity Pathway Analysis was used.

Western Blot Analysis

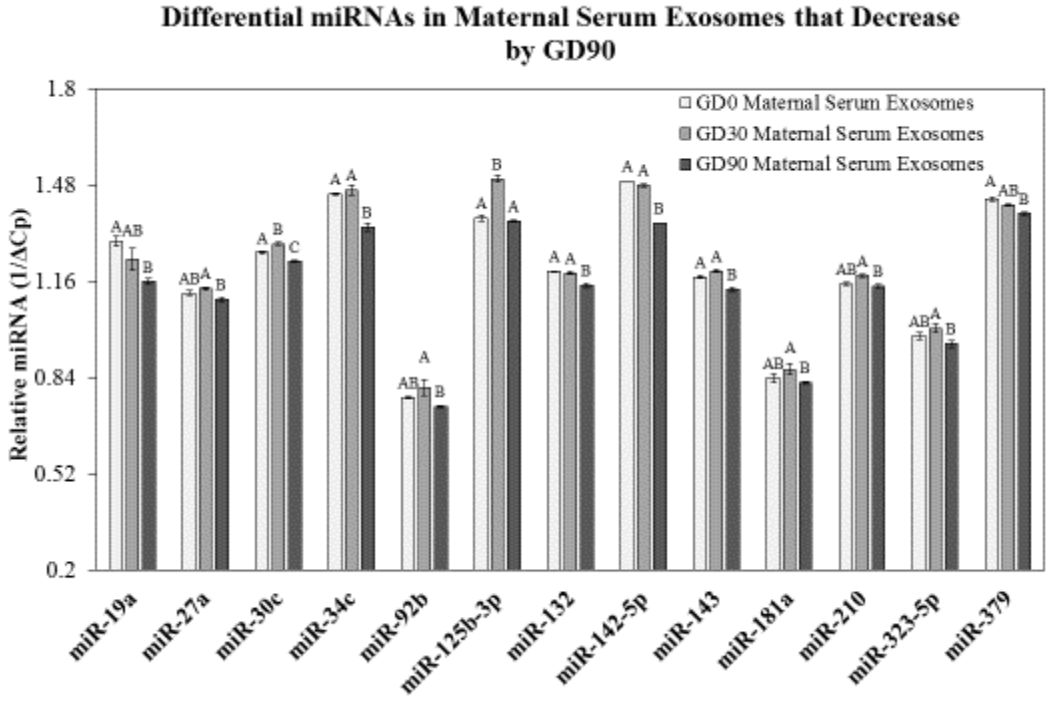
Serum exosomal protein was isolated using TRI Reagent BD as per the manufacturer's protocol (Molecular Research), resuspended in 8M urea, then quantified with a Bradford assay (BioRad) as previously described (da Silveira et al 2012). Placentome protein was isolated from pulverized tissue and approximately 2 mg of pulverized tissues were added directly to 2mL of ice cold lysis buffer containing 0.48M Tris pH7.5, 10mM EGTA pH8.6, 10mM EDTA pH8.0, and 0.1% (w/v) PMSF and protease inhibitor. Samples were sonicated on ice for 5 minutes and centrifuged at 10,000 rpm for 10 minutes at 4°C. Concentration of placentome protein was determined using standard curves by Bradford assay (BioRad).

150μL of serum exosomal protein or 50μL of protein was diluted in 6x SDS-DTT loading dye with 0.375M Tris pH6.8, 4M glycerol, 0.21M SDS, 0.6M DTT, and 0.06% (w/v) bromophenol blue. β-mercaptoethanol (1.75μL) and water was added to reach a final volume of 35μL. Samples were boiled for 5 minutes after addition of β-mercaptoethanol, then electrophoresed at 95 volts in 10% Tris-HCL polyacrylamide gels (BioRad). Protein was electrophoresed in an ice cold running buffer containing 50mM Tris, 384mM glycine, and 7mM SDS. Protein was transferred onto 0.2 μm nitrocellulose membranes (Protran) for 1 hour at 200 milliamps at 4°C in transfer buffer containing 2mM Tris, 150 mM glycine, 5M methanol, and 3.5mM SDS. After protein transfer, blots were blocked for non-specific binding with 2% milk-TBST for 1 hour at room temperature. After blocking, blots were washed with TBST and left overnight at 4°C with primary antibody diluted in 2% milk-TBST. Rabbit anti-cytochrome-C (SantaCruz sc7159) and anti-HSP70 (SantaCruz sc1060-R) were used at 1:200 dilutions in 2% milk-TBST.

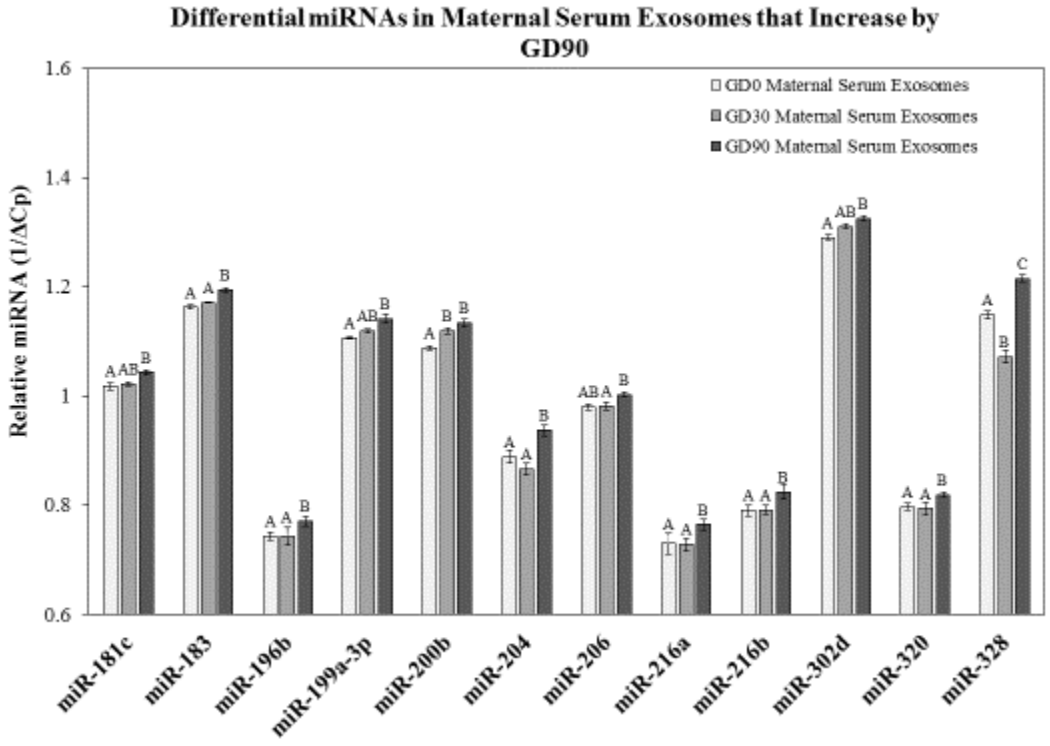
After incubation with primary antibody, blots were washed in TBST and incubated for 1 hour at room temperature with secondary antibodies, goat-anti-rabbit-HRP (Abcam ab6721, 1:1000). Blots were subsequently washed in TBST and ECL Prime Western Blotting Detection System (Amersham Biosciences) was applied to detect immunoreactivity. Chemiluminescent bands were detected using the Chemidoc MP System (BioRad).



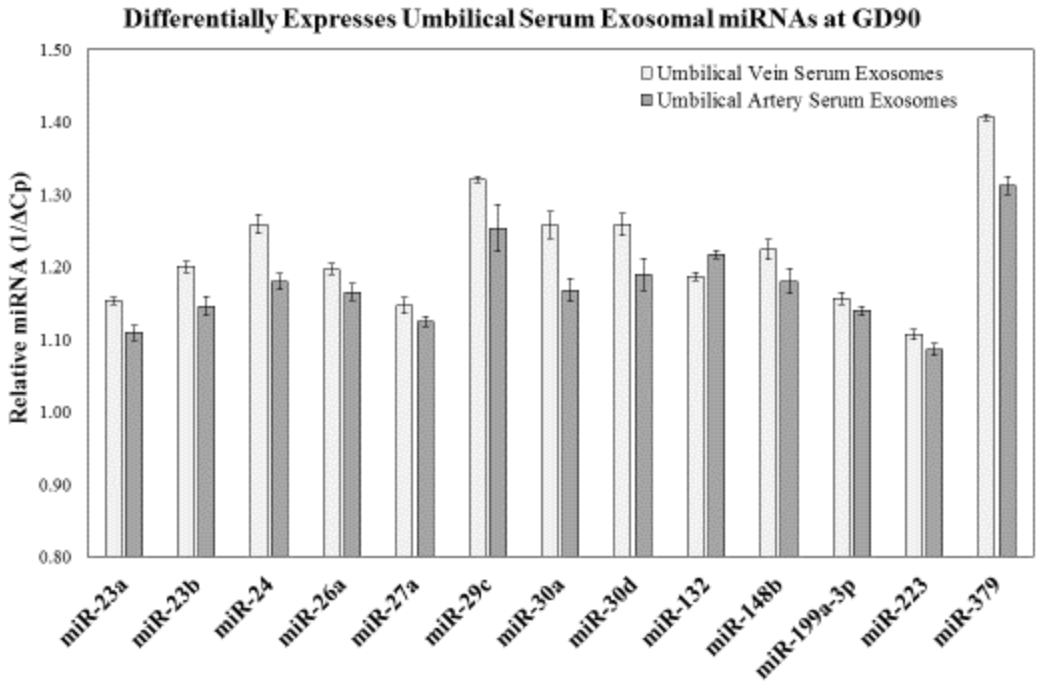
APPENDIX FIGURE I.I. Western blot of serum exosomal and placentome protein. Cytochrome C presence in cellular protein from ovine GD90 placentomes. Hsp70 presence in isolated serum exosomal protein fraction from ovine jugular and uterine vein serum.



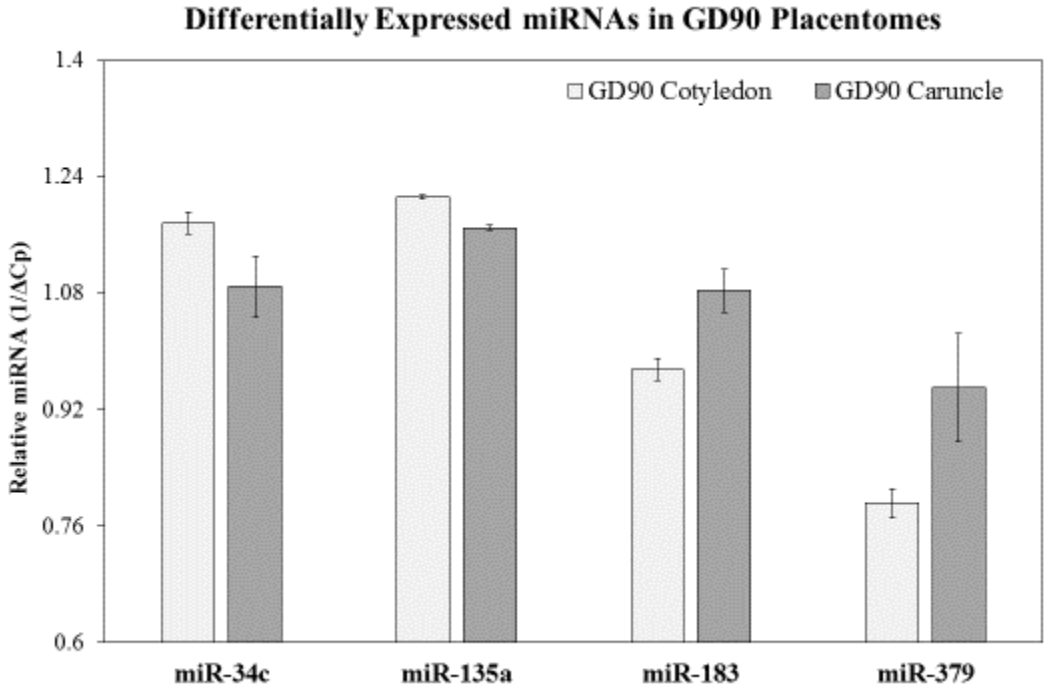
APPENDIX FIGURE I.II. Maternal serum exosomal miRNAs that decrease by GD90 ($P<0.05$).



APPENDIX FIGURE I.III. Maternal serum exosomal miRNAs that increase by GD90 ($P<0.05$).



APPENDIX FIGURE I.IV. Serum exosomal miRNAs that are differentially expressed in umbilical vein and artery at GD90 ($P<0.05$).



APPENDIX FIGURE I.V. miRNAs differentially expressed in placentome tissue of fetal (cotyledon) and maternal (caruncle) origin at GD90 ($P<0.05$).

APPENDIX TABLE I.I. Pathways associated with differentially expressed miRNAs, their associated p-value, and number of molecules in pathway.

Pathways Associated with Down-Regulated Exosomal miRNAs in Maternal Serum at GD90

Diseases and Disorder	**p-Value**	**Molecules**
Cancer	5.29E-14 – 4.58E-02	10
Organismal Injury and Abnormalities	5.29E-14 – 3.45E-02	11
Reproductive System Disease	5.29E-14 – 3.41-02	9
Endocrine System Disorders	6.20E-13 – 2.75E-02	11
Gastrointestinal Disease	6.20E-13 – 3.92E-02	11
Molecular and Cellular Functions	**p-Value**	**Molecules**
Cell Cycle	3.33E-07 – 2.90E-02	4
Cellular Development	1.20E-05 – 4.45E-02	8
Cellular Growth and Proliferation	4.50E-05 – 4.45E-02	8
Cellular Death and Survival	9.87E-05 – 4.97E-02	8
Cell-to-Cell Signaling and Interaction	1.21E-03 – 1.21E-03	2
Physiological System Development and Functions	**p-Value**	**Molecules**
Digestive System Development and Function	6.63E-07 – 6.63E-07	3
Hepatic System Development and Function	6.63E-07 – 6.63E-07	3
Organ Development	6.63E-07 – 3.45E-02	6
Respiratory System Development and Function	6.05E-04 – 6.05E-04	1
Tumor Morphology	6.05E-04 – 9.04E-03	3

Pathways Associated with Up-Regulated Exosomal miRNAs in Maternal Serum at GD90

Diseases and Disorder	**p-Value**	**Molecules**
Organismal Injury and Abnormalities	4.91E-13 – 3.69E-02	11
Reproductive System Disease	4.91E-13 – 3.69E-02	9
Cancer	3.30E-12 – 3.69E-02	10
Gastrointestinal Disease	1.37E-11 – 3.69E-02	9
Hepatic System Disease	1.27E-06 – 2.49E-03	6
Molecular and Cellular Functions	**p-Value**	**Molecules**
Cell-to-Cell Signaling and Interaction	6.05E-04 – 6.64E-03	3
Cellular Assembly and Organization	6.05E-04 – 6.05E-04	1
Cellular Compromise	6.05E-04 – 6.05E-04	1
Cellular Death and Survival	9.59E-04 – 4.97E-02	7
Cellular Development	1.21E-03 – 4.45E-02	7
Physiological System Development and Functions	**p-Value**	**Molecules**
Organ Development	5.40E-04 – 5.40E-04	2
Nervous System Development and Function	6.05E-04 – 6.05E-04	1
Tissue Development	6.05E-04 – 1.26E-02	3
Cardiovascular System Development and Function	1.21E-03 – 1.92E-02	2
Skeletal and Muscular System Development and Function	1.21E-03 – 1.02E-02	2

APPENDIX TABLE I.I Continued. Pathways associated with differentially expressed miRNAs, their associated p-value, and number of molecules in pathway.

Pathways Associated with Differentially Expressed Umbilical Serum Exosomal miRNAs at GD90:

Diseases and Disorder	**p-Value**	**Molecules**
Cancer	4.36E-15 – 4.62E-02	10
Endocrine System Disorders	1.79E-12 – 2.75E-02	9
Gastrointestinal Disease	1.79E-12 – 2.75E-02	11
Metabolic Disease	1.79E-12 – 2.75E-02	10
Infectious Disease	9.12E-12 – 4.21E-02	5
Molecular and Cellular Functions	**p-Value**	**Molecules**
Cellular Development	1.68E-06 – 4.45E-02	10
Cellular Growth and Proliferation	1.68E-06 – 4.45E-02	9
Cellular Compromise	6.05E-04 – 6.05E-04	1
Cell Cycle	1.21E-03 – 6.64E-03	4
Cell-to-Cell Signaling and Interaction	1.21E-03 – 6.64E-03	3
Physiological System Development and Functions	**p-Value**	**Molecules**
Organismal Development	9.12E-12 – 2.10E-02	6
Organ Development	9.98E-04 – 1.80E-02	4
Tissue Development	1.21E-03 – 1.21E-03	1
Nervous System Development and Function	1.81E-03 – 1.81E-03	1
Tumor Morphology	2.42E-03 – 4.83E-03	1

Pathways Associated with Differentially Expressed miRNAs in Ovine Placentomes at GD90:

Diseases and Disorder	**p-Value**	**Molecules**
Cancer	1.23E-07 – 4.75E-02	3
Organismal Injury and Abnormalities	1.37E-05 – 3.09E-02	4
Reproductive System Disease	1.37E-05 – 3.09E-02	3
Endocrine System Disease	1.42E-05 – 3.73E-02	4
Gastrointestinal Disease	1.42E-05 – 4.32E-02	4
Molecular and Cellular Functions	**p-Value**	**Molecules**
Cellular Development	1.31E-06 – 4.75E-02	2
Cell Cycle	2.20E-04 – 5.93E-03	1
Cell-to-Cell Signaling and Interaction	4.40E-04 – 4.40E-04	1
Cell Growth and Proliferation	4.40E-04 – 4.75E-02	1
Cellular Function and Maintenance	1.10E-03 – 1.10E-03	1
Physiological System Development and Functions	**p-value**	**Molecules**
Embryonic Development	1.00E-04 – 4.14E-02	2
Respiratory System Development and Function	2.20E-04 – 2.20E-04	1
Tumor Morphology	4.40E-04 – 4.40E-04	1
Connective Tissue Development and Function	5.65E-04 – 5.64E-04	2
Cell-mediated Immune Response	1.10E-03 – 1.10E-03	1

APPENDIX TABLE I.II. List of p-values associated with differentially expressed miRNAs.

ANOVA p-Values for Differentially Expressed miRNAs in Maternal Jugular Serum Exosomes	
miR-19a	P=0.035
miR-27a	P=0.027
miR-30c	p=0.001
miR-34c	p=0.009
miR-92b	p=0.003
miR-125b-3p	p=0.007
miR-132	p=0.002
miR-142-5p	p=0.009
miR-143	p=0.001
miR-181a	p=0.036
miR-181c	p=0.025
miR-183	p=0.002
miR-196b	p=0.022
miR-199a-3p	p=0.017
miR-200b	p=0.003
miR-204	p=0.001
miR-206	p=0.039
miR-210	p=0.020
miR-216a	p=0.015
miR-216b	p=0.015
miR-302d	p=0.048
miR-320	p=0.015
miR-323-5p	p=0.04
miR-328	p<0.001
miR-379	p=0.033
T-Test p-Values for Differentially Expressed miRNAs in GD90 Umbilical Vein and Artery Serum Exosomes	
miR-23a	p=0.036
miR-23b	p=0.029
miR-24	p=0.012
miR-26a	p=0.018
miR-27a	p=0.044
miR-29c	p=0.063
miR-30a	p=0.012
miR-30d	p=0.057
miR-132	p=0.003
miR-148b	p=0.024
miR-199a-3p	p=0.007
miR-223	p=0.001
miR-379	p=0.059
List of T-Test p-Values for Differentially Expressed miRNAs in GD90 Cotyledon and Caruncle Placentome Tissue	
miR-34c	p=0.057
miR-135a	p=0.038
miR-183	p=0.068
miR-379	p=0.050

APPENDIX TABLE I.III. List of forward primers used in real time PCR.

List of miRNA Forward Primers	
let-7a	UGAGGUAGUAGGUUGUAUAGUU
let-7d	AGAGGUAGUAGGUUGCAUAGUU
let-7e	UGAGGUAGGAGGUUGUAUAGUU
miR-15a	UAGCAGCACAUAAUGGUUUGUG
miR-15b	UAGCAGCACAUCAUGGUUUACA
miR-16	UAGCAGCACGUAAAUAUUGGCG
miR-17	CAAAGUGCUUACAGUGCAGGUAG
miR-19a	UGUGCAAAUCUAUGCAAAACUGA
miR-19b	UGUGCAAAUCCAUGCAAAACUGA
miR-20a	UAAAGUGCUUAUAGUGCAGGUAG
miR-20b	CAAAGUGCUCAUAGUGCAGGUAG
miR-21	UAGCUUAUCAGACUGAUGUUGA
miR-22	AAGCUGCCAGUUGAAGAACUGU
miR-23a	AUCACAUUGCCAGGGAUUUCC
miR-23b	AUCACAUUGCCAGGGAUUACC
miR-24	UGGCUCAGUUCAGCAGGAACAG
miR-25	CAUUGCACUUGUCUCGGUCUGA
miR-26a	UUCAAGUAAUCCAGGAUAGGCU
miR-27a	UUCACAGUGGCUAAGUUCCGC
miR-29b	UAGCACCAUUUGAAAUCAGUGUU
miR-29c	UAGCACCAUUUGAAAUCGGUUA
miR-30a	UGUAAACAUCCUCGACUGGAAG
miR-30b	UGUAAACAUCCUACACUCAGCU
miR-30c	UGUAAACAUCCUACACUCUCAGC
miR-30d	UGUAAACAUCCCCGACUGGAAG
miR-34c	AGGCAGUGUAGUUAGCUGAUUGC
miR-92a	UAUUGCACUUGUCCCGGCCUG
miR-92b	UAUUGCACUCGUCCCGGCCUCC
miR-99a	AACCCGUAGAUCCGAUCUUGUG
miR-100	AACCCGUAGAUCCGAACUUGUG
miR-101a	UACAGUACUGUGAUAACUGAA
miR-107	AGCAGCAUUGUACAGGGCUAUCA
miR-122	UGGAGUGUGACAAUGGUGUUUG
miR-125b-3p	ACGGGUUAGGCUCUUGGGAGCU
miR-132	UAACAGUCUACAGCCAUGGUCG
miR-135a	UAUGGCUUUUUAUUCCUAUGUGA
miR-138	AGCUGGUGUUGUGAAUCAGGCCG
miR-142-3p	UGUAGUGUUUCCUACUUUAUGGA

APPENDIX TABLE I.III Continued. List of forward primers used in real time PCR.

List of miRNA Forward Primers	
miR-142-5p	CAUAAAGUAGAAAGCACUACU
miR-143	UGAGAUGAAGCACUGUAGCUC
miR-146b	UGAGAACUGAAUUCCAUAGGCU
miR-148a	UCAGUGCACUACAGAACUUUGU
miR-148b	UCAGUGCAUCACAGAACUUUGU
miR-149	UCUGGCUCCGUGUCUUCACUCCC
miR-150	UCUCCCAACCCUUGUACCAGUG
miR-152	UCAGUGCAUGACAGAACUUGG
miR-181a	AACAUUCAACGCUGUCGGUGAGU
miR-181b	AACAUUCAUUGCUGUCGGUGGGU
miR-181c	AACAUUCAACCUGUCGGUGAGU
miR-182	UUUGGCAAUGGUAGAACUCACACCG
miR-183	UAUGGCACUGGUAGAAUUCACU
miR-191	CAACGGAAUCCCAAAAGCAGCUG
miR-192	CUGACCUAUGAAUUGACAGCC
miR-193	AACUGGCCUACAAAGUCCCAGU
miR-193b	AACUGGCCCACAAAGUCCCGCU
miR-194	UGUAACAGCAACUCCAUGUGGA
miR-195	UAGCAGCACAGAAAUAUUGGC
miR-196a	UAGGUAGUUUCAUGUUGUUGGG
miR-196b	UAGGUAGUUUCCUGUUGUUGGG
miR-199a-3p	ACAGUAGUCUGCACAUUGGUUA
miR-199a-5p	CCCAGUGUUCAGACUACCUGUUC
miR-199b	ACAGUAGUCUGCACAUUGGUUA
miR-200b	UAAUACUGCCUGGUAAUGAUGA
miR-200c	UAAUACUGCCGGGUAAUGAUGGA
miR-204	UUCCCUUUGUCAUCCUAUGCCU
miR-206	UGGAAUGUAAGGAAGUGUGUGG
miR-210	CUGUGCGUGUGACAGCGGCUGA
miR-211	UUCCCUUUGUCAUCCUUUGCCU
miR-212	UAACAGUCUCCAGUCACGGCCA
miR-214	ACAGCAGGCACAGACAGGCAGU
miR-216a	UAAUCUCAGCUGGCAACUGUGA
miR-216b	AAAUCUCUGCAGGCAAAUGUGA
miR-219	UGAUUGUCCAAACGCAAUUCU
miR-221	AGCUACAUUGUCUGCUGGGUUUC
miR-222	AGCUACAUCUGGCUACUGGGU
miR-223	UGUCAGUUUGUCAAAUACCCCA

APPENDIX TABLE I.III Continued. List of forward primers used in real time PCR.

List of miRNA Forward Primers	
miR-296-3p	GAGGGUUGGGUGGAGGCUCUCC
miR-296-5p	AGGGCCCCCCCUCAAUCCUGU
miR-301a	CAGUGCAAUAGUAUUGUCAAAGC
miR-301b	CAGUGCAAUGGUAUUGUCAAAGC
miR-302d	UAAGUGCUUCCAUGUUUGAGUGU
miR-320	AAAAGCUGGGUUGAGAGGGCGA
miR-323-3p	CACAUUACACGGUCGACCUCU
miR-323-5p	AGGUGGUCCGUGGCGCGUUCGC
miR-328	CUGGCCCUCUCUGCCCUUCCGU
miR-335-5p	UCAAGAGCAAUAACGAAAAAUGU
miR-339-3p	UGAGCGCCUCGGCGACAGAGCCG
miR-340-3p	UCCGUCUCAGUUACUUUAUAGC
miR-342-3p	UCUCACACAGAAAUCGCACCCGU
miR-342-5p	AGGGGUGCUAUCUGUGAUUGAG
miR-377	AUCACACAAAGGCAACUUUUGU
miR-379	UGGUAGACUAUGGAACGUAGG
Mouse U6 snRNA	UGGCCCCUGCGCAAGGAUG
RNU43 (snoRNA)	CUUAUUGACGGGCGGACAGAAAC
Hm/Ms/Rt U1 snRNA	CGACUGCAUAAUUUGUGGUAGUGG

REFERENCES

Bentwich I, Arniel A, Karov Y, Aharonov R, Gilad S, Barad O, Barzilai A, Einat P, Einav U, Meiri E, Sharon E, Spectro Y, Bentwich Z 2005 Identification of hundreds of conserved and nonconserved human microRNAs. Nat Genet 37:766-770

Burger D, Schock S, Thompson CS, Montezano AC, Hakim AM, Touyz RM 2013 Microparticles: biomarkers and beyond. Clin Sci 124:423-441

Burns G, Brooks K, Wildung M, Navakanitworakul R, Christenson LK, Spencer TE 2014 Extracellular vesicles in luminal fluid of the ovine uterus. PLoS ONE 9(3):e90913

Cai Y, Yu X, Hu S, Yu J 2009 A brief review on the mechanisms of miRNA regulation. Genomics Proteomics Bioinformatics 7(4):147-154

Camussi G, Deregibus MC, Bruno S, Cantaluppi V, Biancone L 2010 Exosomes/microvesicles as mechanism of cell-to-cell communication. Kidney Int 78:838-848

da Silveira JC, Veeramachaneni DNR, Winger QA, Carnevale EM, Bouma GJ 2012 Cell-secreated vesicles in equine ovarian follicular fluid contain miRNAs and protein: a possible new form of all communication within the ovarian follicle. Biol Reprod 86(3):71, 1-10

da Silveira JC, Carnevale EM, Winger QA, Bouma GJ 2014 Regulation of ACVR1 and ID2 by cell-secreted exosomes during follicle maturation in the mare. Reprod Biol Endocrinol 12:44

Delorme-Axford E, Donker RB, Mouillet J-F, Chu T, Bayer A, Ouyang Y, Wang T, Stolz DB, Sarkar SN, Morelli AE, Sadovsky Y, Coyne CB 2013 Human placental trophoblasts confer viral resistance to recipient cells. Pro Natl Acad Sci 110(29)12048-12053

Donker RB, Mouillet JF, Chu T, Hubel CA, Stolz DB, Morelli AE, Sadovsky Y 2012 The expression profile of C19MC microRNAs in primary human trophoblast cells and exosomes. Mol Hum Reprod 18(8):417-424

Fevrier B, Raposo G 2004 Exosomes: endosomal-derived vesicles shipping extracellular messages. Curr Opin Cell Biol 16(4):415-421

Gyorgy B, Módos K, Pálóczi K, Pásztói M, Misják P, Deli M, Sipos Á, Szalai A, Voszka I, Polgár A, Tóth K, Csete M, Nagy G, Gay S, Falus A, Kittel Á, Buzás ET 2011 Detection and isolation of cell-derived microparticles are compromised by protein complexes resulting from shared biophysical parameters. Blood 117(4):e39-e48

Keller S, Sanderson MP, Stoeck A, Altevogt P 2006 Exosomes: from biogenesis and secretion to biological function. Immunol Lett 107(2):102-108

Laddha SV, Nayak S, Paul D, Reddy R, Sharma C, Jha P, Hariharan M, Agrawal A, Chowdhury S, Sarkar C, Mukhopadhyay A 2013 Genome-wide analysis reveals downregulation of miR-379/miR-656 cluster in human cancers. Biol Direct 8(10): 1-14

Lancaster GI, Febbraio MA 2005 Exosome-dependent trafficking of HSP70: a novel secretory pathway for cellular stress proteins. J Biol Chem 280:23349-23355

Luo S-S, Ishibashi O, Ishikawa G, Ishikawa T, Katayama A, Mishima T, Takizawa T, Shigihara T, Goto T, Izumi A, Ohkuchi A, Catsubara S, Takeshita T, Takizawa T 2009 Human villous trophoblasts express and secrete placenta-specific microRNAs into maternal circulation via exosomes. Biol Reprod 81: 717-729

Lu J, Clark AG 2012 Impact of microRNA regulation on variation in human gene expression. Genome Res 22:1243-1254

Montenegro D, Romero R, Kis SS, Tarca AL, Draghici S, Kusanovic JP, Kim JS, Lee DC, Erez O, Gotsch F, Hassan SS, Kim CJ 2009 Expression patterns of microRNAs in the chorioamniotic membranes: a role for microRNAs in human pregnancy and parturition. J Pathol 217(1):113-121

Morales Prieto DM, Markert UR 2011 MicroRNAs in pregnancy. J Reprod Immunol 88:106-111

Morales Prieto DM, Ospina-Prieto S, Chaiwangyen SW, Markert UR 2014 Elsevier Trophoblast research award lecture: Origin, evolution and future of placenta miRNAs. Placenta 28:S39-S45

Ouyang Y, Mouillet J-F, Coyne CB, Sadovsky Y 2014 Rewiew: placenta-specific microRNAs in exosomes—good things come in nano-packages. Placenta 28:S69-S73

Racicot K, Schmitt A, Ott T 2012 The myxovirus-resistance protein, MX1, is a component of exosomes secreted by uterine epithelial cells. Am J Reprodu Immunol 67(6):498-505

Redman CWG, Sargent IL 2007 Microparticles and immunomodulation in pregnancy and pre-eclampsia. J Reprod Immunol 76:61-67

Seabrook JL, Cantlon JD, Cooney AJ, McWhorter EE, Fromme BA, Bouma GJ, Anthony RV, Winger QA 2013 Role of LIN28 in mouse and human trophoblast cell differentiation. Biol Reprod 89(4):95, 1-13

Sonkoly E, Ståhle M, Piravesi A 2008 MicroRNAs and immunity: novel players in the regulation of normal immune function and inflammation. Semin Cancer Biol 18(2):131-140

Southcombe J, Tannetta D, Redman C, Sargent I 2011 The immunomodulatory role of syncytiotrophoblast microvesicles. PLoS ONE 6(5):e20245

Taylor DD, Akyol S, Gercel-Taylor C 2006 Pregnancy-associated exosomes and their modulation of T cell signaling. J Immunol 176:1534-1542

Théry C, Zitvogel L, Amigorena S 2002 Exosomes: composition, biogenesis and function. Nat Rev Immunol 2:569-579

Théry C, Clayton A, Amigorena S, Raposo G 2006 Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol 3.22.1-3.22.29

Torley KJ, da Silveira JC, Smith P, Anthony RV, Veeramachaneni DNR, Winger QA, Bouma GJ 2011 Expression of miRNAs in ovine fetal gonads: protential role in gonadal differentiation. Reprod Biol Endocrinol 9:2-11

Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JL, Lötvall JO 2007 Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9:654-659

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3(7):0034.1-0034.11

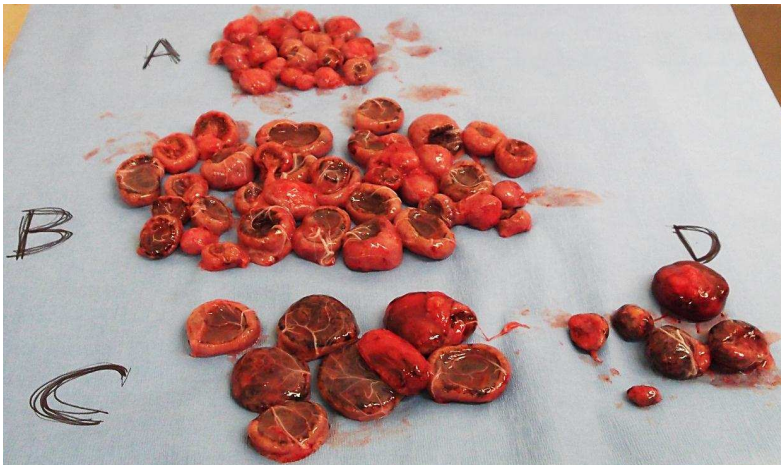
Wilfred BR, Wan WX, Nelson PT 2007 Energizing miRNA research: a review of the role of miRNAs in lipid metabolism, with a prediction that miR-103/107 regulates human metabolic pathways. Mol Genet Metab 91(3):29-217

Xu P, Zhao Y, Liu M, Wang Y, Wang H, Li YX, Zhu X, Yao Y, Wang H, Qiao J, Ji L, Wang YL 2014 Variations of microRNAs in human placentas and plasma from preeclamptic pregnancy. Hypertension 63(6):1276-1284

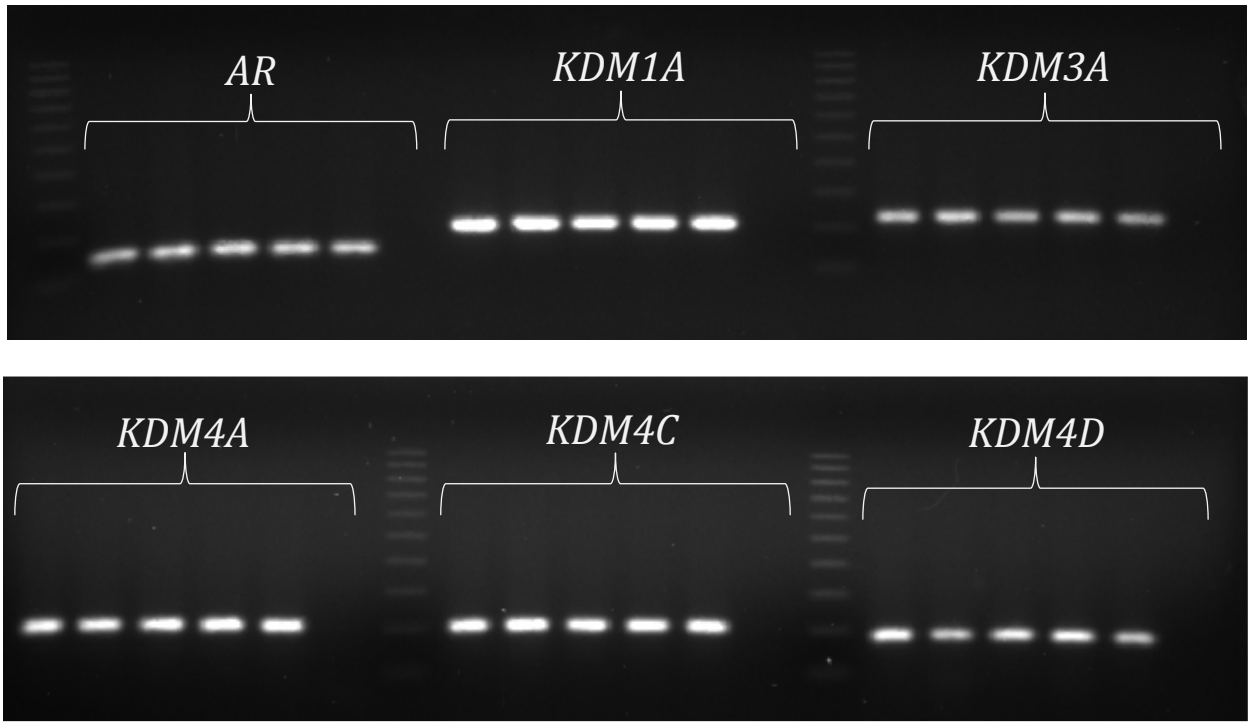
Zhang R, Wang Y-Q, Su B 2008 Molecular evolution of a primate-specific microRNA family. Mol Biol Evol 25(7):1493-1502

Zhang Y, Fei M, Xue G, Zhou Q, Jia Y, Li L, Xin H, Sun S 2012 Elevated levels of hypoxia-inducible microRNA-210 in pre-eclampsia: new insights into molecular mechanisms for the disease. J Cell Mol Med 16(2):249-259

APPENDIX II: SUPPLEMENTARY FIGURES

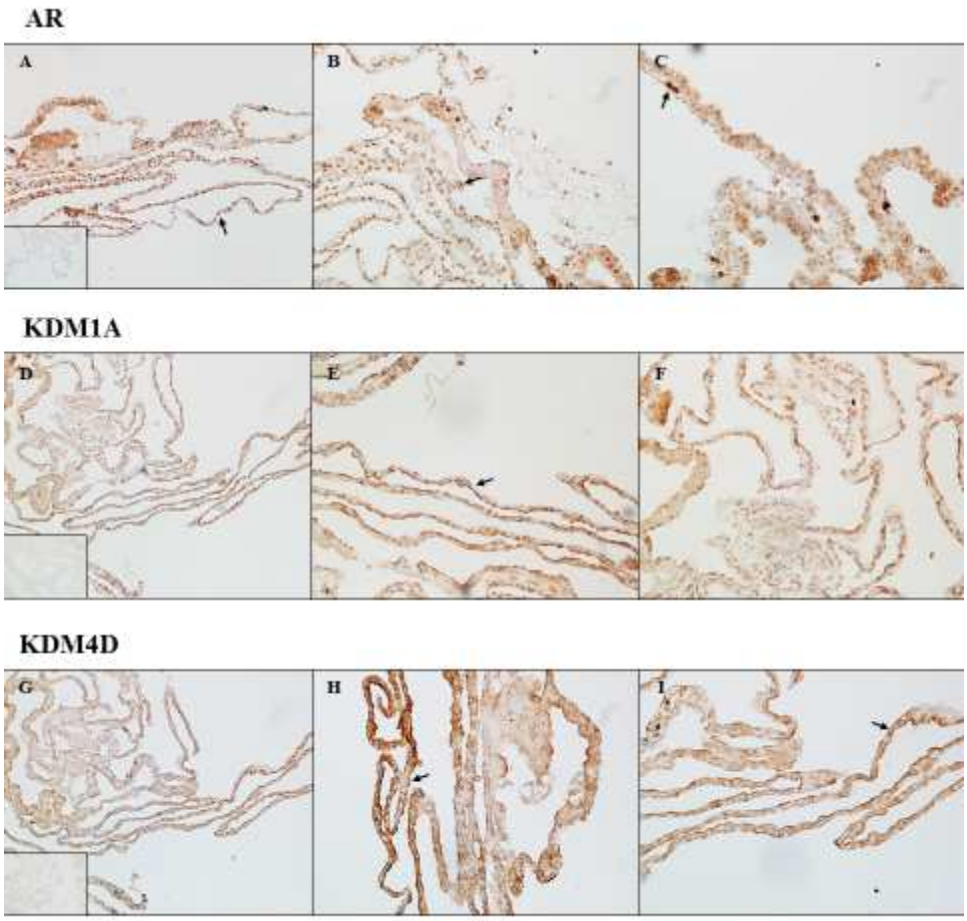


APPENDIX FIGURE II.I. Classification of ovine placentomes collected at GD90 from control and prenatal androgenized ewes.

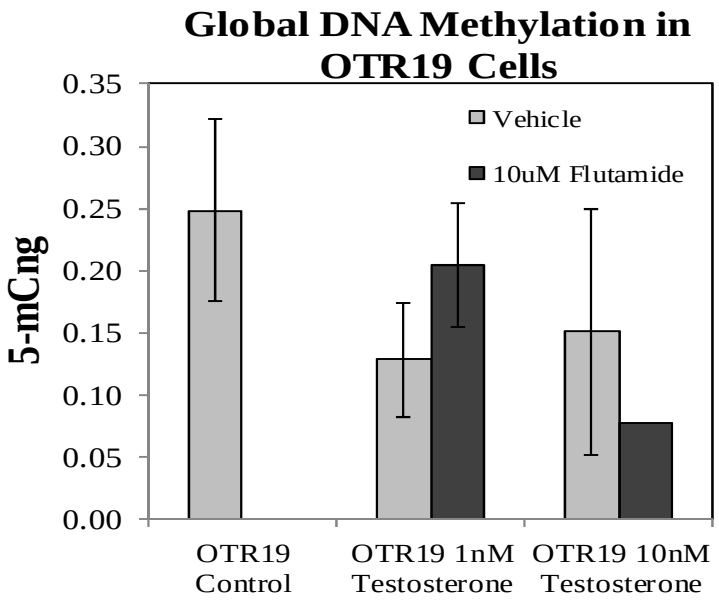


APPENDIX FIGURE II.II. End-point PCR for *AR* and *KDMs* in GD16/17 sheep conceptus. Samples provided by Dr. Quinton Winger. Primers utilized are those reported in Chapter II. Order of samples for each primer set:

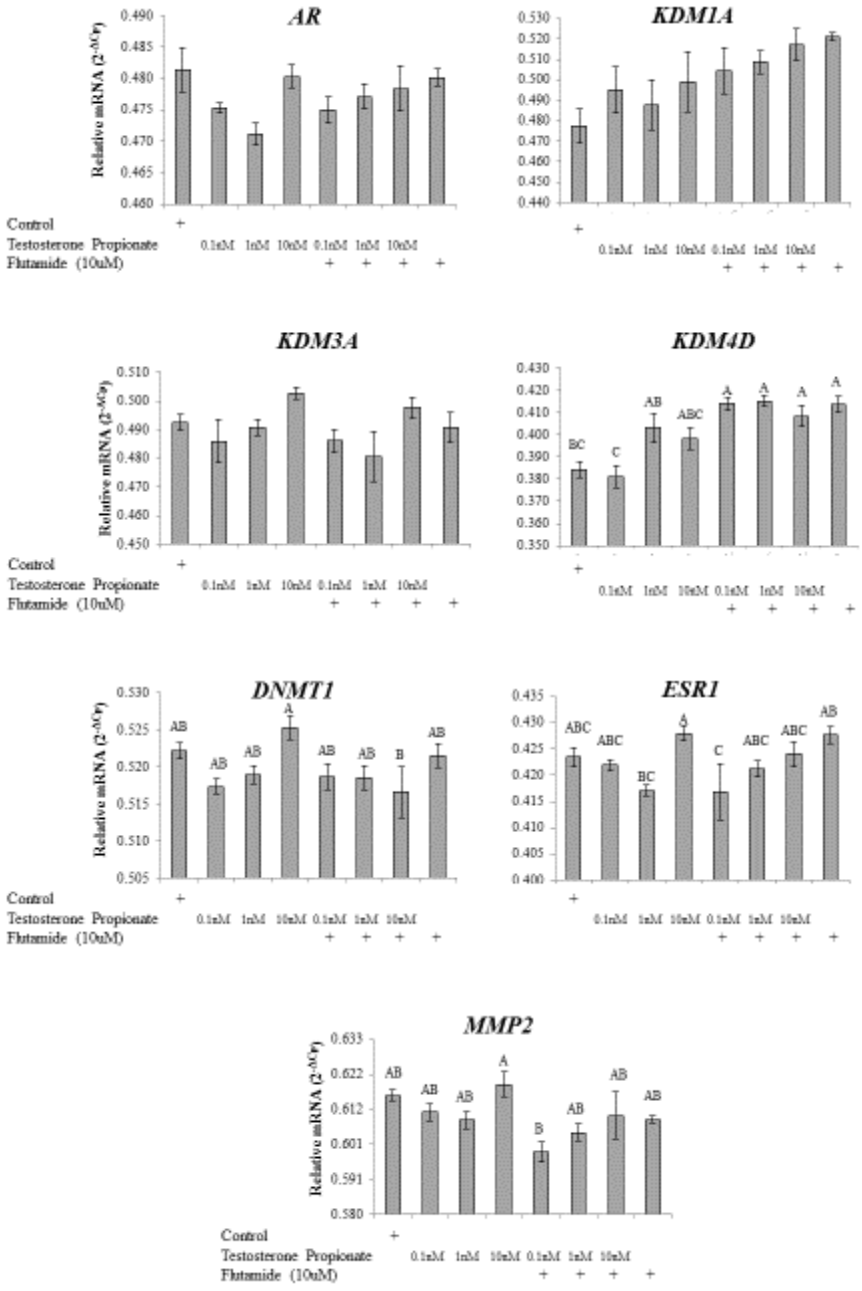
1. GD16 Ewe 163 fetus #1
2. GD16 Ewe 161 fetus #1
3. GD16 Ewe 145 fetus #1
4. GD16 Ewe 123 fetus #1
5. GD17 Ewe 622 fetus #B
6. PCR control using control from reverse transcription reaction



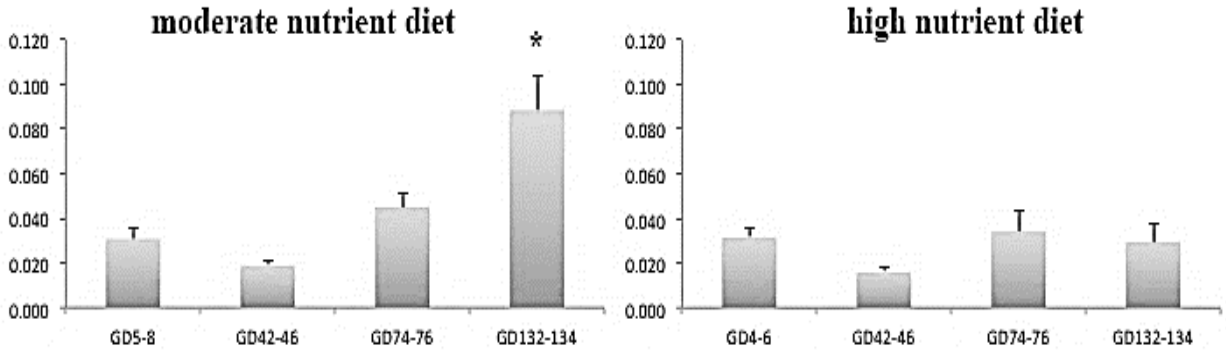
APPENDIX FIGURE II.III. Immunolocalization of AR, KDM1A, and KDM4D in gestational day 17 sheep conceptus. Samples provided by Dr. Quinton Winger. AR immunolocalized to **A)** nuclei of the trophectoderm (arrow), **B)** cytoplasm of uncharacterized or differentiating cells in trophectoderm (arrow), and **C)** in the nuclei of uncharacterized, specialized cells, possibly binucleate cells (arrow). **D-F)** KDM1A immunolocalized to nuclei of the trophectoderm (arrow), **G-I)** as did KDM4D (arrows). Inserts represent staining on control slides. Protocol followed that described in Chapter II.



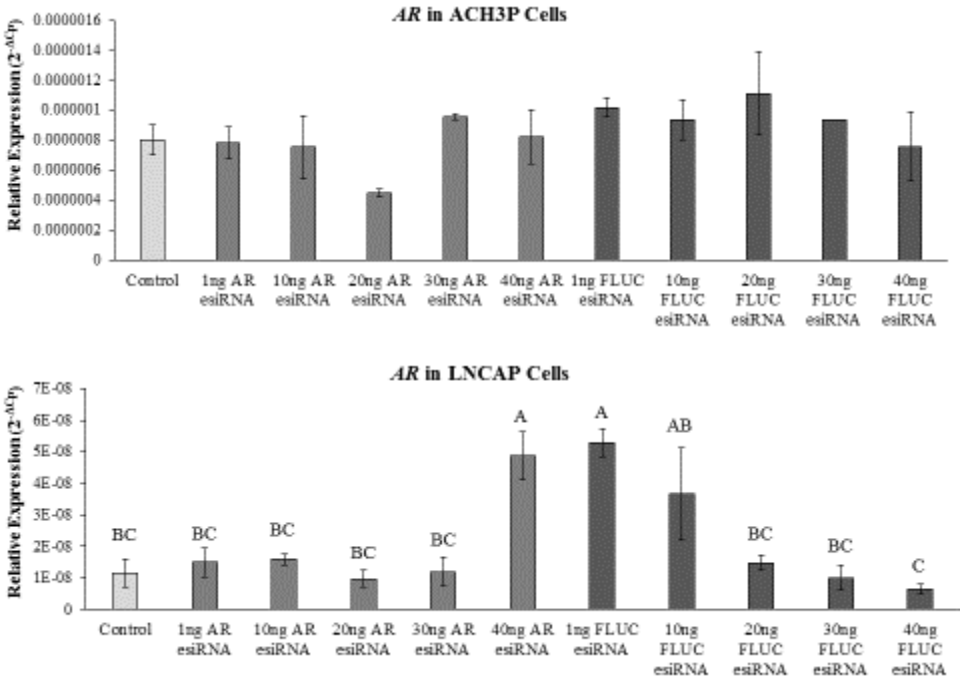
APPENDIX FIGURE II.IV. Changes in global DNA methylation in testosterone propionate treated OTR19 cells. Protocol for DNA methylation ELISA followed that described in Chapter II.



APPENDIX FIGURE II.V. Changes in relative mRNA in OTR19 cells 48 hours after testosterone propionate treatment. Difference in letter represents a statistical difference with $p<0.05$ as determined by ANOVA with Tukey's pair-wise comparison (Minitab16).



APPENDIX FIGURE II.VI. Maternal serum testosterone levels throughout gestation in the ewe fed moderate and high nutrient diet. Samples provided by Dr. Russell Anthony. RIA for testosterone was performed by Dr. Terry Nett's lab.



APPENDIX FIGURE II.VII. Relative *AR* mRNA levels with esiRNA treatment. Treatment followed protocol in Appendix IX. *AR* relative mRNA expression was normalized to *18s* in ACH-3P cells and to the geometric mean of *18s* and *GAPDH* in LNCAP cells. Treatment appeared ineffective at altering *AR* mRNA levels at the doses and times used as described in Appendix III. Difference in letter signifies a statistical difference of $P<0.05$ with absence of letters signifies no statistical difference ($P>0.05$). LNCAP, prostate cancer cell line

APPENDIX III. ACH-3P AND LNCAP TRANSFECTION TEST PROTOCOL

AR: EHUO25951-20UG SIGMA esiRNA
FLUC: EHUFLUC-20UG SIGMA esiRNA

1. Plate cells overnight in 12 well plate so that they will be 30% to 50% confluent the following morning using charcoal stripped, phenol free media
   Plate 150,000 live cells per well (assuming $4x10^5$ cells when confluent)
2. Gently add desired amount of esiRNA to Opti-MEM I Reduced Serum Medium (Or other media without serum and antibiotic) to reach a final volume of 90µL as listed below:

uL of esiRNA [200ng/µl]	**ng of esiRNA**	**Volume of Opti-MEM I Reduced Serum Medium**
0.005 µL	1 ng	89.999 µL
0.05 µL	10 ng	89.99 µL
0.1 µL	20 ng	89.98 µL
0.15 µL	30 ng	89.97 µL
0.2 µL	40 ng	89.96 µL
Control		90 µL

3. Gently mix Oligofectamine Reagent before use, then dilute 2µL with 8µL Opti-MEM I Medium to reach a final volume of 10µL per reaction. Mix gently and incubate for 5-10 minutes at room temperature
4. Combine the diluted esiRNA with the diluted Oligofectamine from step 3 to make a total volume of 100µL per well. Mix gently and incubate for 15-20 minutes at room temperature (the solution may appear cloudy)
5. While complexes are forming, remove the growth medium from the cells and wash once with media lacking serum and antibiotics. Then add 400µL of serum/antibiotic-free medium to each well.
6. Gently mix the 100 uL of complexes from step 4 to the 400uL of media in each well to get 500µL total volume per well
7. Incubate cells at 37°C for 4 hours
8. Add 250µL of growth medium containing 3x the normal concentration of serum (charcoal stripped) without removing the transfection mixture
9. Assay for gene activity at 48 hours post transfection