PARACRINE EFFECTS OF INTERFERON GAMMA ON THE PORCINE UTERINE ENDOMETRIUM

A Dissertation

by

BRYAN ANTHONY MCLENDON

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Chair of Committee,	Gregory A. Johnson
Co-Chair of Committee,	Fuller W. Bazer
Committee Members,	Robert C. Burghardt
	Guoyao Wu
Head of Department,	Todd O'Hara

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ABSTRACT

Pregnancy is a complex period that requires many different biological processes, ultimately resulting in an inefficient system. A large portion of individual conceptus loss occurs during the peri-implantation period and later, during the period when the uterine-placental interface is folding. To determine how pregnancies can be affected, our lab studied the immunology of pregnancy during the peri-implantation period and the expression of aquaporins throughout gestation. Results of the present studies with pigs revealed: (1) accumulation of immune cells and apoptosis of stromal cells within the endometrium at sites of implantation during the period of IFNG secretion by conceptuses; (2) accumulation of proliferating cell nuclear antigen (PCNA)-positive T cells within the endometrium at sites of implantation; (3) significant increases in expression of T cell co-signaling receptors and chemokines within the endometrium at sites of implantation; (4) significant increases in T cell co-signaling receptors and the chemokine CXCL9 in the endometrium of cyclic gilts infused with IFNG; (5) identification of CD4+ (22.59%) as the major T cell subpopulation, with minor subpopulations of CD8+ (1.38%), CD4+CD25+ (1.08%), and CD4+CD8+ (0.61%) T cells within the endometrium at sites of implantation; (6) AQPs 1, 5, 8, and 9 show differential and discrete temporal cell-type specific protein expression by key tissues at the uterine-placental interface; and (7) AQPs 1 and 8 mRNA expression increases as pregnancy progresses to potentially provide the conceptus with water and nutrients necessary for development. Our results provide new insights into the complex events occurring during gestation, potential mechanisms

explaining how the conceptus is signaling to the endometrium, and how the endometrium provides the conceptus with water and nutrients needed for growth and survival.

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CHAPTER I

INTRODUCTION

Infertility is a significant problem within livestock species, with implantation failure during the peri-implantation period being one of the major causes of infertility (Bazer *et al.* 1983, Johnson *et al.* 2009). The paracrine interactions between the conceptus (embryo and associated placental membranes) and the uterus are critical for the growth and development of the conceptus during gestation. Establishment and maintenance of pregnancy begins with several notable events, including 1) the conceptus secreting a pregnancy recognition signal so that the uterus does not terminate the pregnancy, 2) the uterus secreting histotroph (a collection of nutrients such as sugars, amino acids, hormones, and transcription factors) for utilization by the conceptus, and 3) remodeling of the endometrium in response to conceptus attachment for implantation (Frank *et al.* 1977, Bazer *et al.* 1977, Godkin *et al.* 1984, Vallet *et al.* 1988, Ashworth *et al.* 1989, Glasser *et al.* 1993, Gray *et al.* 2001b, Burghardt *et al.* 2002). Each of these events are complex and require large amounts of energy, which provide an explanation for the high rates of embryonic/fetal loss during this early period of gestation (Bazer *et al.* 1983, Johnson *et al.* 2009).

After the pig embryo hatches from the zona pellucida around Day 11 of gestation, it begins to rapidly elongate, changing from a tubular and then into a filamentous structure by Day 16. As it elongates, the conceptus trophectoderm secretes estradiol, which is considered the pregnancy recognition signal in pigs, allowing for the continued secretion

of progesterone (P4) and the maintenance of pregnancy. The conceptus trophectoderm also secretes other proteinaceous paracrine factors during elongation including, but not limited to, interleukin 1 beta (IL1B), prostaglandin E2 (PGE2), transforming growth factor beta (TGFB), and the interferons delta (IFND) and gamma (IFNG) that act in a paracrine manner to effect uterine function (La Bonnardiere *et al.* 1991, Christenson *et al.* 1994, Gupta *et al.* 1996, Ross *et al.* 2003). Unlike other livestock species, pigs are unique in that their conceptuses secrete both type I and type II interferons (IFNs), with the type II IFNG being the major species (Mirando *et al.* 1990, La Bonnardiere *et al.* 1991, Lefevre *et al.* 1998). As the conceptus is elongating and secreting these paracrine factors, it also begins the process of implantation. Implantation in the pig is a prolonged process that occurs from Days 13 to 25 of pregnancy. This is termed the peri-implantation period. The pig placenta is epitheliochorial, meaning that the porcine conceptus does not invade into the endometrium and all epithelial layers remain intact throughout pregnancy. As previously stated, this period of gestation is complex and correlates with high rates of fetal loss. The formation of areolae on the chorioallantois begins towards the end of the peri-implantation period. Areolae are areas of the conceptus chorioallantois that form opposite the openings of uterine glands to create pockets above the opening of uterine glands that are important for increased transport of water and histotroph that is secreted by or transported by the glandular epithelium into the lumen of the uterine glands. In addition, extensive folding at the uterine-placental interface develops during gestation in order to increase surface area for transport of nutrients from the mother to the fetus. The development of areolae and the extensive folding of the uterine-placental interface overcome the physical barrier of

epitheliochorial placentation, which has the most layers for nutrients and gases to traverse of all placental types.

IFNG is an inflammatory cytokine first discovered in activated human peripheral blood lymphocytes in 1981 (Gray *et al.* 1982). It is the only type II interferon and is known predominantly for being involved in immune responses with antiviral, antimicrobial, and immunomodulatory activities. However, during porcine pregnancy, IFNG is secreted by the conceptus trophectoderm and is believed to exert paracrine effects on the endometrium. Unlike interferon tau (IFNT), which has an anti-luteolytic effect on the corpus luteum (CL) in ruminants, IFNG appears to have no effect on the CL, and is not considered to be a signal for pregnancy recognition. Instead, porcine conceptus IFNG has been proposed to act in a paracrine manner on the endometrium to promote remodeling of the endometrium necessary for implantation. IFNG and IFND are also proposed to increase expression of IFN stimulated genes (ISGs), including major histocompatibility complex (MHC) class I and MHC class II molecules in the pig endometrium (Joyce *et al.* 2008, Kim *et al.* 2012). The porcine conceptus expresses the IFNG receptor starting around Day 16 of pregnancy, suggesting a delayed autocrine effect (D'Andrea and LaBonnardiere 1998). However, the main hypothesis is that conceptus IFNG acts on the endometrium to prepare for conceptus implantation and pregnancy.

Aquaporins (AQPs) are water-selective channels that function as pores for water transport through the plasma membrane (Agre *et al.* 2002). To date, 13 isoforms of AQPs have been found in mammals, with some AQPs transporting other molecules in addition to water. The movement of water and nutrients across cell membranes is crucial for

reproduction and is likely influenced by the expression of AQPs in tissues of the reproductive tract (Zhu *et al.* 2015). The placenta and uterus are the major organs responsible for supporting embryonic/fetal development, and thus play essential roles in assuring survival and growth of the conceptus. Key roles of the uterus and placenta are to regulate the transport of nutrients and water across the uterine-placental interface during pregnancy recognition, conceptus implantation, and placental development/placentation (Wilson 2002). Expression of AQPs in the uteri of different species, including humans (Li *et al.* 1994), mice (Richard *et al.* 2003), rats (Lindsay and Murphy 2006, 2007), dogs (Aralla *et al.* 2012), and pigs (Skowronski 2010) has been reported, but the spatio-temporal expression of AQPs has not been determined throughout gestation for pigs.

The uterus is a tubular organ composed of the endometrium and myometrium. The myometrium is composed of an inner circular layer and an outer longitudinal layer. While the endometrium focuses on the growth and development of the embryo/fetus, the myometrium builds cell-cell and cell-extracellular matrix (ECM) connections in order to create a cohesive population of cells that act similar to a syncytium. This allows for rapid communication between the smooth muscle cells of the myometrium for numerous functions including, but not limited to, muscle contractions and expelling of the fetus and placenta during parturition. These cell-cell and cell-ECM connections are known as integrin adhesion complexes (IACs) and are composed of ECM proteins, intracellular adaptor proteins, and integrins.

IACs are dynamic macromolecular complexes comprised of heterodimeric transmembrane integrin receptors that connect ECM proteins to the actin cytoskeleton,

along with a diverse array of cell signaling intermediates (Sastry & Burridge 2000, Wozniak *et al.* 2004, Larsen *et al.* 2006). Therefore, IACs function to transmit force at cell adhesion sites and serve as signaling centers from which numerous intracellular pathways can regulate cell growth, proliferation, survival, gene expression, development, tissue repair, migration, and invasion (Burghardt *et al.* 2009). In sheep, integrins and ECM proteins have been identified in the myometrium during gestation, but little is known about the localization and function of IACs during the pre- and postpartum periods. In light of the function of smooth muscle cells in the myometrium and information available on IACs, it has been hypothesized that IACs are important for coordinated contractions necessary for expulsion of the fetus and placenta at the end of gestation.

CHAPTER II

LITERATURE REVIEW

Estrous Cycle in Pigs

The estrous cycle in pigs lasts approximately 21 days and begins with the onset of estrus, or standing heat. This period is marked by peak estradiol (E2) secretion from developing follicles and ends with ovulation in response to luteinizing hormone (LH) and this begins the metestrus period, when E2 begins to decrease and progesterone (P4) from the developing corpora lutea (CL) begins to increase. After the follicles ovulate, the theca and granulosa cells begin to undergo a remodeling process called luteinization to form CL. The CL are responsible for synthesizing and secreting P4, the hormone of pregnancy. The diestrus period begins once P4 levels are high and the corpora lutea are fully functional. This is the longest period of the estrous cycle and lasts until the CL are destroyed and undergo luteolysis in response to prostaglandin $F_{2\alpha}$ (PGF) exposure. PGF is secreted from the endometrial luminal epithelium (LE) in a pulsatile manner in response to pituitary oxytocin binding the oxytocin receptors on the LE and enters the utero-ovarian vein to be transported to the ovary, where it initiates lysis of the CL (Moeljono *et al.* 1976, Mirando *et al.* 1995). Luteolysis results in termination of diestrus and the beginning of proestrus. During this period, P4 levels decrease and E2 and follicle stimulating hormone (FSH) levels begin to increase due to the removal of P4 inhibition and there is continued development of growing follicles. This continues until peak E2 secretion, marking the start of estrus and the beginning of a new cycle.

Pig Pregnancy

Pigs are polytocous, polyestrus animals, meaning they can give birth to multiple young year-round and their pregnancy generally lasts 114 Days. The pig placenta is macroscopically diffuse, having chorionic villi distributed throughout the placenta, and microscopically epitheliochorial as each layer of the endometrium and placenta remains intact. The uterine-placental interface is a substantial physical barrier for nutrients and gases to cross, which is why it is necessary for pig conceptuses to elongate and increase the surface area of the trophectoderm/chorion in contact with the endometrium.

Early conceptus development

Early embryogenesis is similar across mammalian species. The oocyte is fertilized and undergoes cleavage divisions until it becomes a morula around Day 3. The cells of the morula continue to divide, but at this stage a blastocoele, or fluid-filled cavity, begins to form and the cells differentiate into an inner cell mass (ICM), which becomes the embryo, surrounded by a single layer of cells called the trophoblast, which becomes the chorion of the placenta. The conceptus (embryo and associated placental membranes) is now called a blastocyst, which hatches from the zona pellucida around Day 6. This time point is where the livestock species begin to differ from mice and humans. After hatching, the blastocyst begins to migrate throughout the uterus and elongate from a spherical shape into a tubular and filamentous form. Again, pigs are litter-bearing species, so they have multiple conceptuses within the uterus. These blastocysts grow and elongate very rapidly after hatching, starting at 0.5 to 1 mm in diameter and increasing in size to Day 10 of pregnancy,

where they transition into large spheres of 10 to 15 mm. By Day 11 of gestation, these conceptuses are 1 mm by 100-200 mm filamentous structures and continue to elongate at 30 to 45 mm/h due to cellular remodeling and hyperplasia, reaching lengths of 800 to 1000 mm by Day 15 of pregnancy (Bazer and Johnson 2014). Hatching of the blastocyst and subsequent elongation is necessary for continued development and differentiation into a conceptus. Elongation and migration of the conceptuses serve to produce enough pregnancy recognition signal for the maintenance of pregnancy and provide sufficient surface area necessary for the transfer of nutrients and gases from the mother to conceptus/fetus (Geisert *et al.* 2017).

Conceptus elongation is regulated by different mechanisms depending on the species. In pigs, it was originally proposed that conceptus elongation occurs through rapid remodeling of the conceptus, rather than an increase in mitotic activity (Perry 1981). Cellular hyperplasia is reduced as the conceptus transitions from an ovoid to filamentous shape and the movement of cells, along with changes in structure, begins at the epiblast, forming a dense region along the embryonic pole of the conceptus (Geisert *et al.* 1982b). This region is termed the elongation zone and it is proposed that the trophectoderm near the epiblast loosen their junctional complexes, allowing cells to transition into a columnar shape (Geisert *et al.* 2015). This cellular fluidity allows for movement toward the tips of the conceptus as the underlying endoderm cells migrate and pull the trophectoderm toward the elongation zone, continuing to elongate until implantation (Geisert *et al.* 2017). Elongation is not an estrogen-dependent process, but interleukin 1 beta 2 (IL1B2) has been implicated in conceptus elongation in the pig. Development of IL1B2 null embryos is not

affected to the blastocyst stage, but they fail to elongate or survive in utero, confirming the importance of IL1B2 in the remodeling and elongation of pig conceptuses (Whyte *et al.* 2018).

Porcine conceptus implantation

As the pig conceptus is elongating and preparing for attachment, it secretes numerous paracrine and endocrine factors that act on the endometrium in order to prepare for implantation and the maintenance of pregnancy. One major secretory product of the conceptus is E2, which is generally considered the pregnancy recognition signal in pigs. This theory is based on observations that the LE secretes PGF, which is responsible for lysing the CL, and the conceptus secretes antiluteolytic estrogens. As previously stated, during the cycle PGF is secreted in a pulsatile manner into the vasculature to induce luteolysis, resulting in a decrease of P4 and restarting the cycle. However, during pregnancy E2 redirects PGF secretion into the uterine lumen and is potentially metabolized to prevent luteolysis, maintaining P4 production and pregnancy (Bazer and Thatcher 1977). Recently, the ablation of aromatase, utilizing CRISPR/Cas9 genome editing, showed that E2 was not essential to maintain early gestation, but was essential to maintain pregnancy after Day 30, suggesting that another mechanism could be responsible for maintaining CL function (Meyer *et al.* 2019). Prostaglandin E2 (PGE2) has a proposed role in pregnancy signaling because PGE2 synthase expression by the conceptus trophectoderm and endometrium has been shown to decrease production of PGF in favor of PGE2, supporting CL maintenance (Waclawik *et al.* 2017). In addition to pregnancy

recognition, conceptus estrogens modulate uterine gene expression (Geisert *et al.* 1982c, Johnson *et al.* 2009, Waclawik *et al.* 2017), and the early exposure of the pregnant uterus to estrogens on Days 9 and 10 results in degeneration of all conceptuses by Day 15 (Ross *et al.* 2007). During pregnancy, the pig endometrium rapidly converts estradiol to estrone and, subsequently, estrone sulfate. Estrone sulfate is the biologically inactive form and is found in high concentrations in the lumen of pregnant pigs (Flood 1974). The conceptus trophectoderm has the sulfatase enzyme that restores the activity of estrogen, allowing for a localized effect of estrogen to up-regulate estrogen-stimulated genes within the pig endometrium (Johnson *et al.* 2009).

In response to the pregnancy recognition signal, P4 production and secretion is maintained in order to support gestation. P4 is known as the hormone of pregnancy and exerts its actions on tissues through the nuclear P4 receptor (PGR) (Wetendorf and DeMayo 2014). PGR is encoded by a single gene that has three isoforms (A, B, and C), each with differing lengths and activities (Mulac-Jericevic and Conneely 2004). PRB is expressed in the LE, GE, stroma, and myometrium through Day 7 of the estrous cycle and pregnancy, but prolonged exposure to P4 downregulates the PGR in the LE by Day 10 and in the superficial GE by Day 12 of both the estrous cycle and gestation. PGR protein expression returns in the LE by Day 17 of the cycle, but is not expressed by the LE between Days 25 and 85 of pregnancy. PGR expression is maintained within the deep GE throughout early pregnancy, but decreases through Day 50 of gestation, with some expression within the necks of uterine GE that open into areolae. This suggests that these regions of the uterine glands are excretory ducts that aid in transport of histotroph into the

lumens of areolae. Areolae are out pockets of the chorionic epithelium of the conceptus trophectoderm that collect secretions from the uterine glands and allow for increased transport of histotroph from the mother to the conceptus/fetus. Unlike the endometrial epithelia, the stroma and myometrium continue to express PGR protein through Day 85 of gestation (Geisert *et al.* 1994, Sukjumlong 2005, Steinhauser *et al.* 2017). As stated, progesterone acts through the PGR to stimulate the production and secretion of histotroph. Interestingly, PGR are not expressed in the epithelia that secrete histotroph during the peri-implantation period (Geisert *et al.* 1994, Sukjumlong 2005, Steinhauser *et al.* 2017). Induction of genes may require the downregulation of PGR for the expression of progesterone-regulated genes, or the expression of genes by P4 could be mediated through paracrine-acting factor(s), termed progestamedins, synthesized and secreted by PGR-positive cells (White *et al.* 2005).

As the conceptus is elongating and both the trophectoderm and endometrium are communicating through paracrine factors, implantation is occurring. Porcine conceptuses do not invade into the uterine endometrium, rather they attach to the endometrial LE and all layers of the placenta and endometrium remain intact throughout gestation. The initial stages of implantation and placentation are common across species and are known as the "Adhesion Cascade for Implantation" (Dantzer 1985, Guillomot 1995, Burghardt *et al.* 2002). This process begins when the uterine LE and conceptus trophectoderm develop the ability to initiate adhesion during the period, termed the "window of receptivity" (Fazleabas *et al.* 2004, Spencer *et al.* 2007b, Bazer *et al.* 2011). P4 plays a major role in the adhesion cascade by downregulating the expression of PGR in the LE after 10 days of

pregnancy, which results in the downregulation of the anti-adhesive molecule mucin 1 (MUC1), a transmembrane mucin that inhibits attachment of the trophectoderm to the LE (Geisert *et al.* 1994, Steinhauser *et al.* 2017, Brayman *et al.* 2004, Bowen *et al.* 1996). Down-regulation of MUC1 exposes potential low affinity carbohydrate ligand binding molecules that are proposed to contribute to the initial attachment of the conceptus to the LE (Kimber *et al.* 1995, Kimber and Spanswick 2000, Spencer *et al.* 2004). It is likely that the porcine conceptus undergoes a series of attach-and-release events that results in apposition of the trophectoderm to the LE, similar to the "Rolling and Tethering" that occurs during leukocyte adhesion to the endothelium for extravasation (Kling *et al.* 1992). These low affinity contacts are then replaced by more stable, adhesive interactions between integrins, such as secreted phosphoprotein 1 (SPP1), and extracellular matrix (ECM) proteins, marking the firm attachment phase. Integrin-mediated adhesion results in the formation of dynamic macromolecular complexes, termed integrin adhesion complexes (IACs), between the conceptus trophectoderm and the uterine LE on Days 20 and 25 of pregnancy (Erikson *et al.* 2009, Frank *et al.* 2017). After stable attachment of the conceptus due to the inter-α-trypsin inhibitor heavy chain-like (IαIH4) protein, Latency Associated Peptide (LAP) of transforming growth factor beta (TGFB), fibronectin, vitronectin, and osteopontin (OPN), the final stage of implantation begins (Geisert *et al.* 1998, Massuto *et al.* 2009a, Bowen *et al.* 1996, Johnson *et al.* 2003, Johnson *et al.* 2014). This final stage is characterized by the development of folds between the uterine LE and conceptus trophectoderm to increase adhesion between the layers and the transition into placentation (Vallet and Freking 2007, Vallet *et al.* 2009; Seo *et al.* 2020).

Placentation in pigs

As stated, the uterine-placental interface begins to fold, undergoing considerable morphological changes to increase surface area between the uterine LE and chorionic epithelium (CE) for the exchange of gases and nutrients (Vallet and Freking 2007, Vallet *et al.* 2009). The early folds occur during the transition from implantation to placentation around Day 25 of pregnancy and increase in complexity through Day 35, and again between Days 50 and 60 (Seo *et al.* 2020). The microarchitecture of these folds was first described at Days 30 and 58 of gestation (Friess *et al.* 1980). At Day 30, the chorioallantoic and endometrial surfaces interdigitate and form endometrial ridges and chorioallantoic troughs. The trophectoderm cells located towards the bottom of the troughs are 40 μm in height and are columnar in shape, while the cells at the sides and tops of the troughs are 20 μm in height. The apical surfaces of the trophectoderm cells also possess microvilli which interdigitate with the apical surfaces of the uterine LE cells and there is a layer of connective tissue separating the sub-epithelial capillaries of both the chorioallantois and endometrium from the epithelial basal laminae (Friess *et al.* 1980). By Day 58 the height of these cells changes, with cells at the bottom of the chorionic troughs reaching 35 μm in height that are described as high columnar and narrow in width. The LE cells and the trophectoderm cells at the sides and tops of the chorionic troughs both measure 15 μm in height and the LE cells at the bottom of the chorionic troughs are 25 μm. The sub-epithelial capillaries now indent into the trophectoderm cells and begin to protrude into some of the trophectoderm cells. These cells in which the capillaries are protruding have a height of 2 μm and are designed for the transport of hemotroph, or blood borne nutrients (Friess *et al.*

1980). Recently, it has been proposed that dilation of these sub-epithelial blood vessels increases blood flow to the uterine-placental interface, creating protrusive forces that trigger IAC formation between the uterine LE and placental CE (Seo *et al.* 2020). In addition to the increased blood flow, endometrial fibroblasts differentiate into myofibroblasts in order to pull the connective tissue inward, both contributing to the creation of the folds at the uterine placental interface (Seo *et al.* 2020).

Placentation also involves the formation of areolae to provide histotrophic support for the growing and developing fetus. Areolae are out pockets of the CE that form at the openings of the mouths of uterine glands and have specialized epithelial cells that are tall columnar and have vacuoles that contain uterine gland secretions, or histotroph (Dempsey *et al.* 1955). The anatomy of areolae allows for the transport of histotroph by fluid-phase pinocytosis across the placenta and into the fetal-placental circulation. It has been estimated that there are around 2,500 areolae distributed over the entire chorioallantois of each conceptus and the number of areolae is correlated with fetal weight (Knight *et al.* 1977). These tall columnar cells of the areolae upregulate genes associated with high affinity and high transport capabilities for molecules including hexose sugars and proteins such as uteroferrin, suggesting that areolae allow for increased transport of histotroph from the uterus to the placenta (Renegar *et al.* 1982, Kramer *et al.* 2020).

Overview of Interferons

Interferons (IFNs) are a group of cytokines that are essential for the immune system to respond to pathogens. There are three classes of interferons, termed type I, type II, and type III, and they all share the ability to elicit antiviral activity when bound to their respective receptors (Negishi *et al.* 2018). Type I IFNs were initially discovered by Isaacs and Lindenmann (1957), when they found that a viral infection provided resistance to subsequent viral infections (Isaacs and Lindenmann 1957). The three types of IFNs are expressed by different cell types, with type I IFNs primarily expressed by innate immune cells, the single type II IFNG typically expressed by T cells and natural killer (NK) cells, and type III IFNs not highly expressed in hematopoietic cells because they are restricted in their tissue distribution and act predominantly at epithelial surfaces (Hervas-Stubbs *et al.* 2011, Wheelock 1965, Gray *et al.* 1982, Sheppard *et al.* 2003, Iversen and Paludan 2010). They are generally classified by receptor specificity, with type III IFNs being the least characterized.

Type I interferons

The type I IFNs are the largest group containing multiple IFN-α subtypes, IFN-β, IFN-ω, IFN-τ (IFNT), and IFN-δ (IFND). These IFNs are all structurally related and bind the heterodimeric receptor IFNAR, composed of IFNAR1 and IFNAR2 chains. Type I IFNs are produced by most cells, with hematopoietic cells secreting mainly IFN-α and IFN-ω, and fibroblasts secreting the majority of the IFN-β (Bach *et al.* 1997). IFNT expression has only been reported in ruminants and is interesting because it is the

pregnancy recognition signal for ruminants. As the conceptus elongates, the mononuclear trophectoderm cells secrete IFNT which acts to prevent luteolysis and maintain P4 production. The current theory is that IFNT silences transcription of the *ESR1* gene and prevents E2-induced expression of *OXTR* in the uterine LE, superficial glandular epithelium (sGE), and GE to abrogate endometrial production of oxytocin-induced luteolytic pulses of PGF. IFNT silencing of *ESR1* expression also prevents E2 from inducing PGR in the epithelia and loss of the PGR by uterine epithelia is necessary for expression of P4-induced and nonclassical IFNT-stimulated genes that support implantation and development of the conceptus (Bazer *et al.* 2018). IFNT has also been implicated in a "servomechanism" that activates and maintains endometrial remodeling, secretory function, and uterine growth during pregnancy. This servomechanism was initially derived from studies to determine if the antiluteolytic effects of IFNT to extend the life of the CL were reinforced by placental lactogen (CSH1) and/or placental growth hormone (GH1). It was determined that the response of the GE to CSH1 and GH1 only occurs after the uterus is first exposed to IFNT (Bazer *et al.* 2018). Therefore, sequential exposure of the endometrium to E2, P4, IFNT, CSH1, and GH1 is necessary for servomechanism function and operates mainly in the uterine glands to increase production of histotroph for fetal-placental growth. IFNT also regulates conceptus elongation and implantation indirectly via paracrine effects of interferon stimulated genes (ISGs) on the endometrium, i.e., ISG15, major histocompatibility complex (MHC), and beta 2 microglobulin (B2M) (Bazer *et al.* 2018). The last type I IFN was discovered recently in

the pig uterus. IFND could not be included into any other type I IFN family, so it constituted a new, fifth type I IFN family (Lefevre *et al.* 1998).

Type II interferon gamma

As previously stated, IFNG is the only type II IFN and is structurally unrelated to type I IFNs, binds to a different receptor, and is encoded by a separate chromosomal locus (Schroder *et al.* 2004). IFNG was originally believed to be involved solely in the immune response because it upregulates the expression of chemokines and adhesion molecules, is a highly inflammatory cytokine that is a major product of T lymphocytes, and can be secreted by numerous other immune and antigen-presenting cells. This was challenged in 1991 when IFNG was discovered within the uterus of pigs (La Bonnardiere *et al.* 1991). Because IFNG is important in the immune system and has been studied extensively, it is known to be regulated by many factors. Interleukin (IL)-12 and IL-18 are the most notable stimulatory molecules regulating IFNG production, while IL-4, IL-10, and transforming growth factor-β are a few negative regulators (Fukao *et al.* 2001, Sen 2001, Schroder *et al.* 2004). Additionally, IFNG can be post-transcriptionally regulated by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH can bind IFNG mRNA directly, inhibiting translation of the protein. Loss of IFNG or IFNGR in mice has no adverse developmental effects and the immune system develops normally, but the mice show deficiencies in resistance to bacterial, parasitic, and viral infections (Buchmeier and Schreiber 1985, Suzuki *et al.* 1988, Huang *et al.* 1993, Kamijo *et al.* 1993, van den Broek *et al.* 1995, Pearl *et al.* 2001). In addition to a dysfunctional immune response, IFNGR1

knockout (KO) mice and cells treated with IFNG-neutralizing antibodies exhibit compromised tumor rejection (Dighe *et al.* 1994, Kaplan *et al.* 1998, Tannenbaum and Hamilton 2000).

IFN receptors

As stated, the type I IFN receptor, IFNAR, is composed of two chains, IFNAR1 and IFNAR2. These chains recruit the Janus-activated kinases (JAKs) TYK2 and JAK1, respectively. Binding of the IFNs to the receptor causes phosphorylation of signal transducer and activator of transcription (STAT)1 and STAT2, which forms a trimeric complex with interferon regulatory factor 9 (IRF9) to form IFN-stimulated gene (ISG) factor 3 (ISGF3) (Taniguchi and Takaoka 2001, Pestka *et al.* 2004, Decker *et al.* 2005, Ivashkiv and Donlin 2014). Translocation of ISGF3 to the nucleus initiates gene transcription by binding IFN-stimulated response elements (ISREs) in the gene promoters of ISGs. In contrast, IRF2 is an inhibitory transcription factor that regulates gene expression and the absence of IRF2 results in an excess of type I IFN signaling (Hida *et al.* 2000). Binding of type I IFNs to the IFNAR also results in activation of STAT1 homodimers that can bind IFNG activated sequence (GAS) motifs, stimulating gene transcription (Decker *et al.* 2005).

The type II IFNG receptor (IFNGR) is composed of two ligand-binding IFNGR1 chains and two signal-transducing IFNGR2 chains, with the IFNGR2 chain being the limiting factor in IFNG responsiveness (Bernabei *et al.* 2001). IFNGR1 associates with JAK1 and IFNGR2 associates with JAK2 and activation of these kinases results in STAT1

homodimerization and nuclear translocation that targets GAS DNA sequences. IFNG signaling can also activate ISGF3 weakly and, in fact, effective IFNG signaling is dependent on weak type I IFN signaling due to low constitutive production of type I IFNs (Takaoka *et al.* 2000). It was originally thought that the two receptor subunits did not strongly associate with each other in the absence of a ligand, but new techniques have allowed for the discovery that the receptor complex is assembled before ligand binding (Kaplan *et al.* 1996, Bach *et al.* 1996, Gessani and Belardelli 1998, Krause *et al.* 2002).

The IRF transcription factors

The IRF transcription factor family is primarily involved with regulating gene expression through IFN signaling and responses. In mammals, there are nine IRF proteins that all share a similar structure, with IRF1, IRF2, and IRF9 (p48, ISGF3-γ) participating in IFNG signaling (Negishi *et al.* 2018). Both IRF1 and IRF9 are stimulatory, while IRF2 is inhibitory. The function of IRF1 was elucidated using IRF1 KO mice, where many genes were sub-maximally induced compared to their wild-type (WT) littermates (Hobart *et al.* 1996, Salkowski *et al.* 1999). IRF2 has been implicated in pregnancy because it inhibits the expression of IFN-stimulated genes (ISGs) in a cell-type specific manner, i.e. uterine LE and superficial GE (Bazer *et al.* 2008).

Interferons in pig pregnancy

The porcine conceptus trophectoderm is unique in secreting type I and type II IFNs during the peri-implantation period. Peak antiviral activity occurs on Days 14 and 15 of

gestation, with type I IFND being the minor species, accounting for only 25 percent of the antiviral activity, while type II IFNG accounts for 75 percent (La Bonnardiere *et al.* 1991, Mirando *et al.* 1990, Lefevre *et al.* 1998, Joyce *et al.* 2007). IFNG and IFND mRNAs are present by Day 10 of pregnancy and there is a 567-fold increase in IFNG mRNA around Day 14 during the transition from spherical to filamentous conceptuses (La Bonnardiere *et al.* 1991). On Day 15 of gestation, IFNG localizes to what is believed to be the Golgi network, within trophectoderm cells in contact with the LE (Lefevre *et al.* 1998, Cencic and La Bonnardiere 2002). Unlike ruminant IFNT, the pig IFNs do not appear to have an antiluteolytic effect, but it has been proposed that they have paracrine and autocrine effects due to the IFNAR1 and IFNGR1 localizing to the endometrial epithelia and conceptus trophectoderm (La Bonnardiere *et al.* 1991, Niu *et al.* 1995, D'Andrea and La Bonnardiere 1998). IFNG and IFND also increase secretion of prostaglandin E2 (PGE2) and are known to increase IFN-responsive genes in the stroma and GE of the endometrium, including STAT1 and STAT2, interferon regulatory factor 1 (IRF1), multiple major histocompatibility complex (MHC) class I molecules, beta-2-microglobulin, and MHC class II molecules (Joyce *et al.* 2007, Joyce *et al.* 2008, Kim *et al.* 2012). IFNG is also known to upregulate the expression of chemokines, which recruit immune cells to sites of inflammation (Schroder *et al.* 2004). Recently, IFNG knockout (KO) conceptuses were created to determine the effects on porcine pregnancy (Johns *et al.* 2021). They showed that ablation of IFNG in pig conceptuses caused degeneration of the conceptuses and endometrial inflammation, suggesting that lack of IFNG could result in elevated type I IFND levels, which could potentially have a pathogenic effect.

IFNs are known to induce the expression of ISGs within the endometrium of the pig, but IFNs can also be regulated. Estrogen from porcine conceptuses has been shown to induce the expression of IRF2, which is an inhibitor of ISG transcription. In pigs, as in sheep, the LE and sGE express IRF2, inhibiting gene transcription in these cell types and restricting the expression of ISGs to the endometrial stroma and GE (Joyce *et al.* 2007). P4 is permissive to the actions of IFNs and, as previously stated, the PGR, along with the ESR1, are downregulated in the uterine LE and sGE during the peri-implantation period. This suggests another mechanism may be involved in regulating the function of the uterine LE and sGE for uterine receptivity and conceptus development. One theory involves progestamedins, which include fibroblast growth factor 7 (FGF7), FGF10, and hepatocyte growth factor (HGF). These growth factors exert paracrine effects on the uterine epithelia and conceptus trophectoderm, which express the receptors FGFR2IIIb and MET (Bazer *et al.* 2009). Because many ISGs are P4 induced and IFN stimulated and IRF2 is upregulated in the LE and sGE, it is important that the actions of progestamedins and IFNs act in nonclassical cell signaling pathways depending on cell type. Two important pathways are the MAPK pathway and the PI3K pathway and these are proposed to affect gene expression necessary for the establishment and maintenance of pregnancy, uterine receptivity to implantation of the conceptus, and secretion of histotroph (Bazer *et al.* 2009).

Overview of Aquaporins

Aquaporins (AQPs) are a family of transmembrane water-selective channel glycoproteins that function as pores for water transport through the plasma membrane (Agre et al. 2002). The first AQP (AQP1) was initially discovered in erythrocytes in 1987 and more recently in endothelial cells throughout the body, and to date, 13 AQPs, named AQPs 0 through 12, have been described in mammals (Agre *et al.* 1987, Mobasheri and Marples 2003). AQPs are classified into three subgroups: classic AQPs, aquaglyceroporins, and super-AQPs. Functional AQPs have a homotetrameric organization in the plasma membrane and each monomer works independently as a hydrophilic pore (Jung *et al.* 1994, Walz *et al.* 1997, Murata *et al.* 2000). This allows all AQPs the ability to rapidly transport water across biological membranes, but also allows AQPs 3, 7, and 9 (aquaglyceroporins) to transport other small molecules such as glycerol, lactate, and urea, and AQP 6 to transport nitrate (Verkman 2002, Ikeda *et al.* 2002, King *et al.* 2004, Prat *et al.* 2012). In addition to transporting water, AQP1 can transport hydrogen peroxide and AQP8 can transport ions and ammonia.

Aquaporins in pregnancy

The movement of water and nutrients across cell membranes is crucial for reproduction and is likely influenced by the expression of AQPs in tissues of the reproductive tract (Zhu *et al.* 2015). The placenta and uterus are the major organs responsible for supporting embryonic/fetal development, and thus play essential roles in assuring survival and growth of the conceptus. Key roles of the uterus and placenta are to

regulate the transport of nutrients and water across the uterine-placental interface during pregnancy recognition, conceptus implantation, and placental development/placentation (Wilson 2002). Placentation in pigs initially involves rapid expansion and development of the chorion (trophectoderm) and allantois between Days 18 and 30 of gestation in pigs due to the accumulation of water within membranes (Bazer and Johnson 2014). The driving force for expansion of the allantois, and in turn the chorioallantois, is the rapid accumulation of water from about 1 ml on Day 18, to 200-250 ml on Day 30 of gestation. Allantoic fluid volume increases from Day 20 (3.7 ml) to Day 30 (189 ml), decreases to Day 45 (75 ml), and then increases again to Day 58 (451 ml). Thereafter, it decreases to Day 112 (24 ml). AQPs play an important role in the transport of water from mother to fetus. The uterus is the site of development of the conceptus and implantation in mammals and there is evidence for the expression of AQPs in the uteri of humans, rats, mice, dogs, sheep, horses, and pigs (Ducza *et al.* 2017). For example, AQP1, 5, 7, 8, and 9 transcripts were detected in the uterine GE of the rat uterus (Lindsay and Murphy 2007), and there is also evidence for the expression of AQP1, AQP5, and AQP9 in the ovary, oviduct, and uterus of gilts on Days 17 and 19 of the estrous cycle (Skowronski *et al.* 2009).

Overview of IACs

Integrins play an important role in cell adhesion to the extracellular matrix (ECM). Numerous integrin-ECM interactions have been described, providing direct physical contact between the ECM and the intracellular actomyosin cytoskeleton (Brakebusch and Fassler 2003). These interactions are termed integrin adhesion complexes (IACs) and they

organize into linear strands called dense plaques within the smooth muscle of hollow organs (Gabella 1984, Eddinger *et al.* 2007). IACs are involved in many cellular processes including cellular adhesion, migration, and stimulation of signal transduction pathways. IACs are composed of integrins, which makeup an integrin receptor, extracellular matrix proteins, and cytoplasmic adaptor proteins. One of the most well characterized integrin receptors is the ITGA5B1, which is made of the alpha 5 and beta 1 integrin subunits. This is a classic mechanosensory receptor for the matricellular protein fibronectin (FN1). FN1 in the ECM allows binding of cells to the ECM, and other cytoplasmic adaptor proteins, such as talin and vinculin, allow communication and mechanotransduction between cells (Robinson *et al.* 2004, Vogel 2006, Larsen *et al.* 2006). In vitro studies have been performed and these studies determined that the size and the specific protein components of IACs are highly dependent on the rigidity of the ECM and the internal or external mechanical forces applied to the integrin-ECM complex (Katz *et al.* 2000, Galbraith *et al.* 2002). Further, it has been proposed that IACs can sense and transduce mechanical forces because multiple adaptor, cytoskeletal, and signaling proteins located within IACs are dependent on applied forces in order to generate downstream signals (Vogel 2006). Specific mechanosensory components of IACs have been associated with ECM and cytoskeletal proteins bound to their integrin receptors, and these proteins are mechanically altered by forces applied to them, resulting in generation of cytoplasmic signals via phosphorylation. Changes in these mechanosensory proteins are then transmitted to signal generators such as focal adhesion kinase (FAK) in order to activate specific signaling pathways (Giannone and Sheetz 2006)

Overview of the myometrium

The uterus is made up of the endometrium and the myometrium, with the majority of research focusing on the endometrium. However, the myometrium plays important role in pregnancy. The myometrium is made up of an inner circular and outer longitudinal layer of muscle, similar to most tubular organs. The major components of the myometrium are smooth muscle cells, with fibroblasts, blood and lymphatic vessels, immune cells, and connective tissue located throughout. The connective tissue is important because it provides a supportive matrix for the bundles of smooth muscles and supports the uterus as it expands during gestation (Rehman *et al.* 2003). The smooth muscle cells of the myometrium increase in number during the first few weeks of gestation, but the predominant growth of the myometrium is by stretch-induced hypertrophy (Ramsey 1994). As pregnancy progresses, smooth muscle cells form gap junctions as a method of communication and aid in myometrial activation (Garfield *et al.* 1988). Myometrial activation marks the beginning of parturition and this period is characterized by the ability of the myometrium to contract and produce force (Rehman *et al.* 2003). This occurs when the myometrium loses the inhibitory signals of P4 and gains the stimulatory signals associated with E2. Additionally, it has been shown that the myometrium changes expression from ERβ to ERα during parturition to aid in the switch from myometrial quiescence to myometrial activation and expulsion of the fetus and placenta (Rehman *et al.* 2003). In addition to using gap junctions to communicate among cells, the smooth muscle cells of the myometrium create connections with the ECM and other cells by forming IACs. As stated, these IACs are able to sense mechanical forces and activate

signaling pathways in order to respond to different stimuli during gestation (Burghardt *et al.* 2009).

IACs in pregnancy

Although IACs are rarely found in vivo due to their small size in strain-shielded extracellular matrices, there have been some recent studies showing ITGA5 integrin gene and protein expression increase within the rat myometrium during late gestation and similar observations for multiple integrins have been discovered in nonlaboring and laboring myometrium of women (Williams *et al.* 2005, Burkin *et al.* 2013). IACs and osteopontin have been localized to the uterine-placental interface of both pigs and sheep, suggesting that IACs aid in attachment of the conceptus trophectoderm to the endometrial LE (Frank *et al.* 2017, Seo *et al.* 2019, Seo *et al.* 2020). The ITGA5 integrin and multiple adaptor proteins have also been localized to the myometrium of sheep during gestation, but little is known about IACs located within the sheep myometrium during the pre- and postpartum periods (Burghardt *et al.* 2009). This is important because fetal size is similar between sheep and humans, resulting in significant growth and stretching of the uterus in both species. Parturition also requires similar coordinated smooth muscle contractions in both species. Therefore, because it is difficult to acquire human myometrial tissues over the course of gestation and rats have much smaller fetuses with less uterine stretching occurring, the sheep is potentially a better model for understanding IAC development and parturition in humans.

CHAPTER III

PIG CONCEPTUSES SECRETE INTERFERON GAMMA TO RECRUIT T CELLS TO THE ENDOMETRIUM DURING THE PERI-IMPLANTATION PERIOD[*]

Introduction

Communication and reciprocal responses between the conceptus (embryo and associated placental membranes) and uterine endometrium are essential for conceptus survival during the peri-implantation period of pregnancy. These interactions also lay the critical physiological and anatomical groundwork for subsequent development of functional uterine luminal (LE) and glandular (GE) epithelia, stroma, trophectoderm/chorion, and allantois required to maintain growth and development of the conceptus throughout pregnancy. In pigs (Bazer and Johnson 2014), the conceptus enters the uterus at 48–56 h after fertilization. Blastocysts form when cells segregate into the embryonic disk, trophectoderm, extra-embryonic endoderm, and formation of the blastocoel occurs. Pig blastocysts are 0.5–1 mm diameter spheres when they "hatch" from the zona pellucida and increase in size to Day 10 of pregnancy (2–6 mm) before undergoing a morphological transition to large spheres of 10–15 mm diameter and then tubular (15 mm by 50 mm) and filamentous (l mm by 100–200 mm) forms on Day 11. There is subsequent growth and elongation of the conceptus to 800–1000 mm in length by

[*] McLendon BA, Seo H, Kramer AC, Burghardt RC, Bazer FW, Johnson GA. Pig conceptuses secrete interferon gamma to recruit T cells to the endometrium during the peri-implantation period. Biol Reprod 2020; 103(5):1018-1029. By permission of Oxford University Press.

Day 15 (Bazer and Johnson 2014). The period of rapid elongation of pig conceptuses is accompanied by secretion of estrogens (Meyer *et al.* 2019) and several cytokines and effector molecules including, but not limited to, interleukin 1 beta (IL1B), prostaglandin E2 (PGE2), transforming growth factor beta (TGFB), and the interferons delta (IFND) and gamma (IFNG) (La Bonnardiere *et al.* 1991, Christenson *et al.* 1994, Gupta *et al.* 1996, Ross *et al.* 2003) by trophectoderm. At this time there is also attachment of the trophectoderm to the uterine LE for implantation, and remodeling of the endometrial stroma to provide optimal immune and vascular support (Burghardt *et al.* 1997).

Pig conceptus trophectoderm is unique in secreting both type I and type II IFNs during the peri-implantation period of pregnancy. Peak antiviral activity in uterine flushings or conceptus explant culture medium occurs on Days 14 and 15 of pregnancy (Mirando *et al.* 1990). The major species (75% of antiviral activity in pig conceptus secretory proteins) is type II IFNG and the other (25%) is the novel type I IFND (La Bonnardiere *et al.* 1991, Lefevre *et al.* 1998, Joyce *et al.* 2007). IFNG and IFND mRNAs are detected as early as Day 10, and abundant IFNG mRNA is detectable in porcine trophectoderm between Days 13 and 20 of pregnancy when there is a 567-fold increase in IFNG mRNA during the transition from spherical to Day 14 filamentous conceptuses (La Bonnardiere *et al.* 1991, Ross *et al.* 2003, Lefevre *et al.* 1998, Joyce *et al.* 2007). On Day 15 of pregnancy, immunoreactive IFNG localizes within the cell cytoplasm near the apex of the nucleus, in what is thought to be the Golgi network, within clusters of conceptus trophectoderm cells in contact with endometrial LE (Lefevre *et al.* 1998, Cencic and La Bonnardiere 2002). In contrast to sheep conceptuses, in which type I IFN tau (IFNT) is

the pregnancy recognition signal, IFNs produced by pig conceptuses do not appear to be antiluteolytic (Cencic *et al.* 2003). Intrauterine infusion of conceptus secretory proteins between Days 12 and 15 of the estrous cycle does not affect inter-estrous interval or temporal changes in concentrations of progesterone in plasma (Harney and Bazer 1989, Lefevre *et al.* 1998). Although pig conceptus IFNs are not known to influence pregnancy recognition, paracrine and autocrine effects for IFNs are suggested by localization of both the interferon alpha and beta receptor subunit 1 (IFNAR1, which binds IFND) and the type II IFN gamma receptor 1 (IFNGR1) on endometrial epithelia and conceptus trophectoderm (La Bonnardiere *et al.* 1991, Niu *et al.* 1995, D'Andrea and La Bonnardiere 1998). IFND and IFNG in conceptus secretory protein preparations increase secretion of prostaglandin E2 (Christenson *et al.* 1994), and increase expression of several known IFN-responsive genes in the stroma and GE of endometrium, including, but not limited to, signal transducer and activator of transcription 1 (STAT1) and STAT2, interferon regulatory factor 1 (IRF1), swine leukocyte antigens 1, 2, 3, 6, 7, 8 (SLAs 1, 2, 3, 6, 7, 8/MHC class I molecules), beta-2-microglobulin, and SLA-DQA and SLA-DQB (MHC class II molecules) (Joyce *et al.* 2007, Joyce *et al.* 2007, Joyce *et al.* 2008). Using endometrial explant cultures from Day 12 of the estrous cycle, expression of SLA-DQA and SLA-DQB mRNAs increase in response to IFNG treatment (Kim *et al.* 2012).

Our current assumption about the immunology of pregnancy is that the conceptus is a foreign semi-allogeneic (graft) that requires an immunosuppressive state within the intrauterine environment to protect the conceptus from the maternal (host) immune system (Hunt 2006). However, the inflammatory cytokine, IFNG (Levy *et al.* 1990), is the second

most highly upregulated gene in the elongating conceptuses of pigs (La Bonnardiere *et al.* 1991, Ross *et al.* 2003, Joyce *et al.* 2007), and there is precedent for IFNG having roles in placentation. The decidua of both human and murine implantation sites are populated with large numbers of uterine natural killer (uNK) cells that produce abundant amounts of IFNG that is hypothesized to induce spiral artery angiogenesis and remodeling required for proper nutritional support of the conceptus (Ashkar and Croy 2001). In pigs, Joyce et al. (2008) reported that conceptus secretory proteins containing IFNG increased expression of the major histocompatibility (MHC) class I molecules and beta-2-microglobulin (B2M) in the endometrial stroma, suggesting that IFNG induces immune responses in the endometrium during the peri-implantation period. Therefore, the host versus graft model of pregnancy that describes pregnancy as an immunosuppressed state appears to be incompatible with the secretion of pro-inflammatory IFNG by porcine conceptuses and uNK cells (Mor *et al.* 2017).

T cells orchestrate the immune response and play a critical role in infection, inflammation, and autoimmunity. IFNG, secreted by T helper 1 (Th1) cells, is conventionally thought to be a major stimulator of cell-mediated immune activation. For full activation, naive T cells require two signals: T cell receptor (TCR) signaling and co-stimulatory signaling. TCR signaling, following interaction with peptide–MHC complexes, confers specificity to T cell activation, but results in T cell unresponsiveness in the absence of co-stimulatory signaling which is delivered from co-stimulatory ligands expressed on antigen presenting cells (APCs) to receptors expressed on T cells. There are many co-stimulatory molecules, with the B7/CD28 family being the best characterized

(Chen and Flies 2013). In this family, ligands expressed on APCs include CD274 (PD-L1), CD80, CD86, and inducible T cell co-stimulator ligand (ICOSLG), and receptors expressed on T cells include programmed cell death 1 (PDCD1), CD28, cytotoxic T-lymphocyte associated protein 4 (CTLA-4), and ICOS. The co-signaling on T cells directs T cell function and determines T cell fate (Chen and Flies 2013).

Our hypothesis is that porcine conceptus IFNG is a paracrine signal directed to the endometrium that affects pregnancy by inducing expression of genes that are critical to establishment of a uterine environment that enhances conceptus development and survival. A key component of this environment is the alteration of the local immune system to support tissue remodeling and acceptance of the fetal allograft. In this study, we examined conceptus and endometrial tissues from the peri-implantation period of pregnancy for: (1) inflammatory responses, including the accumulation of immune cells and apoptosis of cells at sites of implantation; (2) expression of T cell co-signaling molecules and chemokines in the endometrium at sites of implantation; and (3) subpopulations of T cells within the endometrium at sites of implantation. Further, we infused IFNG into the uterine lumen of cyclic pigs to determine effects of IFNG on the expression of T cell co-signaling molecules and chemokines.

Materials and Methods

Ethics Statement

All experimental and surgical procedures followed the Guide for Care and Use of Agricultural Animals and approved by the Institutional Agricultural Animal Care and Use Committee of Texas A&M University.

Study 1: Assessment of normal pregnancy

Sexually mature gilts approaching their first estrous cycle (F1 crosses of Yorkshire X Landrace) were housed in small groups (10–15 per appropriately sized pen) in an open covered facility, and fed twice daily (0700 and 1800 h) 1 kg of a corn- and soybean meal-based diet. Gilts were observed daily for signs of estrus and exhibited at least two estrous cycles of normal duration (18–21 days) prior to being used in these studies. Gilts were initially immobilized with Telazol (2 mg/kg, Zoetis, Kalamazoo, MI, USA), maintained on isoflurane (3%) until sacrificed via injection of Beuthanasia-D (30 ml, Merck Animal Health, Madison NJ, USA) into the heart and were hysterectomized on either Day 10, 12, 15, 18, 20, 24, or 30 of pregnancy (n = 3–5 gilts/day). For confirmation of pregnancy prior to implantation (Days 10–15), the lumen of each uterine horn was flushed with 20 ml physiological saline and examined for the presence of morphologically normal conceptuses. Cross sections of uterine-placental interface were: (1) embedded in OCT compound, frozen in liquid nitrogen, and stored at −80 ◦C; and (2) fixed in 4% paraformaldehyde (PFA) and paraffin-embedded. Additionally, endometrium was

physically dissected from the myometrium, snap-frozen in liquid nitrogen, and stored at −80 ◦C for RNA extraction.

Study 2: Assessment via flow cytometry

Gilts were observed for estrus and bred to boars as previously described. Gilts were hysterectomized on Day 16 of pregnancy (n = 5). Endometrium was dissected from the myometrium and placed on ice. Approximately 500 mg of tissue was then digested with 4 ml Accutase (ThermoFisher Scientific, Waltham, MA, USA) for 30 min at 37 ◦C, 1 ml of fetal bovine serum (FBS) was added to stop the tissue digestion, and the tissue was placed on a 70 μm cell strainer. The tissue was then rinsed three times with 5 ml phosphate buffered saline (PBS). The tissue was homogenized with the rubber plunger end of a 3 ml syringe after each rinse. The cell suspension was then centrifuged and the supernatant discarded. The cells were then frozen in the freezing medium containing 10% FBS and 10% dimethyl sulfoxide (DMSO) until used.

Study 3: Infusion of IFNG into the uterine lumen of estrogen-induced pseudopregnant pigs

Gilts were observed for estrus as previously described. Gilts were then injected (i.m.) with 5 mg of 17 beta-estradiol benzoate (5 mg in 5 ml of corn oil; (Sigma-Aldrich, St. Louis, MO, USA) on Days 11–14 post-estrus to induce pseudopregnancy. On Day 13 post-estrus, gilts were subjected to mid-ventral laparotomy, and both uterine horns ligated proximal to the uterine body. A vinyl catheter (0007760; Durect Corporation, Cupertino,

CA, USA), connected to a preloaded and equilibrated Alzet 2ML1 osmotic pump (Durect Corporation), was then inserted about 1 cm distal to the utero-tubal junction into the lumen of the uterine horn. The pump was then secured within the ovarian bursa via suture and then affixed to the mesosalpinx supporting the oviduct by suturing the mesosalpinx to the perimetrium of the uterine horn using coated vicryl suture (Ethicon Inc., Cincinnati, OH, USA) (see Supplemental Figure S1). For each gilt (n = 4/treatment), each ligated uterine horn was assigned randomly to receive either recombinant porcine IFNG (50 μg; Cat# 985-PI-050/CF; R&D Systems, Minneapolis, MN, USA) or porcine serum albumin (PSA; Sigma-Aldrich). The pumps continuously delivered 10 μl/h (240 μl/day containing 1.5 × 105 IU of antiviral activity/day) of fluid into the uterine horns. The rationale for how much IFNG to infuse was based on published antiviral activities in uterine luminal flushings and previous infusion of IFNG and IFND in which ~1.5 × 105 IU/Day of IFNG and ~2.0 × 105 IU/Day IFND were infused into pigs (La Bonnardiere *et al.* 1991, Lefevre *et al.* 1998). Gilts were hysterectomized on Day 18 post-estrus, at which time CL appeared to be normal, for evaluation of effects on endometrial gene expression.

RNA extraction, cDNA synthesis, and quantitative PCR

Total RNA was extracted from endometrial and chorioallantoic tissue samples using Trizol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendations. First strand cDNA was synthesized from 5 μg total RNA using a Superscript III First Strand Kit (Life Technologies) according to the manufacturer's instructions. MIQE guidelines were followed when performing the qPCR.

The qPCR assays were performed using PerfeCta SYBR Green Mastermix (Quanta Biosciences, Gaithersburg, MD, USA) in 10 µl reactions with 2.5 mM of each specific primer, on a Roche 480 Lightcycler (Roche, Basel, Switzerland) with ~60 ng of cDNA per reaction. The PCR program began with 5 min at 95 ◦C followed by 40 cycles of 95 ◦C denaturation for 10 s and 60 ◦C annealing for 30 s, and 72 ◦C extension for 30 s. A melt curve was produced with every run to verify a single gene-specific peak. Negative controls with reverse transcriptase omitted during cDNA synthesis were run for each sample to confirm the absence of genomic contamination. Additionally, no template controls (water) were run for each primer set. Primers were designed to overlap exon-exon boundaries when possible. Primer information and slopes and efficiencies of standard curves are summarized in Supplemental Table S1. Ribosomal protein L7 (RPL7) was used to normalize data from endometrial tissue (Seo *et al.* 2012). The $2^{-\Delta\Delta Ct}$ method was utilized to normalize data and the fold-changes were subjected to statistical analyses.

Immunofluorescence analyses

All immunofluorescence analyses were performed on tissues from all animals as previously described (Seo *et al.* 2019). Briefly, immunoreactive IFNG and CD3 proteins were localized in paraffin-embedded sections from pregnant pigs using immunofluorescence microscopy. Sections (5 µm thick) were deparaffinized and rehydrated in an alcohol gradient. Antigen retrieval was performed using boiling citrate. Sections were then blocked in 10% normal goat serum for 1 h at room temperature. These sections were incubated overnight at 4 ◦C with the following primary antibodies: rabbit

anti-IFNG polyclonal antibody (US Biological, Salem, MA; USA, Cat# I7662-16N8; 1:200) and rabbit anti-CD3 polyclonal antibody (Agilent; Santa Clara, CA; USA, Cat# A045229-2; 1:100). Normal rabbit IgG (EMD Millipore; Billerica, MA, USA, 12-370), at a concentration equal to that for the primary IgG, was used as the negative control. Immunoreactive proteins were detected using the goat anti-rabbit Alexa Fluor 488-conjugated secondary antibody (Life Technologies, Grand Island, NY, USA) for 1 h at room temperature at a dilution of 1:250. Tissue sections were then washed three times for 5 min/wash in PBS. Slides were counterstained with Prolong Gold Antifade reagent containing DAPI (Life Technologies) and coverslipped. Images were taken using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY, USA) interfaced with an Axioplan HR digital camera.

Frozen tissue sections of uterine-placental interface (8 μm) were fixed in methanol at −20 ◦C and washed in PBS. These sections were then blocked in 10% normal goat serum diluted in antibody dilution buffer for 1 h at room temperature. Rabbit anti-CD45 polyclonal antibody (Abcam; Cambridge, MA, USA, ab10559; 1:400) was added and tissue sections were incubated overnight at 4 ◦C in a humidified chamber. Tissue sections were then washed three times for 5 min/wash in PBS. Goat anti-rabbit IgG Alexa 488 (Life Technologies; 1:250) was added to tissue sections and incubated for 1 h at room temperature. Tissue sections were then washed three times for 5 min/wash in PBS, and slides were processed for counterstaining and imaging as described previously.

For dual immunofluorescence staining for CD3 and proliferating cell nuclear antigen (PCNA; Abcam; ab29; 1:200), we followed the same procedures as described for

normal immunofluorescence staining except that we added the two primary antibodies simultaneously on the first day and added the two secondary antibodies (goat anti-rabbit-Alexa Fluor 488-conjugated and goat anti-mouse-Alexa Fluor 594-conjugated) simultaneously on the second day.

TUNEL staining to assess apoptosis of cells

Apoptosis was assessed using a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay (Promega, Madison, WI, USA) according to the manufacturer's instructions for frozen tissue. Briefly, frozen tissue sections of uterine–placental interface (8 μm) were fixed in 4% methanol free PFA in PBS for 25 min at 4 ◦C. The tissue sections were then washed with PBS, and the slides were covered with equilibration buffer for 10 min at room temperature followed by incubation with TdT incubation buffer (containing TdT and nucleotide mix) for 1 h at 37 ◦C in a humidified chamber. The reaction was terminated by submersion of slides in 2× SSC for 15 min at room temperature. The sections were then washed with PBS, counterstained with Prolong Gold Antifade reagent containing DAPI (Life Technologies), and coverslipped. Images were taken using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) interfaced with an Axioplan HR digital camera.

Endometrial cell preparation and flow cytometry

The cryopreserved endometrial cells were thawed and resuspended in cell-staining buffer (PBS with 5% FBS). The average viability of the gated cell population after thawing

was 84.8%. For multicolor staining, the cells were incubated with an unconjugated mouse anti-CD25 IgG1 (Bio-Rad, Hercules, CA; clone K231.3B2; 1:200) for 20 min on ice. Cells were then washed with the cell staining buffer and incubated with: (1) allophycocyanin (APC)-conjugated rat anti-mouse IgG1 (Thermo Fisher Scientific; clone M1-14D12; 1:200) for indirect CD25 staining; (2) mouse anti-CD4 IgG2b-flourescein isothiocyanate (FITC) (Bio-Rad, clone MIL17; 1:500); and (3) mouse anti-CD8 IgG2a-phycoerythrin (PE) (Bio-Rad, Hercules, CA, USA, clone MIL12; 1:500) for 20 min on ice. After washing with the cell-staining buffer, the live-dead marker 7-amino-actinomycin D (7-AAD) was added to exclude non-viable cells from flow cytometric analyses. Cells were sorted using a Beckman Coulter Moflo Astrios Cell Sorter and analyzed using FlowJo software. OneComp eBeads (Invitrogen, Waltham, MA, USA; 0.125 µl/1 drop beads) were used to compensate for spectral overlap, fluorescence minus one (FMO) controls were used, as well as other gating controls (see Supplemental Figure S2).

Statistical analyses

All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA). Data from quantitative PCR for effect of days of pregnancy and flow cytometry for T cell subpopulations were subjected to one-way ANOVA followed by a post-hoc Tuckey analysis. Data from quantitative PCR analyses for effect of IFNG infusion were analyzed using the Students t-test. All data are presented as mean ± SEM with significance set at $P < 0.05$.

Results

IFNG is expressed by conceptus trophectoderm during the peri-implantation period of pregnancy

Immunofluorescence staining for IFNG at the endometrial–placental interface was performed on Days 15, 18, and 20 of pregnancy. Strong immunostaining for IFNG protein was detected in the conceptus trophectoderm on Day 15 of pregnancy, and then decreased through Day 20 of gestation (Figure 1). IFNG appeared to be present in the cytoplasm of the trophectoderm cells.

Accumulation of immune cells within the endometrial stroma and apoptosis of endometrial stromal cells coincide with the period of IFNG secretion

Immunofluorescence staining for CD45, a common marker for leukocytes, was performed to examine changes in the distribution of immune cells within the endometrium during peri-implantation period of pregnancy. CD45-positive cells were present within the endometrial stroma on Days 15 and 18, and then decreased by Day 20 of gestation (Figure 2A). TUNEL staining to determine whether endometrial cells were undergoing apoptosis during the peri-implantation period of pregnancy revealed the absence of TUNEL-positive endometrial LE or GE cells. However, TUNEL-positive cells were present within the endometrial stroma on Days 15 and 18, and numbers decreased by Day 20 of gestation (Figure 2B).

Accumulation of proliferating T cells within the endometrial stroma coincides with IFNG secretion

Having determined that immune cells accumulate within the endometrium on Days 15 and 18 of gestation, we next performed immunofluorescence staining for CD3, to identify T cells, and PCNA, to identify proliferating cells, to determine whether activated T cells accumulate within the endometrial stroma during the peri-implantation period of pregnancy. CD3-positive cells were found to accumulate within the endometrial stroma on Days 15 and 18 of pregnancy (Figure 3A), and some of the CD3-positive cells were also positive for PCNA immunostaining, indicating the presence of proliferating and, therefore, activated T cells within the endometrial stroma during the peri-implantation period of pregnancy in pigs. (Figure 3B).

Expression of T cell co-signaling molecules and chemokines within the endometrium coincides with IFNG secretion

Full activation of T cells requires a co-stimulatory signal from co-stimulatory ligands expressed on APCs to receptors on T cells. Therefore, we examined expression of T cell co-signaling molecules in the endometrium during the peri-implantation period of pigs using real-time PCR analyses. Expression of all receptors we examined increased on Day 15 and/or Day 18 of pregnancy (Figure 4A) including PDCD1 (Day 10 vs Day 15, $P < 0.001$; Day 10 vs Day 18, $P < 0.001$), CD28 (Day 10 vs Day 18, $P < 0.05$), CTLA-4 (Day 10 vs Day 18, $P < 0.05$), and ICOS (Day 10 vs Day 18, $P < 0.01$). Among the ligands examined, expression of CD80 mRNA increased on Days 18 and 24 of pregnancy (Day

10 vs Day 18, $P < 0.05$; Day 10 vs Day 24, $P < 0.05$), whereas expression of mRNAs for CD274 (PD-L1), CD86, and ICOSLG were not affected by day of pregnancy (Figure 4B).

Since IFNG is known to increase expression of C-X-C motif chemokine ligand 9 (CXCL9), CXCL10, CXCL11 to recruit T cells to inflamed tissues (Campbell and Koch 2011, Han *et al.* 2017), we next examined expression of these chemokines in the endometrium during the peri-implantation period of pregnancy. Expression of CXCL9 (Day 10 vs Day 18, $P < 0.01$), CXCL10 (Day 10 vs Day 18, $P < 0.001$), and CXCL11 (Day 10 vs Day 18, $P < 0.01$) increased in the endometrium on Day 18 of pregnancy (Figure 4C).

The majority of T cells within the endometrium are CD4+

We utilized flow cytometry to determine the percentage of CD4+, CD8+, and CD4+ CD25+ cells located within the endometrium on Day 16 of pregnancy. The percentage of CD4+ (T helper) cells ranged from 11.59 to 33.19%, which made them the largest T cell subpopulation within the endometrium ($P < 0.001$), while the percentage of CD4+CD25+ (regulatory T) cells was lower, ranging from 0.416 to 1.676% (Figure 5). The percentage of CD8+ (cytotoxic T) cells was also low, ranging from 0.58 to 3.49% (Figure 5). Interestingly, there were also CD4+CD8+ T cells in the endometria of some pigs (Figure 5).

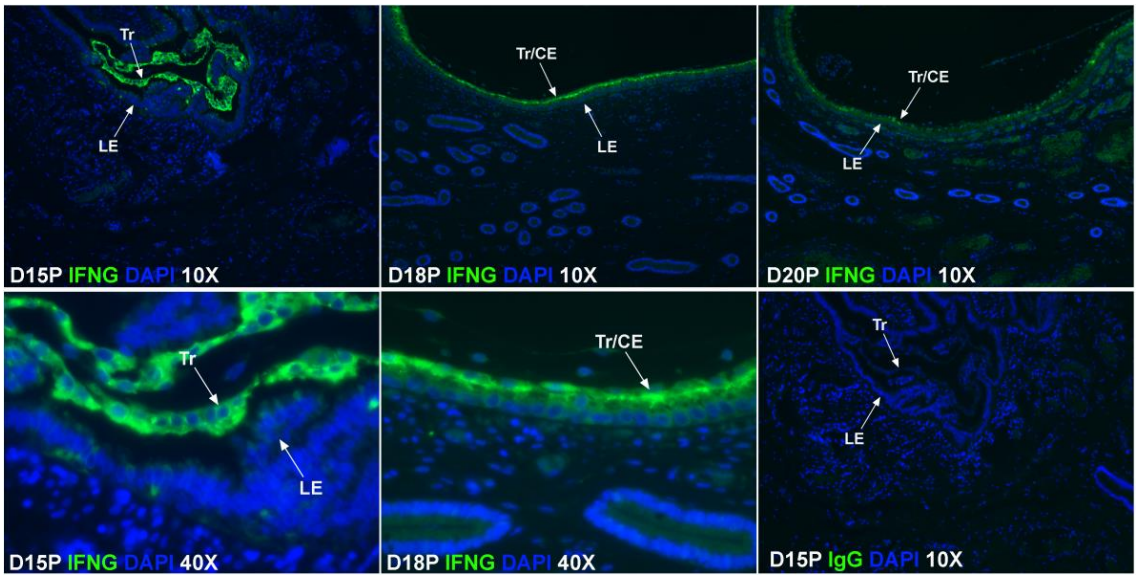


Figure 1. Immunofluorescence staining for IFNG protein (green color) on paraffin embedded cross-sections of the endometrial-placental interface on Days 15, 18, and 20 of pregnancy (P).

IFNG is localized to the conceptus trophectoderm (Tr). A non-relevant IgG replacing the primary antibody served as the immunostaining control. Nuclei are stained with DAPI for histological reference. Width of fields is 890 μm. LE, endometrial luminal epithelium; CE, chorionic epithelium.

IFNG increases expression of T cell co-signaling molecules and chemokines in the endometrium of pseudopregnant pigs

We infused recombinant IFNG protein into the uterine horn of pseudopregnant pigs to determine effects on endometrial expression of T cell co-signaling receptors, T cell co-signaling ligands, and chemokines. Real-time PCR analyses demonstrated that IFNG infusion did not affect expression of the T cell co-signaling ligands, but IFNG did increase expression of the T cell co-signaling receptors PDCD1 ($P < 0.01$) and ICOS ($P < 0.05$), as well as the chemokine CXCL9 ($P < 0.05$) (Figure 6).

Discussion

IFNs were originally discovered as agents that interfere with viral replication (Isaacs and Lindenmann 1957). They are classified into type I and type II IFNs according to receptor specificity and sequence homology. IFNG is the only type II interferon and it was initially believed to be exclusively produced by immune cells. However, Lefèvre et al. first reported that pig conceptuses secrete large amounts of IFNG, with peak synthesis occurring on Days 15–16 of pregnancy (Lefevre *et al.* 1990). In agreement with this, our results confirm strong immunostaining for IFNG protein in the conceptus trophectoderm. On Day 15 of pregnancy, IFNG protein was present throughout the entire cytoplasm; however, by Day 18 of pregnancy, IFNG protein was predominantly localized to the apical half of the conceptus trophectoderm cells. Lefevre et al. reported that IFNG localizes in the cytoplasm near the apex of the nucleus in cells, in what they hypothesized to be the Golgi network (Lefevre *et al* 1998). The present results do not conflict with this prior report of IFNG localization in porcine conceptus trophectoderm, Interestingly, in the present study, IFNG was not detected in the endometrium; however, Tayade et al. isolated endometrial lymphocytes from Day 20 of gestation and reported that they are an abundant source of IFNG mRNA at sites of implantation in pigs (Tayade *et al.* 2007). Further Joyce et al., using in situ hybridization analyses, detected cells scattered within the endometrium that expressed IFNG mRNA (Joyce *et al.* 2007). It is possible that alternative splicing of IFNG mRNA precursor might occur to produce different subtypes of IFNG proteins in the trophectoderm and endometrial lymphocytes.

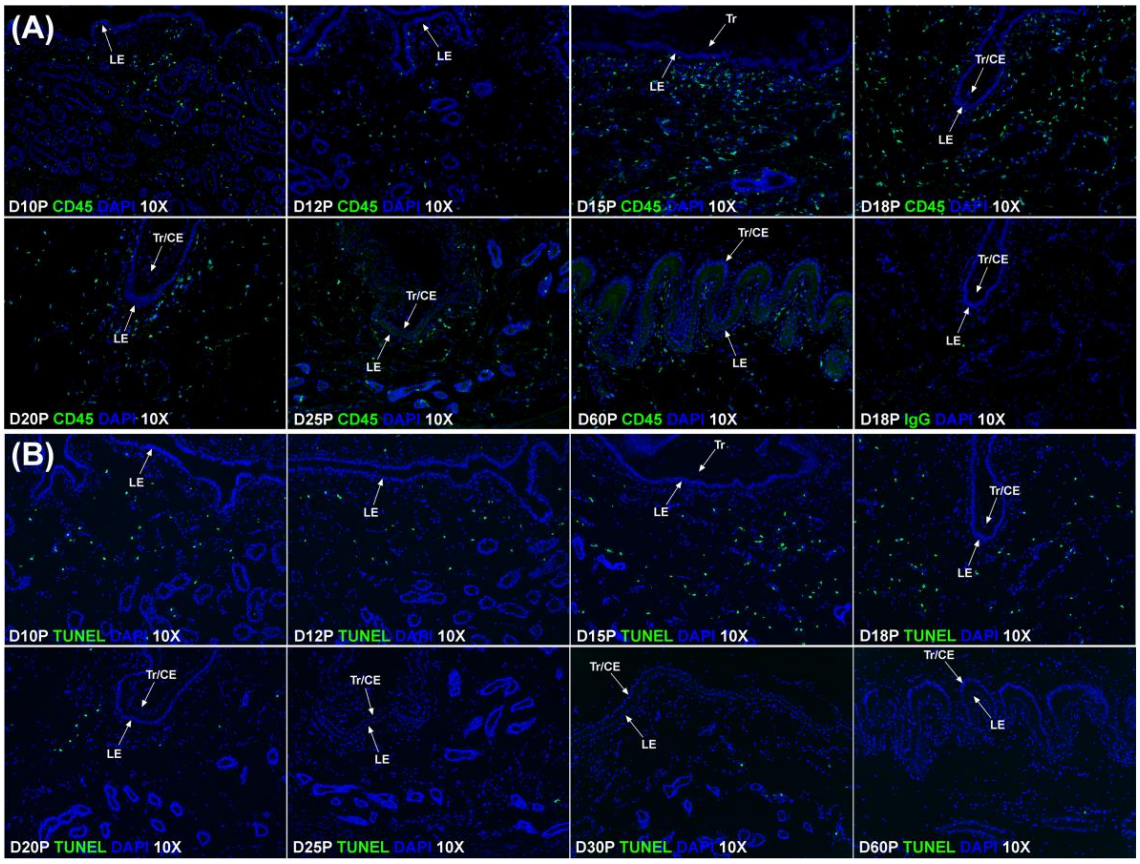


Figure 2. Immunofluorescence staining for CD45 and TUNEL throughout gestation.

Immune cells and cells undergoing apoptosis increase in the uterine stroma on Days 15 and 18 of pregnancy. A non-relevant IgG replacing the primary antibody served as the immunostaining control. Nuclei are stained with DAPI for histological reference. Width of fields is 890 μm. LE, endometrial luminal epithelium; Tr, trophectoderm; CE, chorionic epithelium.

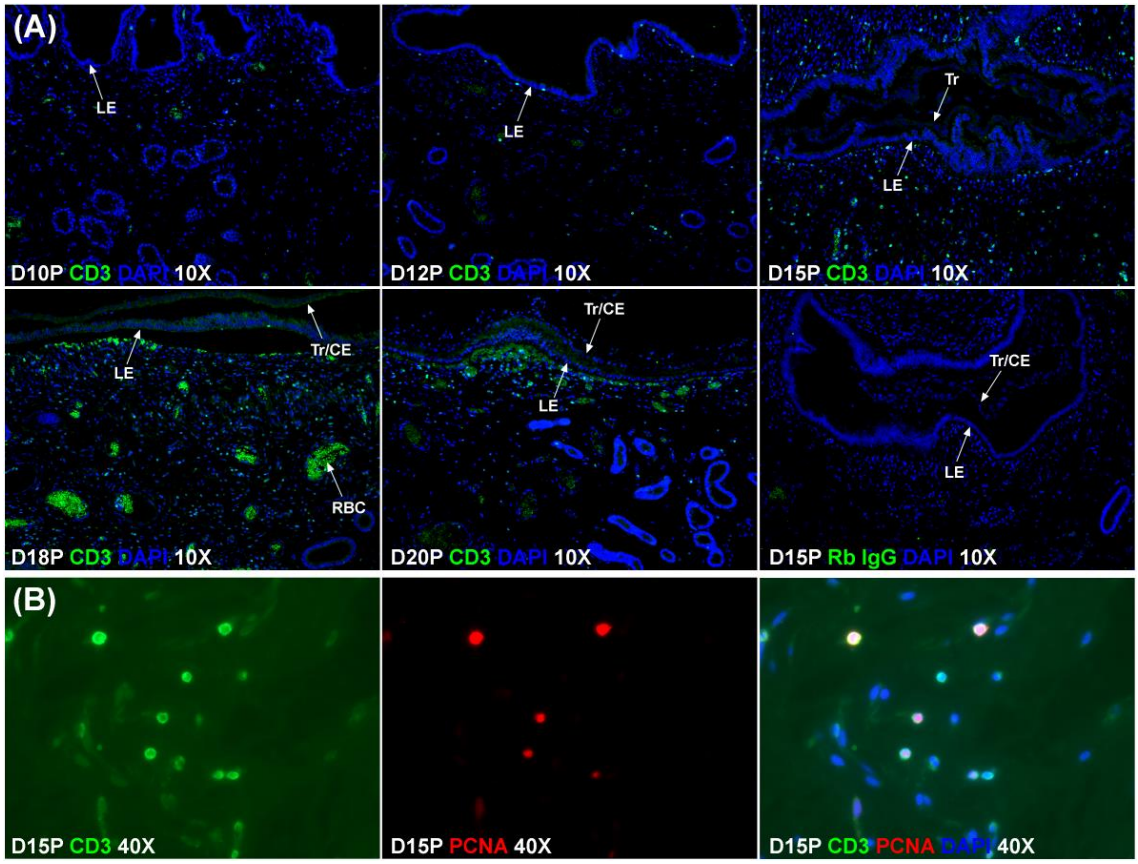


Figure 3. Immunofluorescence staining for CD3 and co-localization with PCNA throughout the peri-implantation period.

Proliferating T cells accumulate within the endometrial stroma on Days 15 and 18 of pregnancy. A non-relevant IgG replacing the primary antibody served as the immunostaining control. Nuclei are stained with DAPI for histological reference. Width of fields for the 10× panels is 890 µm, and for the 40× panels is 220 µm. Note that the red blood cells (RBC) within the tissue sections auto-fluoresce and that is not immunostaining. LE, endometrial luminal epithelium; Tr, trophectoderm; CE, chorionic epithelium.

Pig placentation is epitheliochorial, as uterine endometrial LE remains intact throughout pregnancy. Conceptus trophectoderm/chorion cells attach directly to the endometrial LE and, in view of immunology, these two epithelia likely serve as barriers to migration of maternal immune cells. In contrast, the trophoblast cells of mouse and

human placentae have direct contact with immune cells in maternal blood (Moffett and Loke 2006). The added layers of maternal tissue in pigs provide a physical anatomical advantage that minimizes exposure of the semi-allogeneic antigens expressed by the conceptus to maternal immune cells. Indeed, neither endometrial LE nor the trophectoderm/chorion of pigs express MHC class I (SLA) molecules and beta-2-microglobulin (B2M) membrane glycoproteins involved in the discrimination of self from non-self, that present peptide antigens to T cell receptors, and bind to inhibitory and activating receptors on natural killer cells and other leukocytes. It is accepted that the cell-type specific down-regulation of SLA and B2M in placental and endometrial tissues helps to provide an immunologically favorable environment for survival of the fetal–placental semi-allograft (Joyce *et al.* 2008). However, results from the present study suggest that immune responses at the endometrial–placental interface are dynamic and, at least in part, are controlled by placental secretions in the form of IFNG.

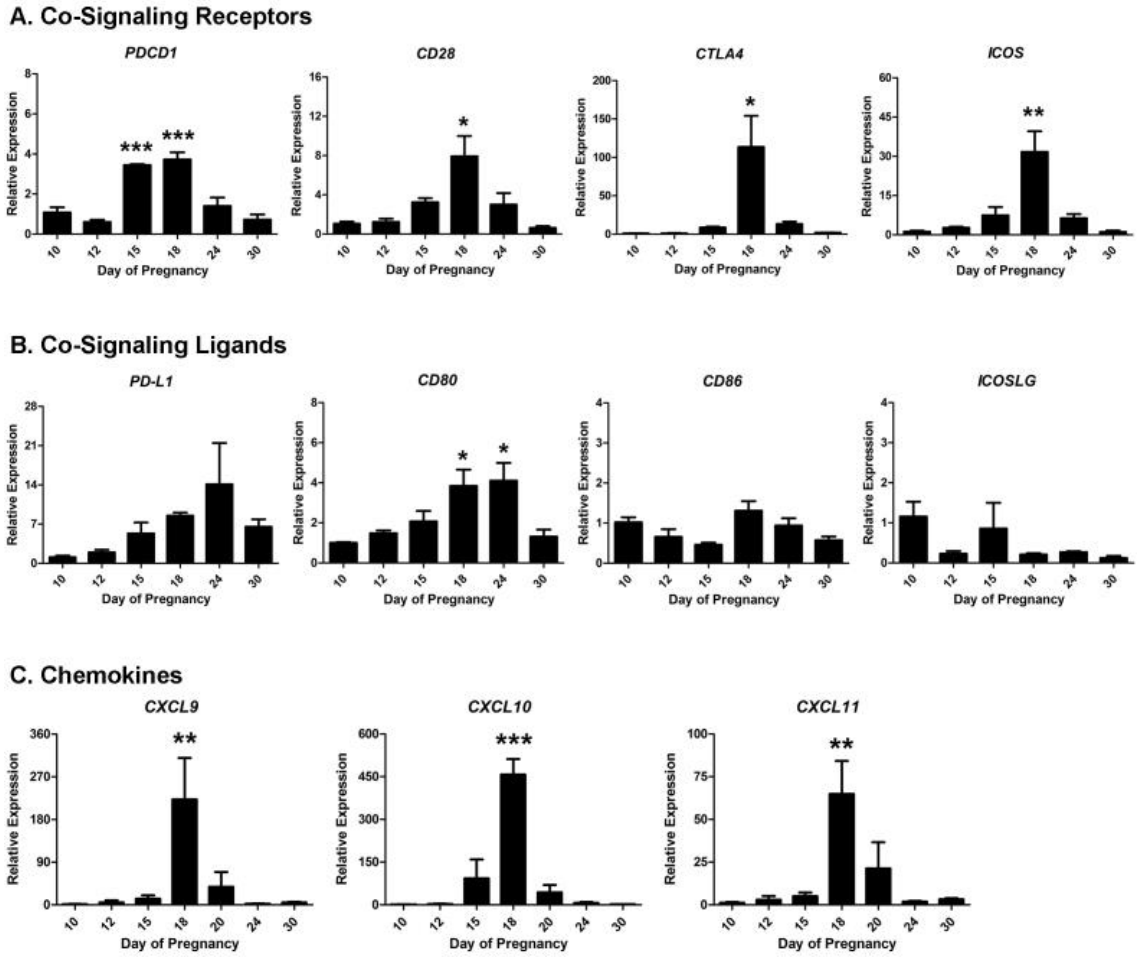


Figure 4. Quantitative real-time PCR showing mRNA expression of T cell co-signaling receptors, co-signaling ligands, and chemokines during pregnancy.
Abundances of T cell co-signaling receptors were significantly increased in the endometrium during the peri-implantation period of pregnancy. Data are presented as mean ± SEM. ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$.

Implantation and early placentation in mice and humans are highly invasive, resulting in destruction of endometrial epithelial, stromal, and vascular tissues, similar to that occurring in an open wound. Wound healing requires a strong inflammatory response, and it is proposed that implantation and placentation in mice and humans are pro-inflammatory events (Mor *et al.* 2017). Although implantation and early placentation in

pigs lack the same degree of tissue damage as is observed for mice and humans, endometrial tissue must still be remodeled to facilitate the growing vasculature and actively secreting GE that supply the hemotrophic and histotrophic support, respectively, for growth and development of the conceptuses (Seo *et al.* 2020). Therefore, pigs may conform to the idea, held for mice and humans, that implantation and early placentation are inflammatory events. Certainly, the trophectoderm cells of elongating and implanting pig conceptuses secrete large amounts of pro-inflammatory cytokines and effector molecules including IL1B, IFNG, and prostaglandins into the endometrium (Bazer and Johnson 2014). Results of the present study reveal that the production of IFNG by implanting conceptuses coincides with an accumulation of immune cells within the endometrium and increased apoptosis of endometrial stromal cells at sites of implantation, both hallmarks of inflammation. Therefore, implantation and early placentation in pigs likely do not progress in an immunologically suppressed environment, rather, the endometrium undergoes a controlled inflammatory response to favor stromal remodeling, tissue injury and subsequent repair, and extensive vascularization required for a successful pregnancy. Once implantation and early placentation are complete, it appears that the endometrium may transform into an anti-inflammatory state, because IFNG is no longer secreted by the conceptus trophectoderm and there are decreases in endometrial immune cells and cells undergoing apoptosis in the endometrium at sites of conceptus implantation by Day 25 of pregnancy.

If the porcine endometrium is undergoing a controlled inflammatory response during implantation of the conceptus, it raises the question of what factor(s) triggers this

inflammatory response under physical and anatomical conditions that limit the exposure of semi-allogeneic antigens to maternal immune cells, i.e., epitheliochorial placentation, and the endometrial LE barrier to direct exposure of the conceptus to the endometrial stroma. Damage-associated molecular patterns (DAMPs) are endogenous molecules released by dying cells in damaged tissues and detected by receptors on various cells of the innate immune system (Gong *et al.* 2020). The recognition of DAMPs promotes sterile inflammation, which is important for tissue repair and regeneration (Gong *et al.* 2020). Trophoblast cells express pattern recognition receptors such as toll-like receptors (TLRs) and NOD-like receptors (NLRs) that recognize DAMPs (Hunt 2006, Janeway and Medzhitov 2002, Costello *et al.* 2007). It is proposed that these trophoblast cells sense and respond to signals from the microenvironment to support decidual differentiation, trophoblast migration and invasion, angiogenesis and spiral artery remodeling, and placental and fetal development (Mor *et al.* 2017). We detected cells undergoing apoptosis in the endometrial stroma at sites of implantation in pigs, suggesting minor damage to endometrial tissues during the process of early placentation. Because a variety of TLRs are expressed at the maternal–fetal interface of pigs (Linton *et al.* 2010, Yoo *et al.* 2019), it is possible that dying cells in the endometrial stroma trigger a controlled inflammatory response during the peri-implantation period of pregnancy.

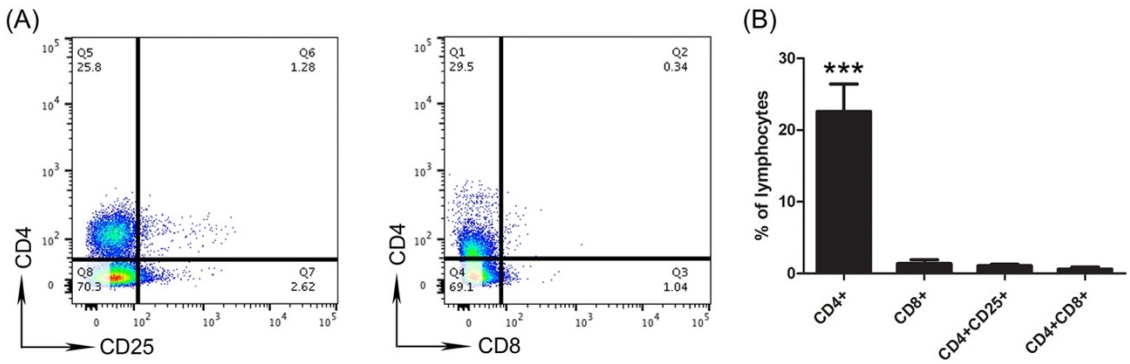


Figure 5. Representative images for flow cytometry and data showing percentages of T cell subtypes in the pig endometrium on Day 16 of gestation.

The majority of the T cells were CD4+, with smaller populations of CD8+, CD4+CD8+, and CD4+CD25+ cells.

T cells play critical roles in inducing tissue inflammation and maintaining tissue homeostasis. For full activation, T cells require binding of co-signaling receptors to ligands expressed on APCs. Our results showed that CD3+ T cells accumulate within the endometrium at sites of implantation, and that some of these CD3+ T cells express PCNA, indicating an accumulation of activated T cells. Because co-signaling directs the function and fate of T cells, it is important to identify the repertoire of co-signaling receptors expressed on T cells within the endometrium. In general, CD28 and ICOS are co-stimulatory for the activation of naive T cells, while CTLA-4 and PDCD1 are inhibitory. CD28 can bind the CD80 and CD86 ligands on APCs, but prefers to bind CD86, because it exists as a monomer on the cell surface (Nagai and Azuma 2019). CTLA-4 can also bind CD80 and CD86, but prefers to bind CD80 because it exists predominantly as a dimer. Because these co-receptors share the same ligands, they intrinsically compete with each other, and this is illustrated by the fact that CD28 stimulates T cell activation and

proliferation, while CTLA-4 delivers inhibitory signals that induce cell cycle arrest and inhibit cytokine production (Nagai and Azuma 2019). ICOS stimulates T cells more effectively than CD28 and, as the name suggests, ICOS is not expressed constitutively on resting T cells, but is induced after TCR stimulation and/or CD28 stimulation (Parry *et al.* 2003, McAdam *et al.* 2000). ICOS has one ligand, ICOSLG, and the predominant phenotype in ICOS- and ICOSLG-deficient mice is a reduction in the number and size of germinal centers within immune organs (Dong *et al.* 2001). The last co-receptor we examined was PDCD1, which has two ligands, PD-L1 and PD-L2. PDCD1 is another inhibitory co-signaling molecule and upon binding with its ligands can downregulate TCR signaling, along with other methods of inhibition (Nagai and Azuma 2019). Our results show that T cell co-signaling receptors CD28, ICOS, CTLA-4, and PDCD1 are significantly upregulated within the endometrium during the peri-implantation period of pregnancy. Upregulation of both stimulatory and inhibitory co-receptors suggests that activation and suppression of effector T cells occur dynamically within the endometrium during the peri-implantation period of pregnancy, and that co-existence and balance of T cells with different co-signaling receptors may provide a tightly controlled inflammatory environment.

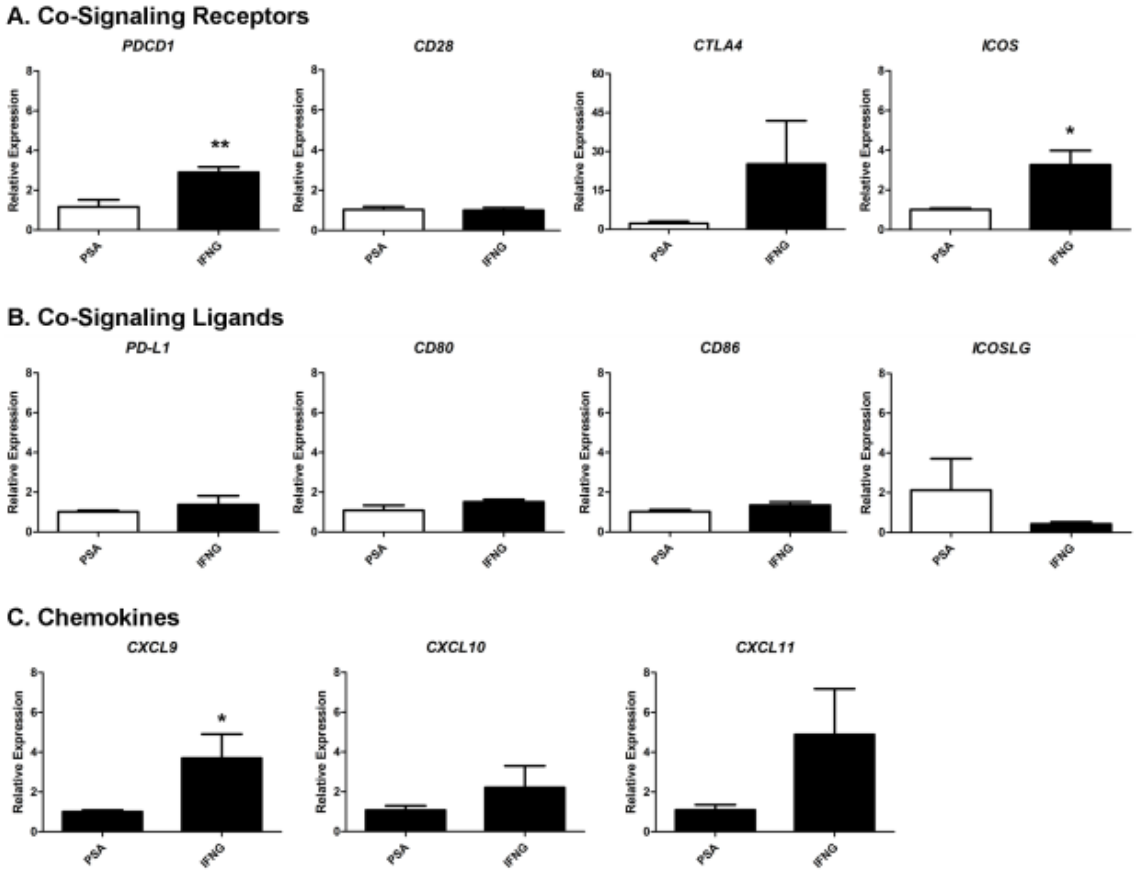


Figure 6. Real-time quantitative RT-PCR showing mRNA expression of T cell co-signaling receptors, T cell co-signaling ligands, and chemokines in the endometrium of pseudopregnant pigs in which either recombinant IFNG protein or PSA was infused into the uterine lumen.

Data are presented as mean ± SEM. ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$.

T cells are produced mainly in the thymus and differentiate into T helper (CD4+), cytotoxic T (CD8+), or regulatory T (CD4+ CD8− CD25+) cells. Naïve (CD4+ CD8+) T cells circulate throughout the blood and lymph to facilitate contact with their specific antigens (Hall 2015). Once an APC presents an antigen to a naïve T cell and co-signaling molecules are bound, activation occurs. These activated (effector) T cells then circulate throughout the body to search for cells expressing this specific antigen (Hall 2015). Most

T cells are programmed to be effector T cells and express either CD4 or CD8, but there is a small population of regulatory T cells (Tregs). These Tregs act as "regulators" and suppress effector T cell function (Hall 2015). The proportions of the different T cell subpopulations that inhabit the endometrium at sites of implantation in pigs is important for determining if, and what kind, of immune response the endometrium is undergoing. Similar to our results with the co-signaling molecules, we found that all T cell subpopulations were present within the endometrium. The major population of T cells were CD4+, suggesting that the maternal immune system is responding to some stimulus within the endometrium. It is not surprising that T helper cells were the major subpopulation within the endometrium because they are important for inducing other inflammatory cells such as macrophages and cytotoxic T cells to increase the effectiveness of the immune response. However, it is necessary that T helper cells are not the only activated T cells, otherwise excessive inflammation would occur without the presence of regulatory T cells to suppress the effector T cell functions. Tregs account for a small proportion of T cells within the inflamed endometrium; however, considering the increase in the total number of overall T cells in the endometrium during conceptus implantation, the influence of these Tregs is likely considerable. CTLA-4 is one of the most widely used markers for Tregs, and CTLA-4 on Tregs induces tryptophan catabolism to suppress effector T cells (Miyara and Sakaguchi 2007). Therefore, the accumulation of Tregs and upregulation of CTLA-4 suggest a possible role for Treg-mediated immunosuppression in the inflamed endometrium of pigs. Considering the high percentage of CD4+ cells and low percentage of CD4+CD25+ cells, it is likely that the T helper cells support

endometrial inflammation, and that this inflammatory response is limited by CD4+CD25+ regulatory T cells to prevent hyperinflammation that would be detrimental to the establishment of pregnancy.

Another subpopulation of T cells identified within the endometrial tissue at sites of implantation in pigs were the CD4+CD8+ T cells. These cells were originally believed to be naïve T cells that escaped from the thymus with increasing age and deterioration of the thymus because only a small number of these cells were detected (Blue *et al.* 1985, Lee *et al.* 2003). Recently, CD4+CD8+ T cells have been detected in large percentages in the peripheral blood of pigs compared with humans and mice and were reported to have characteristics of memory T cells and to differentiate from CD4+ cells (Zuckermann 1999, Okutani *et al.* 2018). Overgaard et al. (2015) reported that these cells may also play different roles depending on the species and tissue in which they are located. They suggested that CD4+CD8+ T cells can act as immune suppressors or as cells with high cytotoxic potential because they are found in rats with inflammatory bowel disease or certain cancers, respectively. Further research regarding the CD4+CD8+ T cells is required to determine factors controlling their differentiation and function within the porcine endometrium.

The function of IFNG secreted by the conceptus trophectoderm is not fully understood, but immune-related functions are likely. IFNG is known to increase expression of chemokines to recruit T cells to inflamed tissues (Campbell and Koch 2011, Han *et al.* 2017), and results from in vitro studies indicate that treatment of porcine endometrial explant tissue with IFNG increases expression of CXCL9, CXCL10, and

CXCL11, and that treatment of peripheral blood mononuclear cells (PBMCs) with those chemokines increases migration of T cells and NK cells (Han *et al.* 2017). In the present study, we determined secretion of pro-inflammatory IFNG by pig conceptuses coincides with the accumulation of T cells and upregulation of chemokines CXCL9, CXCL10, and CXCL11. Thus, we hypothesized that conceptus IFNG acts on the endometrium to increase chemokines to recruit T cells. In support of this, IFNG receptors IFNGR1 and IFNGR2 have been previously localized to cells scattered within the endometrial stroma of pregnant pigs (Yoo *et al.* 2019). Interestingly, T cells and the co-signaling receptor PDC1 increased in the endometrium on Days 15 and 19 of gestation; however, significant increases in the chemokines were not observed until Day 18 of gestation. It is noteworthy that each of the chemokines shows a tendency to increase on Day 15, and this increase might be enough to begin the recruitment of T cells to the endometrium. Further, although all gilts were handled in the same way during breeding, there is minor variation in when the gilt actually ovulates during estrous, and this can complicate interpretation of daily changes in gene expression. Indeed, our results showed that uterine infusion of IFNG protein into pseudopregnant pigs significantly increased the expression of chemokine CXCL9 in the endometrium. Uterine infusion of IFNG protein also increased expression of the T cell co-signaling receptors PDCD1 and ICOS. IFNG might directly affect the expression of PDCD1 and ICOS genes in T cells within the endometrium or might increase the number of T cells that express those receptors by increasing chemokines that then recruit T cells, or both. Taken together, these results suggest that conceptus IFNG

promotes inflammatory responses in the endometrium by increasing expression of endometrial chemokines to recruit T cells at sites of implantation in pigs.

CHAPTER IV

INTEGRIN ADHESION COMPLEX ORGANIZATION IN SHEEP MYOMETRIUM REFLECTS CHANGING MECHANICAL FORCES DURING PREGNANCY AND POSTPARTUM

Introduction

Throughout pregnancy the myometrium undergoes hypertrophy in response to the developing fetus and associated placental membranes (Shynlova *et al.* 2007). This includes cell proliferation, increased ECM production and remodeling of integrin-focal adhesion (FA) contacts (Williams *et al.* 2005). FAs, a type of integrin adhesion complex (IAC), are consistently seen in cultured cells (Burridge and Chrzanowska-Wodnicka, 1996, Humphries *et al.* 2006), although are less obvious in vivo.

The onset of parturition is initiated by the fetus and relies on both endocrine and mechanical signaling pathways caused by uterine stretch in the myometrium. In sheep, the endocrine signaling pathway involves a change in the ratio of estrogen to progesterone secretion that primes the uterus for myometrial activation critical for delivery of the fetus (Challis *et al.* 2000). The simultaneous increase of estrogen and decrease of progesterone increases the contractility of the myometrium, in part, by increasing contraction associated proteins (including gap junctions, comprised of connexin 43 (Cx43), oxytocin receptor, and others) which allow for direct electrical and chemical communication between the muscle fibers [6]. In addition to endocrine signaling, mechanotransduction by integrins plays an important role in parturition, with mechanical stretch promoting extensive uterine

hypertrophy and remodeling in pregnant and postpartum animals (Cullen and Harkness, 1968, Goldspink and Douglas, 1988, Csapo *et al.* 1965).

Integrin activation through binding with the ECM leads to assembly of IACs that relay a variety of signals involved in many cellular processes such as cellular adhesion, migration, and stimulation of numerous signal transduction pathways (Wozniak *et al.* 2004). Each integrin is a heterodimer that includes an alpha and beta subunit, and the alpha/beta heterodimers specify the ligand-binding abilities of the integrin. For example, the alpha 5 integrin subunit (ITGA5) partners exclusively with the beta 1 integrin subunit (ITGB1) forming the ITGA5/ITGB1 (ITGA5B1) integrin receptor (Robinson *et al.* 2004). This is a classic mechanosensory receptor for the matricellular protein fibronectin (FN1). FN1 is a multimodular force-bearing ECM protein that can exhibit a wide range of conformations in the ECM based upon substrate rigidity (Vogel, 2006). It is secreted by cells and assembled into a matrix that binds to cell surface integrin receptors, which under tension, can lead to conformational changes of FN1 into different functional signaling states. Binding of FN1 to ITGA5B1 can promote FN1 fibrillogenesis to provide a dense meshwork of interconnected ECM supporting strong intercellular cohesion (Robinson *et al.* 2004, Vogel, 2006, Wierzbicka-Patynowski and Schwarzbauer, 2003). Talin (TLN1) and vinculin (VCL) are interacting cytoplasmic adaptor proteins that connect the ITGA5B1 integrin receptor to the actin cytoskeleton, allowing for communication and mechanotransduction between cells and/or cell-ECM contacts (Vogel, 2006, Sastry and Burridge, 2000, Larsen *et al.* 2006).

In vitro studies revealed that size, as well as specific protein components, of IACs are dependent on the rigidity of the ECM and internal or external mechanical forces applied to the integrin-ECM complex (Katz *et al.* 2000, Galbraith *et al.* 2002). IACs organize into linear strands called dense plaques within the smooth muscle cells of hollow organs (Eddinger *et al.* 2007, Gabella, 1984). Smooth muscle IACs are present in many organs, including the smooth muscle surrounding blood vessels, to create a cohesive functional syncytium that can coordinate contractility of the organ or vessel wall (Williams *et al.* 2005). Studies in rats showed that ITGA5 integrin gene and protein expression increase within the myometrium and incorporate into IACs during late pregnancy and labor (Williams *et al.* 2005). Similar observations have been made for multiple integrins in the nonlaboring and laboring myometrium of women (Burkin *et al.* 2013). The ITGA5 integrin and multiple adaptor proteins have been localized in myometrium of sheep during gestation (Burghardt *et al.* 2009). However, less is known about IACs located within the sheep myometrium during the pre- and post-partum periods.

Fetal size is similar between sheep and humans. The uterus of both species undergoes significant growth during late pregnancy resulting in uterine stretch and parturition requires similar coordinated smooth muscle contractions. Procurement of myometrial tissues from women over the course of pregnancy is problematic for ethical reasons. The temporal assembly of IACs within the myometrium throughout pregnancy has only been reported for the rat, a species with far smaller fetuses and proportionately less stretch of the uterine wall (Williams *et al.* 2005). We hypothesized that the uterus of sheep develops the mechanical strength to expel the fetus and placenta at parturition by

assembling highly ordered IACs between myometrial cells during late pregnancy. These IACs subsequently disassemble after parturition. Therefore, we examined the spatial expression of ITGA5, ITGB1, FN1, TLN1 and VCL proteins, all components of IACs, in the myometrium of sheep throughout gestation and after parturition. We also examined the same IAC components in myometrial cells grown on either uncoated or FN1-coated rigid substrates. Results of the present study indicate that sheep are similar to humans regarding the assembly of IACs in the pregnant myometrium, and suggest that IACs may form much earlier in human gestation than was previously implied by the rat model. Results highlight the continued value of the sheep model as a flagship gynecological model for understanding parturition in humans (Challis *et al.* 2000).

Materials and Methods

Animals and tissue collection

All animal experiments complied with the Guide for Care and Use of Agricultural Animals and were approved by the Institutional Agricultural Animal Care and Use Committee of Texas A&M University. Ewes (*Ovis aries*) were observed daily for estrus in the presence of vasectomized rams. Cyclic ewes were ovariohysterectomized on Day 15 of the estrous cycle (n=4). Ewes were mated to intact rams three times at 12 h intervals beginning at onset of estrus (Day 0). Pregnant ewes were ovariohysterectomized on either Day 15, 40, 80, 120, or 140 of pregnancy and on Day 1, 7, or 14 postpartum (n=4 ewes/day). Myometrial tissue was dissected from the endometrium and stored at −80°C until processed for western blot analyses. In addition, several sections (~ 1.5 cm) from the

middle of each uterine horn were embedded in Tissue-Tek Optimal Cutting Temperature (OCT) Compound (Miles, Oneonta, NY), frozen in liquid nitrogen vapor, and stored at −80°C until processed for immunofluorescence analyses.

Cell culture

For in vitro studies we utilized the human myometrial cell line PHM1-41, which was derived from term-pregnant human myometrium (patients not in labor) and immortalized using a vector expressing human papillomavirus E6 and E7 proteins (Monga *et al.* 1996). Cells were cultured in DMEM-F12 plus 10% fetal calf serum.

Western blot analyses

Myometrial samples were homogenized in lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 10 mM Tris, 1 mM EDTA, 0.1 mM EGTA, 0.2 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 30 mM $Na_4P_2O_7$, 1 mg/ml leupeptin, 1 mg/ml pepstatin) at a ratio of 1 g tissue per 5 ml buffer. Cellular debris was cleared by centrifugation (12,000 ×*g*, 15 min, 4°C). The protein concentration of the supernatant was determined using a Bradford protein assay (Bio-Rad Laboratories, Hercules, CA) with BSA as the standard. Western blot analyses were performed as described previously [24]. Briefly, proteins (10 μg) were denatured in Laemmli buffer, separated on 8% SDS-PAGE gels, and transferred to nitrocellulose. Blots were blocked in 5% nonfat milk/TBST (Tris-buffered saline, 0.1% Tween-20) at room temperature for 1 h, incubated with either rabbit anti-ITGA5 (#AB1928; 1:1000) or rabbit anti-ITGB1

(#AB1952; 1:1000) from Chemicon (Temecula, CA, USA) [22], or normal rabbit IgG (Sigma Aldrich, St. Louis, MO; 1:1000) in 2% nonfat milk/TBST overnight at 4°C. Blots were then rinsed three times for 10 min each with TBST at room temperature, incubated with goat anti-rabbit IgG horseradish peroxidase conjugate (1:20,000 dilution of 1 mg/ml stock; Kirkegaard & Perry Laboratories, Bethesda, MD), and then rinsed three times for 10 min each with TBST. Immunoreactive proteins were detected using enhanced chemiluminescence (SuperSignal West Pico Luminol System, Pierce Chemical Co., Rockford, IL) according to the manufacturer's recommendations using a FluorChem IS-8800 120 imager (Alpha Innotech, San Leandro, CA). Blots were quantified using AlphaEase FC software (Alpha Innotech).

Immunofluorescence analyses

For immunofluorescence staining, primary antibodies included rabbit anti-ITGA5 (#AB1928; 1:200), rabbit anti-ITGB1 (#AB1952; 1:200), and mouse anti-FN1 (#MAB88916; 1:200) from Chemicon (Temecula, CA), and mouse anti-talin clone 8d4 (#T3287; 1:100), mouse anti-vinculin clone hVN-1 (#V9131; 1:200), normal rabbit IgG (#15006; 1:200) and normal mouse IgG (#15381; 1:100 or 1:200) from Sigma Aldrich (St. Louis, MO). The secondary antibodies included fluorescein-conjugated goat anti-rabbit IgG (Chemicon; 1:250), fluorescein-conjugated goat anti-mouse IgG (Zymed, San Francisco, CA, USA; 1:250), as well as Texas Red-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA).

Immunostaining of frozen sections of myometrium allowed for localization of ITGA5, ITGB1, FN1, TLN1, and VCL proteins as previously described [25]. Briefly, frozen sections (~ 10 μm) of uterine wall, including the myometrium, were cut with a cryostat (Hacker-BrightOTF, Hacker Instruments, Inc., Winnsboro, SC, USA) and mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA). Sections were fixed in −20°C methanol for 10 min, permeabilized at room temperature with 0.3% Tween-20 in 0.02 M PBS (rinse solution), and blocked in 10% normal goat serum for 1 h at room temperature. Sections were then dipped in rinse solution at room temperature and incubated overnight at 4°C with each primary antibody, and detected with fluorescein-conjugated secondary antibody. Slides were then overlaid with a cover-glass and Prolong antifade mounting reagent (Molecular Probes). Negative controls included the appropriate normal rabbit or mouse IgG at the same concentration as primary antibodies. For co-localization of proteins, frozen sections were prepared as described above.

Immunostaining of cultured human myometrial PHM1-41 cells involved seeding cells on Lab-Tek glass chambered slides that were either untreated or pretreated overnight with 50 □g/ml of human fibronectin 120 kDa α chymotryptic fragment containing the cell attachment region (Sigma Aldrich, St. Louis, MO). Myometrial cells were then added and allowed to attach in DMEM-F12 plus 10% fetal calf serum. Cells were then washed in PBS, chambers were removed leaving a silicone gasket surrounding each well, and fixed in -20°C methanol for 10 min. Immunofluorescence co-localization of proteins was then performed as described previously (Muniz *et al.* 2006). After washing with PBS

containing 0.3% (vol/vol) Tween-20, slides were incubated overnight at 4°C with initial primary antibody (either anti-ITGA5, -ITGB1, -FN1, or -VCL IgG at dilutions described above). Following three washes in 4°C rinsing solution for 10 min each, slides were incubated with initial secondary antibody (either FITC- or Texas Red-conjugated anti-mouse IgG at dilutions described above) for 4 h at room temperature and washed in 4°C rinsing solution 6 times for 10 min each. Slides were then incubated overnight at 4°C with the second primary antibody (either anti-ITGA5, -ITGB1, -FN1, or -VCL IgG at dilutions described above). Following six washes in 4°C rinsing solution for 10 min each, slides were incubated with 2 μg/ml of the second secondary antibody (either FITC- or Texas Red-conjugated anti-rabbit IgG at the dilutions described above) for 2 h at 4°C, washed 6 times in 4°C rinsing solution for 10 min each, and dipped in distilled-deionized H_2O. Gaskets were removed and antifade mounting reagent was added prior to overlaying a coverslip. Immunofluorescence images were acquired using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY, USA) interfaced with an Axioplan HR digital camera.

Statistical Analyses

Data were subjected to least-squares ANOVA using the general linear models procedures of the Statistical Analysis System (SAS, Cary, NC). The abundances of ITGA5 and ITGB1 proteins were determined by western blotting and evaluated within each blot to control for any differences in exposure times. All tests of significance were performed using the appropriate error terms according to the expectation of the mean squares for error. Data are presented as least-squares means with overall standard errors.

Results

ITGA5 and ITGB1 proteins increase in the myometrium during mid-gestation and are maintained postpartum

Temporal changes in the expression of ITGA5 and ITGB1 proteins in the myometrium of pregnant and postpartum ewes were determined using Western blotting (Figures 1 and 2). ITGA5 migrated to ~130 kDa on all Days examined, whereas ITGB5 migrated to ~128 kDa on all Days examined, however a lower MW band was also observed on Day 80 of gestation. The lower molecular weight form has not been reported in the literature, but is observed for the product quality control Western blots for the anti-ITGB5 IgGs from some companies. At present we do not speculate on the identity of this immunoreactive protein. On Day 15 of gestation, ITGA5 and ITGB1 proteins were expressed at low levels. However, on Day 80, abundance of ITGA5 and ITGB1 proteins in the myometrium increased significantly compared with Day 15, and abundant levels of ITGA5 and ITGB1 proteins were maintained for the remainder of pregnancy and through Day 14 postpartum (Figures 1 and 2).

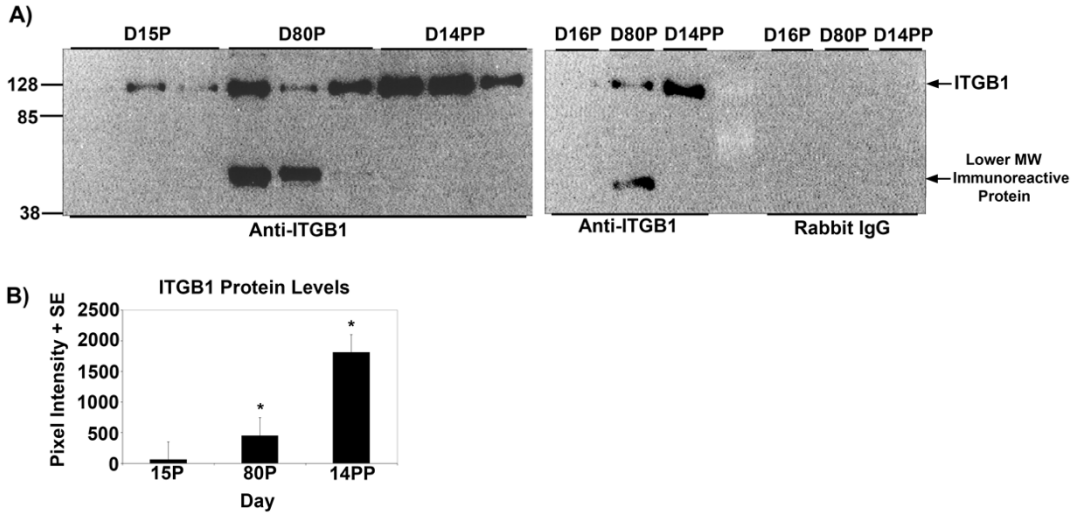


Figure 7. Western blot detection and quantification of alpha 5 integrin protein.

A) Western blot detection of the alpha 5 integrin (ITGA5, 8% 1D-PAGE) in ovine myometrial extracts (10 μg/lane) from different Days of pregnancy (P) or postpartum (PP). Each lane represents a sample from a different ewe. Immunoreactive proteins were detected using a rabbit anti-ITGA5 IgG or irrelevant rabbit IgG. The positions of pre-stained molecular weight standards are indicated on the left of the gels. B) Quantification of total ITGA5 protein in myometrial tissue as detected by Western blotting. ITGA5 protein increased on Day 80 of gestation, and expression remained elevated through Day 14 postpartum.

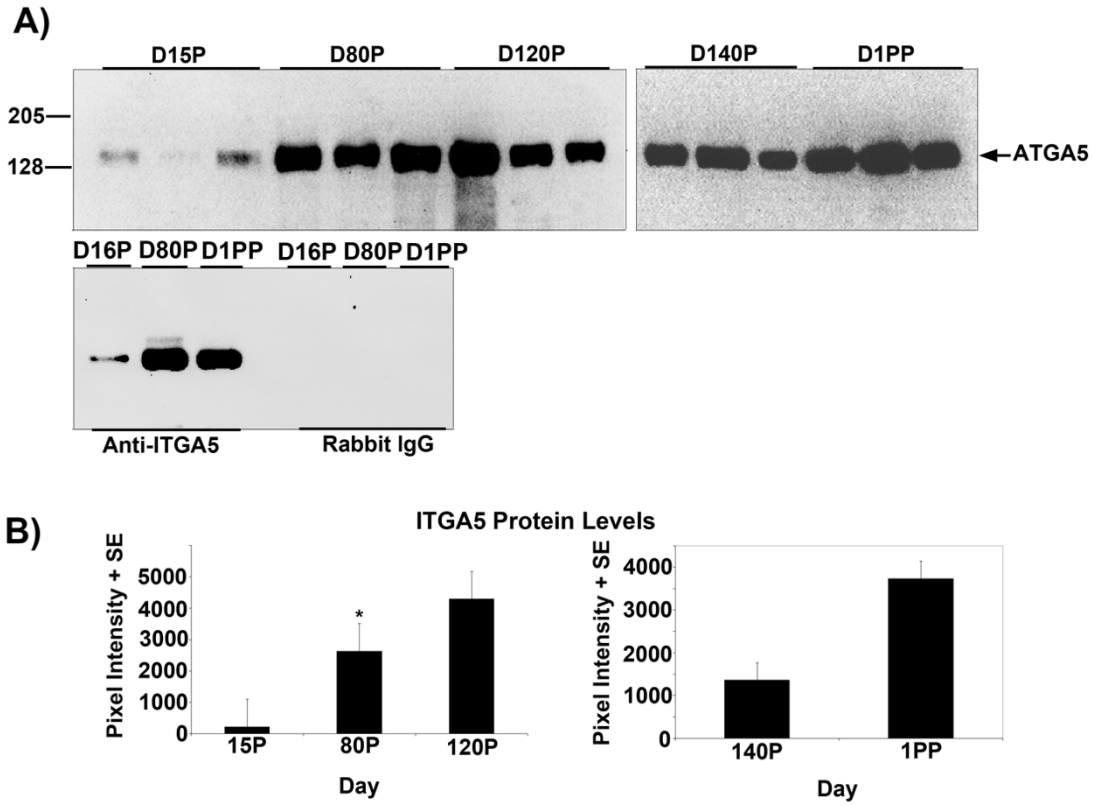


Figure 8. Western blot detection and quantification of beta 1 integrin protein.

A) Western blot detection of the beta 1 integrin (ITGB1, 8% 1D-PAGE) in ovine myometrial extracts (10 μg/lane) from different Days of pregnancy (P) or postpartum (PP). Each lane represents a sample from a different ewe. Immunoreactive proteins were detected using a rabbit anti-ITGB1 IgG or irrelevant rabbit IgG. The positions of pre-stained molecular weight standards are indicated on the left of the gels. B) Quantification of total ITGB1 protein in myometrial tissue as detected by Western blotting. ITGB1 protein increased on Day 80 of gestation, and expression remained elevated through Day 14 postpartum.

ITGA5 and ITGB1 proteins assemble into longitudinally oriented IACs at the surface of myometrial cells during late pregnancy that disperse postpartum

The temporal and spatial localization of ITGA5 and ITGB1 in the myometrium of pregnant and postpartum sheep were determined by immunofluorescence staining (Figures 3 and 4). Scattered punctate immunostaining for ITGA5 and ITGB1 was detected at the surface of myometrial cells in both longitudinal and circular layers of Day 15 cyclic (shown for ITGB1, Figure 4) and pregnant sheep myometrium (Figures 3 and 4). The punctate staining was more obvious in the Day 15 pregnant myometrium. Longitudinal strands of IACs developed at the surface of the myometrial cells by Day 40 of pregnancy; and increasingly ordered IACs were present in Day 80, Day 120, and Day 140 myometrium (Figures 3 and 4). ITGA5 and ITGB1 continued to be highly expressed in postpartum myometrium although they were no longer organized into longitudinal IACs at the surface of myometrial cells on postpartum Day 1, Day 7, or Day 14 (Figures 3 and 4).

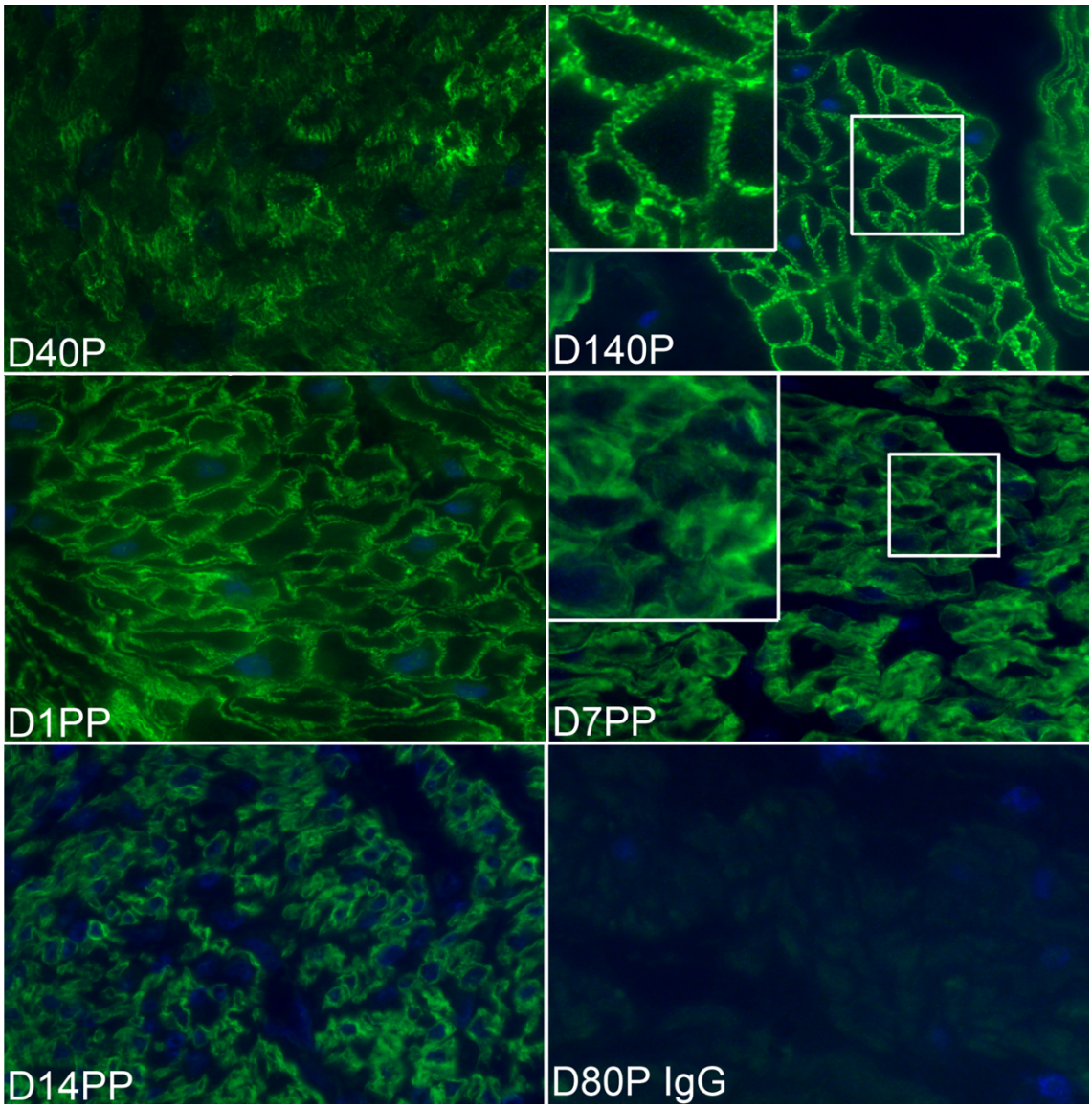


Figure 9. Immunostaining for the alpha 5 integrin in the myometrium of ewes throughout pregnancy and postpartum.

Note the linear strands of IACs that appear as regularly distributed spots at the myometrial cell surface present in some cells on Day 40, which are present and highly ordered on all myometrial cells by Day 140 of pregnancy. These highly ordered IACs are no longer present in the myometrium of postpartum ewes. Insets provide for comparison surface views of myometrial cells during late pregnancy and postpartum. An irrelevant rabbit IgG serves as a negative control. Width of each field is 140 μm; inset is 25 μm.

The ITGA5 subunit partners exclusively with the ITGB1 subunit forming the ITGA5B1 receptor (Shynlova *et al.* 2007), and the spatial pattern of expression of ITGA5 and ITGB1 precisely overlapped in the myometrium of Day 80 pregnant ewes, confirming the presence of the ITGA5B1 integrin receptor at the surface of myometrial cells (Figure 5). Further similar spatial immunostaining patterns were observed for the mechanosensory ECM protein FN1 and ITGA5, and for ITGA5 and the intracellular mechanosensory cytoskeletal protein VCL in the myometrium of Day 80 pregnant ewes, confirming the assembly of IACs at the surface of myometrial cells (Figure 6). It is noteworthy that the overlap of expression for ITGA5 with FN1 and VCL was not as exact as the overlap of expression for ITGA5 and ITGB1. ITGA5 and ITGB1 both span the cell membrane. However, FN1 is present external to the cell in the ECM, and VCL is located about 40-60 nm deep within the cytoplasm of the cell (Kanchanawong *et al.* 2010). Therefore, the expression of FN1 and VCL do not precisely overlap spatially with the expression of ITGA5, and a yellow immunofluorescence signal is not observed. FN1, VCL, and the intracellular mechanosensory cytoskeletal protein TLN1 no longer assembled into IACs in the myometrium of postpartum ewes (Figure 7).

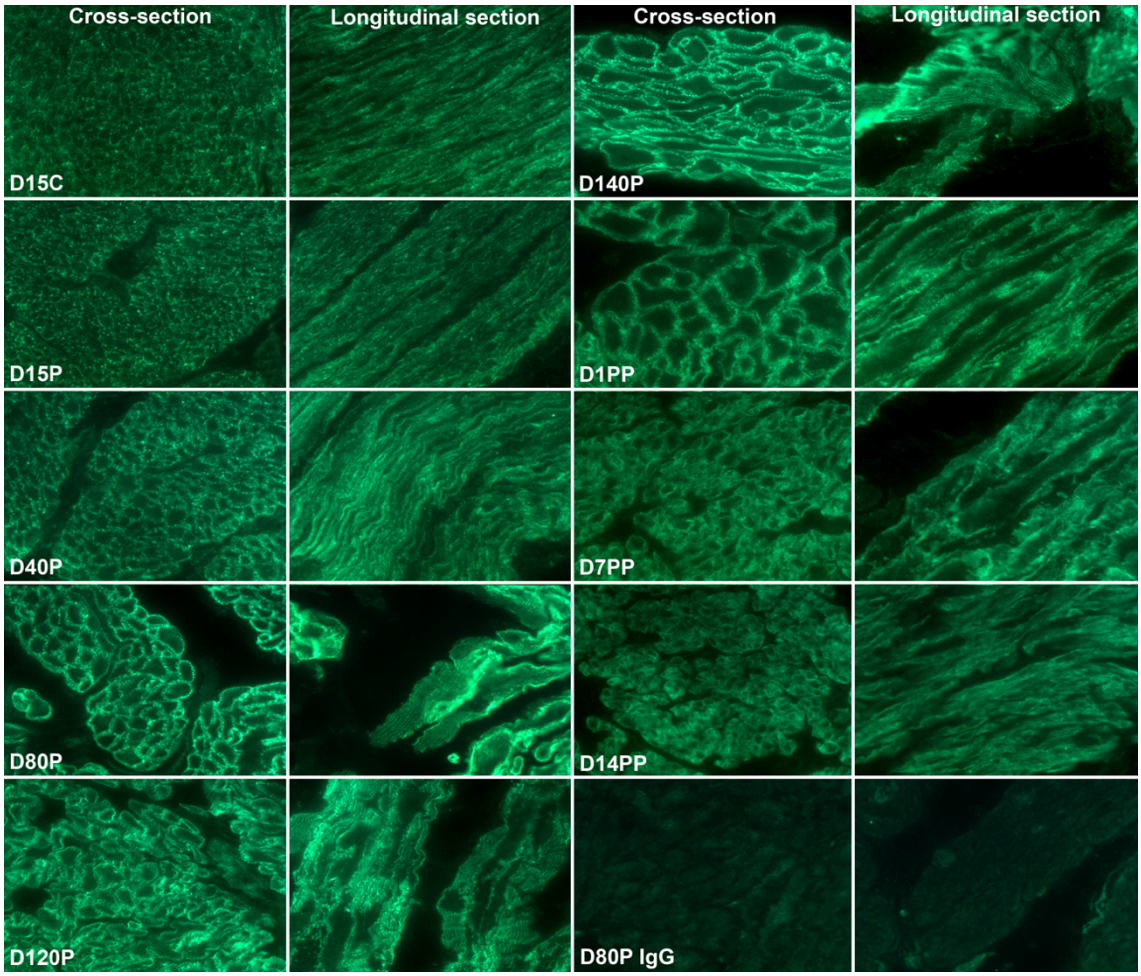


Figure 10. Immunostaining for beta 1 integrin in the myometrium of ewes throughout pregnancy and postpartum.

Note the linear strands of IACs that appear as regularly distributed spots at the myometrial cell surface present in some cells on Day 40, which are present and highly ordered on all myometrial cells on Days 80, 120, and 140 of pregnancy. These highly ordered IACs are not present on the myometrial cells on Day 15 of the estrous cycle or pregnancy and are not present in the myometrium of postpartum ewes. Views of myometrial cells cut in cross-section and in longitudinal section are shown. An irrelevant rabbit IgG serves as a negative control. Width of each field is 140 μm.

FN1 stimulates activation of ITGA5, ITGB1, and TLN1 to rapidly form IACs

As a corollary to the increasing order of IACs during expansion of the uterine wall and greater forces applied between FN1, the fibronectin receptor, and cytoskeletal adaptor protein TLN1, we compared the attachment of human myometrial cells to uncoated and FN1-coated culture dishes following seeding of cells into culture medium containing fetal bovine serum. While serum contains growth and attachment factors and cultured cells secrete ECM, there was an increased spreading of cells, plus the number and size of IACs in cells attaching to FN1-coated slides compared with non-coated slides (Figure 8). Immunostaining of ITGB1 and TLN1 at the basal surface of the cells indicated that rapid in vitro assembly of IACs on a FN1 rigid substrate was occurring (data not shown). Comparable results showing co-distribution of ITGA5 with TLN1 at the basal surface of the cells confirms the rapid in vitro assembly of IACs to FN1 on a rigid substrate (Figure 8).

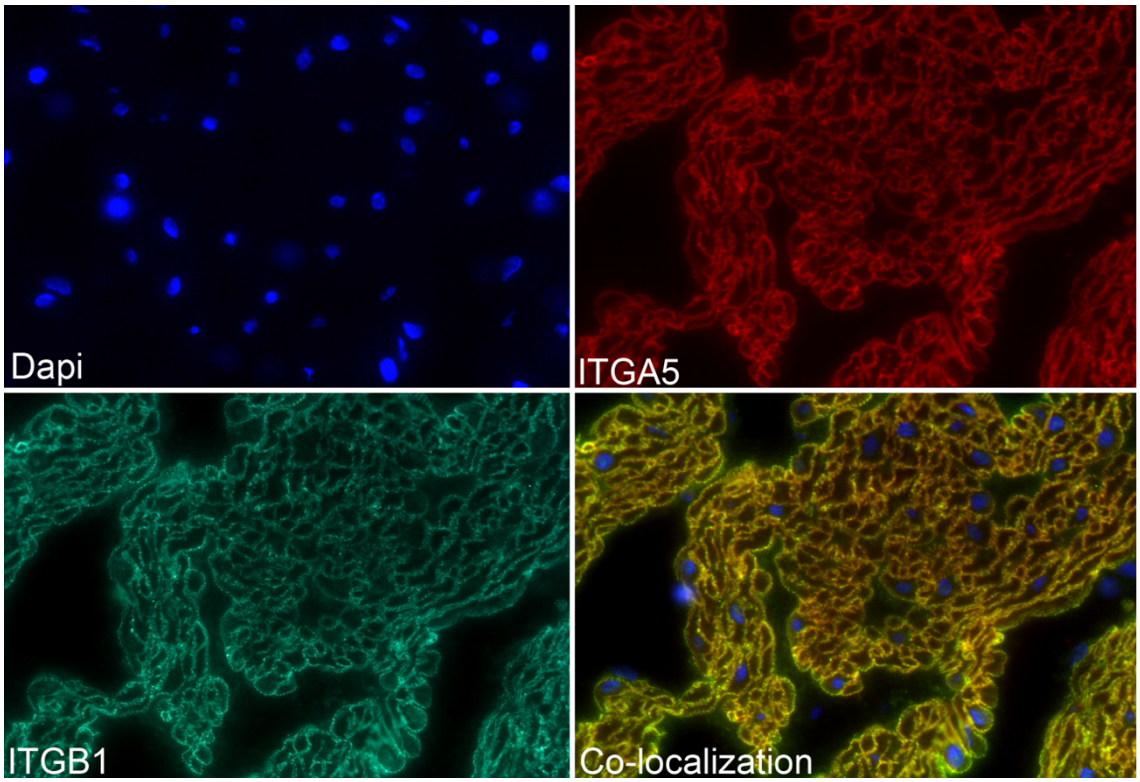


Figure 11. Double-immunostaining for alpha 5 and beta 1 integrins in the myometrium of ewes on Day 80 of pregnancy.

ITGA5 and ITGB1 precisely co-localized to the same regions on the surface of the myometrial cells (yellow color), confirming the presence of the ITGA5B1 integrin receptor. DAPI was used to stain nuclei for histological reference. Width of each field is 220 μm.

Discussion

Constant changes in the lumen diameter of hollow organs generate forces that strain the intercellular connections between smooth muscle cells within the muscularis externa (Bershadsky *et al.* 2006). Cells within tissue-level compartments respond to extracellular mechanical forces by integrin-activated assembly of IACs that dynamically respond to those forces (Zhang *et al.* 1999, Chen, 2008). IACs are complex structures composed of transmembrane integrins activated by attachment to ECM proteins that connect to the cytoskeleton and serve as signaling centers that can regulate numerous physiological processes (Humphries *et al.* 2019). As external forces sensed through integrins increase, IACs grow and mature, which leads to actin remodeling that transduces intracellular forces necessary to balance changing external forces (Zhang *et al.* 1999, Chen, 2008). The myometrium of the pregnant uterus is exposed to external forces that differ over the course of pregnancy from those forces applied to the smooth muscle cells of blood vessels or tubal organs that undergo peristalsis. As pregnancy progresses in women, the uterus can increase 500- to 1000-fold in volume and 24-fold in weight. Therefore, the uterine wall must adapt to increases in fetal growth, placental fluid volumes, blood flow, and hormonal profiles in order to transform it into an organ that can forcefully expel the fetus and placental membranes during parturition (Ono *et al.* 2007).

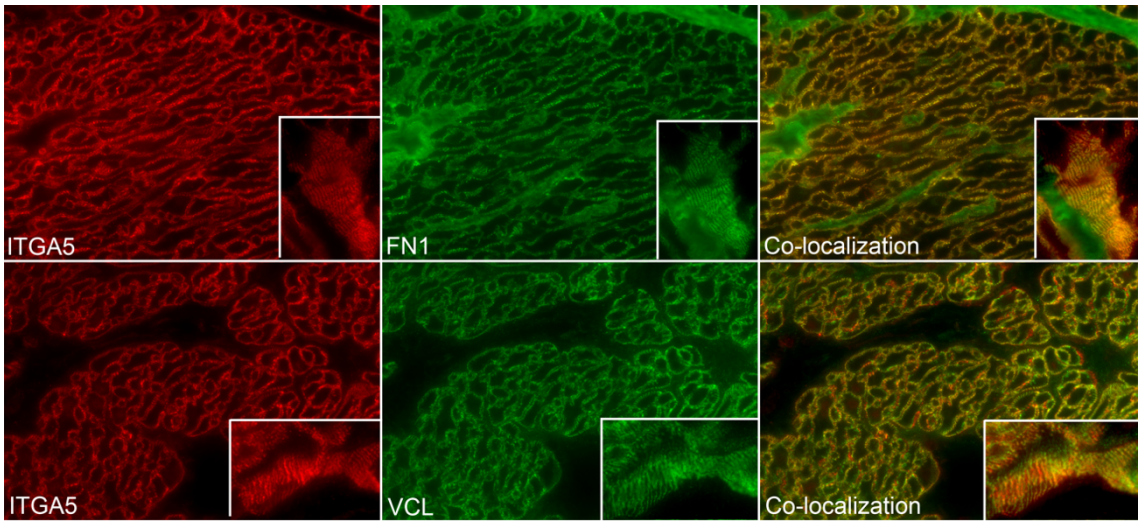


Figure 12. Assembly of integrin adhesion complexes (IACs) in sheep myometrium.

Immunostaining for alpha 5 integrin (ITGA5, red color), fibronectin (FN1, green color), and vinculin (VCL, green color) in the myometrium of ewes on Day 80 of pregnancy is shown. ITGA5 and FN1, and ITGA5 and VCL co-distributed to the same regions on the surface of the myometrial cells (note), confirming the presence of IACs. Note that while ITGA5 and VCL are co-localized, VCL is located deeper within the cytoplasm therefore fluorophores do not always overlap. Width of each field is 220 μm; inserts are between 20-30 μm wide.

In response to these unique forces in rodents, myometrial cells assemble IACs, but these IACs develop during late pregnancy. Both the hormones of pregnancy and mechanical stretch upregulate the expression of FN1, the integrin receptor ITGA5B1, and other IAC constituents, including the cytoskeletal mechanosensor TLN1 (Shynlova *et al.* 2007, Williams *et al.* 2005, Shynlova *et al.* 2004). Both TLN1 and vinculin (VCL) are recognized as cytoplasmic tension-transducing proteins whose signaling activity is modulated by force-induced conformational changes in IACs (Hytonen and Vogel, 2008,

Mykuliak *et al.* 2018). FN1 is a multimodular force-bearing ECM protein that can exhibit a wide range of conformations in the ECM based upon substrate rigidity (Vogel, 2006). It is secreted by cells and assembled into a matrix that binds to cell surface integrin receptors, which under tension can lead to conformational changes of FN1 into different functional signaling states (Robinson *et al.* 2004, Vogel, 2006, Wierzbicka-Patynowski and Schwarzbauer, 2003). FN1-mediated focal adhesion kinase activation through the FN1 receptor is dependent on mechanical tension which, in contrast to the collagen I receptor, is decoupled from substrate rigidity or mechanical tension (MacPhee *et al.* 2001, Seong *et al.* 2013). The growth of myometrial IACs is sensitive to rigidity and strength of adhesion to the ECM (Nicolas *et al.* 2004) and the IAC linkage to the ECM and the myometrial actomyosin complex provides sufficient force to expel the fetus at term (Macphee and Lye, 2000).

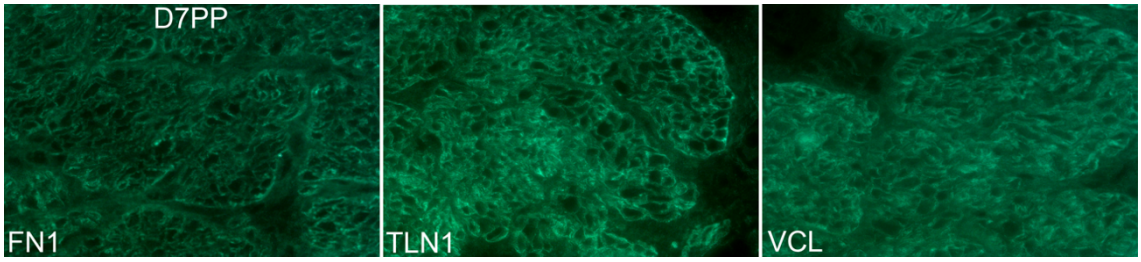


Figure 13. Integrin adhesion complexes in sheep myometrium disassemble postpartum.

Immunostaining for fibronectin (FN1), talin (TLN1), and vinculin (VCL) in the myometrium of ewes on Day 7 postpartum (D7PP) is shown. Similar to results for the alpha 5 integrin (ITGA5) and the beta 1 integrin (ITGB1) shown in Figures 3 and 4, the multimodular, mechanosensory ECM protein FN1 and intracellular mechanosensory cytoskeletal proteins TLN1 and VCL no longer assemble into IACs in the myometrium of postpartum ewes. Width of each field is 220 μm.

Human fetuses are larger than those of rats, and therefore in women, mRNA and protein for the alpha 1 (ITGA1), alpha 3 (ITGA3), ITGA5, alpha 7 (ITGA7), alpha v (ITGAV), ITGB1, beta 2 (ITGB2), beta 3 (ITGB3), and beta 5 (ITGB5) are significantly higher in term myometrial samples than in nonpregnant control samples (Burkin *et al.* 2013). Therefore, it is likely that their presence within the uterus generates more significant mechanical stretch of the uterine wall than rats. The present study is the first to comprehensively examine, across the extent of pregnancy, IAC assembly within the myometrium of a species larger than rats, where the stretch forces applied to the myometrium during gestation are expected to be amplified. Results of the present study indicate that a similar process of IAC assembly takes place in sheep myometrium as occurs in rodents and humans during pregnancy. Interestingly, punctate staining of ITGA5 and ITGB1 subunits was detected in cyclic ewes on Day 15, but punctate staining of these integrin subunits was greater in Day 15 pregnant animals. This punctate staining in cyclic animals has not been reported in rodents. Immunostaining of the integrin subunits increased and was co-distributed with FN1, VCL, and TLN1 on the surface of myometrial cells by Day 40 of pregnancy, indicating the assembly of IACs. Therefore, well-defined IACs begin to assemble in the myometrium of sheep during the first trimester, which is significantly earlier than IACs assemble during pregnancy in rodents. The magnitude and organization of these IACs continue to increase concurrently with the increasing accumulation of fluids in the allantois and amnion and growth of the fetus, suggesting that mechanical stretch of the uterine wall contributes sufficient continuous local force by Day 40 of pregnancy to initiate the development of ordered IACs in the myometrial cells of

sheep. Because sheep have a fetus near the size of the human fetus, first trimester assembly of IACs in the myometrium of women may occur in a manner similar to described here for the sheep. This highlights the continued value of the sheep model as a flagship gynecological model for understanding parturition in humans (Challis *et al.* 2000). Further, these IACs disassemble by Day 1 postpartum although expression of the proteins was maintained for at least 14 days, presumably due to the size of the gravid uterus and duration of uterine involution.

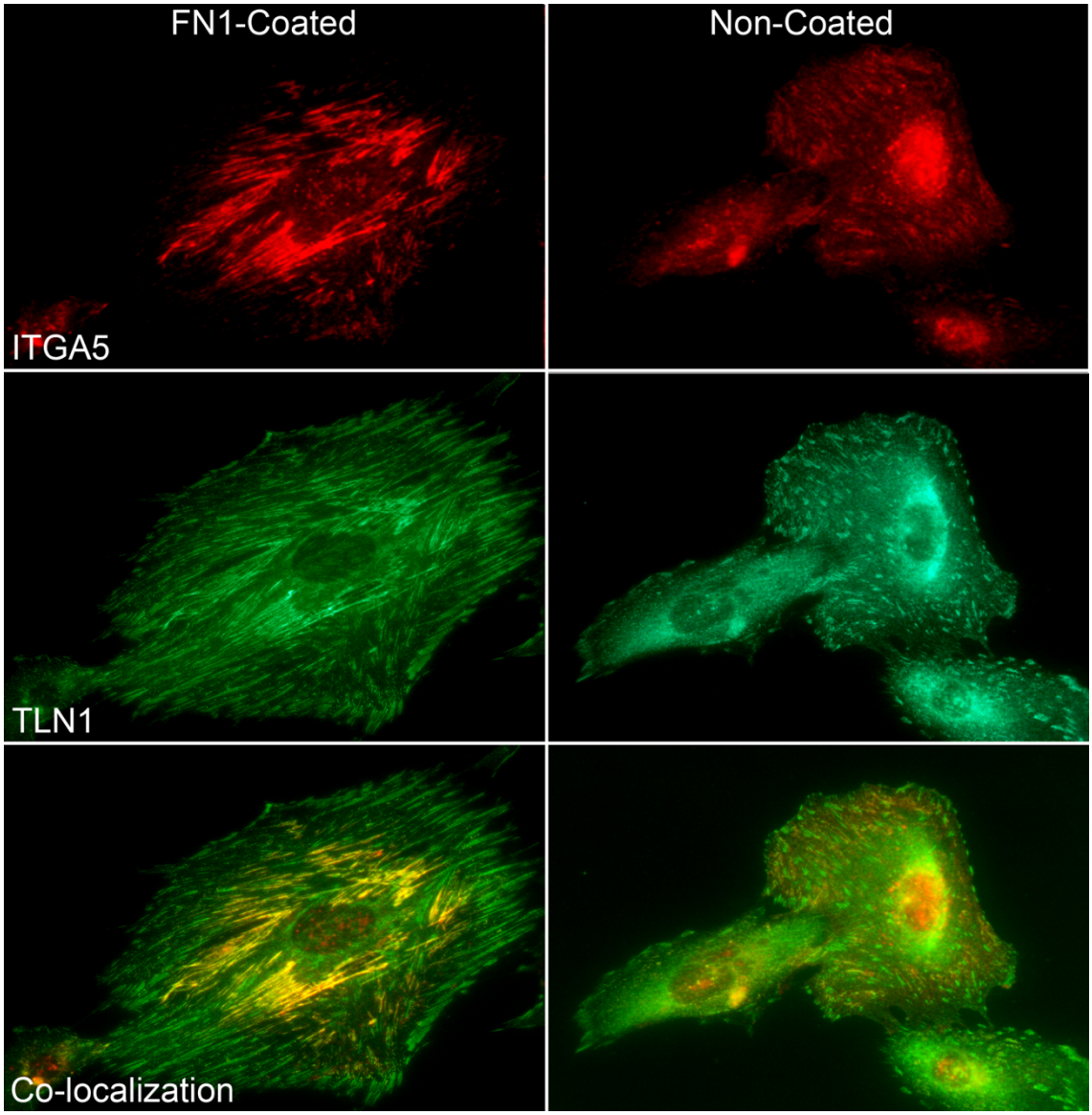


Figure 14. Immunofluorescence localization of the alpha 5 integrin and talin on human myometrial cells bound to fibronectin.

Cells were seeded on coverglass chambered slides coated with FN1 and immunostained with antibodies to ITGA5 and TLN1. Both ITGA5 and TLN1 were observed in large aggregates as components of integrin adhesion complexes (IACs) at the basal surface of myometrial cells as they attach to the FN1-coated slides. Although cultured cells secrete ECM and bind to that ECM at their basal surface while attaching to slides, note the increased number of IACs for cells attaching to FN1-coated versus non-coated slides. Width of each field is 140 μm.

Earlier studies have shown that other tissue compartments within the uteri of domestic species exhibit tissue-specific organization of IACs during pregnancy. First, the subepithelial uterine stromal cells of sheep and pigs differentiate into a myofibroblast phenotype associated with the upregulation of the cytoskeletal proteins desmin, vimentin, and alpha smooth muscle actin to augment the contractility of fibroblasts (Johnson *et al.* 2003, Seo *et al.* 2020). These stromal cells also express ITGAV and ITGB3 integrins, as well as the ECM proteins secreted phosphoprotein 1 [SPP1, also known as osteopontin (OPN)], FN1, and vitronectin (VTN). The stromal ITGAVB3 receptor binds VTN, FN1, and SPP1 to form IACs, and the diffuse spatial pattern of localization of these proteins within the stroma suggests they are organized into 3D matrix adhesions that developed in a mechanically stressed but more strain-shielded environment (Burghardt *et al.* 2009). Second, in pigs it is hypothesized that folding at the interface between the uterine luminal epithelium (LE) and the non-invasive placental chorionic epithelium (CE) is driven by external forces delivered to this interface. Subepithelial endometrial blood vessels deliver increased blood flow that pushes upward on the interface between the uterine LE and the placental CE to trigger focal IAC assembly, and endometrial fibroblasts differentiate into contractile myofibroblasts that pull connective tissue downward and inward to sculpt folds at the uterine-placental interface (Seo *et al.* 2020). Third, it is proposed that SPP1 binds integrin receptors expressed by the uterine LE and placental trophectoderm of both sheep and pigs to form IACs that adhere the uterus to the placenta during the attachment phase of implantation and the development of synepitheliochorial and epitheliochorial placentation, respectively (Frank *et al.* 2017, Erikson *et al.* 2009, Kim *et al.* 2010).

In the present study, the ability of myometrial cells to dynamically adapt to changes in extracellular force was also illustrated by the formation of IACs in human myometrial cells on FN1-coated glass chambered slides. Results showed that the ITGA5 integrin subunit was abundantly expressed at the basal surface of cultured myometrial cells and aggregated to IACs at sites of cell anchorage to the substrate. Pre-coating of the substrate with the cell attachment fragment of FN1 enhanced the size and length of in vitro IACs compared to the untreated substrate, illustrating the dynamic ability of cells to respond to ECM stiffness through the FN1 receptor.

In summary, IACs begin to assemble within the sheep myometrium during early-to-mid gestation in response to increased stretch of the uterine wall and continue to increase as pregnancy progresses. FN1 is essential for IAC assembly and these IACs contribute to a mechanical syncytium that allows for the sensing of mechanical forces from both inside and outside of the cell in order to sustain powerful contractions during labor. After parturition, IACs are disassembled, but the integrin subunits ITGA5 and ITGB1 remain expressed at the protein level at least two weeks postpartum, indicating that turnover of these proteins is much slower than their synthesis and suggesting that integrins may contribute to the involution process of an organ capable of remarkable myometrial hypertrophy and hyperplasia. Results of the present study indicate that sheep are similar to humans regarding the assembly of IACs in the pregnant myometrium, and suggest that IACs may form much earlier in human gestation than was previously implied by the rat model. Results highlight the continued value of the sheep model as a flagship gynecological model for understanding parturition in humans (Challis *et al.* 2000).

CHAPTER V

TEMPORAL AND SPATIAL EXPRESSION OF AQUAPORINS 1, 5, 8, AND 9 WITHIN THE PORCINE UTERUS AND PLACENTA DURING GESTATION

Introduction

The volumes of allantoic and amniotic fluids in mammalian conceptuses are rapidly changing during gestation to support embryonic/fetal growth and development (Bazer 1989). Aquaporins (AQPs) are water-selective channels that function as pores for water transport through the plasma membrane (Agre *et al.* 2002). To date, 13 isoforms of AQPs have been found in mammals, with some AQPs transporting other molecules in addition to water. In the current study, we focused on three aquaporins and one aquaglyceroporin, which is known to transport other molecules including glycerol and lactate. The movement of water and nutrients across cell membranes is crucial for reproduction and is likely influenced by the expression of AQPs in tissues of the reproductive tract (Zhu *et al.* 2015). The placenta and uterus are the major organs responsible for supporting embryonic/fetal development, and thus play essential roles in assuring survival and growth of the conceptus (embryo/fetus and associated placental membranes and fluids). Key roles of the uterus and placenta are to regulate the transport of nutrients and water across the uterine-placental interface during pregnancy recognition, conceptus implantation, and placental development/placentation (Wilson 2002). Expression of AQPs in the uteri of different species, including humans (Li *et al.* 1994), mice (Richard *et al.* 2003), rats (Lindsay and Murphy 2006, 2007), dogs (Aralla *et al.*

2012), and pigs (Skowronski 2010) has been reported. Further, mice lacking AQP5 and AQP8 have an abnormal accumulation of intrauterine luminal fluid and aberrant implantation of conceptuses (Zhang *et al.* 2015). At present, there is a paucity of information regarding the expression of AQPs at the uterine-placental interface of mammals, including pigs. Therefore, the current study was conducted to determine the temporal cell-type specific expression of AQPs 1, 5, 8, and 9 at the uterine-placental interface of gilts during gestation.

Materials and methods

Chemicals and reagents

Primary antibodies of AQPs 1 (Millipore), 5 (Sigma Chemicals), 8 (Sigma Chemicals), and 9 (GeneTex) were purchased. Mouse and rabbit IgG (Millipore) antibodies were used as negative controls for immunofluorescence analyses. Other reagents were obtained from Sigma Chemicals.

Animals and tissue collection

This study was approved by the Texas A&M University's Animal use and Care Committee. Twenty gilts (Yorkshire × Landrace) were fed daily 2 kg of a corn- and soybean meal-based diet (Li *et al.* 2014) and bred to boars of known fertility during the second period of estrus. The day of breeding was designated as Day 0 of gestation. Hysterectomy was performed on Days 9, 12, and 15 of the cycle and Days 10, 12, 15, 20, 25, 30, 35, 40, 60, 85, and 90 of gestation (5 gilts/Day), as described previously (Li *et al.*

2014). Tissue sections (~1 cm thick) from the middle of each uterine horn of gilts on Days 9, 10, 12, and 15, and from implantation sites beginning Day 20, were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2) and embedded in Paraplast-Plus (Oxford Laboratory, St. Louis, MO).

Progesterone and Estrogen Models

To evaluate effects of estrogen (E2) and E2-induced pseudopregnancy on expression of AQPs 1, 5, 8, and 9 mRNAs in endometria, gilts were detected in estrus (Day 0) and assigned randomly to receive daily intramuscular injections of either estradiol benzoate (E2 in corn oil (E2); n = 4) or corn oil alone (CO; n = 4) on Days 11, 12, 13, and 14 of the estrous cycle to induce pseudopregnancy (Frolova *et al.* 2009). All gilts were euthanized and then ovariohysterectomized on Day 15. Endometrial tissues were collected as previously described.

To evaluate effects of long-term treatment with progesterone (P4) on expression of AQPs 1, 5, 8, and 9 mRNAs in endometria, gilts were ovariectomized on Day 12 of the estrous cycle and assigned randomly to receive daily intramuscular injections of either CO (4 ml) or P4 (200 mg in 4 ml CO) on Days 12 through 39 post-estrus (n = 3/treatment) (Waugh and Wales 1993). All gilts were hysterectomized on Day 40 post-estrus and endometrial tissues collected as previously described.

Immunofluorescence Microscopy

Paraffin embedded sections (5 μm) of the uterine-placental interface were adhered to slides, deparaffinized and rehydrated in CitriSolv, ethanol, and water. For antigen unmasking, the sections were brought to a boil in a 10mM Sodium Citrate Buffer solution. The sections were then washed in PBS 3 times for 5 min each, blocked with 10% normal goat serum, and then the sections were incubated with either rabbit anti-AQP1 (2.5 μg/ml), rabbit anti-AQP5 (2 μg/ml), mouse anti-AQP8 (5 μg/ml), or rabbit anti-AQP9 (2.5 μg/ml) overnight at 4°C in a humidified chamber. Normal rabbit or mouse IgG was substituted for a primary antibody and served as a negative control. Expression was detected with either fluorescein-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:250) for one hour (Chemicon International). Slides were then overlaid with Prolong Gold Anti-fade mounting reagent containing DAPI (Molecular Probes) and a coverslip. Images were taken using an Axioplan 2 microscope and a Zeiss Imager.M2, (Carl Zeiss, Thornwood, NY) interfaced with an Axioplan HR and an AxioCam HRm digital camera, respectively. Photographic plates were assembled using Adobe Photoshop (version 6.0, Adobe Systems Inc., San Jose, CA).

RNA Extraction, cDNA Synthesis, and Primer Design

Total RNA was isolated from frozen endometrium using Trizol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's recommendations. First strand cDNA was synthesized using a Superscript III First Strand Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. First strand cDNA was

diluted 5x for the qPCR reaction. Primers for qPCR were designed using NCBI Genbank sequences and submitted to BLAST (http://www.ncbi.nlm.nih.gov/) to confirm specificity against the known porcine genome.

Quantitative PCR assays were performed using PerfeCta SYBR Green Master mix (Quanta Biosciences, Gaithersburg, MD) in 10 µl reactions with 2.5 mM of each specific primer, on a Roche 480 Lightcycler (Rochem Life Sciences) with approximately 60 ng cDNA per reaction. The PCR program began with 5 min at 95°C followed by 40 cycles of 95°C denaturation for 10 sec and 60°C annealing/extension for 30 sec. A melt curve was produced with every run to verify a single gene-specific peak. Standard curves using pooled cDNA with 2-fold serial dilutions were run to determine primer efficiencies. All primer correlation coefficients were greater than 0.95 and efficiencies were 100-112%. Ribosomal protein 7 (Rpl7) was used to normalize data from endometrial tissues (Seo *et al.*, 2012). The primer sequences for AQP1 were, forward 5'-tcaactaaagaagaggcaaaaacac-3'and reverse 5'-gcccaaaatattacctctctttctc-3'. The primer sequences for AQP5 were, forward 5'- ccatcctctacttctacctgctc-3' and reverse 5'- ttcgctgtcatctgttttccttctt-3'. The primer sequences for AQP8 were, forward 5'- attctccatcggcttctctgt-3' and reverse 5'- tccctttagaattaggcgagttttc-3'. The primer sequences for AQP9 were, forward 5'- tcattatagtcttcgctgtttttga-3' and reverse 5'- ctacaggaatccaccagaagttatt-3'. The $2^{-\Delta\Delta Ct}$ method was used to normalize data, and fold-changes were subjected to statistical analyses.

Statistical analysis

All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA). Data from quantitative PCR for effect of days of pregnancy were subjected to one-way ANOVA followed by a post-hoc Tukey analysis. Data from quantitative PCR analyses for effect of P4 and E2 treatments were analyzed using the Students t-test. All data are presented as mean ± SEM with significance set at $P < 0.05$.

Results

AQP1 mRNA and protein expression increase at the uterine-placental interface throughout the estrous cycle and gestation

AQP1 protein was highly expressed in all endothelial cells of both the uterine and placental vasculatures from Day 15 to Day 90 of pregnancy (Figure 1). AQP1 protein expression was also observed in red blood cells (RBCs) and the smooth muscle cells of the myometrium. Interestingly, only the smooth muscle cells in the myometrium showed expression of AQP1, as there was no expression of AQP1 protein in the smooth muscle cells of the tunica media of blood vessels. AQP1 mRNA expression showed a significant increase in both the cycle and throughout gestation.

AQP5 is expressed in the placental areolae throughout gestation

AQP 5 protein was expressed in small sections of the chorionic epithelium beginning on Day 20 of gestation. By Day 25, it was apparent that AQP5 was being localized to the chorionic epithelium of areolae and this expression continued through Day

90 of pregnancy (Figure 2). AQP5 mRNA showed no change in expression levels throughout pregnancy.

AQP8 mRNA and protein expression increase at the uterine-placental interface throughout gestation

Endometrial expression (Figure 3A and 3B). AQP8 protein was expressed by the endometrial glands and glandular epithelium (GE), and in the tunica media and adventitia of blood vessels beginning on Day 25 of pregnancy and continued to be expressed through Day 60. AQP8 expression also localized to the stroma adjacent to the luminal epithelium beginning on Day 30 of gestation. There appeared to be a decrease in AQP8 expression on Days 35 and 40 of gestation but an increase on Day 60. Although there was no expression in the smooth muscle cells of the myometrium, AQP8 staining was expressed in the connective tissue of the myometrium (Figure 3D).

Conceptus expression (Figure 3A and 3C). AQP8 protein expression was localized to the conceptus trophectoderm on Days 15 and 20 of pregnancy, but by Day 25 its expression was limited to the placental areolae. AQP8 was also expressed in the tunica media and adventitia of placental blood vessels and within the stroma by Day 30 of gestation and appeared to decrease on Days 35 and 40, but expression increased on Day 60 of gestation.

mRNA expression levels (Figure 3E). AQP8 mRNA expression showed no significant changes during the cycle or in the placenta throughout gestation. However,

endometrial mRNA expression increased starting at Day 15 of pregnancy and continued until Day 40 where it remained until Day 60 of pregnancy.

AQP9 protein expression is localized to the endometrial LE and placental allantoic epithelium and mRNA expression increases in the placenta

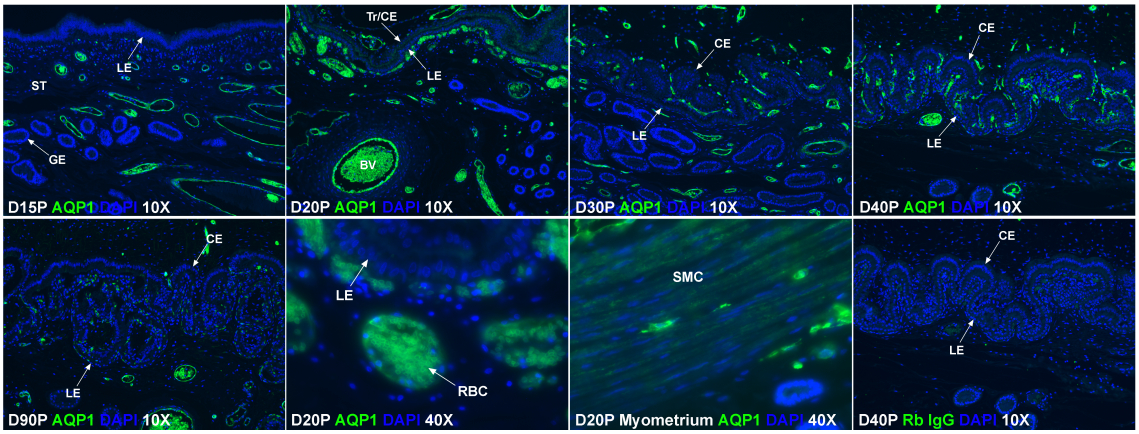
AQP9 protein was expressed in the uterine luminal epithelium (LE), GE, and areolae throughout pregnancy (Figure 4). In the uterine LE, AQP9 was localized to the apical surface of the cells during early pregnancy, with a shift in expression towards the basal surface of the cells by Day 40. Expression within the uterine GE was low throughout gestation and the allantoic epithelium showed AQP9 protein expression on Day 20 of gestation. AQP9 mRNA expression decreased during the cycle and increased in the placenta during gestation.

Discussion

Placentation in pigs initially involves rapid expansion and development of the chorion (trophectoderm) and allantois between Days 18 and 30 of gestation in pigs due to the accumulation of water within membranes (Bazer and Johnson 2014). The driving force for expansion of the allantois, and in turn the chorioallantois, is the rapid accumulation of water from about 1 ml on Day 18 to 200-250 ml on Day 30 of gestation. Allantoic fluid volume increases from Day 20 (3.7 ml) to Day 30 (189 ml), decreases to Day 45 (75 ml), and then increases again to Day 58 (451 ml). Thereafter, it decreases to Day 112 (24 ml). AQPs play an important role in the transport of water from mother to fetus. The uterus is

the site of implantation and development of the conceptus in mammals, and there is evidence for the expression of AQPs in the uteri of humans, rats, mice, dogs, sheep, horse, and pigs (Ducza 2017). For example, AQP1, 5, 7, 8, and 9 transcripts were detected in the uterine GE of the rat uterus (Lindsay and Murphy 2007). There is also evidence for the expression of AQP1, AQP5, and AQP9 in the ovary, oviduct, and uterus of gilts on Days 17 and 19 of the estrous cycle (Skowronski *et al.* 2009). We have previously shown that AQPs 1, 3, 5, and 9 were expressed in the uterine endometrium, as well as the placenta of gilts at both mRNA and protein levels on Day 25 of gestation (Zhu *et al.* 2015). The current work identified the cell-specific expression of AQPs 1, 5, 8, and 9 mRNA and protein at the porcine uterine-placental interface during gestation.

A) AQP1 Protein Expression



B) AQP1 mRNA Expression

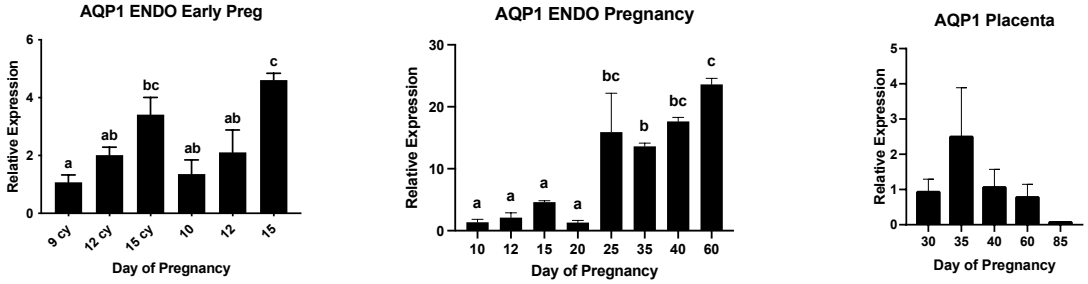


Figure 15. Aquaporin 1 protein and mRNA expression throughout gestation

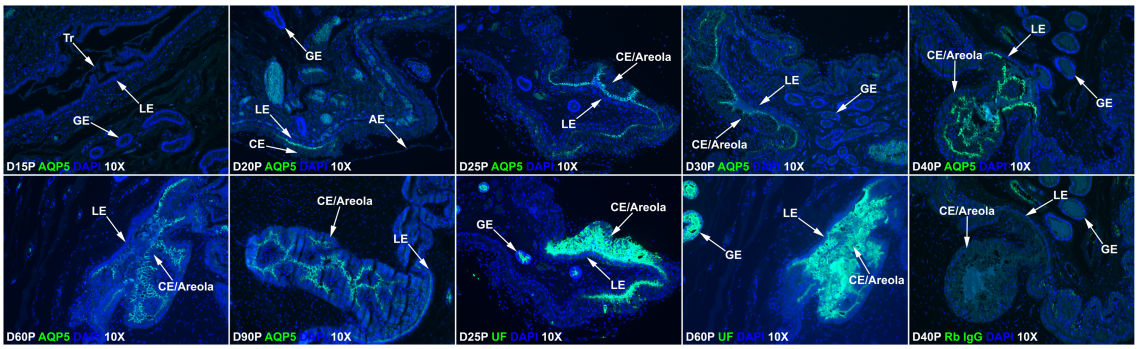
A) Immunofluorescence microscopy for aquaporin 1 (AQP1; green) at the uterine-placental interface of gilts on Days 15 (D15), 20, 30, 40, and 90 of pregnancy. AQP1 protein is localized to all endothelial cells within both uterine and placental tissues, to the myometrium and within red blood cells. Nuclei are stained with DAPI for histologic reference. The D40 rabbit IgG (Rb IgG) panel serves as the negative control. Width of fields for microscopic images captured at 10X is 940 μm. Width of fields for microscopic images captured at 40X is 230 μm. Legend: D, day; P, pregnancy; LE, luminal epithelium; GE, glandular epithelium; ST, stroma; BV, blood vessel; Tr, trophectoderm; CE, chorionic epithelium; RBC, red blood cells; SMC, smooth muscle cells. B) AQP1 mRNA expression throughout gestation in the endometrium and placenta.

The expression of AQP1 in the porcine endothelium (Figure 1) was expected because it was initially discovered in erythrocytes and later found in endothelial cells throughout the human body (Agre *et al.* 1987, Mobasheri and Marples 2004). This also explains why the mRNA expression increased because as pregnancy progresses, there is a higher requirement for nutrients to be transported to the fetus, resulting in increased angiogenesis. AQP1 is responsible for the high water permeability of the endothelium to maintain water and ion homeostasis for numerous functions, including cell differentiation, proliferation, secretion, and apoptosis (Ishibashi *et al.* 2011). Expression of AQP1 in the RBCs (Figure 1) of pigs was not unprecedented. There are two hypotheses about the function of AQP1 in the membranes of RBCs. The first suggests that AQP1 contributes to undulations or "flickering" of the cell membrane, which helps in moving the RBCs through capillaries. The second suggests that the high permeability of RBC membranes allows for concomitant displacement of water molecules when rapid exchange of ions and solutes occurs (Benga 2012). We also observed AQP1 expression in the myometrium of

pigs (Figure 1). Lindsay and Murphy (2004) have shown AQP1 in the myometrium of rats and this expression is believed play a role in decreasing the size of the uterine lumen to assist in positioning of the blastocyst during implantation (Lindsay and Murphy 2004). In rodents, the uterine lumen closes down to form an implantation chamber and previous reports have speculated that AQP1 in the myometrium could allow water into the cells, leading to swelling of the smooth muscle and closing of the lumen (Gannon *et al.* 2000). Increased expression of AQP1 in the mesometrial smooth muscle rather than the antimesometrial myometrium could initiate contraction or cause swelling, which could contribute to the antimesometrial location of the implanting blastocyst (Lindsay and Murphy 2004). Although AQP1 mRNA and protein have been previously reported to be expressed in the myometrium of pigs in explant cultures by real-time PCR and Western blot analyses, respectively (Skowronski *et al.* 2009, Skowronska *et al.* 2015), the localization of AQP1 protein in the smooth muscle cells of the porcine myometrium (Figure 1) is a novel finding. AQP1 in the smooth muscle cells of the myometrium could act similarly to rodents and allow swelling of the myometrium in order to increase contact with the conceptuses or to displace them throughout the lumen. Pope *et al.* (1986) found that porcine embryos migrated through the uterus in response to uterine contractions and in vitro studies with rat smooth muscle cells have shown that AQP1 transports hydrogen peroxide into smooth muscle cells, which then leads to hypertrophy of those cells (Ghouleh *et al.* 2013). Additionally, AQP1 has been shown to upregulate beta-catenin protein levels, which promotes smooth muscle cell proliferation in vitro (Yun *et al.* 2017).

These data suggest potential roles of AQP1 in the myometrium of pigs, but further studies are necessary to determine the effects of AQP1.

A) AQP5 Protein Expression



B) AQP5 mRNA Expression

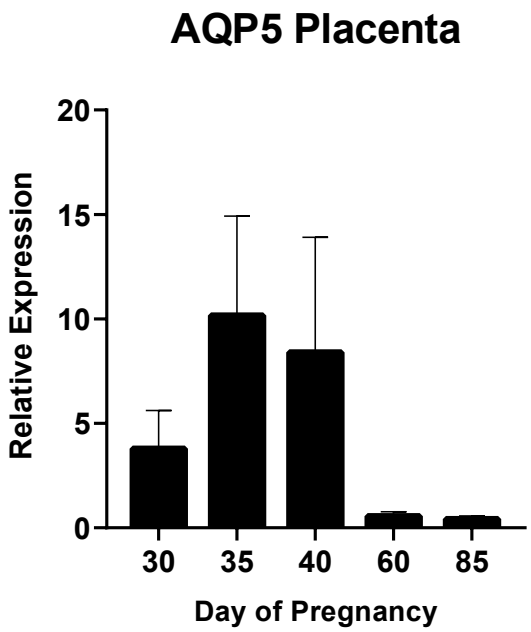
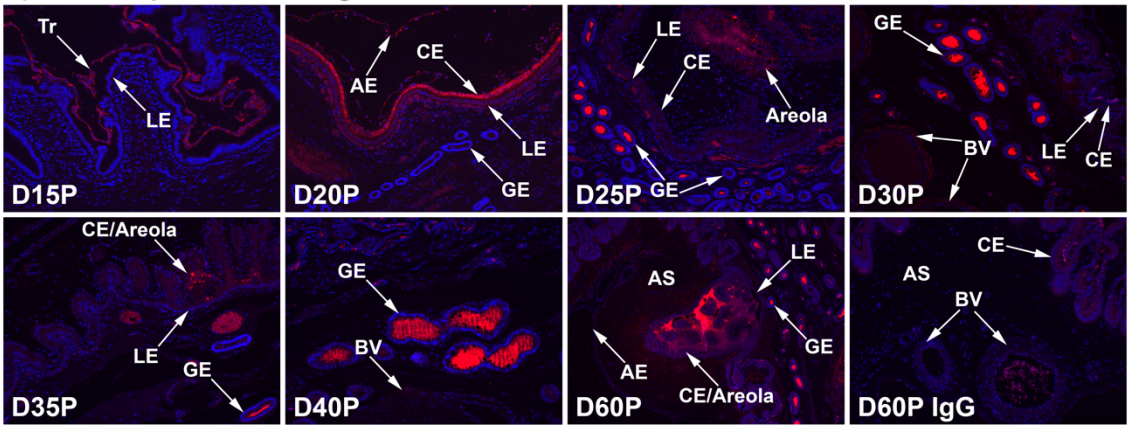


Figure 16. Aquaporin 5 protein and mRNA expression throughout gestation.
A) Immunofluorescence microscopy for aquaporin 5 (AQP5; green) at the uterine-placental interface of gilts on Days 15 (D15), 20, 25, 30, 40, 60, and 90 of pregnancy. AQP5 protein is localized to the chorionic epithelium on Day 20 and this expression is maintained through Day 90 in the areolae. Day 25 and Day 60 have serial sections stained with uteroferrin to confirm areolae. Nuclei are stained with DAPI for histologic reference.

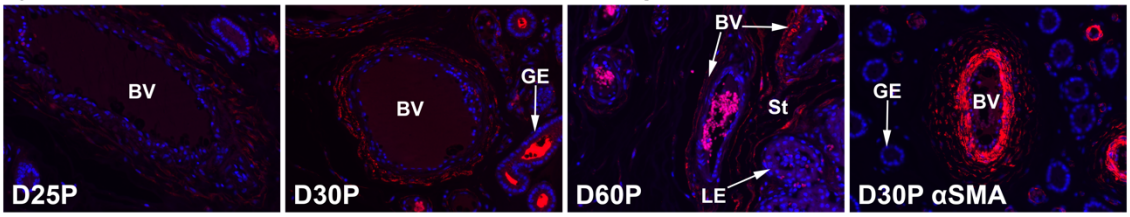
The D40 rabbit IgG (Rb IgG) panel serves as the negative control. Width of fields for microscopic images captured at 10X is 895 μm. Legend: D, day; P, pregnancy; LE, luminal epithelium; GE, glandular epithelium; Tr, trophectoderm; CE, chorionic epithelium; AE, allantoic epithelium. B) AQP5 mRNA expression throughout gestation in the placenta.

AQP5 has been described throughout the human body and, along with water, it has been proposed to transport gases and ions (Yool and Weinstein 2002, Boassa *et al.* 2006, Wang *et al.* 2007, Hub *et al.* 2009, Wang and Tajkhorshid 2010, Direito *et al.* 2016). AQP5-null mice show decreased water permeability across the alveolar membranes (Ma *et al.* 2000), suggesting an important role in water transport across membranes. Interestingly, AQP5 expression is upregulated in numerous cancers throughout the body and it is has been shown that downregulation of AQP5 leads to a higher susceptibility of apoptosis in cancer cells (Direito *et al.* 2016, Chae *et al.* 2008). In the current study, we found that AQP5 was expressed solely in the chorionic epithelium where areolae were located (Figure 2). This expression is important because areolae allow for large amounts of histotroph to be transported from mother to fetus. Because there is significant transport of water and nutrients through the areolae during gestation, it is likely that AQP5 plays a crucial role in providing the fetus with water and other nutrients necessary for growth and survival.

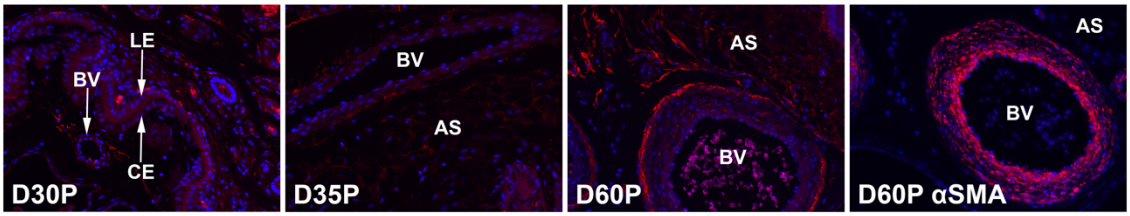
A) AQP8 Expression throughout Gestation



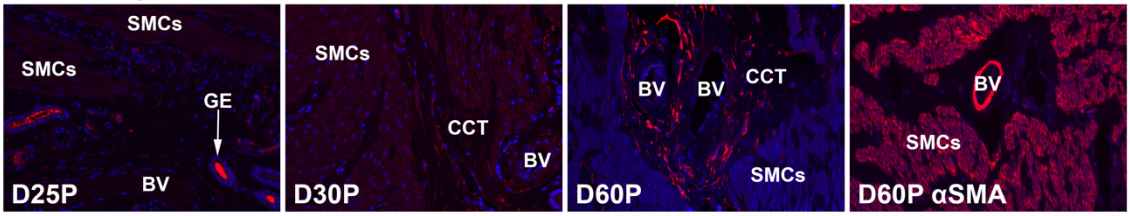
B) AQP8 Endometrial Blood Vessel and Stromal Expression



C) AQP8 Placental Blood Vessel and Stromal Expression



D) AQP8 Myometrial Expression



E) AQP8 mRNA Expression

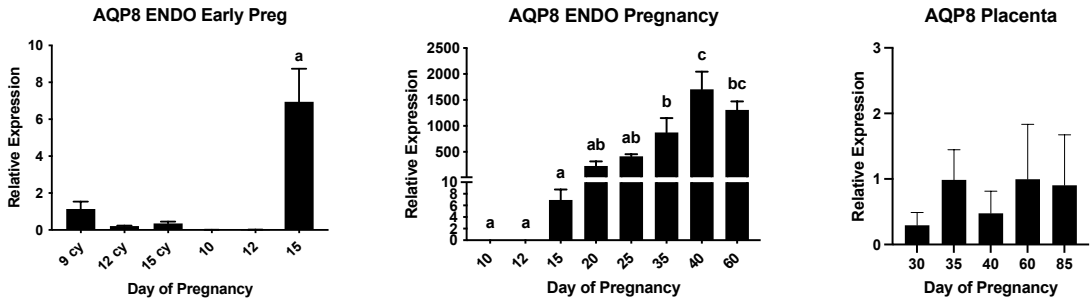


Figure 17. Aquaporin 8 protein and mRNA expression throughout gestation and specific cell-type localization.

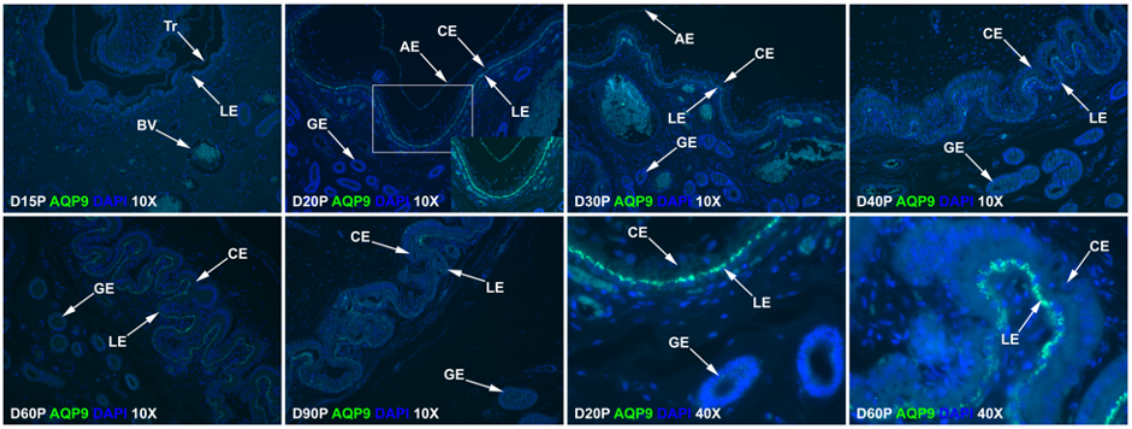
A-D) Immunofluorescence microscopy for aquaporin 8 (AQP8; red) at the uterine-placental interface of gilts on Days 15 (D15), 20, 25, 30, 35, 40, and 60 of pregnancy. AQP8 protein is localized to the trophectoderm on Days 15 and 20, the areolae, endometrial glands, and glandular epithelium beginning on Day 25, the tunica media and tunica adventitia of blood vessels within both uterine and placental tissues, and stromal cells within the allantois and endometrium beginning on Day 30 of gestation. The connective tissue within the myometrium also expressed AQP8 protein beginning on Day 25 of pregnancy. Alpha smooth muscle actin was shown to denote the smooth muscle cells within the myometrium and blood vessel walls. Nuclei are stained with DAPI for histological reference. The Day 60 mouse IgG (Ms IgG) panel serves as the negative control. Width of fields for microscopic images captured at 10X is 895 μm. Width of fields for microscopic images captured at 20X is 448 μm. Legend: D, day; P, pregnancy; LE, luminal epithelium; AE, allantoic epithelium; GE, glandular epithelium; BV, blood vessel; Tr, trophectoderm; CE, chorionic epithelium; AS, allantoic stroma; St, stroma; SMCs, smooth muscle cells; CCT, collagenous connective tissue. E) AQP8 mRNA expression throughout gestation in the endometrium and placenta.

Based on quantitative real-time PCR data, ovine conceptuses express AQP8 mRNA beginning on Day 27 of gestation and its expression is maintained throughout the remainder of gestation (Liu *et al.* 2004). Expression of AQP8 mRNA in the endometrium of mares on Day 14 of pregnancy has also been reported (Klein *et al.* 2013). However, the present results are the first to reveal the cell-specific localization of AQP8 in the uteri and

conceptuses of pigs. In addition to water, AQP8 has been shown to transport ions and ammonia, both of which are important nutrients for cell homeostasis and growth (Saparov *et al.* 2007). It has been proposed that AQP8 in the rodent uterus is responsible for shuttling water between the myometrium and stroma of the endometrium (Kobayashi *et al.* 2010), and that AQP8 in the sheep conceptus is partially responsible for the high water permeability of the placenta (Liu *et al.* 2004). Our novel results suggest that AQP8 in the porcine uterus, specifically the trophectoderm cells of the early conceptus, cells of blood vessel walls, endometrial glands, placental areolae, endometrial and allantoic stromal cells, and the allantoic epithelium may play a role similar to that in the rat uterus and sheep conceptus for the transport of water from the maternal circulation to the chorioallantois during pregnancy. In addition, the localization of AQP8 protein in epithelial cells of the amnion and allantois of canines has been suggested to mediate fluid transfer across fetal membranes (Aralla *et al.* 2012). There are also dynamic changes in allantoic fluid volume in pigs throughout pregnancy and those changes may be responsible for expanding the chorioallantoic membranes and allowing them to establish intimate contact with a maximum amount of endometrial surface area (Knight *et al.* 1977). This agrees with our immunofluorescence results showing AQP8 protein in the allantois of pigs during later pregnancy (Figure 2) and suggests that it is involved in water and ammonia transport within the placenta. The increase in mRNA expression supports this idea that AQP8 is involved in water and nutrient transport because the expression increased as pregnancy progressed. Lastly, the localization of AQP8 protein in the placental areolae associated with the openings of the uterine glands in the pig endometrium is novel and important

because there is substantial transport of water and other nutrients through the areolae throughout pregnancy (Bazer *et al.* 2012). We propose that AQP8 is at least partly responsible for the transport of water and other nutrients through the chorionic epithelium of the areolae.

A) AQP9 Protein Expression



B) AQP9 mRNA Expression

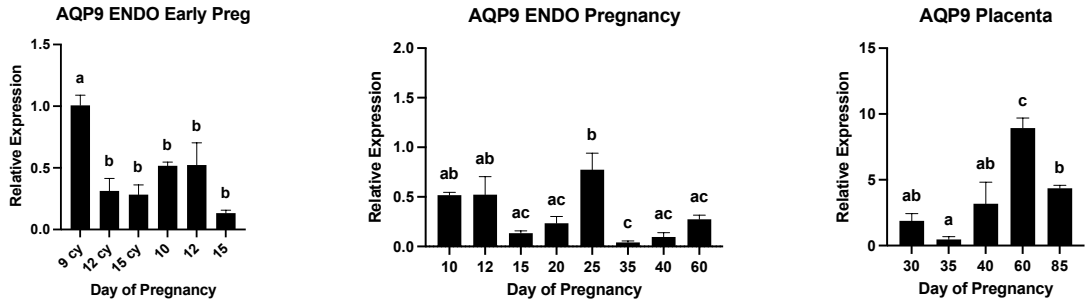


Figure 18. Aquaporin 9 protein and mRNA expression throughout gestation.

A) Immunofluorescence microscopy for aquaporin 9 (AQP9; green) at the uterine-placental interface of gilts on Days 15 (D15), 20, 30, 40, 60 and 90 of pregnancy. AQP9 protein is localized primarily to the uterine luminal epithelium (LE) throughout gestation, changing from an apical expression at the beginning of gestation to a more basal expression around Day 40. AQP9 is also expressed in the allantoic epithelium on Day 20 and decreases thereafter. It is also localized to the glandular epithelium starting on Day 20

and maintains low expression throughout gestation. Nuclei are stained with DAPI for histologic reference. The D60 rabbit IgG (Rb IgG) panel serves as the negative control. Width of fields for microscopic images captured at 10X is 895 μm. Width of fields for microscopic images captured at 20X is 448 μm. Width of fields for microscopic images captured at 40X is 224 μm. Legend: D, day; P, pregnancy; LE, luminal epithelium; GE, glandular epithelium; Tr, trophectoderm; BV, blood vessel; CE, chorionic epithelium; AE, allantoic epithelium. B) AQP9 mRNA expression throughout gestation in the endometrium and placenta.

Aquaporin 9 has been investigated in a number of species for multiple roles (Ducza *et al.* 2017; Prat *et al.* 2012). Here, we focus primarily on the expression of AQP9 protein at the porcine uterine-placental interface and its novel localization within the endometrial LE, GE, and allantoic epithelium. The expression of AQP9 protein in the uterine GE is important because the GE is responsible for the secretion of histotroph, which is critical for the developing conceptus (Bazer *et al.* 2012). Because the uterine GE expresses AQP9 protein, certain solutes like glycerol (a product of lipolysis) and lactate (a product of glycolysis) are able to be transported from the maternal endometrium to the conceptus which is able to utilize these nutrients due to its expression in the allantoic epithelium. Glycerol and lactate are important for many biological processes and along with water provide essential nutrients for the growth and development of the conceptus (Maciolek *et al.* 2014; Xue *et al.* 2017). It has been reported that, in pigs, there are two periods of fluid accumulation in the allantoic sac, the first being between Days 20 and 30 of gestation, and the second between Days 40 and 60. This is accompanied with an increase in protein within the allantoic sac, followed by a decline in both protein and fluid volume to Day 100 (Knight *et al.* 1977). Interestingly, we observed a spatial-temporal localization of AQP9

protein in the uterine LE that suggests AQP9 may play a role in the accumulation and subsequent decrease in fluid volume at these time points. The mRNA data agrees with the idea of changing the expression pattern because there is an increase in expression from Day 20 to 25 in the endometrium when fluid accumulation in the conceptus is increasing and a subsequent decrease in mRNA expression on Day 35, when there is a corresponding decrease in fluid accumulation within the conceptus (Bazer and Johnson 2014). During the period of fluid accumulation within the allantoic sac, AQP9 protein is expressed at the apical surface of the uterine LE, typical of a secretory phenotype. As the allantoic fluid volume decreases, the location of the expression of AQP9 protein changes to the basal surface of the uterine LE, consistent with an epithelium that absorbs fluid from the lumen. This novel localization may provide a mechanism to explain the transportation of water and other nutrients from the porcine maternal endometrium to the conceptus, and then back from the conceptus to the endometrium. This is also supported by our AQP1, AQP5, and AQP8 results. Therefore, using AQP1, AQP5, AQP8, and AQP9, cells can potentially transport water and other nutrients from the uterine vasculature, through the tunica intima, tunica media, and tunica adventitia, across the uterine LE and chorion, and either through the tunica adventitia, tunica media, and tunica intima of placental blood vessels or across the allantoic stroma and into the allantois, via the allantoic epithelium, for utilization within the stromal compartment of the placenta (Figure 4). Indeed, the reverse is also possible, and may explain the changing volumes of allantoic fluid and hydration of placental connective tissues during pregnancy in pigs (Knight *et al.* 1977).

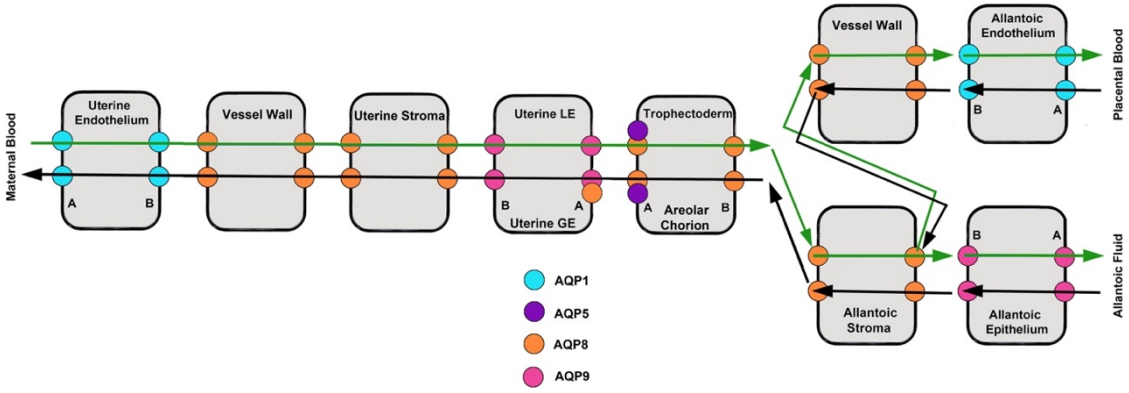


Figure 19. Model depicting the different cell layers in epitheliochorial placentation and where each aquaporin is located.

Model depicting the different cell layers in epitheliochorial placentation and where each aquaporin is located. Based on these data, transportation of water and other nutrients can cross each layer from the mother to fetus as a result of 1) AQP1 expression in the uterine endothelium; 2) AQP5 expression in the trophectoderm and areolar chorionic epithelium; 3) AQP8 expression localized to the endometrial vessel walls, the endometrial stroma, the conceptus trophectoderm and areolar chorionic epithelium, the allantoic stroma, and finally the placental vessel walls; 4) AQP9 expression within the luminal epithelium and the allantoic epithelium; and 5) AQP1 expression localized to the placental endothelium for transport into the fetal circulation.

In conclusion, our results reveal the temporal cell-type specific expression of AQP 1, 5, 8, and 9 proteins within the porcine uterus and placenta during gestation. The expression of these AQPs in the endometrium and myometrium of the uterus, and the chorioallantois is likely key to the rapid changes in volumes of the fetal fluids that are critical for implantation and placentation in pigs. These findings provide new knowledge and insight about the transport of water and other nutrients from mother to fetus in support of conceptus growth, development, and survival in mammals in general, and pigs in particular.

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