DISSERTATION

GENOMIC AND NON-GENOMIC ANDROGEN SIGNALING IN HUMAN PLACENTA CELLS

Submitted by

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ABSTRACT

GENOMIC AND NON-GENOMIC ANDROGEN SIGNALING IN HUMAN PLACENTA CELLS

The placenta is a transitory endocrine organ critical in regulating fetal growth and development. Not only can the placenta produce and secrete steroid hormones such as progesterone, androgens, and estrogens, but it also a target for these hormones. These hormones play an imperative role throughout pregnancy and in placental development by decidualization, regulating uterine blood flow, angiogenesis and cytotrophoblasts differentiation. However, the exact mechanism of sex steroid signaling in placental development and function is unknown. Steroid receptors exhibit genomic effects involving translocation of the hormone-receptor complex to the nucleus and subsequent gene regulation. However, steroid hormones are also known to have rapid non-genomic effects through various membrane receptors. Receptors for both estrogens and androgens have been described within the placenta but surprisingly little is known about their signaling in trophoblast cell differentiation and function, especially in the case of androgens. A solid understanding of these processes is critical, as improper cytotrophoblast differentiation can have detrimental effects on placental development, maternal and fetal health, and lead to pregnancy-related disorders such as preeclampsia (PE) and intrauterine growth restriction (IUGR).

These pregnancy disorders are significant causes of infant and maternal disease, affecting up to 15% of pregnancies in the United States. Interestingly, women diagnosed with PE and IUGR

have increased circulating androgens and placentas evaluated from these patients show increased AR levels compared to those from normal pregnancy. Therefore, abnormal androgen signaling could play a role in placental pathologies accompanying these disorders. A better understanding is necessary regarding the role and function of androgen signaling in order to provide insight into these placental diseases. Our overall goal of is to characterize and demonstrate a role for androgen signaling in placental development and function. We hypothesize that androgen signaling regulates trophoblast cell differentiation through genomic and non-genomic actions. The first chapter provides an overview about sex steroid signaling in the placenta. Chapter II focusses on the molecular regulation of androgen receptor (AR) by the pluripotency factor LIN28. LIN28 is an RNA-binding protein necessary for maintaining pluripotency in stem cells, and contains two paralogs, LIN28A and LIN28B. We previously found that LIN28A plays an important role in cytotrophoblast differentiation, however, LIN28B appears to be the predominant form expressed in sheep and human trophoblast cells. Furthermore, studies in human prostate cancer cells indicate AR is regulated by LIN28B through the miRNA let-7c. Because of the many similarities between cancer cells and trophoblast cells, we hypothesize that LIN28B regulates AR through let-7 miRNA in trophoblast cells leading to cell differentiation. We demonstrate that LIN28B regulates AR *in vitro* using the immortalized human first trimester trophoblast cell line ACH-3P. We observed that lenti-viral knockdown of LIN28B resulted in increased levels of let-7 and decreased levels of AR. Additionally, overexpression of let-7c decreased both LIN28B and AR, increased expression of syncytiotrophoblast marker ERVW-1 and stimulated hCG secretion. Therefore, genomic androgen signaling through AR appears to be involved in trophoblast cell differentiation into syncytiotrophoblast cells.

Finally, chapter III focuses on the non-genomic actions of androgens in placental cells. Specifically, we hypothesized that androgens can bind and stimulate G-protein coupled receptor GPRC6A leading to trophoblast cell fusion and hormone secretion. GPRC6A is involved in binding Osteocalcin (Ocn), an osteoblast-specific hormone that plays a role in bone calcification and calcium ion homeostasis. Ocn also binds GPRC6A in the pancreas, leading to beta cell proliferation and insulin release. More recently, GPRC6A was identified in testicular Leydig cells, and binding of Ocn resulted in testosterone production and secretion. In 2015, research emerged that testosterone activated GPRC6A can stimulate insulin secretion in the pancreas. Related to pregnancy, Ocn serum levels increase in pregnant women, where is crosses the placenta and prevents neuronal apoptosis in the growing fetus.

To demonstrate a role for GPRC6A in mediating possible non-genomic actions of androgens in the placenta, we first described its localization in placental tissues. Similar to AR, GPRC6A was detected in human, sheep, and mouse placenta, where it localizes to the syncytiotrophoblast cells. Using immortalized human first trimester trophoblast cell lines; ACH-3P and Swan-71, we also detected the presence of GPRC6A by western blot. Immunostaining revealed membrane localization of both impermeable FITC-BSA-conjugated testosterone and GPRC6A. Furthermore, non-genomic effects of testosterone-GPRC6A binding was demonstrated by treating cells with FITC-BSA-conjugated testosterone and measuring changes in second messenger pathways. Cyclic-AMP and hCG levels were measured using ELISA and revealed that treatment increased both cAMP and hCG, suggesting that androgen signaling also occurs through non-genomic pathways leading to syncytialization and hormone secretion in ACH-3P cells.

Together, these data suggest that androgen signaling involves both genomic and non-genomic signaling pathways and play a role in trophoblast differentiation and function.

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DEDICATION

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CHAPTER I: REVIEW OF LITERATURE

Summary

The placenta is recognized as an endocrine organ, largely due to its secretions of steroid hormones, including progesterone, androgens, and estrogens. Steroid hormones play an essential role in the progression of pregnancy, fetal development, and growth. Furthermore, steroids are necessary for establishment and maintenance of a normal pregnancy, preparing the endometrium for implantation, stimulating endometrial secretions, and regulating uterine blood flow; however the exact mechanism of sex steroid signaling in placental function is unknown. Estrogen and androgen receptors are known to exhibit genomic effects involving translocation of the hormone-receptor complex to the nucleus and subsequent gene regulation. However, steroid hormones are also known to have rapid non-genomic effects through various membrane receptors. Receptors for both estrogens and androgens have been described within the placenta but surprisingly little is known about their signaling in trophoblast cell differentiation and function. In this review, we will provide an overview of the current knowledge on sex steroid signaling in normal placental development as well as evidence of abnormal estrogen and androgen levels and signaling associated with placental dysfunction.

Placental growth and fetal development

The placenta is a transitory endocrine organ that secretes hormones such as androgens, estrogens, progestins, chorionic gonadotropins and placental lactogen, and is essential to the health and development of a fetus. The placenta is also responsible for the exchange of nutrients, gas, and waste between the fetus and mother, and improper placental development can lead to disorders

such as preeclampsia (PE) and intrauterine growth restriction (IUGR) [Morgan et al, 2016]. Over the past twenty-five years, the maternal morbidity and mortality rate in the United States has continued to rise due to PE and IUGR [CDC, 2016]. Often, these placental disorders are linked to metabolic syndromes, such as gestational diabetes mellitus (GDM), obesity, and polycystic ovarian syndrome (PCOS) [Bartnik et al, 2016; Koster et al, 2015], and abnormal differentiation of trophoblast cells is thought to be the underlying cause of these disorders [Sibai et al, 1997; Goldenberg et al, 2007]. However, not only is the placenta a source of hormones, it also contains receptors and as such, is a target of hormone action.

The formation and development of the placenta is a complex process that involves cell migration, cell proliferation and angiogenesis. Placental steroids have been recognized as regulators of trophoblast differentiation and development by stimulation of vascular endothelial growth factor (VEGF) and angiogenesis during pregnancy [Albrecht et al, 2010]. In humans, a fertilized oocyte develops into a multi-cell blastocyst approximately 96 hours after fertilization. On day three post-fertilization, the blastocyst enters the uterine cavity and the trophectoderm, comprised of trophoblast cells, begin to invade the maternal decidua around seven days after fertilization [Knobil and Neill 1998; Kaufmann et al, 2003]. The trophoblast cells give rise to cytotrophoblast cells, which function as proliferative bipotential progenitor cells of the placenta [Enders 1968; Kaufmann et al, 2003]. Cytotrophoblasts can further differentiate into two sublineages, extravillous trophoblast (EVT) and syncytiotrophoblast (ST). Extravillous trophoblast cells are involved in maternal spiral artery remodeling, to accommodate increased uterine blood flow to the placenta and growing fetus [reviewed in Chang et al, 2018] a continuous, multinuclear layer of syncytiotrophoblast where continued cell fusion leads to development of a

syncytium that will be in direct contact with maternal blood (figure 1) [Brosens et al, 1967; Pijnenborg et al, 1981; Benirschkle and Kaufmann 2000; Hirano et al, 2002; Guibourdenche et al, 2009].

The fetal portion of the placenta, known as the chorion, contains floating and anchoring villi that cover the surface of the placenta and are the functional units that transfer nutrients and waste between mother and fetus [Bonagura et al, 2008; Aberdeen et al, 2010]. Early in placental development, cytotrophoblasts proliferate in a hypoxic environment and begin to invade the uterus. As oxygen levels increase, cells are driven towards differentiation and rapid invasion and migration into the spiral arteries [Red-Horse et al, 2004]. This hypoxic environment stimulates estrogen and progesterone secretion from trophoblast cells in order to support uterine spiral artery remodeling and steroid production throughout pregnancy. For chorionic development to occur in humans, cytotrophoblasts cells must differentiate and invade into the maternal spiral arteries to divert and enhance blood flow to the placenta [Red-Horse et al, 2004].

By week 12 of pregnancy, the arterioles have expanded to adapt to the increase in blood flow. Failed invasion and expansion of the arterioles would lead to an inability of the placenta to support a growing fetus. During the process of spiral artery invasion, cytotrophoblasts express many angiogenesis-related factors including VEGF, insulin-like growth factor (IGF) and placental growth factor (PGF) [Zhou et al, 2002]. IGFs and VEGF are expressed throughout the placenta of various species, including the chorion, cytotrophoblasts and maternal decidualized cells, and function as key regulators of fetal growth [Zhou et al, 2002; Lash et al, 2003]. In pregnancies complicated by IGUR and PE, there is impaired blood flow, potentially due to inappropriate

maternal artery invasion by the extravillous trophoblasts. Using a well-established sheep model for IUGR, it has been reported that IUGR is associated with abnormal vasculature, decreased vessel organization [Regnault et al, 2002], and increased VEGFA levels early in gestation, suggesting a compensatory mechanism to maintain normal blood flow.

Early chorionic villus development involves invasion and migration of trophoblasts into discrete columns composed of fetal vessels and mesenchyme [Kay et al 2011]. Villi are further characterized as primary, secondary or tertiary. Primary villi contain a central core of cytotrophoblasts, secondary contain cytotrophoblasts and a mesenchymal core while tertiary villi have a surface of syncytiotrophoblasts and a complete layer of cytotrophoblasts [Kay et al 2011]. At day 21 post conception, placental vasculogenesis begins in the core of the secondary villi [Kay et al, 2011]. Pluripotent mesenchymal cells are recruited, and can differentiate into hematopoietic/blood cell precursors or angioblastic cells that will form the first vessels [Kay et al, 2011]. At day 32, vasculogenesis gives way to angiogenesis to create the complex network of vessels that comprise the placenta. This is achieved by two main types of angiogenesis, branching and non-branching.

Non-branching angiogenesis refers to the growth of a vessel by extension while branching occurs by either sprouting or intussusception and require additional recruitment of perivascular cells for stabilization and is mediated by many growth factors including; VEGF, PGF, and IGF [Kay et al, 2011]. As pregnancy progresses, *VEGFA* and its receptors, *VEGFR1* and *2* levels decrease and PGF levels begin to rise around 12 weeks post-conception [Regnault et al, 2002; Reynolds et al, 2005; Hagen et al, 2005]. As PGF expression peaks, non-branching angiogenesis

takes over to form the terminal villi [Kaufmann et al, 1985]. Although the role and importance of the placenta as an endocrine organ during pregnancy is well established and receptors for both estrogen and androgen sex steroids have been described within the placenta, surprisingly little is known about estrogen and androgen signaling in trophoblast cell differentiation and function. In this review, we will provide an overview of the role of estrogen-estrogen receptor (ESR) and testosterone-androgen receptor (AR) in placental physiology and dysfunction.

Estrogen signaling

Estrogens are steroid hormones that contain an 18-carbon structure and include; estrone (E_1), estradiol (E2), estriol (E_3), and 16-hydroxy estrone, with the most biologically active estrogen being E2. Estrogen signaling plays an important role in placental development, pregnancy and parturition. During pregnancy, the placenta is responsible for the secretion of large amounts of systemic estrogens necessary for placental cell proliferation, differentiation and angiogenesis [Hall JE., 2011]. Similar to other steroid hormones, estrogen function requires signaling through a member of the nuclear receptor superfamily of transcriptional regulators; estrogen receptor (ESR). There are two ESR's- ESR1 and ESR2 derived from separate genes. *ESR1* is located in the long arm of chromosome 6 and *ESR2* is located on chromosome 14.

As with all members of the nuclear receptor superfamily, these receptors are structurally divided into 6 domains; an N-terminal transactivation function 1 (A and B) domain, a DNA binding (C) domain, a short hinge (D) domain containing nuclear localization signals, a ligand binding and transactivating function 2 (E) domain, and a C-terminal (F) domain [recently reviewed in Arnal et al, 2017]. Estrogen binding to its receptors leads to a conformational change that causes it to dissociate from heat shock proteins and translocation of ligand bound-ER into the nucleus. This

complex binds to estrogen response elements, GGTCAnnnTGACC [Mangelsdorf et al, 1995] near or in the promotor region of target genes to regulate transcription [Lodish et al 2008]. Ligand binding activates multiprotein coactivator complexes through LxxLL motifs to regulate transcriptional activity by forming a homodimer or heterodimer with ESR2. Ligand activated ESR can also interact with DNA binding proteins such as AP1 and SP1 to promoter regions in DNA binding domain-independent fashion.

In addition to its genomic actions via nuclear ESR's, estrogens also produce non-genomic effects, which is common among steroid hormones. These effects are rapid, and occur by binding to membrane bound ESR1/2 or a G-protein-coupled receptor such as GPER1 or GPR30 [Albrecht and Pepe 2010]. Binding to these membrane receptors leads to increased intracellular calcium, cyclic adenosine monophosphate (cAMP) production or phosphorylation of protein kinase B (Akt) or mitogen-activated protein kinase (MAPK) [Björnström et al 2005]. Studies have also shown that nuclear receptors, including ESR and AR can translocate to the plasma membrane when a conserved 9 amino acid motif in the ligand binding domain becomes palmitoylated [Acconcia et al 2005]. Once localized to the plasma membrane, these receptors act as a scaffolding complex for other signaling molecules that are activated by E2 [Acconcia et al 2005].

Estrogen and ESR in placental function

The human placenta is capable of producing large amounts of estrogen necessary for placental and fetal growth as well as for the initiation of parturition [Gibb et al., 2006]. By seventh week of pregnancy, there are adequate numbers of syncytiotrophoblasts to become the main producers of hormones in the placenta [Guibourdenche et al., 2009]. Maternal cholesterol is

incorporated into trophoblast cells via endocytosis, where it can be used as free cholesterol within cellular membranes, stored in lipid droplets, or utilized for steroidogenesis [Guibourdenche et al., 2009]. The syncytiotrophoblasts are incapable of efficiently synthesizing cholesterol via *de novo* synthesis and therefore a maternal source is required during pregnancy [Guibourdenche et al., 2009]. Cholesterol is converted to pregnenolone by P450scc via side-chain cleavage, and then progesterone [Guibourdenche et al., 2009]. ESR1 and 2 have both been localized to specific cells of the placenta (Table I); ESR1 is expressed in differentiating cytotrophoblasts while ESR2 is found in syncytiotrophoblasts and extravillous trophoblasts [Bukovsky et al, 2003; Schiessl et al, 2006; Kumar et al, 2009].

For estrogen synthesis to occur in the human placenta, there needs to be adequate levels of maternal and fetal dehydroepiandrosterone sulfate (DHEA-S) secreted by the adrenal glands. DHEA-S is converted to androstenedione by 3β-HSD and then to testosterone by 17β-HSD. Testosterone is further processed into estradiol by aromatase [Bousquet et al., 1984; Strauss et al., 1996; Wooding et al., 1996; Guibourdenche et al., 2009: Hu et al., 2010]. An hypoxic environment can regulate aromatase activity and as such, estradiol synthesis [Thompson and Siiteri 1974; Goto and Fishman 1977; Zachariah and Juchau 1977]. This contributes to the varying roles of estrogen between pregnancy trimesters where increased hypoxia during the first trimester leads to rapid proliferation and differentiation of the trophoblasts as well as remodeling of the maternal spiral arteries.

Estrogen also influences uterine receptivity and blastocyst implantation by interacting directly with the uterus to regulate growth factors, cytokine release and prostaglandins [reviewed

in Bazer et al 2009]. For example, there is a finite amount of time the uterus is receptive to an implanting embryo, and estrogen and progesterone work in tandem to regulate factors necessary for this to occur [Bazer et al 2008]. Estrogens are also responsible in part, for the regulation of leptin expression in placental cells via genomic and non-genomic pathways [Gambino et al 2010]. Leptin is a nonglycosylated peptide of 146 amino acids that mediates thermogenesis, angiogenesis, arterial blood pressure, hematopoiesis osteogenesis, chondrogenesis as well as immune and neuroendocrine functions [reviewed in Schanton et al 2018]. Leptin is secreted by adipose tissue and the placenta and is a known inducer of trophoblast proliferation [Gambino et al 2012]. In first trimester trophoblast cells treated with E2, there is increased leptin expression and estrogen binding of ESR1 activates the LEP promoter in JEG-3 choriocarcinoma cells [Chardonnens et al 1999; O'Neil et al 2001]. In addition to transcriptional activation of *LEP*, treating mutant MEK or MAPK cells with E2 did not illicit a response in leptin expression, suggesting these pathways are necessary for estrogen-induced leptin synthesis [Gambino et al 2010; Maymo et al 2011].

As the uterine spiral arteries are remodeled, an extensive vascular network is created to allow for appropriate fetal growth. Estrogens stimulate angiogenesis by increasing expression of VEGF as well as increasing vascular permeability and endothelial cell mitosis [Albrect et al 2010, Astwood, 1938; Friederici, 1967]. Early in the second trimester, an increase in estrogen blocks additional differentiation of cytotrophoblasts into EVTs, by down-regulating VEGF [Albrecht et al., 2006; Bonagura et al., 2008]. This process may be due to the increased blood pressure and flow from the now remodeled maternal spiral arteries, and estrogens are no longer required to maintain the established uteroplacental blood flow [Aberdeen et al, 2010]. ESR1 signaling also has a

positive feedback role by regulating aromatase expression through its actions on CYP19A1 transcription and thus promoting placental estrogen synthesis [Kumar et al., 2009].

As indicated above, estrogens also signal through membrane receptors, including the G-protein coupled receptor GPR30. Using placental explants and immortalized human trophoblast cells HTR8/SVneo, Tong and colleagues reported that estrogen signaling through GPR30 activates the PI3K-Akt signaling pathway, up-regulates MMP9 expression and increased trophoblast cell invasion [Tong et al., 2016]. Furthermore, GPR30 levels are lower in placentas from preeclamptic women compared to placentas from uncomplicated pregnancies [Tong et al. 206; Feng et al., 2017].

Estrogen and ESR in placental dysfunction

Estrogens and ESR have also been implicated in various placental disorders such as PE, GDM and IUGR [Poidatz et al 2015]. In 2018, Wan et al described a reduction in systemic estrogen and progesterone plasma levels in 86 women with diagnosed preeclampsia with fetal-growth restriction (FGR). Of these, 35 women were characterized as being severely preeclamptic and 31 with early onset PE [Wan et al 2018]. No significant differences were found in plasma E2 and progesterone in women with mild and severe PE patients or between early versus late onset PE. Interestingly, levels of E2 and progesterone from PE placental explants were lower compared to normal tissue. In first trimester placental extracts incubated with an oxidative species to mimic oxidative stress, they found lower E2 in culture media. These findings suggest that deficiency of the placenta leads to decreased sex steroid secretion [Wan et al 2018]. In women with GDM, placental estrogens and aromatase are decreased [McRobie et al 1997; Uzelac et al 2010].

Decreased levels of E2 and E_3 have been observed in both mild and severe cases of PE and preeclampic placental tissue [Açıkgöz et al 2013; Hertig et al 2010; Jobe et al 2013] while E_1 has generally been observed as decreased only in severe cases of PE [Hertig et al 2010; Jobe et al 2013]. E_3 was also found to be significantly lower in PE placental tissue compared to normal pregnancies [Açıkgöz et al 2013].

The role of ESR in placental dysfunction is less well known and the literature has described conflicting results. For example, Molvarec et al found two *ESR* polymorphisms associated with severe preeclampsia. Peripheral blood samples revealed homozygous T-A haplotype carriers of ESR1 polymorphisms that were associated with an increased risk for severe preeclampsia [Molvarec et al 2006]. Contradictory to this study, no association between ESR polymorphisms and severe PE was described in a study in 2009 [Zhang et al 2009]. Finally, Park and colleagues examined ESR1 and ESR2 expression in preeclamptic placenta, and found that ESR1 expression is reduced and ESR2 activity appeared to be inactivated in preeclamtic placenta [Park et al., 2018]. Currently, it is unknown whether abnormal E2 levels or ESR signaling is a cause, or the result of abnormal placental differentiation or function.

Testosterone signaling

Androgens are a family of 19 carbon steroid hormones that includes adrenal androgens, dehydroepiandrosterone (DHEA) and DHEA-S, androstenedione, androstenediol, testosterone, and dihydrotestosterone (DHT). Similar to progesterone and E2, maternal testosterone plasma levels increase during pregnancy [Castracane et al 1998; O'Leary et al 1991] and the placenta itself is a source of testosterone [Bellingham et al 2012; Padmanabhan and Veiga-Lopez 2014; Rhind et

al 2011]. Recent studies revealed that the endometrium is also a source of the more potent, DHT. In fact, androgens are proposed to play a role in endometrial cell proliferation and decidualization (endometrial stromal cell differentiation) by regulating expression of decidualization marker IGFBP1 and receptivity marker SPP1 [Gibson et al 2016; Simitsidellis et al 2018].

AR has been investigated as both a transcription factor and a signal transducer in a variety of tissues. Notably AR acts as a ligand activated transcription factor to regulate gene expression by binding to the androgen response element (ARE: AGAACAnnnTGTTCT) in promoter regions of AR target genes. AR classically binds androgens to produce genomic effects involving translocation of the androgen-AR complex to the nucleus, leading to regulation of gene transcription. AR can also interact with co-regulators or DNA-bound transcription factors to regulate gene transcription. The AR gene is localized to the X chromosome and encodes a protein made up of 919 amino acids and like ESR contains an N-terminal domain, AF1 transactivation factor domain, DNA-binding and hinge domain, a ligand binding domain, AF2 transactivation domain and C-terminal domain [Park, 2005].

AR signaling was first described in *Xenopus* oocytes as a membrane signaling mechanism through activation by a bound ligand. This ligand-receptor complex repressed G-protein signaling leading to a decrease in cAMP and ERK signaling. Migeon et al [1981] localized AR to the X chromosome by establishing a cell line that expressed the testicular feminization locus (*Tfm*) from mouse kidney cells that lacked androgen binding ability. Experiments using these cells showed that when the AR locus was present, binding affinity was similar to what is observed in human cells [Migeon 1981]. Like estrogens, androgens can also illicit rapid non-genomic effects including

Ca2+ influx and cAMP pathway activation through binding of membrane receptors [Wang et al 2014; Pi et al 2010;]. For example, recent evidence suggests that the G-protein coupled receptor GPRC6A is a membrane receptor for androgens [Wang et al 2014; Pi et al 2010]. GPRC6A is better known for its role in mediating the effects of Osteocalcin, an osteoblast-specific hormone that plays a role in bone calcification and calcium ion homeostasis [Pi et al 2011]. Recent studies have indicated a role for GPRC6A in stimulating testosterone production in Leydig cells [Chamouni et al 2014]. Knock-out of GPRC6A in male mice leads to testicular feminization [Pi et al 2008] while overexpression in HEK 293 cells negative for AR treated with testosterone induced extracellular-related kinase (ERK) activity [Kang et al 2004].

Testosterone and AR in placental function

Very little is known about the androgens and androgen signaling in the placenta. Most pregnancy-related research regarding androgens centers on infertility or placental disorders such as PE. During early embryonic development, testicular androgen production is required for appropriate male sex differentiation [Metzler et al 2017; Hughes et al 2012]. In 1979, Barile et al reported the first evidence for a testosterone-binding protein in human placental tissue. In 1980, Hirota et al found AR in the cytosol of human placental villi and it has since been localized to differentiated syncytiotrophoblast, cytotrophoblasts and placental stroma (Table I) [Iwamura et al, 1994; Hsu et al, 2009]. Research from our laboratory demonstrates that AR binds to an ARE in the VEGF promoter in ovine placental tissue. This data suggests that placental androgens may play and important role in regulating trophoblast function [Cleys et al 2015].

In pregnant women, androstenedioine levels increase starting on day 14 of pregnancy [Castracane et al 1998; O'Leary et al 1991] while testosterone was found to decrease blood flow to the placenta [Abbas and Gupta 2008]. Human fetuses with complete androgen insensitivity syndrome due to mutations in AR 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 AR 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]. This suggests that maternal androgen signaling may be sufficient to maintain androgen-mediated angiogenesis in the placenta during pregnancy.

Alternatively, compensatory mechanisms likely exist to support placental development and differentiation, and possibly include non-genomic signaling, heterodimerization with other nuclear receptors, and/or overlapping functions of both estrogen and androgen receptor signaling. For example, estrogen regulation of LEP expression in trophoblast cells involves both genomic and non-genomic processes and both ESR and AR are known regulators of cell proliferation and angiogenesis. Finally, like androgens, maternal serum levels of osteocalcin also increase during gestation [Maliqueo et al 2016; Seki et al 1991]. Although AR has been localized to the decidua and placental trophoblast cells in humans (table 1), nothing is known about GPRC6A localization in the placenta [Uzelac et al 2010; Srichomkwun et al 2015].

Testosterone and AR in placental dysfunction

There is limited information about a functional role of androgens and AR during pregnancy and atypical placental development. In women with PCOS, increased serum testosterone has a profound impact on insulin action, increased adiposity, increased risk of developing type II diabetes [reviewed Morford et al 2018] and increased adverse pregnancy outcomes, including miscarriage [Bahri et al 2018 and references therein]. Increased maternal glucose has been shown to cause increased fetal birth weight and neonatal adiposity [reviewed by Bahri et al 2018; Meng et al 2016]. In PE patients there is increased serum testosterone levels and decreased aromatase activity compared to normal pregnancies which is non-dependent on fetal gender [Atamer et al 2004; Ghorashi and Sheikhvatan 2008; Hsu et al, 2009; Thoumsin et al 1982]. AR levels have been shown to be increased in the syncytiotrophoblast and stroma of PE patients [Hsu et al., 2009; Sathishkumar et al, 2011] and like ER, polymorphisms in AR have been correlated with an increased risk for the development of PE, miscarriage, and spontaneous pre-term birth [Lim et al 2011; Jahaninejad et al 2013].

Specifically, polymorphisms greater than 16 GGC trinucleotide repeats in the transcriptional activation domain of the *AR* gene can lead to decreased AR function and expression [Lim et al 2011]. PE patients have also been found to have increased free testosterone and androstenedione at both 17 and 33 weeks of gestation [Carlsen et al., 2005] but by six weeks post-partum, testosterone levels in the serum return to normal [Serin et al., 2001]. This association suggests an alternative source of androgens during pregnancy, most likely the placenta. In women with GDM, increased testosterone has been used as an early indicator of developing the disease and increased AR in the placental tissues of these patients has been reported [Uzelac et al 2010;

Gözükara et al 2014]. Decreased *VEGFR2* and *VEGFRA* are also decreased in GDM placentas compared to normal tissue [Uzelac et al 2010] suggesting a derangement in placental angiogenesis, potentially mediated by androgens.

Excess testosterone during pregnancy such as seen in women with PCOS has been reported to negatively affect placental angiogenesis, but not lead to fetal virilisation of female fetuses [O'Leary et al 1991; Castracane et al 1998; Sir-Petermann et al 2002; Fornes et al 2016]. For example, in rats treated with exogenous testosterone, placental VEGFR1 expression was decreased compared to controls [Fornes et al 2016]. Similarly, in a prenatal androgenization PCOS model in sheep increased AR expression in the placenta was observed, as well as increased levels epigenetic regulators including DNMT1 and H19 (Cleys et al., 2015). Interestingly, contrary to the observation in rats treated with testosterone and in GDM placentas that VEGFA receptor decreased, placental expression of VEGFA protein were increased both on maternal and fetal sides in this PCOS model. This suggests potential compensatory exist in trophoblast cells to regulate proper placental angiogenesis.

ESR and AR in placenta of other mammals

Sex steroids and their receptors have also been found in placenta of other species, such as rodents and ruminants, and similar to humans little is known about ESR and AR function in this organ. For example, studies in mice have reported that ESR1 is expressed in uterine stromal and epithelial cells and is important in mediating decidualization and implantation [e.g., Pawar et al., 2015]. However, very little is known about ESR in placenta aside from its reported presences according to RT-PCR [Mouse Genome Informatics reference ID J:46439]. In mouse embryos, it

was found that ESR may regulate trophectoderm cell differentiation [Cheng et al 2016]. Similarly, AR was found to be located in nuclei of placental stroma at day 1-2 of gestation and increased days 3-4. After day 5 of pregnancy, AR protein levels decrease [Xu et al 2015]. *In vivo* AR is inhibited during decidualizion, possibly due to the increase of progesterone during this process, while estrogen was found to increase AR expression in uterine stroma [Xu et al 2015]. In obese mice exposed to DHT at gestational day 15.5, AR protein expression increased in the placenta, similar to what occurs in women suffering from polycystic ovarian syndrome (PCOS) [Fornes et al 2017].

In sheep, both ESR1 and ESR2 have been localized to maternal and fetal placental tissues during early pregnancy (days 14-30) by immunofluorescence [Bairagi et al 2018].-*ESR1* levels are decreased after day 16 of pregnancy in caruncular (maternal) tissue, whereas *ESR2* levels are generally low [Reynolds et al 2015]. Interestingly, *ESR1* levels increase in between day 16 and 20, after which they drop in cotyledons (fetal). Previously, we reported AR presence in sheep placentomes at mid-gestation both in maternal and fetal compartments [Cleys et al., 2015]. Moreover, chromatin immunoprecipitation experiments indicated that VEGFA is a direct target of AR in trophoblast cells [Cleys et al., 2015], suggesting AR signaling may play a role in regulating placental angiogenesis through its action on expression of angiogenic factors such as VEGFA.

Finally, both ESR1 and ESR2 (mRNA and protein) have been reported in bovine placentomes. Interestingly, in cows ESR1 appears to be confined primarily to caruncles, whereas ESR2 is present in cotyledons during mid- and late gestation. ESR1 localized to caruncular epithelial cells and capillary pericytes [Hoffmann & Schuler, 2002]. Due to its higher expression

on the fetal side, and localization in mature trophoblast giant cells and vascular cells, ESR2 is thought to play a role in trophoblast giant cell differentiation and vascular function [Schuler et al., 2002; 2005]. An intracrine function for local ESR signaling in placentomes is further supported by the observation that inactive estrone sulfate entering maternal circulation maybe converted locally into (active) free estrogens by steroid sulfatase present in caruncles, as well as upregulation of aromatase during trophoblast giant cell differentiation [Schuler et al., 2008].

Similarly, AR is present cow placentomes from day 50 until term. Moreover placental testosterone concentrations increase during gestation suggesting a role for androgen signaling through AR in placental physiology [Khatri et al., 2013]. Nuclear staining for AR is observed in trophoblast giant cells, and immunoreactivity appeared to increase in immature and mature trophoblast giant cells, uninucleated trophoblast cells and stromal cells from mid-gestation until term. This overlap in expression between ESR2 and AR in trophoblast giant cells suggest possible complimentary functions in trophoblast cell function or differentiation.

Summary & Conclusion

The placenta is well known for its ability to secrete hormones during pregnancy thereby promoting fetal growth and development as well as maintaining pregnancy. The placenta synthesizes and secretes estrogens and androgens throughout pregnancy, and both ESR and AR localize to different trophoblast cells types suggesting diverse roles in placental development and function. Placenta development and trophoblast differentiation involves many processes commonly seen in cancer, such as proliferation, migration, invasion, and angiogenesis, and ESR and AR signaling are known to be involved in these cellular processes.

Similar to their role in cancer and tumorigenesis, we postulate that estrogen and androgen signaling play a diverse role in trophoblast differentiation (Figure 2). To date studies have demonstrated that E2 through non-genomic or genomic (ESR) signaling regulates LEP expression thereby in turn controlling trophoblast proliferation, and is a known regulator of placental angiogenesis. However its' role in trophoblast differentiation into invasive EVT's or cell fusion and syncytialization is less clear. Very little is known about AR function in the placenta. Recent evidence indicates AR signaling plays a role in endometrial cell differentiation (decidualization), and our own data suggests a role in regulating VEGFA and placental angiogenesis. Whether additional or unique roles exist in trophoblast proliferation, differentiation and/or syncytialization is still unclear.

Finally, the observations that pregnancy disorders such as PE, GDM, and PCOS are associated with placental dysfunction and abnormal estrogen/ESR and androgen/AR levels suggests involvement of these steroids in normal placental physiology. Furthermore, although both ESR's and AR have been localized in placental tissue, the exact contribution of abnormal of ESR and AR signaling in placental dysfunction in these pregnancy disorders remains to be fully explored. Ultimately, this knowledge will provide the foundation for future studies designed to uncover underlying mechanisms in which for example metabolic diseases associated with abnormal steroid levels lead to placental dysfunction. Moreover, it also provides novel avenues that focus on estrogen and androgen signaling that can be targeted/investigated to alleviate pregnancy disorders associated with placental dysfunction and abnormal steroid hormones levels.

Genomic and non-genomic androgen signaling in the placenta; novel studies

Previous studies have indicated that AR is regulated by pluripotency factor LIN28B through small non-coding RNA *let-7c* in prostate cancer. LIN28B is an RNA-binding protein that is involved in cell differentiation and is regulated by the let-7 family of micro-RNAs (miRNAs). Research in our lab has uncovered a role for LIN28A in modulating trophoblast cell differentiation, as trophoblast cells differentiate, LIN28 levels go down and let-7 levels increase [Seabrook et al 2014]. LIN28A and B have been localized to the cytotrophoblast layer of first trimester human placental tissue [Seabrook et al 2014]. This data demonstrates that LIN28, in addition to being a potent regulator of pluripotency in human embryonic stem cells, may have a conserved functional role in trophoblast differentiation. Emerging research has shown that both LIN28A and B work in tandem to directly inhibit the let-7 miRNA family and prevent stem cell differentiation. As Lin28B is a potent regulator of cell differentiation in other cell populations, we hypothesized that Lin28B acts as a regulator of AR through *let-7c* miRNA in human trophoblast cells. The first aim of this work describes the relationship between LIN28B-*let-7c*-AR in first trimester human trophoblast cell line; ACH-3P and overexpression of *let-7c* induces increased levels of syncytialization markers *ERVW-1* and hCG.

Because androgen signaling is poorly understood in the placenta, the second aim will focus on testosterone-activation of membrane receptor GPRC6A. Recently, GPRC6A, a G protein-coupled receptor, was found to bind testosterone and dihydrotestosterone. GPRC6A is involved in binding Osteocalcin (Ocn), an osteoblast-specific hormone that plays a role in bone calcification and calcium ion homeostasis. Ocn also binds GPRC6A in the pancreas, leading to beta cell proliferation and insulin release, as well as stimulating testosterone production by binding Leydig

cells in the testes [Pi et al 2011]. Furthermore, Ocn levels increase in pregnant women, crossing the placenta to prevent neuronal apoptosis in the growing fetus [Seki et al 1991]. We hypothesize that GPRC6A plays a role in mediating the non-genomic effects of androgens to induce syncytialization. The second aim of this work describes testosterone-activation induces differentiation of cytotrophoblast progenitor cells *in vitro*.

This body of work describes genomic and non-genomic androgen signaling in human placental cells. In the following chapters, we describe a regulation mechanism for AR in human placental cell line ACH-3P, as well as a potential role for non-genomic testosterone signaling through membrane receptor GPRC6A, in regulating human trophoblast cell differentiation.

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CHAPTER II: THE LIN28B - LET-7C - AR AXIS IN HUMAN TROPHOBLAST CELL DIFFERENTIATION

Summary

LIN28 is an RNA-binding protein necessary for maintaining pluripotency in stem cells and plays an important role in trophoblast cell differentiation. LIN28B regulates AR signaling through its actions on let-7c in human prostate cancer. We hypothesize that LIN28B regulates AR through let-7 miRNA in trophoblast cells leading to trophoblast cell differentiation. A human first trimester trophoblast cell line, ACH-3P was used to evaluate the regulation of AR through small, non-coding miRNA let-7c. LIN28B and AR mRNA and protein levels were determined by qRT-PCR, Western blot, and immunolocalization respectively. A LIN28B knockdown cell line was constructed using lentiviral-based vectors and overexpression of let-7c using a synthetic miRNA (mimic) were used to examine the regulation of AR in ACH-3P cells. AR mRNA, protein, differentiation markers *ERVW-1* and soluble hCG concentrations were examined in these models. LIN28B knockdown in ACH-3P cells resulted in increased levels of let-7 and decreased levels of AR. Furthermore, addition of let-7c mimic decreased AR in ACH-3P cells. Finally, LIN28B knockdown in ACH-3P and let-7c mimic treatment both resulted in increased expression of syncytiotrophoblast marker ERVW-1 and hCG secretion. These data reveal a molecular interaction between LIN28B and AR through let-7c, which may be necessary for trophoblast cell differentiation into the syncytiotrophoblast sub-lineage.

Introduction

Preeclampsia (PE) and intrauterine growth restriction (IUGR) are significant causes of infant and maternal disease. PE is thought to account for approximately 12% of maternal deaths while IUGR affects up to 8% of pregnancies in the United States (Campbell et al 2013). Over the past 25 years, the maternal mortality rate in the United States has continued to increase and experts believe this association is linked to pre-existing hypertensive diseases, which is a significant risk factor for the development of PE [Bellamy et al 2007]. In humans, impaired trophoblast cell differentiation and invasion into the maternal spiral arteries is thought to be an underlying cause of these placental disorders [Regnault et al 2002]. Placental dysfunction is a significant health problem as it not only can adversely affect maternal and fetal well-being but also has long-term health effects that include hypertension, cardiac disease, and obesity in offspring [Simmons 2012; Sarr el al 2012; Barker et al 1990]. Studies evaluating placentas in women with PE consistently show maternal and fetal vascular abnormalities, which may be related to trophoblast cell differentiation [Kovo et al 2013]. Therefore, understanding the regulation of human trophoblast differentiation and molecular events that control placental growth and development is crucial in understanding underlying causes of placental disorders.

Our research has focused on the RNA-binding protein LIN28 and nuclear transcription factor androgen receptor (AR) in placental development. LIN28 is important in maintaining pluripotency in human stem cells, and our studies have shown a role for LIN28A in trophoblast cell differentiation [Seabrook et al 2013]. Recently, Tummala et al. showed that LIN28, through its actions on small noncoding microRNA let-7, also regulates AR-dependent signaling in human prostate cancer [Tummala et al 2013]. This is significant as PE patients have significantly

increased circulating serum androgens and increased placental expression of AR [Tummala et al 2013; Nadiminty et al 2012]. Moreover, our preliminary studies have revealed that AR binds an Androgen Response Element (ARE) in the promoter region of *VEGFA* [Cleys et al 2014]. Therefore it is possible that similar to what is described in cancer cells, trophoblast differentiation and placental development involves a molecular interaction between LIN28 and AR through let-7 miRNAs.

MicroRNAs (miRNA) are small (~19-22 nucleotide), non-coding RNAs that regulate gene expression by degradation of mRNA or inhibition of translation. This specific family of miRNAs is regulated by LIN28A and B, two homologs of the heterochronic gene, LIN28 [Zhang et al 2016]. LIN28 is an RNA binding protein involved in cell differentiation - and as cells differentiate LIN28 levels decrease, resulting in an increase in let-7 miRNAs. Both LIN28A and B are important in regulating the let-7 family of miRNAs by inhibiting let-7 processing at pri- and pre-miRNA stages, keeping cells in an undifferentiated state [Zhang et al 2016]. Let-7s can target mRNA for degradation or repression [Boyerinas et al 2010]. Zhang et al. demonstrated that LIN28B is active during early reprogramming in human fibroblasts and in conjunction with LIN28A, is important in embryonic stem cell pluripotency and metabolism [Zhang et al 2016].

Preliminary studies performed in our laboratory indicate AR localization in the cytotrophoblast and stroma of first trimester human placenta samples. It has been widely observed that differentiation of the trophoblast during the first trimester occurs in a hypoxic uterine environment and is regulated by steroid hormones, such as estrogen, which also promotes vascularization and angiogenesis [Atamer et al 2004; Carlsen et al 2005]. In this study, we use first

trimester human trophoblast cells line ACH3P to develop a LIN28B knock-down cell line to further investigate the interaction between AR and LIN28B. We hypothesize that, similar to prostate cancer cells, LIN28B regulates AR through the miRNA let-7c in trophoblast cells, leading to trophoblast differentiation.

Materials and Methods

Cell lines and treatments

Two human first trimester trophoblast cell lines were used in this study: ACH-3P (a gift from Ursula Hiden, Medical University of Graz, Austria) immortalized by fusion of primary first trimester human trophoblast cells with human choriocarcinoma cell line, AC1-1 and a telomerase immortalized first trimester trophoblast cell line [Hiden et al 2007], Swan 71 (a gift from Gil Mor, Yale University, USA) [Straszewski-Chavez et al 2009]. ACH-3P cells were cultured in DMEM F-12 medium (HyClone), 10% FBS (HyClone), and 1% PSA (Corning Life Sciences). Swan 71 cells were cultured in DMEM/F12 medium, 10% FBS, 1% PSA, 1 mM non-essential amino acids (HyClone), and 1 mM sodium pyruvate (Corning Cellgro). Syncytialization of ACH-3P cells was induced with 40 μM forskolin (Sigma-Aldrich) treatment for 48 h. Control cells were treated with 0.1% DMSO.

Human first trimester placental samples

Human first trimester (11.5 week) placental samples were obtained from elective terminations from anonymous, non-smoking, non-drug using patients, in accordance with the Colorado State University Institutional Review Board. Samples were stored in sterile PBS upon collection and were transferred to ice cold 4% paraformaldehyde (Fischer Scientific) 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, first trimester (n=3; LIN28B, n=2; AR) samples were available. Tissue sections of 5 µm thickness were taken from the center of paraffin blocks for immunofluorescence or immunohistochemistry.

Immunostaining

To determine cellular localization of selected proteins (AR and LIN28B) in human placental samples, 5 µm sections of embedded tissue were mounted onto charged glass microscope slides (Premiere). Sections were deparaffinized and rehydrated in successive 4 minute baths of Citrasolv (Decon Labs), 100% ethanol, 90% ethanol, 70% ethanol, 50% ethanol, and distilled water. Sections then underwent antigen retrieval using 10 mM sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0) and microwaved for 10 minutes after being brought to a boil. Sections were rinsed 3 times with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) then blocked at room temperature using 6% goat serum in PBS.

Sections were then incubated in a humidity chamber overnight at 4°C in polyclonal antibodies against AR (Abcam 74272) or LIN28B (Cell Signaling 41965), and hCG (NeoMarkers) at 1:100 for immunofluorescence. After washing 3 times in PBS, sections were incubated for 1 hour at room temperature in 1:1000 goat anti-rabbit secondary antibody (Abcam ab6721) for immunohistochemistry or 1:1000 goat anti-rabbit AlexaFluor 488 (Abcam, ab150077) and goat anti-mouse AlexaFluor 594 (Abcam, ab150092) for immunofluorescence. After 1 hour, slides were washed in PBS then dehydrated by successive baths of 50%, 70%, 90%, and 100% ethanol. Diaminobenzedine (DAB) was used as the final chromogen for immunohistochemistry (product

no. SK-4100; Vector Laboratories). Slides used for immunofluorescence were mounted with Prolong Gold containing DAPI (4',6-diamidino-2-phenylindole) (Life Technologies). Secondary antibody only staining was used as a negative control. Slides were visualized using Olympus D73 camera on Nikon Eclipse E800 microscope and CellSense 1.3 software.

LIN28B Knockdown (KD)

LIN28B KD and non-target scramble control cell lines were created using commercially available MISSON shRNA Lentiviral Transduction Particles, lentiviral-based pLKO.1-puro vectors (Sigma-Aldrich TRCN0000122599) with a puromycin resistance gene for selection downstream of a human phosphoglycerate kinase eukaryotic promoter. The mission particles were designed to target human *LIN28B* for degradation. ACH-3P cells were infected in three replicate experiments with either *LIN28B*-targeted particles or non-target control particles at an MOI of 500 viral particles per cell. Infected and non-infected ACH-3P cells were selected by treatment with 8 µg/ml puromycin for 14 days to verify selection. LIN28B mRNA and protein KD was determined by qPCR and Western blot.

Western blot

Western blot analysis was used to determine the presence LIN28B and AR protein in human first trimester trophoblast cells. Cells were lysed in RIPA buffer (20 mM Tris, 137 mM NaCl, 10% glycerol, 1% nonidet P-40, 3.5 mM SDS, 1.2 mM sodium deoxycholate, 1.6 mM EDTA, pH 8) with 10% protease/phosphate inhibitor cocktail (Sigma-Aldrich) and 1 mM phenymethanesulfonyl fluoride. The BCA protein assay kit (Pierce) was used to determine protein concentration. Absorbance was measured at λ 595 nm using a Biotek Synergy 2 Microplate Reader

(Biotek). Protein was electrophoresed in 4-15% Mini-PROTEAN TGX Stain-Free precast gels (Biorad) and transferred to 0.2 μm pore nitrocellulose membrane (Biorad) at 110 volts at 4°C for 1 hour.

Membranes were then blocked in 5% milk-TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6) for 1 hour at room temperature then incubated in a goat polyclonal antibody to AR (Abcam ab19066) or rabbit polyclonal antibody to LIN28B (Bethyl A303-588A) diluted 1:1000. Blots were incubated at 4°C overnight. Membranes were then washed 3 times for ten minutes for a total of 30 minutes in 1x TBST, incubated with a horseradish peroxidase-conjugated secondary antibody rabbit anti-goat (Abcam ab6741) and goat anti-rabbit (Pierce 1858415) for 1 hour at room temperature, diluted 1:2000. Rabbit polyclonal antibodies; GAPDH (paired with AR) and αTubulin (paired with LIN28B), were used as loading controls. Membranes were developed using ECL Western Blotting Detection Reagent chemiluminescent kit (Thermo Fisher Scientific) and imaged using a ChemiDoc XRS+ chemiluminescence system (BioRad). Densitometry was measured using ImageJ software (NIH) to compare relative density of normalized protein samples and student's *t*-test was used to compare relative intensity between ACH-3P and Swan 71 WT cells and between ACH-3P non-target controls and LIN28B KD cells. P values ≤ 0.05 were considered to be statistically significant. All western blot experiments were performed with three bioreplicates per cell line.

Real-Time RT-PCR

Messenger RNA was extracted from cell pellets using mRNeasy Mini Kit (Qiagen) while small RNAs were extracted from cell pellets using miRNA Mini Kit (Qiagen) according to the

manufacturer's directions. Complementary DNA (cDNA) was generated from 1µg of total RNA using qScript cDNA Supermix (Quanta Biosciences) and quantitative real-time RT-PCR (qPCR) of mRNA was performed. Each 20ul qPCR reaction consisted of 10µl SsoAdvanced Universal Probes Supermix (Biorad), 1µl of 150 nM TaqMan Gene Expression Assay (Applied Biosystems), and 9µl of cDNA template diluted to 11ng/uL. Quantitative PCR was performed using the LightCycler480 thermal cycler (Roche) with the following parameters: 10 min pre-incubation at 95°C, 40 cycles of amplification, which included denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds, followed by a final cooling cycle at 40°C for 5 min. Normalization of mRNA in human cells was accomplished using GAPDH.

For miRNA analysis, cDNA was generated from 500 ng of total cellular RNA using miScript II RT Kit (Qiagen). Each 10µl miRNA qPCR reaction consisted of 5µl LightCycler 480 SYBR Green I Master mix (Roche), 1µl of forward primer, 1µl universal reverse primer (Qiagen miScript for human *let-7* miRNA primers), and 8µl of cDNA template diluted to 1ng/uL. MicroRNA qPCR was performed using the LightCycler480 thermal cycler (Roche) with the following cycling parameters: 15min enzyme activation step at 95°C, followed by 45 cycles of amplification, which included denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 70°C for 30 seconds, followed by a melting curve analysis to confirm amplification quality and specificity. Normalization of *let-7* miRNA levels in human cells was calculated using the geometric mean of small nucleolar RNA, C/D box 41 *(SNORD41*), and small nucleolar RNA, C/D box 44 (*SNORD44*) miRNAs. For all qRT-PCR experiments, normalized data was analyzed and plotted as $2^{-\Delta Cp}$. One-way ANOVA was used to compare relative mRNA levels in non-target controls and *LIN28A* KD ACH-3P cells. *P* values $\leq$ 0.05 were considered to be

statistically significant. All qRT-PCR experiments were performed with three bioreplicates per cell line.

Enzyme-Linked Immunosorbent Assay

ACH-3P LIN28B KD and non-target scramble control cells were seeded in six-well culture dishes with 2.5×10^5 cells per well and three biologic replicates per treatment. Cell culture medium (1.5 ml/replicate) and cells were collected and counted at 24, 48, and 72 hours. Concentrations of human chorionic gonadotropin (hCG) were quantified using an hCG ELISA kit utilizing a mouse monoclonal anti-hCG (specific to beta-hCG) conjugated to horseradish peroxidase according to the manufacturer's instructions (ALPCO Diagnostics). Experimental replicates were assayed in triplicate, and absorbance was measured at λ 450 nm. Soluble hCG concentrations (mIU/ml) were calculated by plotting sample mean absorbance values against the mean values calculated from duplicate standard curve values. The standard curve consisted of 0, 5, 20, 50, 150, and 300 mIU/ml hCG standards. Soluble hCG concentrations were then normalized to the number of cells at the time of collection. One-way ANOVA was used to compare hCG concentrations between non-target controls and LIN28B KD ACH-3P cell medium. *P* values ≤ 0.05 were considered to be statistically significant. All ELISA experiments were performed with three bioreplicates per cell line.

Let-7 mimic transfection

ACH-3P WT cells were seeded at 4×10^4 cells per well in 500ul DMEM F-12 on a 24 well plate. Once adhered, cells were transfected with hs-let-7c-5p, hs-let-7e-5p, or hs-let-7f-5p (Qiagen) to mimic human mature miRNA (5'UGAGGUAGUAGGUUGUAUGGUU,

5'UGAGGUAGGAGGUUGUAUAGUU, and 5'UGAGGUAGUAGAUUGUAUAGUU respectively) and 3uL HiPerFect Transfection Reagent (Qiagen) to a final concentration on 5nM of mimic. Control cells were transfected with Mission siRNA Universal Negative Control (sic-002) and 3uL HiPerFect Transfection Reagent at the same concentration. Cell pellets were collected at 48 hours for total RNA and protein. All mimic experiments were performed with three bioreplicates per treatment. Student's t-test was used to compare *let-7* levels between Universal siRNA controls and mimic treated ACH-3P cells.

Statistics

GraphPad Prism 7© software was used to perform unpaired t-tests using qRT-PCR $2^{-\Delta Cp}$ values and Western blot band density comparing ACH-3P and Swan 71 cells, and non-target scramble controls and LIN28B KD cells. A p-value of less than or equal to 0.05 was considered statistically significant.

Results

LIN28B and AR protein and mRNA in the human placenta

Previous studies in our lab demonstrated the presence of LIN28A in mouse placenta and human ACH-3P cells, and revealed a role for LIN28A in trophoblast cell differentiation. Subsequent studies uncovered that ACH-3P cells in fact contain LIN28A and LIN28B, whereas human first trimester placenta primarily contain LIN28B (West et al., manuscript submitted). The goal of this study was to demonstrate if the LIN28B-Let7-AR molecular axis exists in trophoblast cells similar to what has been described in cancer cells. In human first trimester placenta, LIN28B and AR appear to localize to cytotrophoblast cells as indicated by the lack of co-staining for hCG

which is specific to the syncytiotrophoblast cells (figure 3), whereas AR also localized to the trophoblast stromal cells (figures 3B and D). Relative amounts of LIN28B and AR were assessed in two commonly used human first trimester trophoblast cells lines, ACH-3P and Swan 71. Real time PCR analysis revealed significantly higher levels of both *LIN28B* and *AR* in ACH-3P cells versus Swan 71 cells (figures 4A and B). Furthermore, Western blot analysis revealed a single specific band of ~32 kDa for LIN28B only in ACH-3P cells (figure 4C), a single band of approximately 79kDa for AR was detected only in ACH-3P cell lysate (figure 4D).

Effects of ACH-3P LIN28B KD on AR

ACH-3P cells were treated with lentiviral-based pLKO.1-puro vectors (Sigma-Aldrich) designed to specifically target *LIN28B*. Transcript and protein knockdown was assessed using qRT-PCR and Western Blot respectively which revealed both LIN28B mRNA and protein were significantly lower in the KD cells versus the non-target scramble controls (figure 5). Additionally, AR mRNA and protein levels were significantly decreased in ACH-3P LIN28B KD cells versus non-target scramble controls (figure 6).

Effects of LIN28B KD and forskolin treatment on markers of trophoblast cell differentiation

ERVW-1 and *LGALS-13* both have been used as markers of trophoblast cell differentiation and are expressed the syncytiotrophoblast and/or extravillous trophoblast. *ERVW-1* (syncytiotrophoblast) levels were found to be significantly increased in ACH-3P LIN28B KD cells (figure 7A), however *LGALS13* (syncytiotrophoblast and extravillous trophoblast) levels were significantly decreased in ACH-3P LIN28B KD cells compared to non-target scramble controls (figure 7B). Human chorionic gonadotropin (hCG) concentrations in culture media were

determined via ELISA at 24, 48, and 72-hour time points (figure 7C). At all time-points examined, hCG concentrations were increased in cell culture media from LIN28B KD cells compared to scramble controls. Finally, ACH-3P WT cells treated with 40μM forskolin for 48h showed significantly increased *ERVW-1* levels (figure 8A) and significantly decreased *AR* (figure 8B) levels compared to DMSO treated controls. These data suggest LIN28B knockdown leads to trophoblast differentiation to a syncytiotrophoblast cell fate and is associated with decreased AR.

Effects of LIN28B KD on let-7 miRNA

Relative miRNA levels were analyzed using qRT-PCR SYBR Green assay. All let-7 miRNA levels (let-7a, b, c, d, e, f, g, and i) were examined. Let-7 family members; let-7c, let-7e, let-7f, let-7g and let-7i were significantly higher in ACH-3P LIN28B KD cells compared to non-target scramble controls (figure 9).

Let-7 Overexpression

To determine if AR is regulated by specific let-7s, ACH-3P cells were treated with let-7e, f, or c mimic or siRNA universal negative control. Let-7e and f were chosen based on their high and increased levels in the previous experiment. As expected, let-7c, e, and f miRNA was significantly increased in the mimic transfected ACH-3P WT cells versus controls (figure 10). Let-7c overexpression led to significantly decreased *LIN28B* and *AR* levels and significantly decreased AR protein (figure 11). Let-7c overexpression also caused significantly increased *ERVW-1* levels and significantly decreased *LGALS13* levels in ACH-3P WT cells compared to controls (figure 12). Cells treated with let-7e and f mimics did not yield changes in *LIN28B* or *AR* levels (figure 11).

Discussion

We previously demonstrated that LIN28A plays a role in mouse trophoblast cell differentiation, as well as human ACH-3P cells. Subsequent experiments reveal that in addition to LIN28A, ACH-3P cells contain LIN28B. In fact, human first trimester placental tissue contains LIN28B and AR and both appear to be present in cytotrophoblast cells. Moreover, AR also localized to the stromal cells confirming previous reports describing AR localization to the decidua and villous stromal cells [Horie et al 1992; Yoshida et al 2016]. Interestingly, LIN28B through its actions on let-7c regulates AR-dependent signaling in human prostate cancer [Tummala et al 2013; Nadiminty et al 2012] and appears to be do the same in human trophoblast cells. Furthermore, our recent work suggests AR interacts with *VEGFA* ovine placental tissue [Cleys et al 2014] and suggests that the LIN28B-let7-AR axis may also play a role in this process.

Androgen and its role in cancer have been extensively studied and regulates cell differentiation, proliferation and tissue vascularization [Yoshida et al 2016], which are also events that are necessary for proper placental development and function. Though AR has been identified in the placenta, very little is known about its role in placental development and maintenance. Knockdown of LIN28B in ACH-3P cells resulted in significantly increased levels of l*et-7* and complement our previous studies that the let-7 family of miRNAs in human placental cell line ACH-3P is regulated by LIN28 [Seabrook et al 2014]. AR mRNA and protein were also decreased in the LIN28B KD cells compared to non-target scramble controls, suggesting potential regulation of AR by LIN28B through let-7 miRNA in human trophoblast cells.

To further investigate this, *let-7c, e* and *f* were overexpressed using synthetic miRNA transfection in ACH-3P cells. Results indicated decreased levels of both targets *LIN28B* and *AR* in only the let-7c mimic transfected cells. Cells transfected with let-7e and f did not significantly alter *LIN28B* or *AR* levels. These data indicate that similar to what has been observed in prostate cancer cells [Nadiminty et al 2012] that LIN28B regulates AR through let-7c. Cell differentiation was characterized by determining expression of the syncytiotrophoblast *ERVW-1* as well as hCG secretion by ACH-3P in cell culture media and forskolin-induced syncytialization of ACH-3P cells for 48h. Knockdown of LIN28B resulted in increased levels of ERVW-1, as well as secretion of hCG, characteristic of differentiated syncytiotrophoblast cells. *ERVW-1* is an envelope glycoprotein that encodes the fusion gene Syncytin-1 and is necessary for the fusion and differentiation of cytotrophoblast cells to form the syncytiotrophoblast layer of the placenta [Reubner et al 2013].

Alternatively, *LGALS13* levels were decreased in let-7c mimic cells compared to siRNA Universal controls. *LGALS13* encodes Galectin-3, otherwise known as Placental Protein-13 (PP13) and is expressed in the syncytiotrophoblast and extravillous trophoblast [Than et al 2014] and is secreted by the placenta. PP13 has been implicated in abnormal placentation and preeclamptic patients as women with decreased LGALS13 levels during the first trimester of pregnancy are at greater risk for developing this disease [Balogh et al 2011; Kliman et al 2012; Bolnick et al 2016]. At present, the expression of the cell marker ERVW-1 increases and LGALS13 is decreased in LIN28B KD ACH-3P cells and let-7c overexpressed cells in this study, indicating these treatments are inducing cells to differentiate towards a more syncytiotrophoblast-like lineage. It is possible

that knockdown of LIN28B leads to compensatory LIN28A up-regulation [West et al 2018] in ACH-3P cells, which in turn could impact presence of LGALS13.

In vitro forskolin treatment leads to fusion of trophoblast cells, similar to naturally syncytializing cells [Wice et al 1990]. Treatment of ACH-3P cells with 40μM forskolin for 48h led to significantly increased *ERVW-1* levels as expected. *LIN28B* and *AR* levels were both significantly decreased in forskolin treated cells vs DMSO controls. Similar experiments in our lab have also shown that forskolin treatment significantly increased soluble hCG, and significantly decreased LIN28A mRNA and protein levels [Seabrook et al 2014]. These data indicate that as cells differentiate towards syncytiotrophoblast sublineage, LIN28B and AR levels both decrease. When let-7c alone is overexpressed, LIN28B and AR follow a similar pattern, indicating a role for the LIN28B-let-7c-AR axis in trophoblast cell differentiation

In summary, LIN28B and AR in trophoblast cells could interact together and regulate differentiation of these cells towards a more specialized cell population. Our data show that LIN28B is present in the human placenta and with loss of LIN28B, let-7c increases and down-regulates AR, leading to differentiation towards syncytiotrophoblast lineage as indicated by increased secretion of hCG and increased levels of ERVW-1.

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CHAPTER III: ANDROGEN ACTIVATION OF GPRC6A INDUCES SYNCYTIALIZATION IN HUMAN TROPHOBLAST CELLS

Summary

Although the placenta is recognized as an endocrine organ, most notably by its secretion of steroid hormones such as progesterone, androgens and estrogens, the role of steroid hormones in placental development is less clear. Data from our laboratory suggest that androgen receptor interacts with *VEGFA* in the ovine placenta. Androgens signal through androgen receptors (AR), and AR is present in the human, mouse, sheep and horse placenta. The classical genomic effects of androgens involve translocation of the androgen-AR complex to the nucleus, leading to regulation of gene transcription. However, androgens also have rapid non-genomic effects. For example, GPRC6A, a G protein-coupled receptor, was found to regulate testosterone production in Leydig cells.

GPRC6A is known to be a receptor for Osteocalcin (Ocn), an osteoblast-specific hormone that plays a role in bone calcification and calcium ion homeostasis. Ocn also binds GPRC6A in the pancreas, leading to beta cell proliferation and insulin release, as well as stimulating testosterone production by Leydig cells in the testes in addition to binding Ocn, GPRC6A is also a receptor for small amino acids; lysine and tyrosine, and for the androgens; testosterone and dihydrotestosterone, suggesting it potentially mediates non-genomic actions of androgens. This study describes presence of GPRC6A in the human, mouse, and sheep placenta using PCR, Western blot, and immunolocalization. We discovered the presence of GPRC6A in the syncytiotrophoblast layer of human first trimester placental tissue, as well as GPRC6A protein in mouse and sheep placental lysate. Furthermore, we found that testosterone activation of GPRC6A

leads to increased cAMP and hCG, indicating trophoblast cell differentiation toward a more syncytiotrophoblast lineage. These findings suggest that androgen signaling may occur through non-genomic pathways in the placenta. Ultimately, these findings will lead to better understanding of the underlying mechanisms behind placental development and disease.

Introduction

The placenta is considered a multifunctional transitory endocrine organ that is responsible for the exchange of nutrients and waste between mother and fetus. The placenta is capable of producing steroid hormones and is also a target for these hormones that are necessary for the proper function of the placenta. For example, estrogen and estrogen signaling has been found to play an important role in placental cell invasion and migration into the maternal decidua and angiogenesis [Bazer et al 2009; Gude et al 2004]. More specifically, estrogen produced by trophoblast cells in a hypoxic uterine environment acts to drive differentiation of cytotrophoblast to more specialized cells; syncytiotrophoblast and invasive extravillous trophoblasts that further remodel the uterine spiral arteries [Albrecht and Pepe 2010]. In humans, impaired trophoblast differentiation and invasion into the maternal spiral arteries is thought to be an underlying cause associated with placental disorders such as preeclampsia (PE), as studies evaluating placentas in women with PE consistently show maternal and fetal vascular abnormalities [Regnault et al 2002].

Although studies regarding estrogen signaling in the placenta have been performed, very little is known about androgen signaling and its role in placental development and/or function. Changes in androgen levels have been linked to various pregnancy related disorders including PE and gestational diabetes mellitus (GDM) [Bartnik et al 2016; Koster et al 2015; Horie et al 2015].

These patients have significantly increased circulating serum androgens and increased placental expression of androgen receptor (AR) [Hsu et al 2009; Niswender et al 2000; Khodzhaeva et al 2015]. Preliminary studies in our lab have revealed that *AR* interacts with *VEGFA* by binding an androgen response element in the *VEGFA* promoter region in the ovine placenta [Cleys et al 2015]. Therefore, it is possible that trophoblast differentiation and placental development involves androgen signaling. It is widely known that steroid hormones can produce genomic and rapid "non-genomic" responses. Non-genomic signaling leads to generation of second messengers that elicits various physiologic responses including differentiation and proliferation [Falkenstein et al 2000; Zachariades et al 2012; Gellersen et al 2009; Boonyaratanakornkit et al 2001]. Androgens classically bind AR to produce genomic effects involving the translocation of the androgen-AR complex to the nucleus leading to regulation of gene transcription [Quigley et al 1995]. However, androgens also illicit rapid non-genomic effects by binding to cell membrane receptors leading to Ca2+ influx or cAMP pathway activation. Recent evidence suggests that the G-protein coupled receptor GPRC6A is a membrane receptor for androgens [Wang et al 2014; Pi et al 2010; Pe et al 2011].

GPRC6A is better known for its role in mediating the effects of Osteocalcin (Ocn), an osteoblast-specific hormone that plays a role in bone calcification and calcium ion homeostasis. GPRC6A is also activated by basic amino acids such as lysine, arginine and ornithine and small and polar amino acids [Pi et al 2011]. Recent studies revealed GPRC6A activation stimulates testosterone production in Leydig cells [Chamouni and Oury 2014] whereas knockout of GPRC6A in male mice leads to testicular feminization [Pi et al 2008]. Coupling of GPRC6A to Gαi-, or Gαq- has been shown to increase intracellular calcium and ERK-activation in the pancreas [Pi et

al 2012; Wellendorph and Bräuner-Osborne 2004]. However, in Leydig cells, GPRC6A stimulates cAMP production leading to activation of steroidogenic enzymes including StAR and testosterone production and secretion, by coupling to Gαs [Oury et al 2011]. In the placenta, cAMP signaling leads to differentiation of cytotrophoblast (CT) progenitor cells. CT cells fuse to form a large, multinucleated syncytiotrophoblast (ST) layer that secrete and produce hormones, including human chorionic gonadotropin (hCG), necessary for maternal recognition and maintenance of pregnancy. *In vitro,* trophoblast cells treated with forskolin, a cAMP activator, is commonly used to induce trophoblast cell fusion to mimic syncytialization and increased expression of syncytin-1 (*ERVW1*) [Qiao et al 2017]. Furthermore, cell fusion is initiated by activation of cAMP/PKA, leading to phosphorylation of CREB transcription factor that stimulates cell fusion and increase hCG production [Gerbaud et al 2015; Frendo et al 2003]. *ERW-1* is a human endogenous retroviral envelope gene that is expressed in trophoblast cells to mediate this fusogenic process [Chen et al 2008].

During gestation, maternal serum levels of both androgens and osteocalcin increase [Maliqueo et al 2016; Seki et al 1991; Oury et al 2013], suggesting a role for these ligands and GPRC6A/AR. In a recent study, BSA conjugated testosterone activated GPRC6A, which led to a dose-dependent increase in ERK phosphorylation [Oury et al 2013] and cAMP production in pancreatic β-cells, both of which are important pathways in the development of the placenta. For example, loss of *Map2k1* and *Map2k2* that encode kinases integral for ERK activation are embryonic lethal because of severe placental defects [Nadeau and Charron 2014]. These data indicate that testosterone-activated GPRC6A could potentially play an important role in the function of the placenta, more specifically in CT differentiation into ST. The goal of this study is

to examine the presence and localization of GPRC6A in the human placenta and its potential functions in trophoblast cell differentiation. We postulate the testosterone activation of GPRC6A will stimulate cAMP signaling and induce syncytialization in ACH-3P cells

Materials and Methods

Cell lines

Two human first trimester trophoblast cell lines were initially used in this study: ACH-3P (a gift from Ursula Hiden, Medical University of Graz, Austria) and Swan71 (a gift from Gil Moore, Yale University, USA). ACH-3P cells are human first trimester trophoblast cells (CT and EVT) fused with the choriocarcinoma cell line AC1-1. Swan71 cells are immortalized (hTERT) human first trimester trophoblast cells. Cells were cultured at 37°F and 5% CO_2. ACH-3P cells were cultured in DMEM F-12 medium (HyClone), 10% FBS (HyClone), and 1% PSA (Corning Life Sciences). Swan71 cells were cultured in DMEM/F12 medium, 10% FBS, 1% PSA, 1 mM non-essential amino acids (HyClone), and 1 mM sodium pyruvate (Corning Cellgro) [Seabrook et al 2013].

Human first trimester placental 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% paraformaldehyde 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, two first trimester placental tissue samples (an 8.6 weeks of

gestation sample and an 11.5 weeks of gestation sample) were available and repeated in triplicate. Tissue sections of 5µm thickness were taken from the center of paraffin blocks for immunohistolocalization.

Mouse placental samples

Mouse placentae was collected from C57/B6 inbred mice. Date of gestation was determined by fore and hind limb morphology (Theiler 1989). Samples were obtained in accordance with the Colorado State University Institutional Animal Care and Use Committee, and samples were stored in sterile PBS upon collection and were transferred to ice cold 4% paraformaldehyde 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, two sections per slide were stained and repeated in triplicate, from three separate dams. For western blot analysis, protein was extracted from three different placenta and samples were run in triplicate.

Sheep placental samples

Sheep placentae (placentomes) was collected at gestational day 55 in accordance with the Colorado State University Institutional Animal Care and Use Committee. Whole placentomes and cotyledons were separated and flash frozen in liquid nitrogen and then stored at -80°F. For western blot analysis, protein was extracted from three different placenta and samples were run in triplicate.

Western Blot

Western blot analysis was used to determine the presence and quantify GPRC6A protein in two human first trimester trophoblast cell lines and determine its presence in the mouse and

sheep placenta. Cells and tissue samples were lysed in RIPA buffer (20 mM Tris, 137 mM NaCl, 10% glycerol, 1% nonidet P-40, 3.5 mM SDS, 1.2 mM sodium deoxycholate, 1.6 mM EDTA, pH 8) with 10% protease/phosphate inhibitor cocktail (Sigma-Aldrich) and 1 mM phenymethanesulfonyl fluoride. The BCA protein assay kit (Pierce) was used to determine protein concentration. Absorbance was measured at λ 595 nm using a Biotek Synergy 2 Microplate Reader (Biotek). Protein was electrophoresed in 4-15% Mini-PROTEAN TGX Stain-Free precast gels (Biorad) and transferred to 0.2 μm pore nitrocellulose membrane at 110 volts at 4° C for 1 hour.

Membranes were then blocked in 5% milk-TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6) for 1 hour at room temperature then incubated in a polyclonal antibody to GPRC6a (LSBio B-9471) diluted 1:500. Blots were incubated at 4° C overnight. Membranes were then washed 3 times for ten minutes for a total of 30 minutes in 1x TBST, incubated with a horseradish peroxidase-conjugated secondary antibody (Pierce 1858415 for mouse and Abcam ab6741 for human and sheep) for 1 hour at room temperature, diluted 1:2000. Membranes were developed using ECL Western Blotting Detection Reagent chemiluminescent kit and membranes were imaged using a ChemiDoc XRS+ chemiluminescence system (BioRad).

Immunofluorescent localization of GPRC6A in tissues

To determine cellular localization of selected proteins in human placental samples, 5μm paraffin sections were taken from the center of the tissue blocks. Slides were deparaffinized with Citrasolve (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 30-minute

incubation in 5% hydrogen peroxide solution in PBS. Slides were blocked for 30 minutes in 10% goat serum at room temperature. After blocking, slides were washed in PBS. GPRC6A (LSBio LS9471) antibodies were diluted 1:100 in 10% goat serum in PBS.

Primary antibodies were incubated overnight at 4°C. Control slides were incubated with 6% goat serum in PBS with exclusion of primary antibody. GPRC6A blocking peptide (LSBio 9471-P) was used at a 3:1 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 Alexa-fluor 488 secondary antibody (Abcam) was used for GPRC6A detection. Slides used for immunofluorescence were mounted with Prolong Gold containing DAPI (4',6-diamidino-2-phenylindole) (Life Technologies). Secondary antibody only staining was used as a negative control. Slides were visualized using Olympus D73 camera on Nikon Eclipse E800 microscope and CellSense 1.3 software.

Immunofluorescent localization of GPRC6A in cells

ACH-3P cells were seeded at 20% confluency on sterile 4-chamber slides in appropriate cell culture media for 24 hours. Once cells were adhered, they either fixed or treated with 100µM FITC-BSA-conjugated testosterone or 100µM FITC-BSA (control) and incubated at 37°F and 5% CO_2 for 30 minutes. FITC-treated cells were then washed 3 times with sterile 6.7µM PBS and fixed with 4% PFA. After fixing, cells were washed 3 times 6.7µM PBS and cells were then mounted with Prolong Gold with DAPI (Fisher Scientific), stored in the dark at 4°F and imaged 24 hours later. For stained cells, each chamber was incubated with 800µl of blocking

solution (6% rabbit serum in PBS) at room temperature for 45 minutes. Blocking solution was aspirated and cells were incubated in primary antibody solutions for 1 hour at room temperature (1:50 GPRC6A in 6% rabbit serum in PBS). Cells were washed with PBS 3 times for 5 minutes each and incubated with goat-anti rabbit Alexa-fluor 488 secondary antibody in blocking solution (1:1000) for 1 hour at room temperature in the dark. Cells were washed with PBS 4 times for 5 minutes each and slides were mounted with Prolong Gold with DAPI and stored in the dark at 4°F for 24 hours until sealed. Secondary antibody only staining was used as a negative control. Slides were visualized using Olympus D73 camera on Nikon Eclipse E800 microscope and CellSense 1.3 software.

Real-Time RT-PCR

Messenger RNA was extracted from cell pellets using mRNeasy Mini Kit (Qiagen) according to the manufacturer's directions. Complementary DNA (cDNA) was generated from 1µg of total RNA using qScript cDNA Supermix (Quanta Biosciences) and quantitative real-time RT-PCR (qPCR) was performed. Each 10ul qPCR reaction consisted of 5ul SYBR Green (Biorad), 1.5ul 0.5uM primer mix (human GPRC6A forward: TTGTCCTCAGAAGACTCTCCCAGA reverse: TTTCATACCCCAGTTTGACTCCA, Ocn forward: CCTCACACTCCTCGCCCTATT reverse: CCGATGTGGTCAGCCAACTC), 2.5ul RNase/DNase free water, and 1ul cDNA (100ng/reaction). Quantitative PCR was performed using the LightCycler480 thermal cycler (Roche) with the following parameters: 10 min pre-incubation at 95°C, 40 cycles of amplification, which included denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds, followed by a final cooling cycle at 40°C for 5 min. Normalization accomplished using GAPDH.

Syncytialization of ACH-3P cells

ACH-3P cells were seeded at approximately 60% confluency in sterile cell-culture dishes. Cells were treated with 100µM FITC-BSA (negative control), 0.013M lysine (positive control), or 100µM FITC-BSA-conjugated testosterone for 48 hours. In addition, forskolin was used as it is a known activator of cAMP and syncytialization. ACH-3P cells were treated with 40µM forskolin (Sigma-Aldrich) for 48 h. Control cells were treated with 0.1% DMSO. All reagents were purchased from Sigma-Aldrich. Cell culture medium and cell lysate was collected at 24 and 48 hours post treatments.

Enzyme-Linked Immunosorbent Assay

ACH-3P cells were seeded at 60% confluency in 12-well plates with three replicates per treatment. Concentrations of human chorionic gonadotropin (hCG) were quantified using an hCG ELISA kit containing a mouse monoclonal anti-hCG (specific to beta-hCG) conjugated to horseradish peroxidase, according to the manufacturer's instructions (ALPCO Diagnostics). Cellular concentrations of cAMP were quantified using a cAMP ELISA according to the manufacturer's instructions (Invitrogen). Experimental replicates were assayed in triplicate, and absorbance was measured at λ 450 nm for hCG and 405nm for cAMP.

Statistics

GraphPad Prism 7© software was used to perform unpaired t-tests using qRT-PCR $2^{-\Delta Cp}$ values, and Western blot band density comparing ACH-3P and Swan 71 cells. Soluble hCG concentrations (mIU/ml) were calculated by plotting sample mean absorbance values against the mean values calculated from duplicate standard curve values. The standard curve consisted of 0,

5, 20, 50, 150, and 300 mIU/ml hCG standards. Cyclic-AMP levels were calculated by plotting sample mean absorbance values against the mean values calculated from duplicate standard curve values and performing a 4-parameter logistic curve linear regression analysis. The standard curve consisted of 0, 0.78, 3.13, 12.5, 50, and 200 pmol/ml cAMP. 1-way ANOVA was performed to determine statistical significance. *P* values ≤ 0.05 were considered statistically significant.

Results

GPRC6A localization in human placental tissue

Immunostaining was used to localize GPRC6A in human first trimester placental tissue. Immunofluorescence revealed GPRC6A co-localizes with hCG in the syncytiotrophoblast layer and underlying trophoblast cells in human first trimester placenta (figure 13). Secondary only negative controls yielded no immunofluorescence.

GPRC6A mRNA and protein detection in human, mouse, and sheep placenta

Western blot and qRT-PCR (figure 14A and B) demonstrated the presence of both mRNA and protein in ACH-3P and Swan71 cells. GPRC6A mRNA levels in ACH-3P cells were significantly higher compared to Swan-71 cells ($p = 0.004$). GPRC6A protein was detectable in ACH-3P and Swan71 trophoblast cell lines with bands at approximately 100 kDa. Densitometry analysis revealed a significantly higher level of GPRC6A protein in ACH-3P vs Swan-71 ($p = 0.04$). Finally, Western blot analysis was conducted to assess the presence of GPRC6A in mouse d12.5 and sheep d55 placental tissue (figure 14C). A band of approximately 100 kDa was present in all mouse and sheep tissue samples.

GPRC6A localization in ACH-3P cells

Immunofluorescence for GPRC6A validated its localization to the plasma membrane of ACH-3P cells (figure 15). Secondary antibody only negative controls were used. Immunofluorescence revealed membrane staining when ACH-3P cells were treated with 100µM of FITC-BSA-testosterone (figure 16) indicating the ability of membrane binding of FITC-BSA conjugated testosterone. There was no staining in cells treated with FITC-BSA only. GPRC6A was previously localized to the membrane (Figure 14) via immunofluorescence. Co-staining with GPRC6A antibody and FITC-BSA-testosterone was attempted, but only GPRC6A antibody staining was visible.

Testosterone treatment induces syncytialization

Cell lysate from cells treated with membrane-impermeable FITC-BSA-testosterone yielded significantly increased levels of cAMP levels compared to FITC-BSA only controls ($p = 0.007$) (figure 17). Furthermore, cAMP levels in the lysine positive controls were significantly increased compared to no-treatment controls ($p < 0.001$) and FITC-BSA controls ($p < 0.001$). Cell culture media from cells treated with membrane-impermeable FITC-BSA-testosterone yielded significantly higher levels of soluble hCG into cell culture media ($p = 0.04$). Lysine treatment also led to significantly higher hCG levels compared to negative controls ($p = 0.05$, no treatment control; $p = 0.03$, FITC-BSA).

Presence of Ocn in placental cells

Ocn mRNA was detected in ACH-3P cells but was not statistically different compared to Swan-71 cells ($p = 0.11$).

Discussion

This is the first study to report the presence of membrane receptor GPRC6A in human, mouse, and sheep placenta. GPRC6A mRNA and protein was detected using qRT-PCR, Western blot, and immunostaining techniques in human placental tissue and human trophoblast cells, ACH-3P and Swan71. Quantitative-RT-PCR demonstrated increased *GPRC6A* in ACH-3P versus Swan-71 cells. Western blot detection of GRPC6A protein in human placental tissue and human placental cells yielded consistent results across samples with bands corresponding to the size previously reported for GPRC6A in tissues such as pancreas [Pi et al 2012; Pi et al 2008]. Furthermore, using immunofluorescence, GPRC6A was localized to the syncytiotrophoblast layer in first trimester placental tissue, indicated by co-staining with hCG. Based on this localization pattern, GPRC6A could play a role in syncytialization. In ACH-3P cells, GPRC6A localized to the plasma membrane. In addition, ACH-3P cells incubated with FITC-BSA conjugated testosterone exhibited FITC positive membrane staining, suggesting testosterone binding site are present on ACH-3P plasma membrane. Co-staining of GPRC6A and FITC-BSA-testosterone was attempted, but only GPRC6A antibody staining was visible. This possibly is due to the inability of FITC-BSA-testosterone to bind GPRC6A in the presence of a high-affinity GPRC6A antibody. Similar co-staining experiments of GPRC6A and FITC-BSA conjugated testosterone have not been reported.

To determine if binding of GPRC6A leads to activation of cAMP signaling, we treated cells with FITC-BSA-testosterone. In cells treated with FITC-BSA-testosterone, there was significantly increased cAMP from cell lysate compared to negative controls. We also found that there was significantly increased secretion of soluble hCG levels in cell culture media in cells

treated with FITC-BSA-testosterone. This data corresponds with our findings of GPRC6A localization in syncytiotrophoblast cells of human first trimester placenta tissue, indicating that this membrane receptor may be an important mediator in trophoblast cell syncytialization and hormone production. BSA conjugation prevents testosterone from entering the cells, therefore FITC staining of FITC-BSA conjugated testosterone to the plasma membrane indicates possible binding to membrane receptors such as GPRC6A.

Non-genomic effects of steroid hormone signaling results a release of second messengers such as Ca^{2+} or cAMP, and contribute to phosphorylation events, which amplify and propagate the extracellular steroid signal into the cell. Non-genomic signaling is characterized by the production of second messengers, which function to amplify and relay the appropriate signal within a cell, resulting in various physiological responses. Ocn binding to GPRC6A involves activation of cAMP signaling and testosterone production independent of the hypothalamic-pituitary-testis axis [Chamouni and Oury 2014]. In this study, we also found binding to GPRC6A in trophoblast cells leads to cAMP production and increased soluble hCG in cell culture media, indicating that ACH-3P cells are differentiating towards a more ST-like cell line. CT cell differentiation yields two main subpopulations of cells; extravillous trophoblast and syncytiotrophoblast. The differentiation of CT to ST involves fusion of cytotrophoblast to form multinucleated syncytia that produce and secrete hCG, transport nutrients and waste between mother and fetus and is involved with maternal-fetal immunotolerance [Benirschke and Kaufmann 2000]. This fusion is regulated by ERVW-1, which acts through its receptor ASCT2 play a major role in this process [Frendo et al 2003; Blond et al 2000]. CT continue to fuse during pregnancy, to replace ST cells that undergo

apoptosis. Current studies are underway to determine if ERVW-1 is upregulated in testosterone-treated trophoblast cells.

The presence GPRC6A protein in human trophoblast cells and localization to the syncytiotrophoblast in human first trimester placenta suggests that GPRC6A could potentially play a role in placental development and function as a receptor for androgen and/or osteocalcin. Both androgens and Ocn plasma levels increase during pregnancy in women [Seki et al 1991; Carlsen et al 2005; Morford et al 2018], and the placenta itself is a source of testosterone. Interestingly, preliminary data revealed that the placenta also contains Ocn mRNA and possibly is a source for Ocn synthesis. Therefore, both locally produced testosterone and Ocn act on GPRC6A in trophoblast cells and is necessary for ST differentiation and function.

In summary, we demonstrated a potential function for non-genomic actions of androgen in trophoblast cells. Specifically, we postulate that testosterone-mediated signaling through GPRC6A plays a role in CT fusion and differentiation to ST cells. Ultimately, these findings can potentially lead to better understanding of the underlying mechanisms behind placental development and the aberrant changes that lead to improper trophoblast cell differentiation leading to placental diseases.

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CHAPTER IV: SUMMARY AND CONCLUSIONS

The goal of these studies is to better understand the role and regulation of androgen signaling in placental cell differentiation. We specifically focused on AR and GPRC6A, a novel membrane receptor and their function in the placenta. The placenta is capable of producing and secreting steroid hormones, such as estrogens and androgens that are necessary for the maintenance of pregnancy, as well as early placental cell differentiation. Both ESR and AR localize to different trophoblast cells types suggesting diverse roles in placental development and function. A vital component of placental formation is the continual differentiation of cytotrophoblast into syncytiotrophoblast and extravillous trophoblast cells. The syncytiotrophoblast layer functions as the feto-maternal interface and these cells produce human chorionic gonadotropin (hCG) which is required for pregnancy recognition. Extravillous trophoblast cells are involved in trophoblast invasion and vascular remodeling. Improper regulation of trophoblast cell differentiation can lead to insufficient populations of trophoblast sub-lineages. The resulting placental dysfunction is thought to be the underlying cause of placental related diseases such as preeclampsia and IGUR, and causes up to 500,000 maternal deaths per year. These disorders are also associated with androgen and AR levels, indicating a role in placental physiology. The role of androgens in cancer involve angiogenesis and cell proliferation and differentiation, which are important processes in formation of the placenta. Although AR has been identified in the placenta, very little is known about its role in placental development or function.

The studies described in Chapter II identify regulation of AR by LIN28B through *let-7c*. We found that overexpression of *let-7c* downregulates both LIN28B and AR, and induces cell differentiation in first trimester human trophoblast cell line ACH-3P. LIN28B is an RNA binding protein that is present in both human and sheep placental tissue and is important for maintaining pluripotency in progenitor cells. LIN28 also regulates the let-7 family of miRNAs by two distinct mechanisms; translational inhibition and targeted degradation. When cells are undifferentiated, LIN28 levels are high and let-7 levels are low. As cells differentiate, let-7 levels rise, as LIN28 levels fall. We found that AR is a downstream target of LIN28B, and decreases in tandem with LIN28B, as cells move towards a more differentiated state. When LIN28B was knockdown, using lentiviral shRNA constructs, AR levels went down and cells expressed increased levels of syncytiotrophoblast differentiation markers *ERVW-1* and hCG. By using a synthetic *let-7c* miRNA mimic, we showed that *let-7c* overexpression resulted in down-regulation of LIN28B and AR, implying the existence and a role for the LIN28B-let-7c-AR axis in trophoblast cell differentiation. LIN28B and AR in the cytotrophoblast progenitor cells in human placental tissue could interact together and regulate differentiation of these cells towards a more specialized cell population, the syncytiotrophoblast cells.

In addition to genomic actions of androgens through AR, we postulate that similar to other tissues, androgens also have rapid, non-genomic effects on trophoblast cells. Recently, GPRC6A was identified to bind testosterone and activate second messenger systems in non-reproductive tissues. Experiments in Chapter III describe the identification of non-genomic effects of testosterone by binding GPRC6A in placental cells. We hypothesized that GPRC6A plays a role in mediating the non-genomic effects of androgens in the placenta and induces syncytialization.

GPRC6A is known to bind Ocn and mediates calcium homeostasis. We have shown the presence of GPRC6A in human, mouse, and sheep placenta. In human placental tissue, it localizes to hCG positive syncytiotrophoblast cells. Using immortalized human first trimester trophoblast cell lines; ACH-3P and Swan-71, we further identified the presence and membrane localization of GPRC6A by western blot and immunostaining. Non-genomic effects of testosterone-GPRC6A binding were examined by treating cells with impermeable FITC-BSA-conjugated testosterone and measuring changes in cAMP via ELISA. We found that the testosterone treatment increased both cAMP and hCG, suggesting that androgen signaling occurs through non-genomic pathways and leads to syncytialization in ACH-3P cells.

In conclusion, these studies demonstrate the presence of both GPRC6A AR in placental tissue. We postulate that signaling through AR is necessary for trophoblast cell differentiation and complements are previous work demonstrating a likely role in placental angiogenesis. Furthermore, similar to cancer cells, AR in trophoblast cells is regulated by LIN28B and its interaction with let7c. By knocking down LIN28B, there is a targeted-decrease in AR, which and cell differentiation towards syncytiotrophoblast lineage. Our subsequent studies focused on a role for non-genomic androgen signaling in the placenta. We identified the presence of GPRC6A in syncytiotrophoblast cells in human placental tissue. Furthermore, treating ACH-3P cells with impermeable FITC-BSA conjugated testosterone leads to an increase in cAMP and secretion of hCG. Therefore, non-genomic actions of androgens through GPRC6A are possibly involved in regulating syncytiotrophoblast cell function. A better understanding of these mechanisms provides much needed new insight into the role of androgen signaling in placental development and function

which ultimately can lead to a better understanding of the underlying causes and mechanisms of placental dysfunction and pregnancy disorders.

TABLES

Table 1: Localization of ESRs and AR in human trophoblast cells [Iwamura et al, 1994; Hsu et al, 2009; Bukovsky et al, 2003; Schiessl et al, 2006; Kumar et al, 2009]

	Receptor		
Location	AR	ESR1	ESR2
Differentiating Cytotrophoblast	—	++	— +
CT	— +	++	— +
ST	++	—	+
EVT	NA	— +	++

++ Positive Immunolocalization
\+ Limited/Low Immunolocalization
— + Low/Variable Immunolocalization
— Not Present
NA Not Determined

Table 2: Primer sequences for human GPRC6A and Ocn

Primer	Forward	Reverse
GPRC6A	TTGTCCTCAGAAGACTCTCCCAGA	TTTCATACCCCAGTTTGACTCCA
Ocn	CCTCACACTCCTCGCCCTATT	CCGATGTGGTCAGCCAACTC

FIGURES

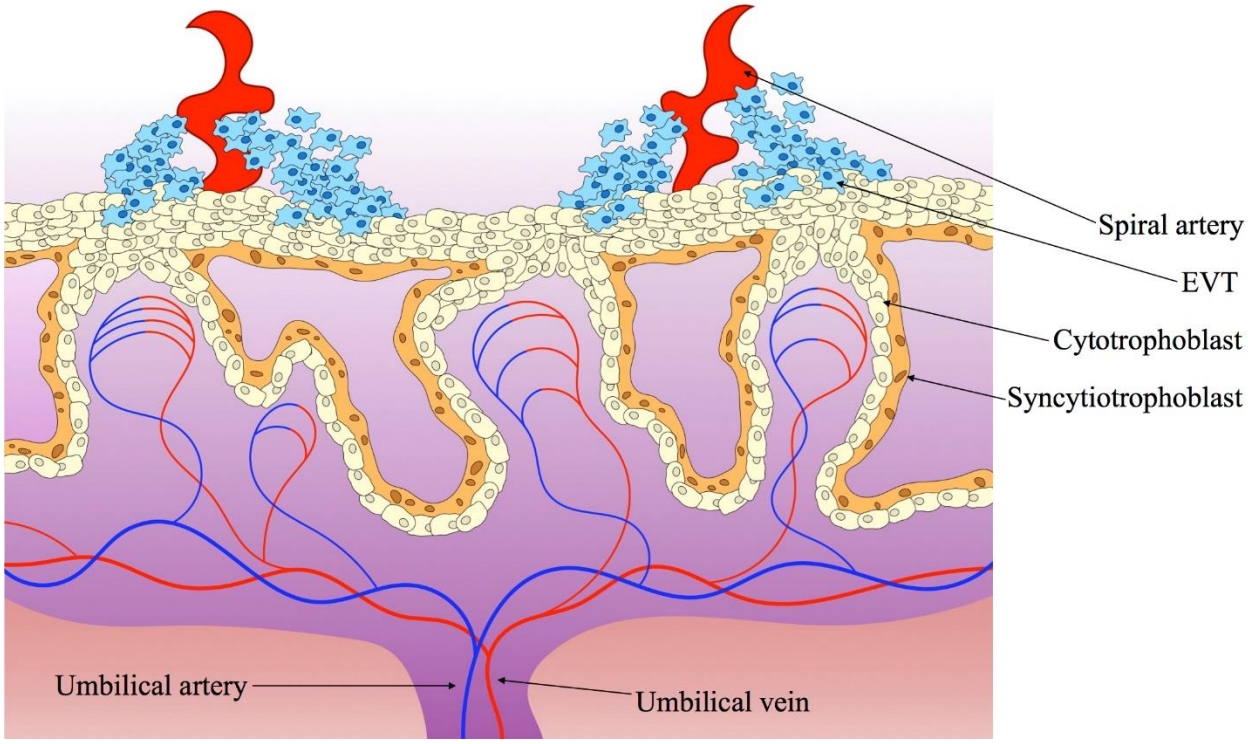

Figure 1: Trophoblast cell differentiation in the human placenta. Proliferating progenitor cytotrophoblast cells differentiate and give rise to extravillous trophoblast (EVT) and multinucleated syncytiotrophoblast (ST). Extravillous trophoblast cells invade maternal interstitial uterine tissue as well as spiral arteries and are important for uterine artery remodeling necessary for increased blood flow to the placenta. Syncytiotrophoblast cells fuse and form a multinucleated syncytium and are critical for nutrient and gas exchange as well as hormone production and secretion.

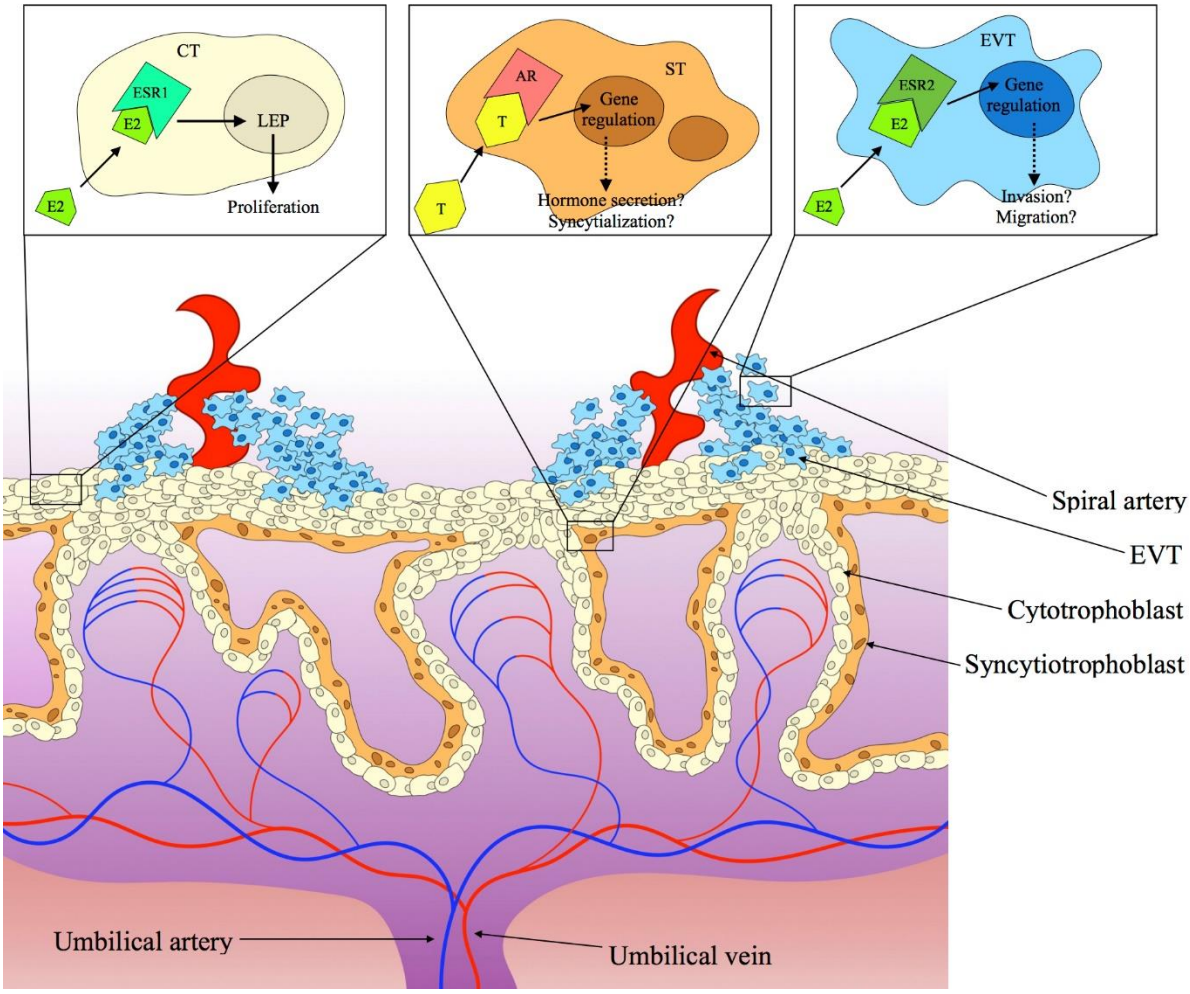

Figure 2: Proposed roles of estrogen (ESR) and androgen receptor (AR) in differentiating trophoblast cells. Solid arrows indicate reported functions, according to the literature. Dashed arrows indicate proposed functions. Based on available expression and localization data we postulate that ESR1 and AR have important functions in cytotrophoblast function (proliferation) and differentiation (syncytialization), respectively. Furthermore, ESR1 expression in extravillous trophoblast cells (EVT) suggests a possible role in EVT functions such as migration and invasion.

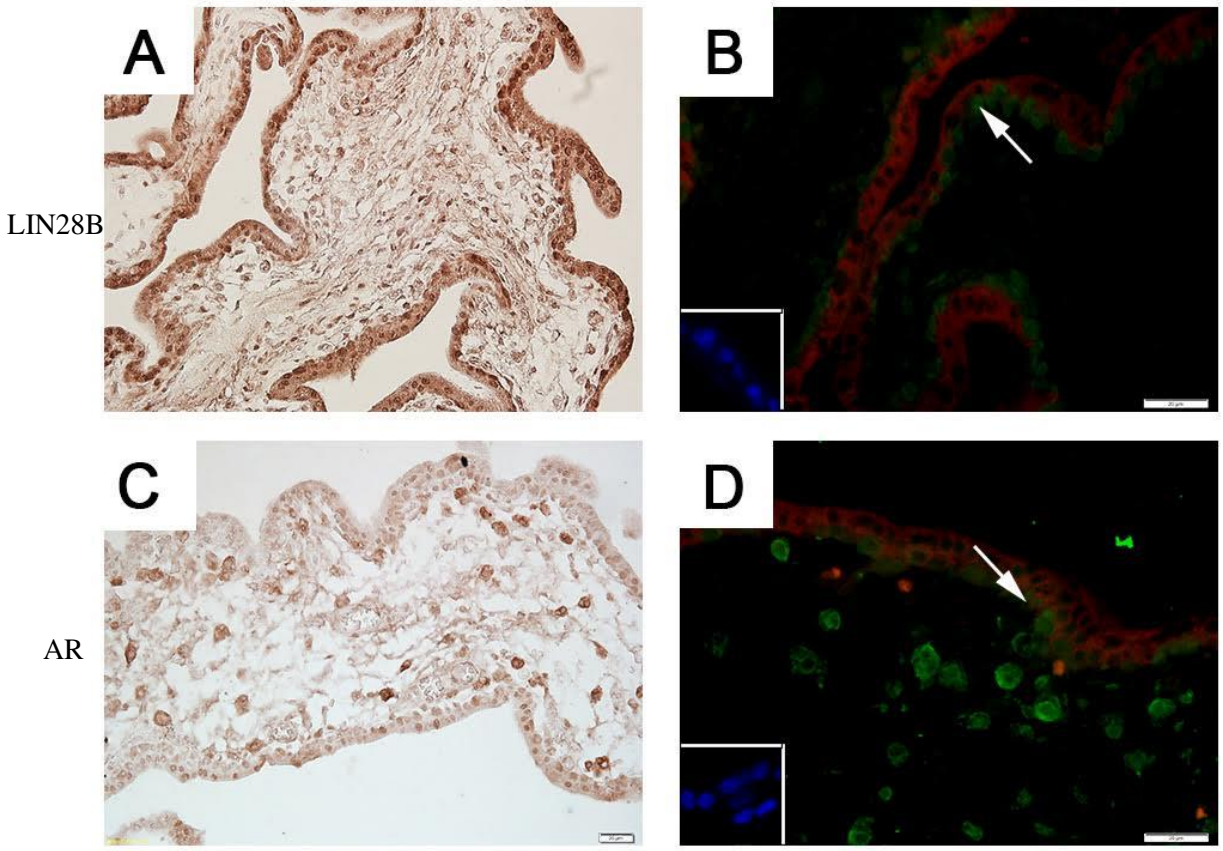

Figure 3: **LIN28B and AR localization in human first trimester placental tissue.** Localization according to immunohistochemistry (A and C, respectively, 20X) and immunofluorescent (B and D, respectively, 40X). Immunofluorescent co-labeling of LIN28B (green) and hCG (red) indicates localization of LIN28B to the cytotrophoblast cells (white arrow; B). Similarly, co-labeling of AR (green) and hCG suggests localization of AR to cytotrophoblast (white arrow) and stromal cells (D). Inserts in B and D indicate DAPI stained, no primary antibody negative control.

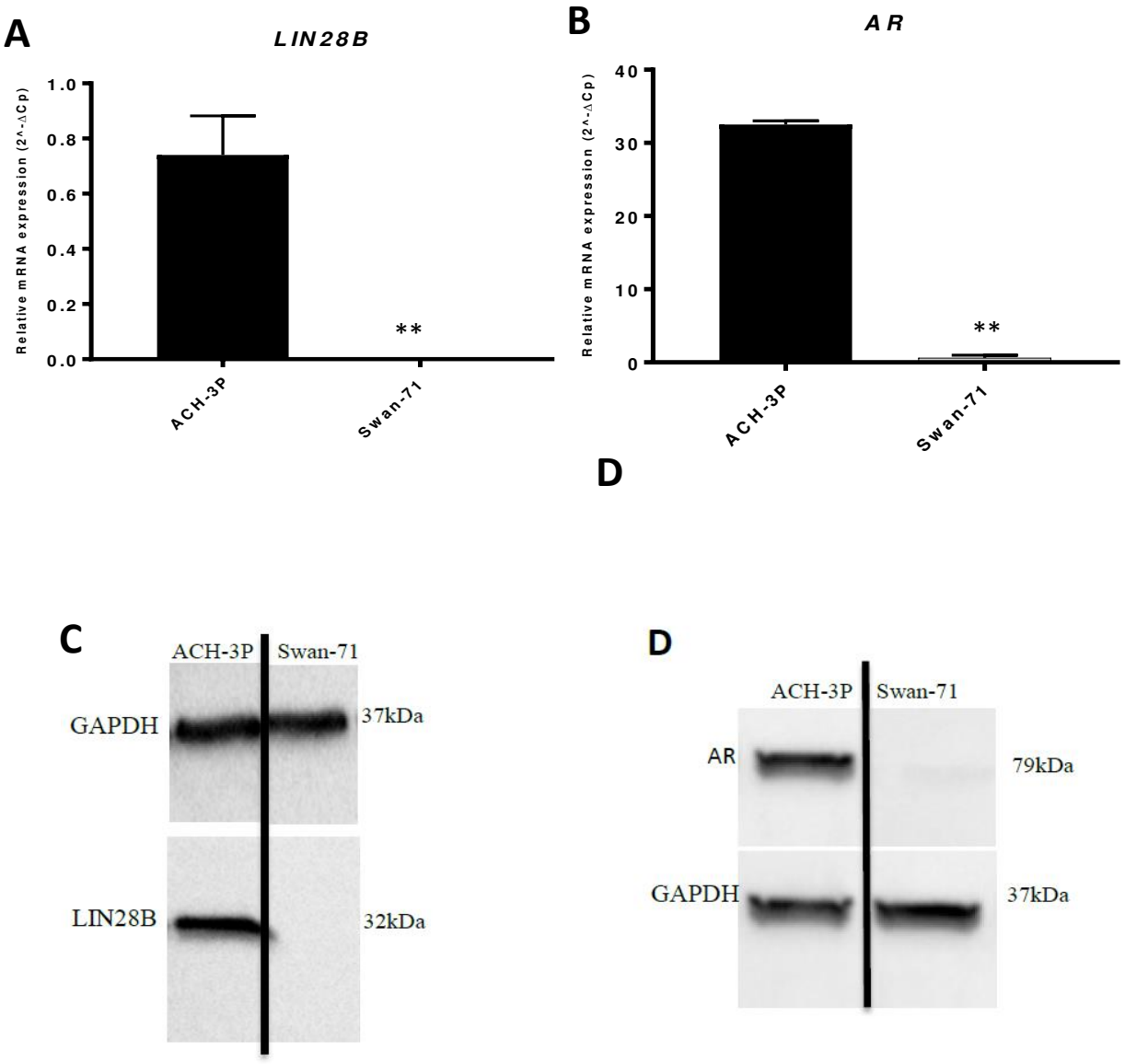

Figure 4: **Relative levels of *LIN28B* (A) and *AR* (B) in human first trimester trophoblast cell lines ACH-3P and Swan-71**. *LIN28B* and *AR* levels are significantly higher in ACH-3P compared to Swan-71 cells. LIN28B (C) and AR (D) protein was also significantly higher in ACH-3P compared Swan-71 cells ** indicates $p \leq 0.01$

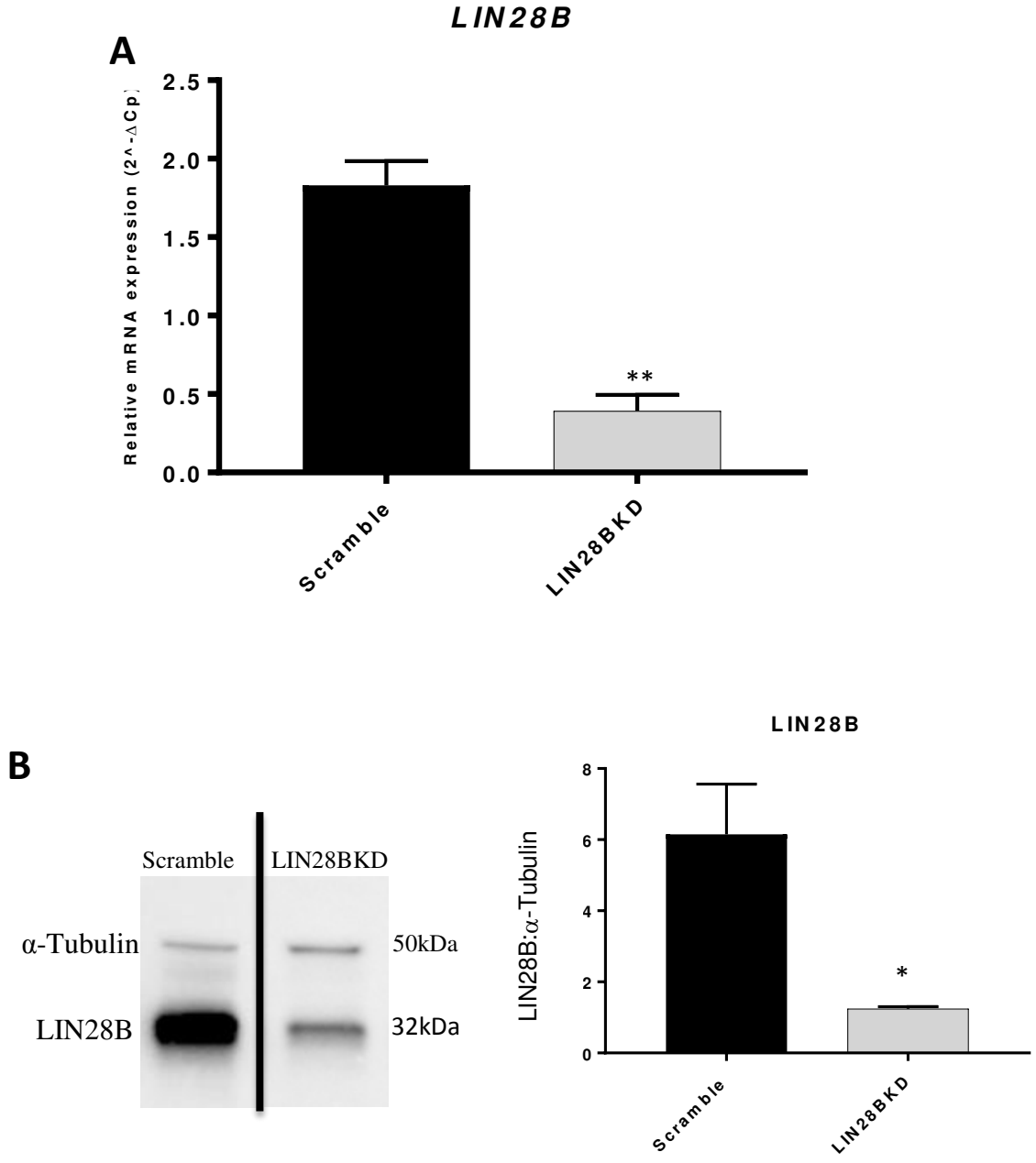

Figure 5: **Relative levels of LIN28B mRNA (A) and protein (B) in ACH-3P scramble control and LIN28B knockdown (KD) cells**. LIN28B mRNA and protein levels are both significantly lower in ACH-3P LIN28B KD cells compared to non-target scramble controls. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$

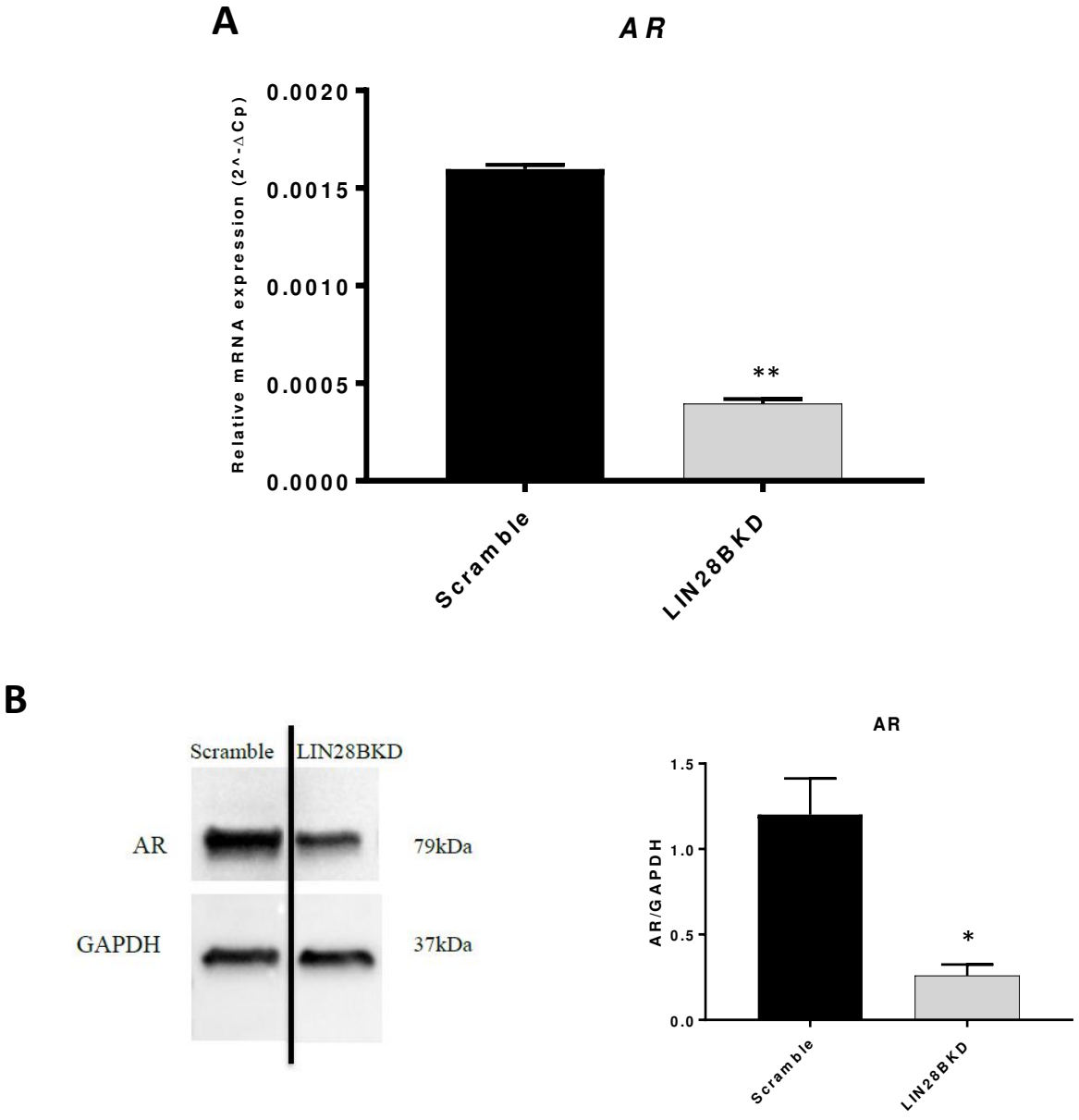

Figure 6: **Relative levels of AR mRNA (A) and protein (B) in ACH-3P scramble control and LIN28B knockdown (KD) cells**. AR mRNA and protein levels are both significantly lower in ACH-3P LIN28B KD cells compared to scramble controls. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$

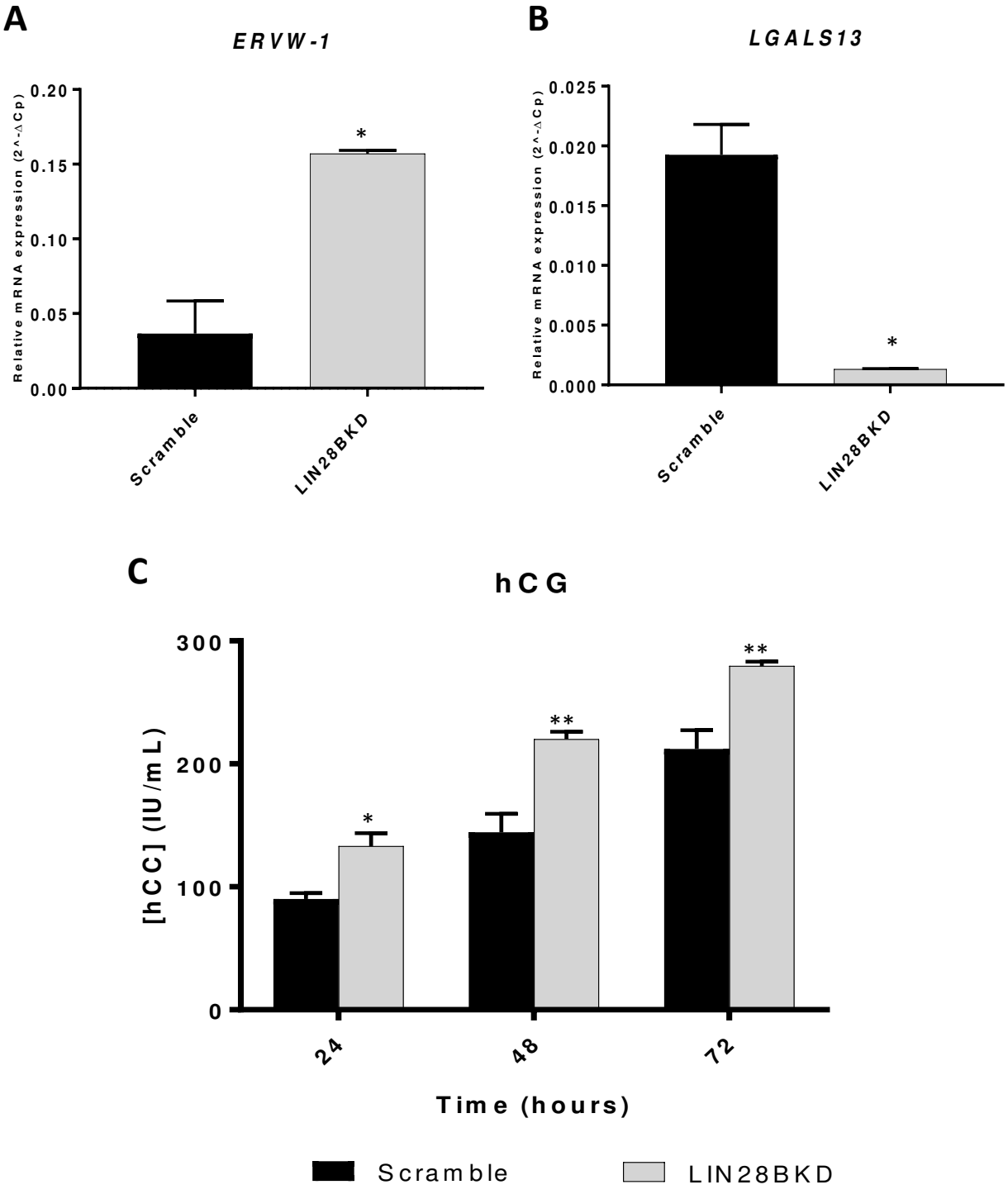

Figure 7: **Relative levels of *ERVW-1* (A) and *LGALS13* (B) and hCG secretion (C) in ACH-**

3P LIN28B KD cells compared to non-target scramble controls. Relative level of *ERVW-1* was significantly higher (A) whereas *LGALS13* level was lower in ACH-3P LIN28B KD cells compared to scramble controls. Cell culture media from ACH-3P LIN28B KD cells contained significantly higher amounts of hCG after 24, 28, and 72 hours of culture. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$.

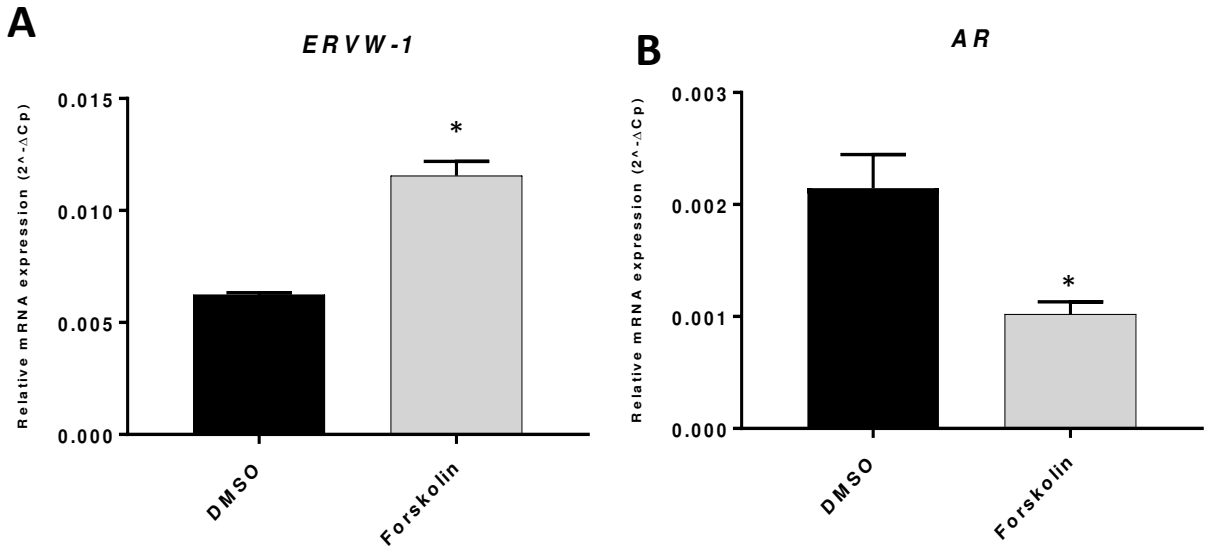

Figure 8: Relative levels of *ERVW-1* (A) and *AR* (B) in forskolin treated ACH-3P cells compared to DMSO controls. Relative levels of *ERVW-1* were significantly higher (A) whereas *AR* levels were significantly lower in forskolin treated cells compared to controls. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$

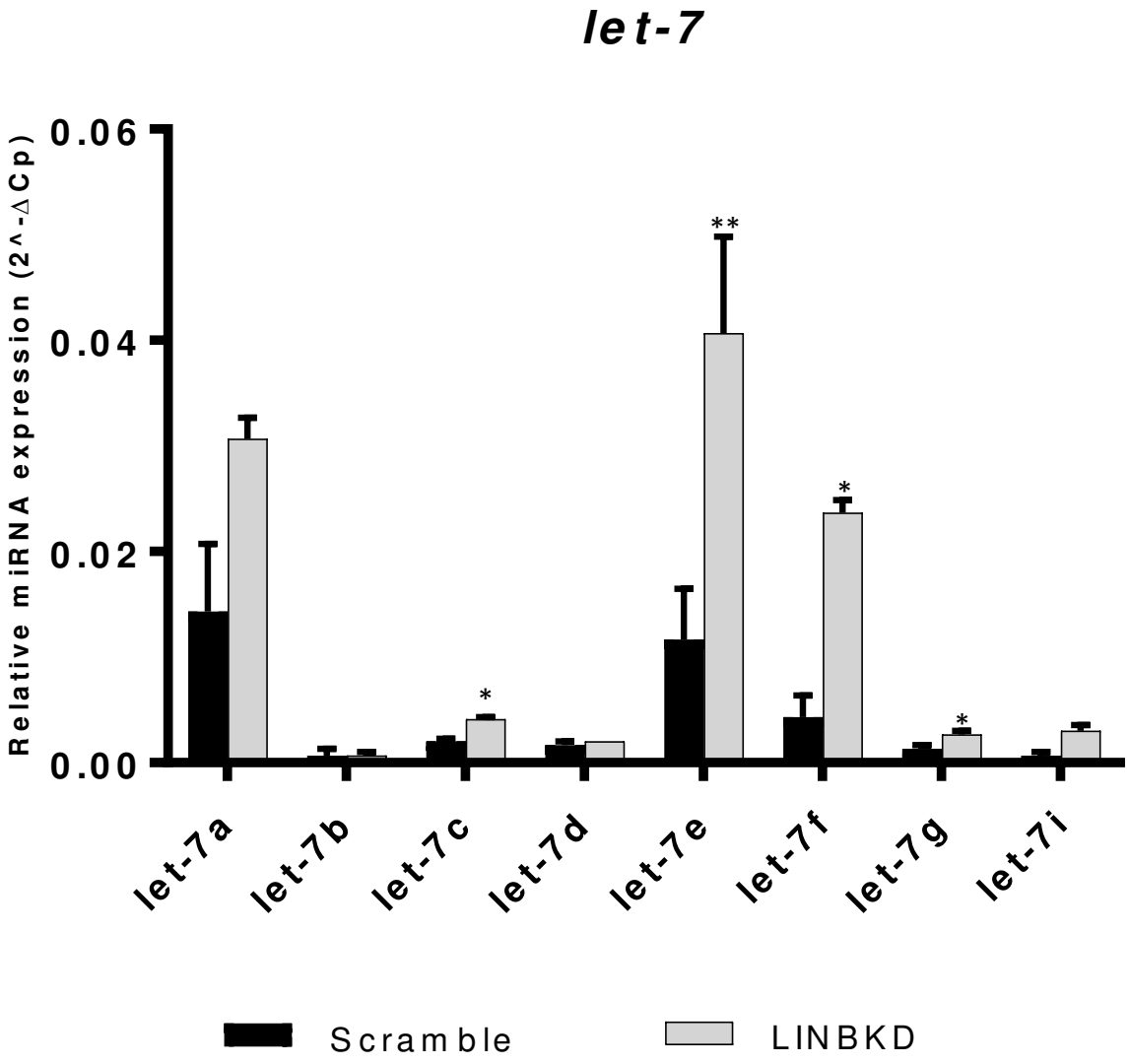

Figure 9: **Relative levels of let-7 miRNA in LIN28B KD cells compared to non-target scramble controls**. *Let-7c, e, f, g,* and *i* miRNA levels were significantly increased in the LIN28B KD cells compared to non-target scramble controls. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$

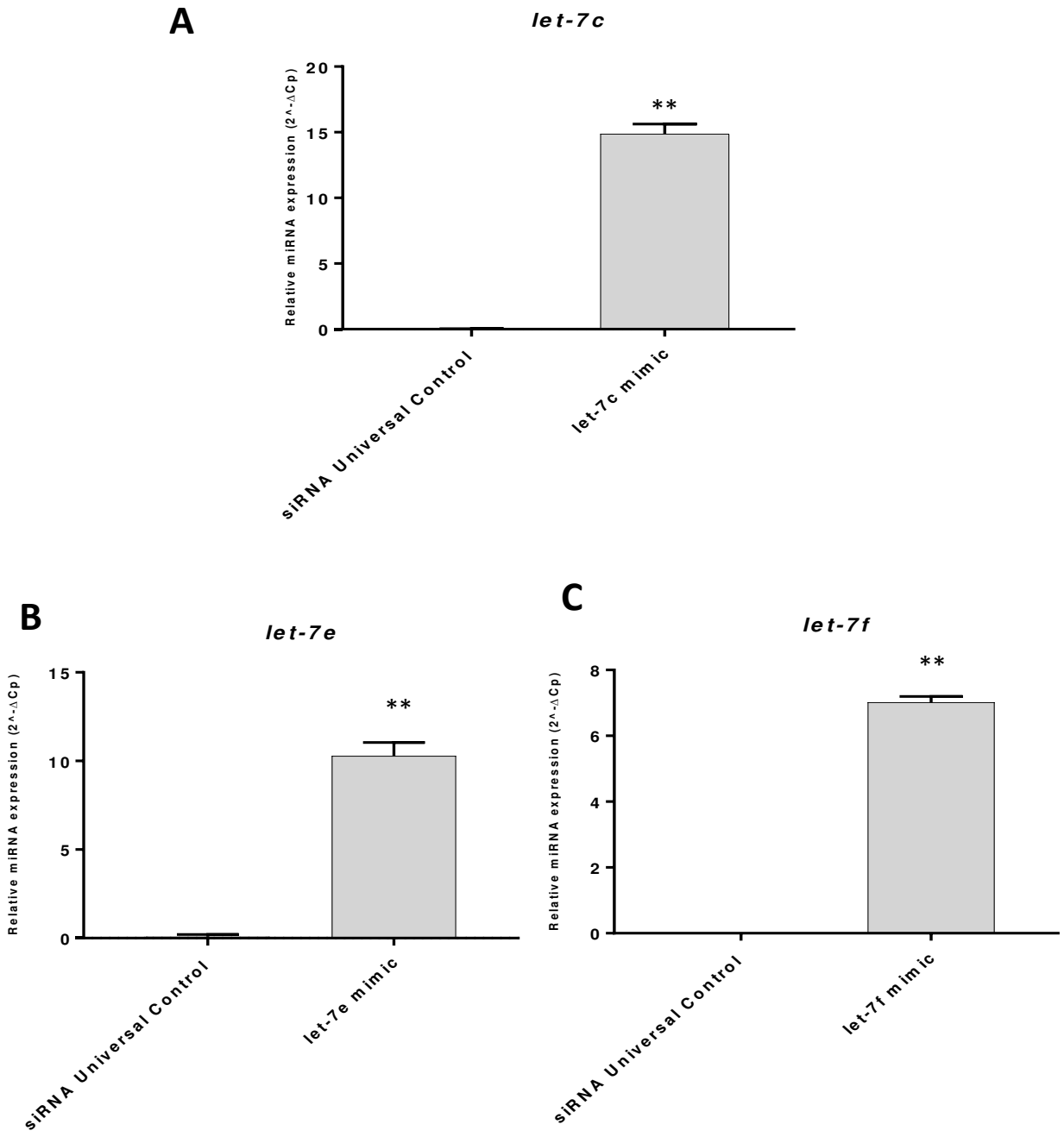

Figure 10: ***Let-7c, e* and *f* overexpression in ACH-3P cells**. A 24-hour treatment of ACH-3P cells with let-7c, e, or f mimic significantly increased respective miRNA levels *let-7c* (A), let-7e (B) and let-7f (C) compared to siRNA Universal controls. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$

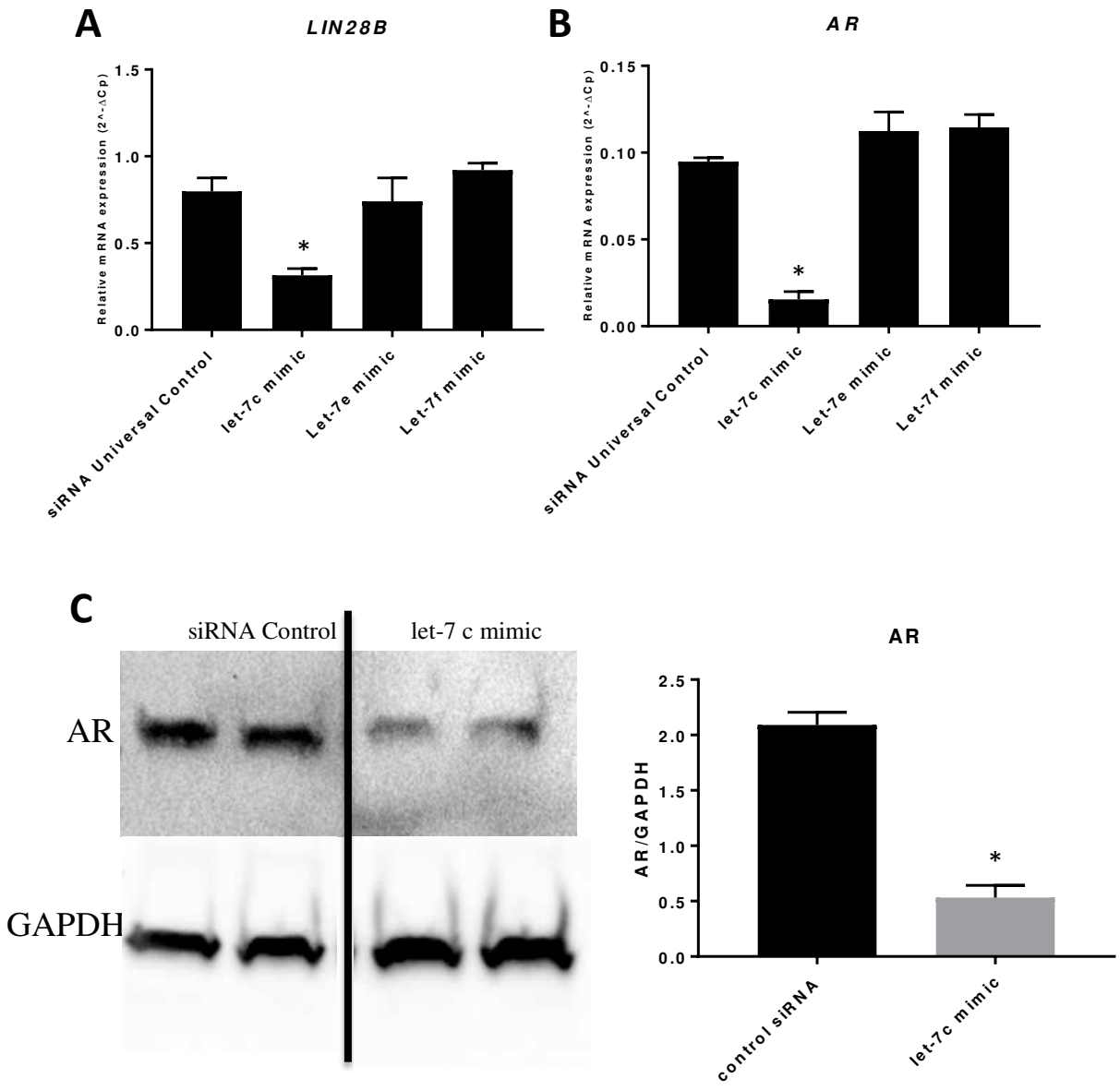

Figure 11: **Effect of *Let-7c, e,* and *f* overexpression in ACH-3P cells on *AR* and *LIN28B***. A 24-hour treatment of ACH-3P cells with let-7c mimic significantly decreased *LIN28B* (A) and *AR* levels (B) compared to siRNA Universal controls. Let-7e and f mimic treatment did not alter *LIN28B* or *AR* levels compared to siRNA Universal controls. Let-7c mimic significantly decreased AR protein compared to siRNA universal controls (C). * indicates $p \leq 0.05$

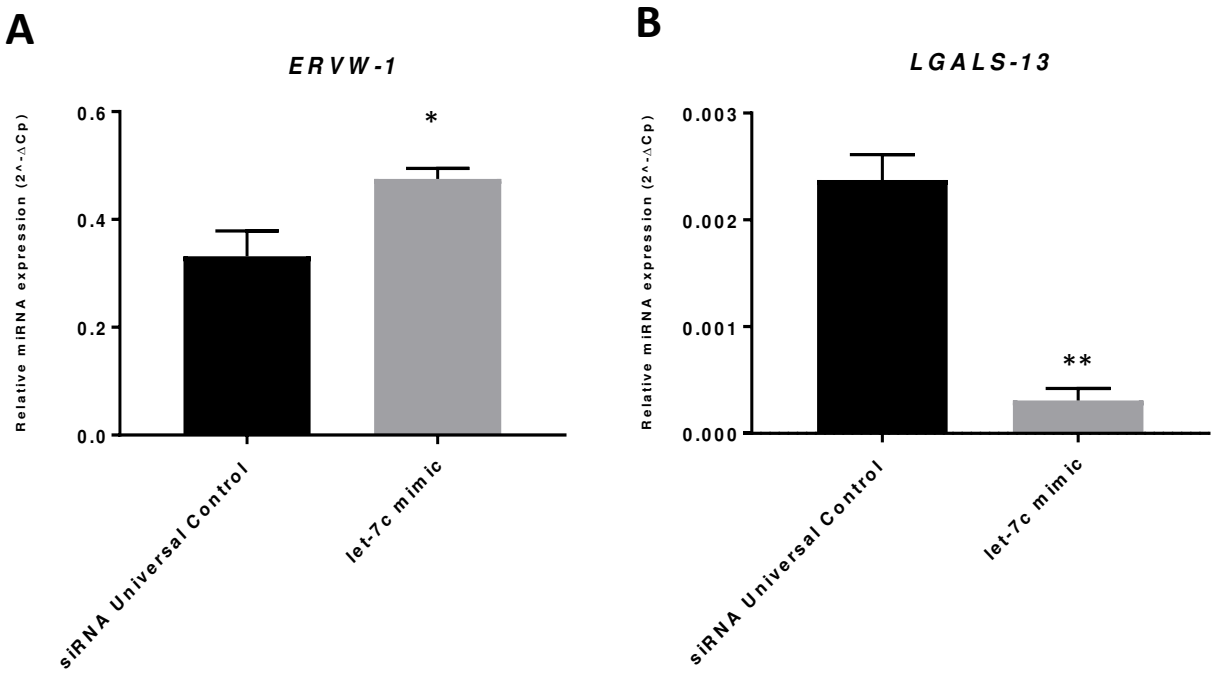

Figure 12: **Effect of *Let-7c* overexpression in ACH-3P cells on *ERVW-1* and *LGALS13***. A 24-hour treatment of ACH-3P cells with let-7c mimic significantly increased *ERVW-1* (A) and significantly decreased LGALS13 levels (B) compared to siRNA Universal controls. * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$

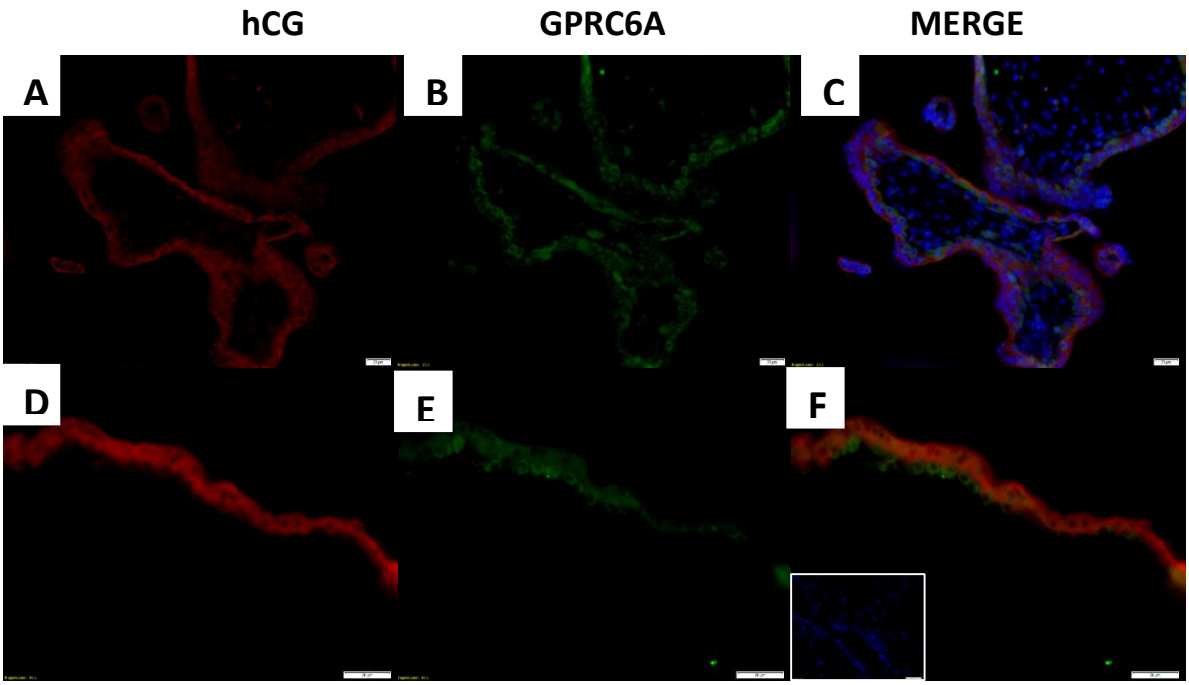

Figure 13: GPRC6A localization in human placenta tissue. Localization according to immunofluorescent (A-C, 20x; D-F 40x). Co-labeling of GPRC6A (green) and hCG (red) suggests localization of GPRC6A to syncytiotrophoblast and underlying trophoblast cells. Insert is rabbit serum negative control with DAPI.

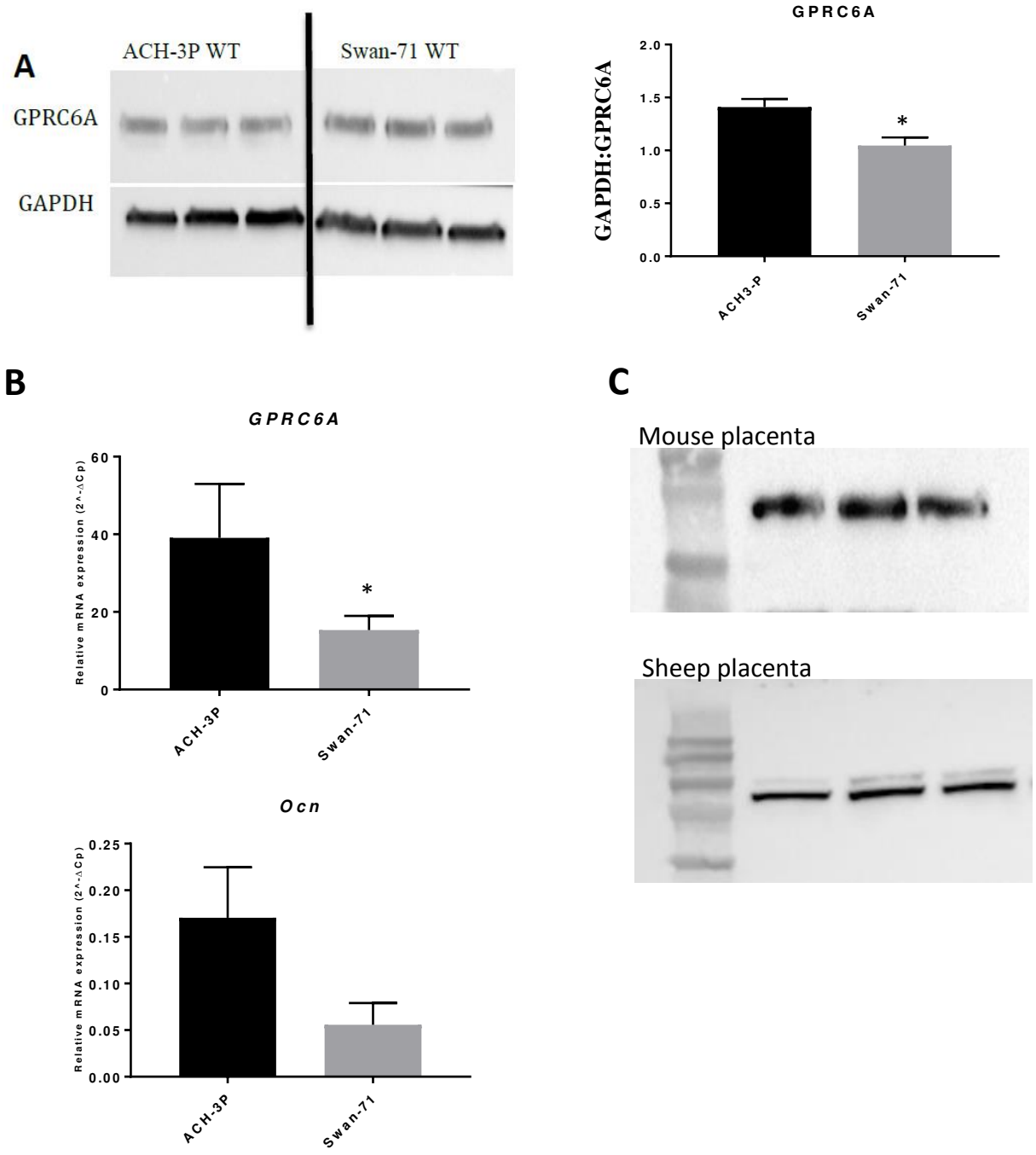

Figure 14: **GPRC6A expression in human, mouse and sheep placenta.** GPRC6A protein (A) and relative mRNA (B) levels are significantly increased in ACH-3P WT cells compared to Swan-71. Ocn mRNA levels were not statistically significant between ACH-3P and Swan cells (p=0.11). GPRC6A protein is present in mouse day 12.5 placental tissue and sheep day 55 placental tissue.

* indicates $p \leq 0.05$, ** indicates $p \leq 0.01$

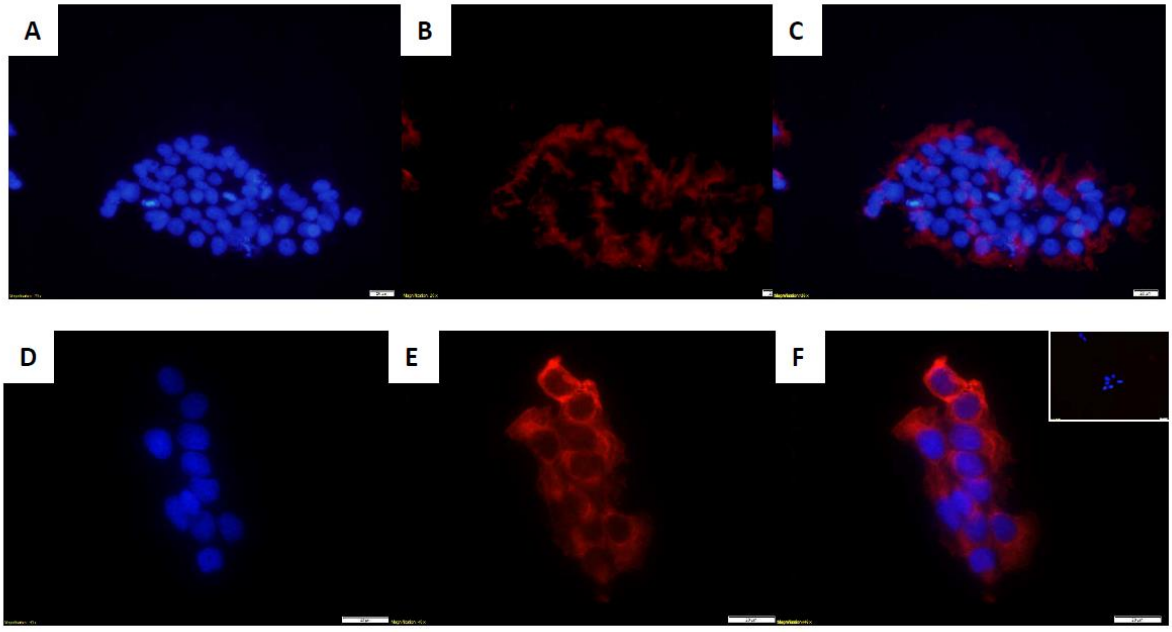

Figure 15: GPRC6A in human placental cells. Immunofluorescence indicates diffuse membrane staining in ACH-3P WT cells (A-C; 20x and D-F; 40x). Panel A/D; DAPI-staining, B/E; GPRC6A, C/F; merge. Insert is secondary only, DAPI-stained negative control.

DAPI

FITC-BSA-T

Overlay

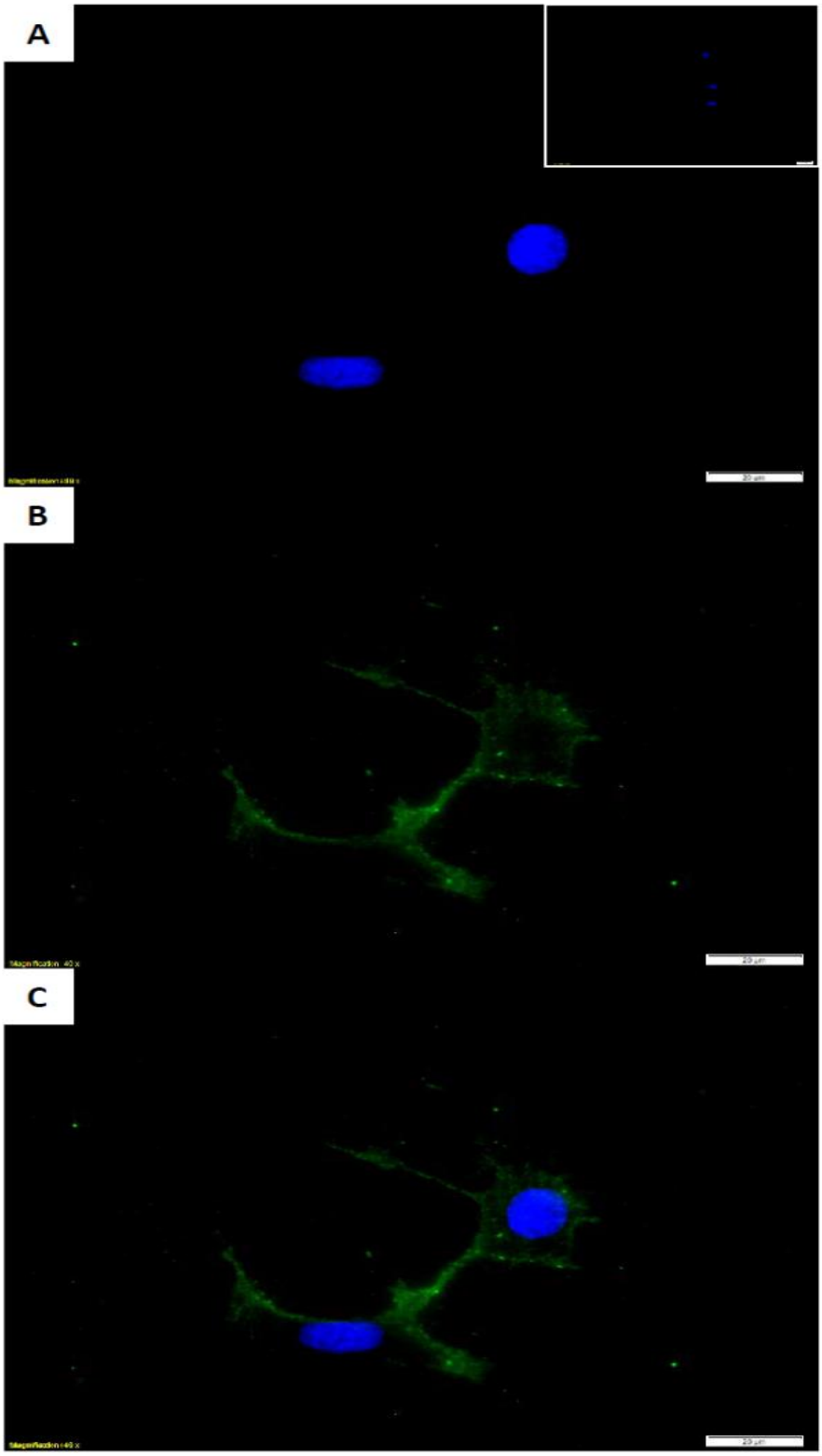

Figure 16: FITC-BSA-testosterone binding in human placental cells. Immunofluorescence indicates membrane FITC staining in ACH-3P WT cells (A-C, 40X). Insert is FITC-BSA control.

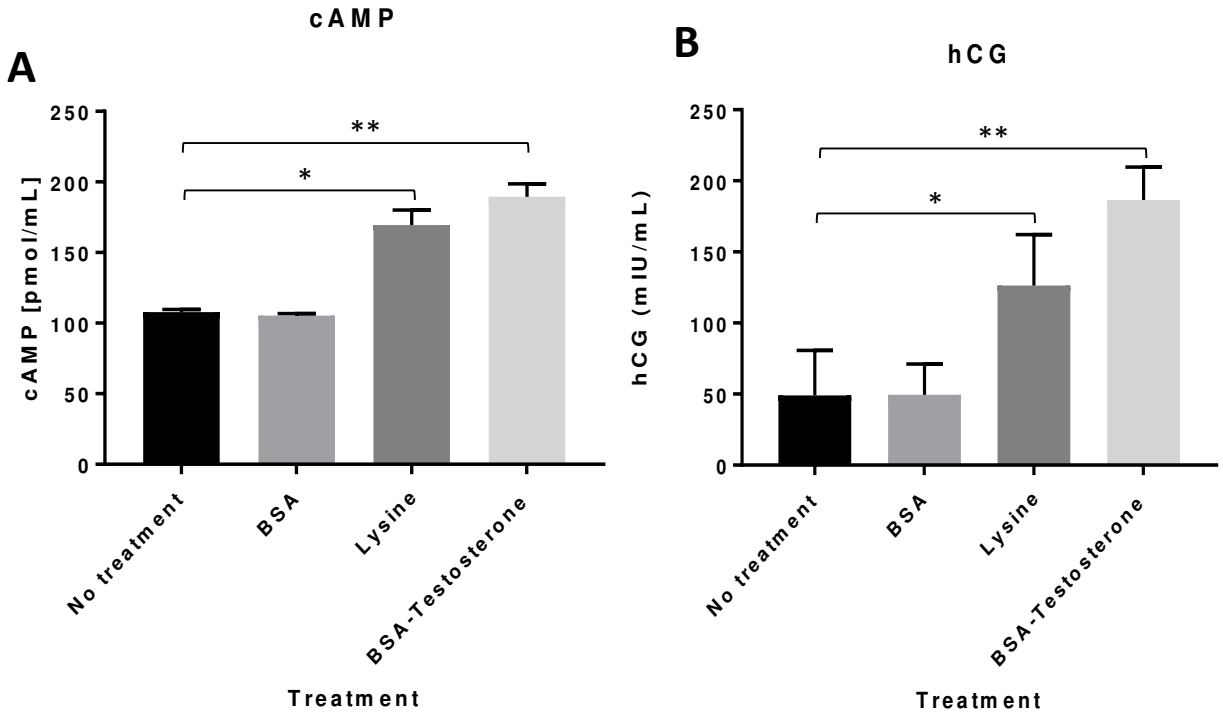

Figure 17: Effects of FITC-BSA-testosterone treatment. Cell lysate from cells treated with FITC-BSA-T and lysine positive control exhibited significantly increased cAMP levels compared to FITC-BSA-only negative controls. Cells treated with FITC-BSA-T and lysine positive control exhibited significantly increased soluble hCG levels in cell culture media compared to FITC-BSA-only negative controls. Cyclic-AMP and hCG levels were not significantly different between lysine controls and FITC-BSA-T treated cells. ** indicates $p \leq 0.01$

APPENDIX: ANDROGEN RECEPTOR (AR) IN HUMAN, MOUSE, AND SHEEP PLACENTA

Summary

Human placentation is a complex process and involves a diverse network of signaling hormones such as progesterone, estrogen, and testosterone. Understanding endocrine action in the placenta will allow for a better understanding underlying placental pathologies associated with pregnancy disorders. Research from our laboratory demonstrates that AR binds to an androgen response element (ARE) in the VEGFA promoter in ovine placental tissue. This data suggests that placental androgens may play and important role in regulating trophoblast function [Cleys et al 2015]. Androgens are 19 carbon steroid hormones that increase during pregnancy [Castracane et al 1998; O'Leary et al 1991]. Androgen metabolism involves the synthesis of dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulphate (DHEAS), androstenedione (A4), testosterone (T) and dihydrotestosterone (DHT) (figure 81). Not only is the placenta a source for these hormones, but it is a target [Bellingham et al 2012; Padmanabhan and Veiga-Lopez 2014; Rhind et al 2011]. AR has been localized to cytotrophoblasts and placental stroma cells [Iwamura et al, 1994; Hsu et al, 2009; Cleys et al., 2015].

AR is mostly commonly recognized as a ligand activated transcription factor that binds AREs in target genes. The previous chapters describe classical versus non-classical androgen signaling. The following addendum contains preliminary data describing the presence and localization of AR in human placenta cells, mouse placenta, and sheep placentomes

Materials and Methods

Human Trophoblast Cells

Two human first trimester trophoblast cell lines were initially used in this study: ACH-3P (a gift from Ursula Hiden, Medical University of Graz, Austria) and Swan71 (a gift from Gil Moore, Yale University, USA). ACH-3P cells are human first trimester trophoblast cells (CT and EVT) fused with the choriocarcinoma cell line AC1-1. Swan71 cells are immortalized (hTERT) human first trimester trophoblast cells. Cells were cultured at 37°F and 5% CO_2. ACH-3P cells were cultured in DMEM F-12 medium (HyClone), 10% FBS (HyClone), and 1% PSA (Corning Life Sciences). Swan71 cells were cultured in DMEM/F12 medium, 10% FBS, 1% PSA, 1 mM non-essential amino acids (HyClone), and 1 mM sodium pyruvate (Corning Cellgro) [30].

Immunofluorescent localization of AR in cells

ACH-3P WT cells were seeded at 20% confluency on sterile 4-chamber slides in appropriate cell culture media for 24 hours. Once cells were adhered, they either fixed or treated with 100µM FITC-BSA-conjugated testosterone or 100µM FITC-BSA and incubated at 37°F and 5% CO_2 for 30 minutes. FITC-treated cells were then washed 3 times with sterile 6.7µM PBS and fixed with 4% PFA. After fixing, cells were washed 3 times 6.7µM PBS. FITC- treated cells were then mounted with Prolong Gold with DAPI (Fisher Scientific), stored in the dark at 4°F and imaged 24 hours later. For stained cells, each chamber was blocked with 800µl of block solution (6% rabbit serum in PBS) and incubated at room temperature for 45 minutes. Block solution was aspirated and cells were incubated in primary antibody solutions for 1 hour at room temperature for 1 hour (1:100 AR in 6% rabbit serum in PBS). Cells were washed with PBS 3 times for 5 minutes each and incubated with rabbit-anti goat Alexa-fluor 488 secondary antibody in 6% rabbit

serum in PBS (1:1000) for 1 hour at room temperature in the dark. Cells were washed with PBS 4 times for 5 minutes each and slides were mounted with Prolong Gold with DAPI and stored in the dark at 4°F for 24hours until sealed. Secondary antibody only staining was used as a negative control. Slides were visualized using Olympus D73 camera on Nikon Eclipse E800 microscope and CellSense 1.3 software.

Mouse placental samples

Mouse day 12.5 placentae was collected from C57/B6 inbred mice. Date of gestation was determined by fore and hind limb morphology (Theiler 1989). Samples were obtained in accordance with the Colorado State University Institutional Animal Care and Use Committee, and samples were stored in sterile PBS upon collection and were transferred to ice cold 4% paraformaldehyde 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, two sections per slide were stained and repeated in triplicate, from three separate dams. For western blot analysis, protein was extracted from three different placenta and samples were run in triplicate.

Sheep placental samples

Sheep placentae (placentomes) was collected at gestational day 55 in accordance with the Colorado State University Institutional Animal Care and Use Committee. Whole placentomes and cotyledons were separated and flash frozen in liquid nitrogen and then stored at -80°F. For western blot analysis, protein was extracted from three different placenta and samples were run in triplicate.

Western Blot

Western blot analysis was used to determine its presence in the mouse and sheep placenta. Cells and tissue samples were lysed in RIPA buffer (20 mM Tris, 137 mM NaCl, 10% glycerol, 1% nonidet P-40, 3.5 mM SDS, 1.2 mM sodium deoxycholate, 1.6 mM EDTA, pH 8) with 10% protease/phosphate inhibitor cocktail (Sigma-Aldrich) and 1 mM phenymethanesulfonyl fluoride. The BCA protein assay kit (Pierce) was used to determine protein concentration. Absorbance was measured at λ 595 nm using a Biotek Synergy 2 Microplate Reader (Biotek). Protein was electrophoresed in 4-15% Mini-PROTEAN TGX Stain-Free precast gels (Biorad) and transferred to 0.2 μm pore nitrocellulose membrane at 110 volts at 4° C for 1 hour.

Membranes were then blocked in 5% milk-TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6) for 1 hour at room temperature then incubated in a polyclonal antibody to AR (Abcam) diluted 1:500. Blots were incubated at 4° C overnight. Membranes were then washed 3 times for ten minutes for a total of 30 minutes in 1x TBST, incubated with a horseradish peroxidase-conjugated secondary antibody (Pierce 1858415 for mouse and Abcam ab6741 for human and sheep) for 1 hour at room temperature, diluted 1:2000. Membranes were developed using ECL Western Blotting Detection Reagent chemiluminescent kit and membranes were imaged using a ChemiDoc XRS+ chemiluminescence system (BioRad).

Results and Discussion

For detection of AR protein, an antibody against N-terminal amino acids 2-16 of human AR was used. A single specific band of ~100 kDa for AR protein was detected in protein isolated from mouse d12.5 placentae while a single, specific band ~75 kDa corresponding to AR protein was detected in ACH-3P and Swan71. This size discrepancy can be explained by detection of AR

splice variants such as AR-V5,6, and 7, that lack the C-terminal androgen binding site, and has been reported previously [Wadosky and Koochekpour 2017]. Real time PCR analysis revealed AR mRNA levels in ACH-3P cells was significantly increased compared to Swan-71 cells ($p < 0.01$). Immunofluorescence in ACH-3P cells, indicated nuclear and cytoplasmic staining. In the mouse placenta, AR was localized the nuclei of giant cells in the spongiotrophoblast as well as nuclei in the labyrinth layer. Based on this localization, AR could play a role in the differentiation of trophoblast progenitor cells to more specialized cell populations. These preliminary studies were performed to determine the potential for more in-depth experiments regarding AR signaling in the human placenta. Understanding the role of androgen signaling in the placenta will lead to better grasp of the mechanisms regarding placental development that could ultimately impact fetal growth.

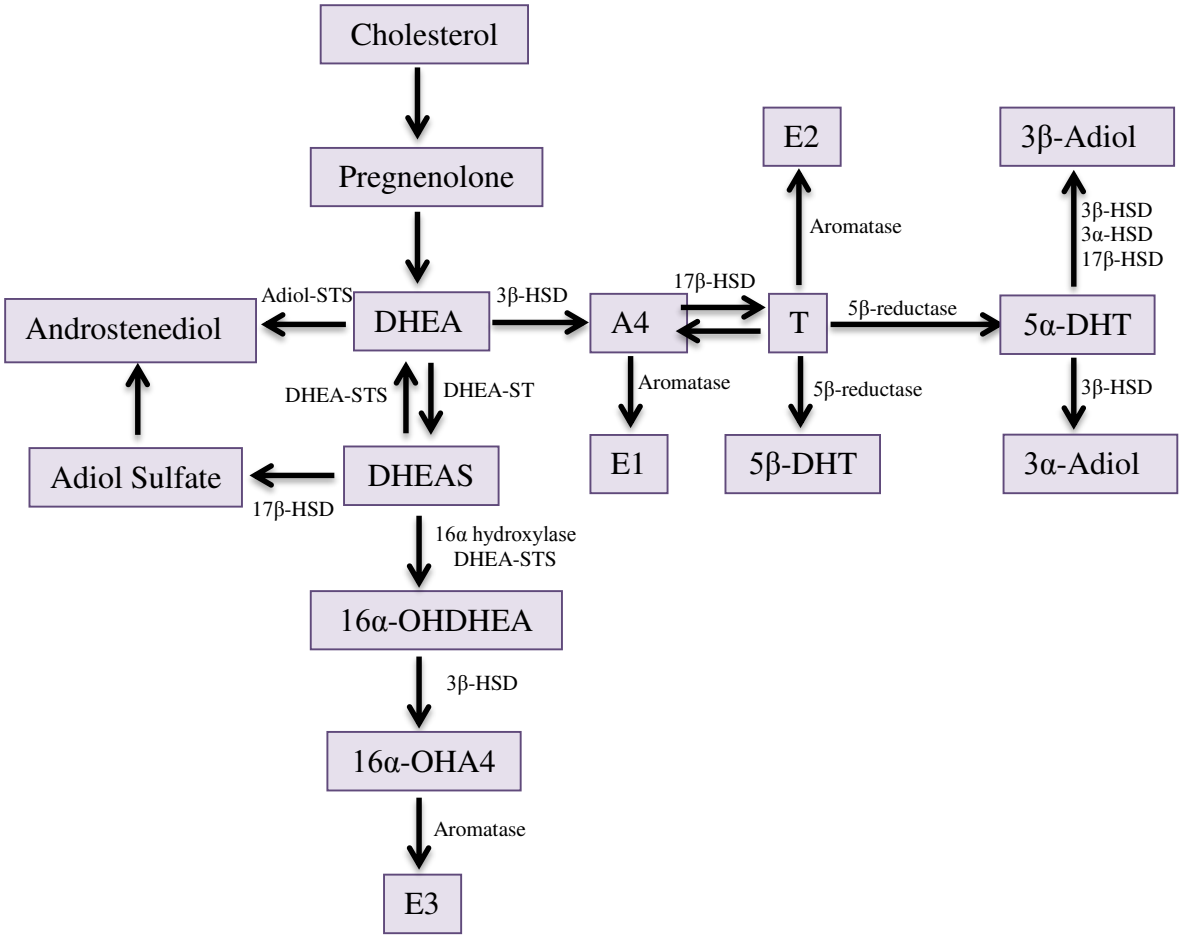

Figure 18: Mammalian androgen synthesis

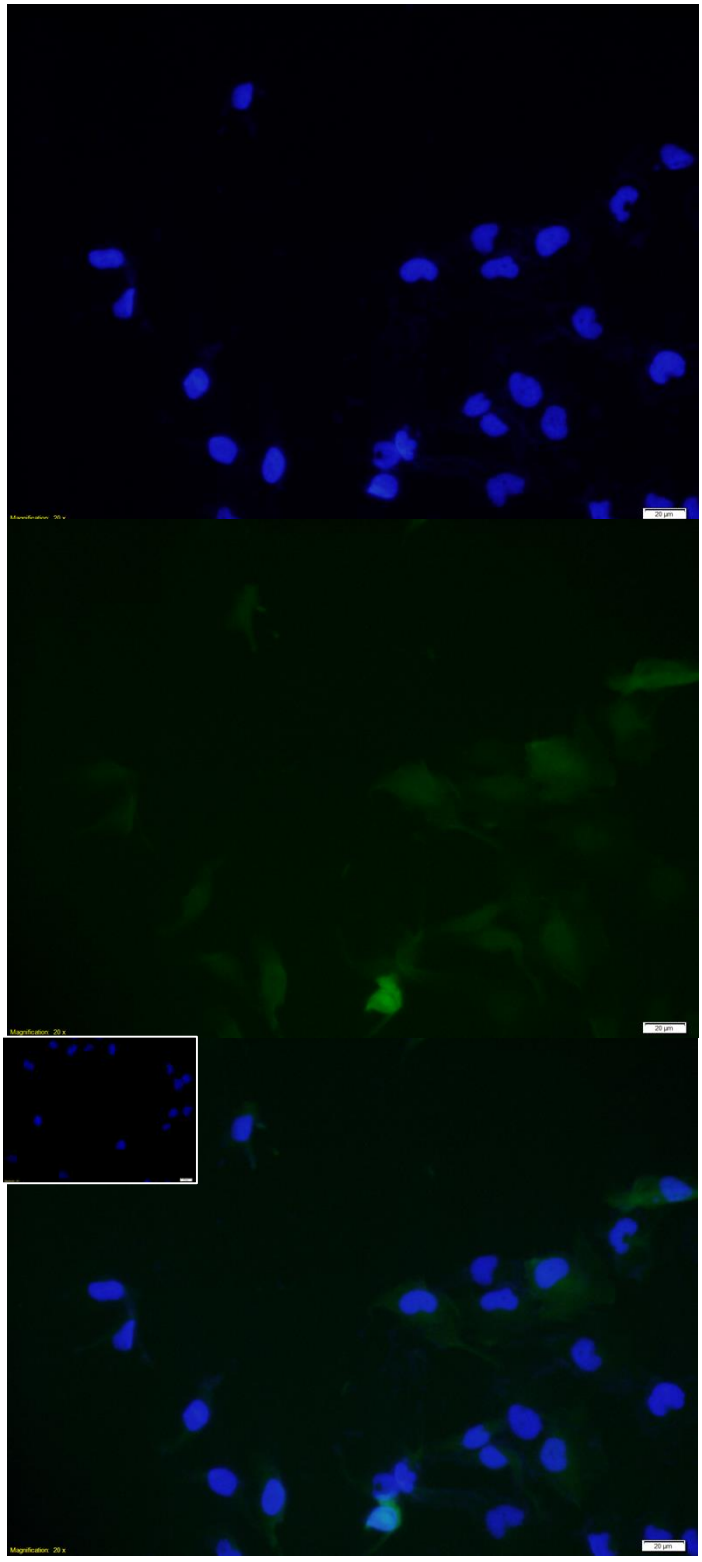

Figure 19: AR in human placenta cells. Localization according to immunocytochemistry. AR

localized to the cytoplasm and nucleus of ACH-3P cells. (20x) Insert is secondary only-negative control.

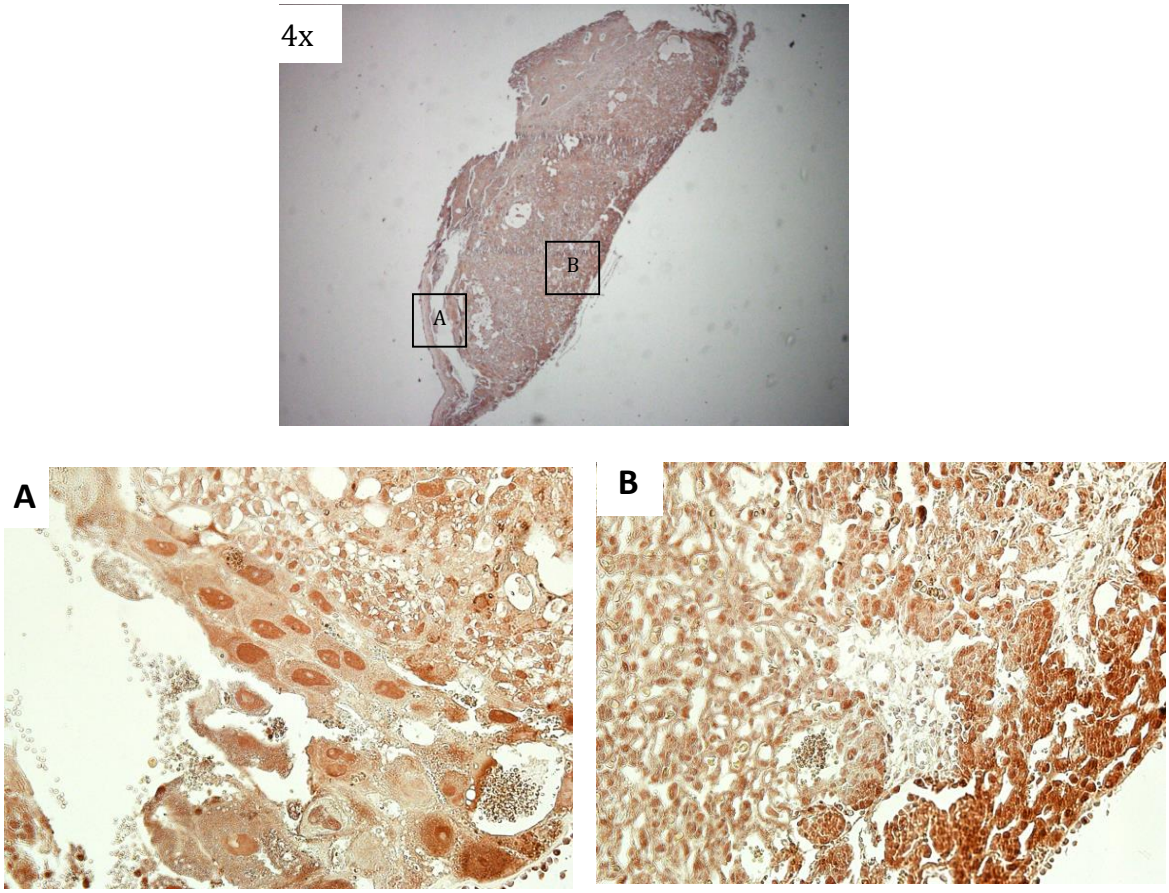

Figure 20: AR localization in mouse placental tissue. Localization according to immunohistochemistry (A and B, 40x) suggests localization of AR to the giant cells in the spongiotrotpblast and labyrinth layer.

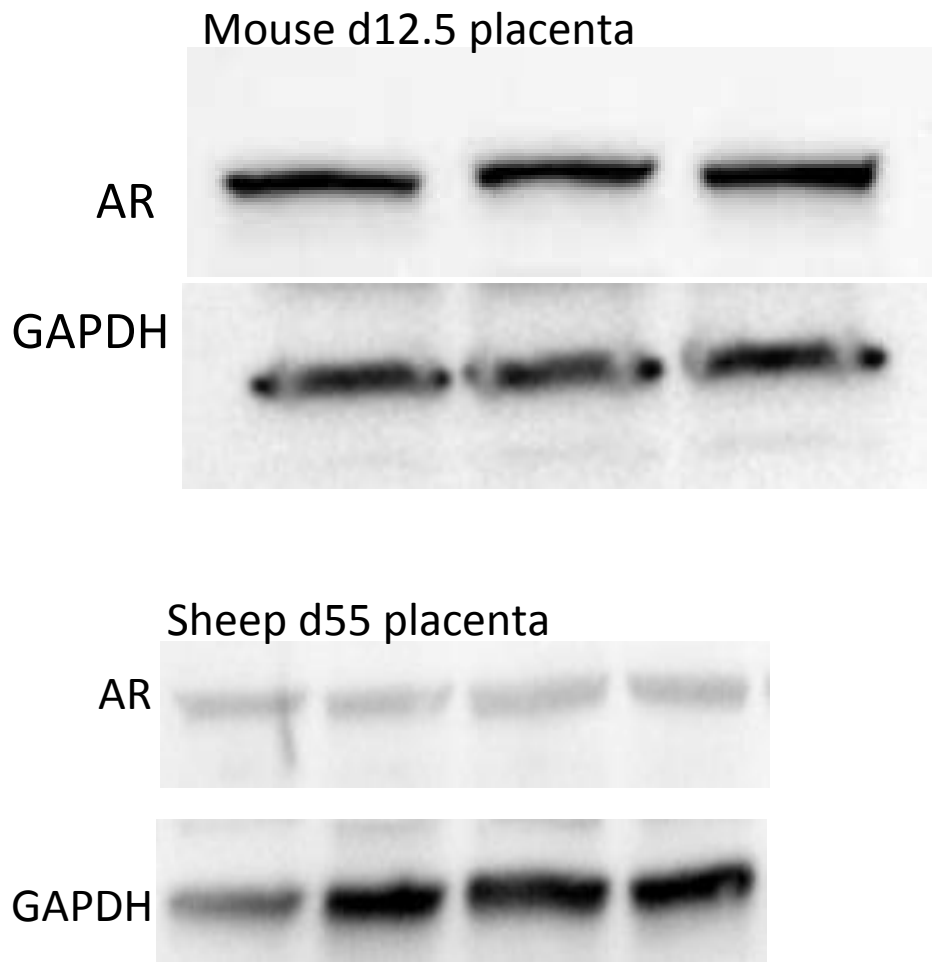

Figure 21: **Androgen receptor protein in mouse and sheep placenta.** AR protein is present in mouse day 12.5 placental tissue and sheep day 55 placental tissue.

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