CHARACTERIZATION OF INTEGRINS AND OSTEOPONTIN AT THE UTERINE-PLACENTAL INTERFACE DURING PREGNANCY IN PIGS AND SHEEP

A Dissertation

by

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ABSTRACT

Establishment of a successful pregnancy is dependent upon proper communication between the conceptus and uterus and provides the critical physiological and anatomical groundwork required to maintain growth and development of the conceptus throughout pregnancy. Osteopontin (OPN) is a soluble extracellular matrix (ECM) protein that interacts with multiple integrin receptors. Integrins are integral membrane proteins that promote cell-to-cell and cell-to-ECM adhesion, cytoskeletal reorganization, and signal transduction through a structure known as a focal adhesion (FA). Osteopontin, and multiple integrins that bind OPN, are observed on the apical surfaces of both trophectoderm (Tr) and uterine luminal epithelium (LE) during pregnancy in pigs and sheep. During the period of implantation in these species, conceptuses elongate and attach to the uterine LE. Placentation creates a unique physiological phenomenon wherein the apical surfaces of two epithelia adhere to one another for an extended period of time. Research for this dissertation investigated interactions between integrins and OPN at this unique interface. We hypothesized that FAs form in response to mechanical stress at the uterine-placental interface and utilize OPN as a bridging ligand to anchor placenta and uterine LE together in pigs and sheep during pregnancy. In the pig ITGAV and ITGB3 subunit proteins aggregate *in vivo* to form FAs during the peri-implantation period of pregnancy. As the uterine-placental interface becomes more folded during placentation, these FAs are no longer observed. We hypothesize that FAs are no longer necessary because folding disperses forces exerted across this interface. In the sheep,

FAs containing integrins ITGAV, ITGA4, ITGA5, ITGB1, and ITGB5, which co-localize with OPN, increase in quantity and organization at the uterine-placental interface as pregnancy progresses. However, ITGB3 is only present during the peri-implantation period of pregnancy. The *in vivo* knockdown of ITGB3 subunit leads to a smaller fetus with decreased OPN and nitric oxide synthase 3 (NOS3) mRNA expression in the placenta of growth retarded fetuses. Additionally, OPN is localized near vascular networks in the placenta during normal sheep pregnancy. These results suggest that delayed embryonic growth in sheep due to ITGB3 knockdown is related to impaired vascular function and/or angiogenesis. In conclusion, integrins and OPN play a crucial role in maintaining attachment of Tr to uterine LE at the uterine-placental interface in response to mechanical forces which are required for normal fetal-placental growth during pregnancy in pigs and sheep.

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CHAPTER I

INTRODUCTION

Implantation and placentation are critical events in pregnancy. Implantation failure during the first three weeks of pregnancy is a major cause of infertility in all mammals (Flint *et al.* 1982, Bazer *et al.* 1983, Johnson *et al.* 2009). Communication and reciprocal responses between the conceptus (embryo/fetus and associated placental membranes) and uterus are essential for conceptus survival throughout pregnancy. The interaction between these two tissues provides the critical physiological and anatomical groundwork for the development of functional uterine luminal epithelium (LE), glandular eptithelium (GE), stroma and placenta required to maintain growth and development of the conceptus throughout pregnancy. The establishment and maintenance of pregnancy in the pig and sheep requires: 1) secretion of estrogen or interferon tau, respectively, from the conceptus to signal pregnancy recognition; 2) secretions from the uterine LE and GE to support attachment, development and growth of the conceptus and 3) cellular remodeling at the maternal-conceptus interface to allow for attachment during 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.* 2001, Burton *et al.* 2002, Burghardt *et al.* 2002). All of these events are orchestrated through endocrine, paracrine, autocrine, and juxtacrine signaling between the conceptus and uterus. The complexity of these events alludes to the high rates of conceptus mortality during the peri-implantation period of pregnancy in these and other species (Bazer *et al.* 1983, Johnson *et al.* 2009).

Osteopontin is a soluble extracellular matrix (ECM) protein that has the ability to bind to multiple integrin receptors (Hynes *et al.* 1987, Dengardt *et al.* 1993, Senger *et. al* 1994, Butler *et al.* 1996, Sodek *et al.* 2000). Integrins are integral membrane proteins composed of an α and β subunits that promote cell to cell and cell to ECM adhesion, cause cytoskeletal reorganization to stabilize adhesion, and transduce signals through a multitude of signaling intermediates (Giancotti *et al.* 1999, Burghardt *et al.* 2002). Integrin-mediated adhesion is focused within a primary mechanotransduction unit of dynamic structure and composition known as a focal adhesion (FA). The size, composition, cell signaling activity and adhesion strength of these FAs is force dependent (Hynes *et al.* 1987, Vogel *et al.* 2006). Integrins have been found to be dominant glycoproteins in many cell adhesion cascades, including well defined roles in leukocyte adhesion to the apical surface of polarized endothelium for extravasation of leukocytes from the vasculature into the surrounding connective tissue (Kling *et al.* 1992). A similar adhesion cascade involving interactions between the ECM and apically expressed integrin receptors on the uterine LE and conceptus trophectoderm (Tr) is proposed as a mechanism for attachment of the conceptus to the uterus for implantation; the initial step for the extensive tissue remodeling that occurs during placentation (Aplin *et al.* 1994). OPN is a leading candidate adhesion molecule for implantation in pigs and sheep (Johnson *et al.* 2014).

It is generally accepted that OPN interacts with apically expressed integrin receptors on uterine LE and conceptus Tr to attach the conceptus to the uterine LE for implantation. Research conducted with pigs and sheep has significantly advanced understanding of the

role(s) of OPN during implantation through exploitation of the prolonged peri-implantation period of pregnancy when elongating conceptuses are free within the uterine lumen requiring extensive paracrine signaling between conceptus and endometrium. This is followed by a protracted and incremental attachment cascade of Tr to uterine LE during implantation, and development of a true epitheliochorial or synepitheliochorial placenta exhibited by pigs and sheep, respectively.

During the period of implantation in pigs, conceptuses elongate and attach to the uterine LE resulting in the juxtaposition of the maternal and fetal micro-vasculatures in order to transport nutrients from the uterus to the developing embryo/fetus. On approximately Day 11 of pregnancy, during the peri-implantation period, rapidly elongating conceptuses begin to secrete estrogen, the maternal recognition signal of pregnancy. Estrogen acts on the uterine epithelia to redirect secretion of PGF2α from the uterine vasculature into the uterine lumen where it is metabolized to allow for maintenance of the CL (Bazer *et al.*1977, Frank *et al.*1977, Spencer *et al.*2004). Besides acting as an antiluteolytic factor during the peri-implantation period in pigs, estrogen has been shown to regulate the expression of OPN in the uterine LE (Garlow *et al.* 2002). OPN mRNA is initially detected in discrete regions of the uterine LE juxtaposed to the conceptus just prior to implantation on Day 13 (White *et al.*2005). OPN expression then expands to the entire uterine LE by Day 20 when firm adhesion of Tr to uterine LE occurs (Garlow *et al.*2002). OPN protein is abundantly present along the apical surface of LE, on conceptus Tr, and along the entire uterine-placental interface during pregnancy (White *et al.*2005). This interface expresses integrin subunits that potentially form heterodimer

receptors that bind OPN, including αvβ3, αvβ1, αvβ5, α4β1, and α5β1 (Burghardt *et al.* 1997*).* It is hypothesized that OPN secreted from uterine LE binds integrin receptors on Tr and uterine LE. This interaction then stimulates changes in Tr morphology and mediates adhesion between Tr and LE that are essential for implantation. Recently a porcine trophoblast cell line (pTr2) and primary porcine uterine epithelial (pUE) cells were used in a series of experiments to examine interactions between membrane integrin receptors and OPN. Affinity chromatography and immunoprecipitation demonstrated binding of OPN to the ITGAV and ITGB6 subunits, and similar experiments using primary porcine uterine epithelial (pUE) cells demonstrated binding of ITGAV and ITGB3 subunits to OPN (Erikson *et al.* 2009). Therefore we hypothesize that OPN selectively binds the αvβ6 integrin heterodimer receptor on conceptus Tr cells and αvβ3 on uterine LE cells. Studies performed for this dissertation sought to better understand the role(s) that integrin receptors αvβ3 and αvβ6, and OPN, play in binding conceptus Tr to uterine LE during the peri-implantation period of pregnancy in pigs. Temporal and spatial mRNA and protein expression demonstrated that although mRNA did not change at the uterine-placental interface, ITGAV and ITGB3, proteins aggregate *in vivo* with OPN to form FAs during the peri-implantation period. As the uterine-placental interface becomes more folded with advancing placentation, these FAs are no longer observed. We hypothesize that these FAs are no longer necessary because this folding disperses forces, including tension, compression, and/or shear, exerted against this interface and there is no longer a need for these FA structures to maintain integrity of attachment between uterus and placenta. Therefore, FA assembly at the uterine-placental interface is

dynamic and specific for a given tissue compartment niche. These studies also determined that knockdown of expression of the ITGAV subunit decreases the ability of porcine Tr cells to bind OPN *in vitro*. These results are the first to directly confirm, in any species, that the ITGAV subunit mediates binding of Tr cells to OPN, and suggests that ITGAV plays a critical role in binding OPN to mediate implantation of the conceptus to the uterine LE in pigs and perhaps other species. Sheep show a very different pattern of FA assembly as FAs increase as pregnancy progresses.

Implantation in sheep involves the apposition and attachment of the elongating conceptus Tr to the uterine LE. Sheep embryos enter the uterus on Day 3, develop to spherical blastocysts and then, after hatching from the zona pellucida, transform from spherical to tubular and filamentous conceptuses between Days 12 and 15 of pregnancy, with extra-embryonic membranes extending into the contralateral uterine horn between Days 16 and 20. During this period of rapid elongation, the mononuclear trophoblast cells of ovine conceptuses secrete interferon tau between Days 10 and 21 of pregnancy, and implantation begins on Day 16 as Tr attaches to the uterine LE (Godkin *et al.* 1984, Spencer *et al.* 2004). In sheep, OPN expression is induced in the GE by progesterone, the hormone of pregnancy (Johnson *et al*. 2000). OPN is then secreted into the uterine lumen where it binds to integrin receptors on conceptus Tr and LE and serves as a bridging ligand that is hypothesized to mediate changes in proliferation, migration, survival, adhesion and remodeling of the conceptus as it elongates, apposes and adheres to the uterine LE (Johnson *et al.*1999, Johnson *et al.*2003). During the period of implantation in sheep, integrins ITGAB, ITGA4, ITGA5, ITGB1, ITGB3 and ITGB5 are

expressed on the apical surface of conceptus Tr and uterine LE *in vivo* (Johnson *et al.*2000). These integrins are constitutively expressed, so receptivity to implantation is believed to be dependent on changes in the expression of ECM proteins, such as OPN, and not temporal or cell-specific patterns of change in expression of integrins (Johnson *et al.*2001). OPN protein is prominent at the uterine-placental interface during the peri-implantation period in ewes. Studies performed for this dissertation comprehensively examined the expression of integrin subunit proteins at the uterine-placental interface throughout pregnancy in the sheep. Results show that multiple integrin subunits are distributed evenly along the entire uterine-placental interface through Day 15 (during attachment for implantation) in a pattern that suggests adhesion, but a lack of assembly into large FAs; whereas by Day 20, a period of instability in the integrity of the endometrial LE that developmentally demarks implantation from placentation, these integrins are found in large intermittent aggregates along the interface. FAs containing integrin subunits ITGAV, ITGA4, ITGA5, ITGB1, and ITGB5 increase in quantity and become more organized at the uterine-placental interface as pregnancy progresses; however ITGB3 subunit is only present during the peri-implantation period of pregnancy suggesting that it is playing a crucial role in initial attachment of the conceptus to the uterine LE. Further, these studies were the first to co-localize OPN with the integrin subunits ITGAV, ITGA4, ITGA5, ITGB1, and ITGB5 and OPN to the large FAs that form at the uterine-placental interface of sheep. These results are the strongest indication, to date, that OPN and integrins interact with one-another at the uterine-

placental interface of sheep and that these interactions are dynamic, changing as the tissue compartment adapts to internal and external forces generated by pregnancy. .

Previous work by our group identified specific integrins that bind to OPN in ovine Tr cells and determined the functional effects of OPN binding to these cells. Using affinity chromatography and immunoprecipitation, it was determined that integrins ITGAV, ITGB3 and ITGA5 directly bound OPN on the surface of ovine Tr cells (Kim *et al.*2010). Because ITGA5 is the only known subunit to dimerize with ITGB1, it was concluded that αvβ3 and α5β1 receptors on ovine Tr cells bind OPN to mediate attachment and implantation (Kim *et al.*2010). Our group examined the effects of OPN on adhesion, migration and proliferation of ovine Tr cells *in vitro* and found that OPN increased adhesion and migration of ovine Tr cells in a dose dependent manner, but did not affect cell proliferation. Whereas the effect of OPN on Tr cell migration was modest, OPN supported very high levels of Tr cell adhesion (Kim *et al.*2010). The previous results were obtained from *in vitro* experiments, but a goal of the studies performed for this dissertation was to begin to assess the *in vivo* functional importance of the integrin receptor αvβ3 in mediating elongation and attachment of the sheep conceptus to the uterine LE. Therefore we performed loss of function by blocking mRNA translation for Tr-expressed ITGB3 by infusing morpholino antisense oligonucleotides (MAOs) into the uterine lumen of pregnant ewes to assess effects on conceptus elongation and attachment for implantation. Results indicated that sheep conceptuses elongate and implant to the uterine wall in the absence of ITGB3 subunit expression on the Tr. Loss of ITGB3 subunit did, however, cause fetal growth retardation when compared to intact controls.

Further, expression of both OPN and nitric oxide synthase 3 (NOS3) were decreased in the placenta of growth retarded fetuses, suggesting that the delayed growth is related to impaired vascular function. Examination of temporal and spatial expression of OPN mRNA and protein in the placenta of pregnant sheep revealed localization of OPN near vascular networks. Collectively these results suggest that delayed embryonic growth is related to impaired vascular function and/or angiogenesis as noted by the decrease in NOS3, and that activation of integrin receptors containing the ITGB3 subunit decreases placental production of OPN leading to fetal growth restriction due to decreased nutrient transfer to the growing conceptus.

CHAPTER II

LITERATURE REVIEW

Justification

Establishment of a successful pregnancy is dependent upon proper communication between the conceptus (embryo and associated placental membranes) and uterus during the peri-implantation period and throughout pregnancy. Paracrine secretions from the conceptus signal to the uterus that pregnancy has been initiated and the uterus responds by continuing to develop an embryotrophic environment that supports conceptus elongation, implantation, and subsequent development of the placenta. These initial events are crucial, and two-thirds of the 20 to 30 percent of pregnancy loss in most mammals occurs during the peri-implantation period of pregnancy. Integrins are dominant glycoproteins in many cell adhesion cascades, and implantation can be characterized as an adhesion cascade involving interactions between the extracellular matrix and apically expressed integrin receptors on the uterine luminal epithelium (LE) and conceptus trophectoderm (Tr) for attachment of the conceptus to the uterus for implantation; the initial step in the extensive tissue remodeling that occurs during placentation. OPN is a leading candidate adhesion molecule for implantation in pigs and sheep.

Integrin Biology

Integrins are a family of cation-dependent heterodimeric intrinsic membrane glycoproteins composed of non-covalently linked α and β subunits that bind to various extracellular matrix (ECM) components and cell adhesion molecules (Giancotti *et al.*1999). Both integrin subunits are composed of an N-terminal domain that is present outside of the cell; this is the longest section of the subunit and encompasses the ligand binding domain. The N-terminal domain of the β subunit is tightly folded and contains internal disulfide loops that contribute to the ligand binding domain of this subunit; whereas, the α subunit contains seven-fold repeats of a homozygous segment where the last three to four repeats contribute to the divalent cation binding region (Gailit *et al.*1988, Kirchhofer *et al.*1990, Calvete *et al.*1991). The binding of divalent cations to the ligand binding domain of the α subunit is necessary for the receptor to bind its specific ligand. Both subunits contribute to the specificity of ligand binding that lies near the interface of the two subunits. This is supported by the observation that changing either subunit can affect the ligand for which the receptor will have the greater affinity. The head of the subunit, that is present at the N-terminal domain, is supported by a stalk that contains extensive disulfide bonds in both the α and β subunits (Calvete *et al.*1989, Calvete *et al.*1991). The stalk traverses through the hydrophobic membrane in its membrane spanning region which gives rise to the cytoplasmic tail. The cytoplasmic portion of the integrin subunit is relatively small in most integrins. The cytoplasmic tail has the ability to attach the receptor to the actin cytoskeleton and several signaling proteins which allow integrin receptors to mediate cytoskeletal reorganization and

transduce cellular signals (Miyamoto *et al.*1995). Integrin receptors are normally present in an inactive conformation when their ligand binding domain is not exposed and thus cannot bind ligands. This inactivated state is due to the formation of a weak clasp by the interaction of the cytoplasmic tails of the α and β subunits. Upon binding of talin to the cytoplasmic tail, interactions between the cytoplasmic tail of the α and β subunits are disrupted causing the integrin receptor to change conformation to a high affinity state by exposing its ligand binding receptor so that it can bind ligands (Larjava *et al.*2008). Besides their ability to mediate cytoskeletal reorganization and transduce cellular signals, integrins are also involved in mediating adhesion, migration, and invasion of cells (Miyamoto *et al.*1995).

Focal adhesions (FA) are specialized sites that allow for interactions between the ECM and actin cytoskeleton through integrin receptors. Within these FAs, integrins cluster into supramolecular complexes with structural cytoskeletal proteins like talin and α-actinin, as well as numerous signaling molecules, including c-Src and focal adhesion kinase (FAK) (Burridge *et al.*1996). These FA complexes act to transmit force placed on the cell by the ECM at sites of adhesion, or vice versa, and serve as signaling centers where cell signaling pathways involved in cell growth, proliferation, survival, gene expression, development, tissue repair, migration, and invasion are activated. Studies with cultured cells identified specific proteins that associate with FAs depending on the composition and rigidity of the ECM along with the degree of force put on the cell by external or internal stressors (Katz *et al.*2000, Galbraith *et al.*2002). The ability of FAs to be maintained as stable structures is dependent on local force from either the outside

or inside of the cell (Bershadsky *et al.*2006). The effects that rigidity of the ECM have on cells has been elucidated in tumor cells and mesenchymal stem cells. For example, proliferation of tumor cells is correlated positively with an increase in the rigidity of the ECM. This increase in matrix rigidity causes activation of Rho that induces Rho generated cytoskeletal tension through myosin II, which activates the ERK-dependent growth of tumor cells (Wang *et al.*1998, Wozniak *et al.*2003). Pertaining to human mesenchymal stem cells, Engler *et al.* (2006) reported that increasing matrix stiffness directs these cells to differentiate along either neuronal, muscle, or bone cell lineages.

Focal adhesions play a major role in migrating cells. In this capacity, the FA interacts with the actin cytoskeleton to give the cell traction as it migrates along an ECM. At the leading edge of the migrating cells, there are nascent, immature, focal complexes that form and then mature into FAs as the cells become stably attached to the ECM and more force is exerted on the focal complex. Actin filaments are tethered to integrins through cross-linking proteins such as talin and vinculin. Talin binds the β integrin subunit through its FERM domain present on its globular N-terminal domain. It then attaches to actin through its multiple actin binding domains present on its flexible rod segment (Hemmings *et al.*1996, Calderwood *et al.*2002). This adhesion of integrin receptors to the actin cytoskeleton can stabilize the binding of talin to vinculin, which then binds to actin. It is through cross linking of talin with vinculin that the integrity of this bond increases. FAs are also involved in actin nucleation and branching; which is key to cell migration. FAK has the ability to act on phosphatidylinositol 3-kinase (PI3K) which induces downstream activation of a guanine nucleotide exchange factor (GEF),

such as Rho associated kinase (ROCK). Downstream of ROCK, cell signaling can activate actin-related protein 2/3 (Arp 2/3) which causes actin branching that, along with actin nucleation, aides in outgrowth of the cytoplasm responsible for movement at the leading edge of a migrating cell (Larsen *et al.* 2006).

Focal adhesions are also linked to signals associated with active transcription in cells. Integrins can associate with FAK through cross-linking proteins talin and vinculin. FAK is a protein tyrosine kinase that has tyrosine residues at positions 397 and 925 that can be phosphorylated and interact with a plethora of different signaling molecules. Following activation of FAK, the Tyr-397 becomes autophosphorylated which results in an SH2 docking site that can bind Src family kinases (Schlaepfer *et al.*1994). Src can then, through binding with Tyr-397, cause phosphorylation of Tyr-925 which allows for the association of growth factor receptor-bound protein 2 (Grb2). Through associating with the nucleotide exchange factor Sos and the small GTP-binding protein Ras, this complex has the ability to activate ERK 1/2 (Schlaepfer *et al.*1994). ERK is known to affect a wide variety of cellular pathways associated with transcriptional regulation.

Osteopontin Biology

Osteopontin (OPN) is a member of the small integrin-binding ligand *N*-linked glycoprotein (SIBLING) family of proteins. Included in this family are bone sialoprotein, dentin matrix protein I, dentin sialophosphoprotein, and matrix extracellular phosphoglycoprotein. These proteins are the product of five clusters of genes on human chromosome 4 (Fisher *et al.* 2001). Osteopontin has been observed in many systems in

the body and has multiple different roles including: a transformation-specific phosphoprotein named 2ar, as an ECM protein of bone with the potential to bridge between cells and hydroxyapatite, and as a cytokine produced by activated lymphocytes and macrophages called early T-cell activation factor 1 (Eta-1) (Senger *et al.*1979, Franzen *et al.*1985, Fisher *et al.*1985, Oldberg *et al.*1987, Prince *et al.*1987, Craig *et al.*1989, Patarca *et al.*1989, Zhang *et al.*1990).

OPN is a monomer that ranges in length from 264 to 301 amino acids and has the ability to undergo tremendous post-translational modification which includes phosphorylation, glycosylation and cleavage (Figure 2-1). This cleavage can result in variants with a molecular mass ranging from 25 to 75 kDa. OPN is made up of a hydrophobic leader sequence characteristic of a secreted protein, a potential calcium phosphate apatite binding region of consecutive asparagine residues, a cell attachment GRGDS sequence, a thrombin cleavage site and two glutamines which give OPN the ability to form monomers through transglutaminase-supported multimer formation (Butler *et al* 1996). The genes that encode OPN have been cloned from eight species to date, including, rat, mouse, human, cow, chicken, rabbit, pig and sheep (Craig *et al.*1989, Kiefer *et al.*1989, Wrana *et al.*1989, Kerr *et al.*1991, Moore *et al.*1991, Tezuka *et al.*1992, Johnson *et al.*1999). When compared between species OPN only has moderate conservation except in the two termini and the GRGDS integrin binging domain (Butler *et al.* 1996). The sequences that are highly conserved among species include: 4 of the 9 or 10 residues in the poly-Asp region, the GRGDS sequence, 15 serine residues that include the SSEEK sequence (residues 26-30), three threonines

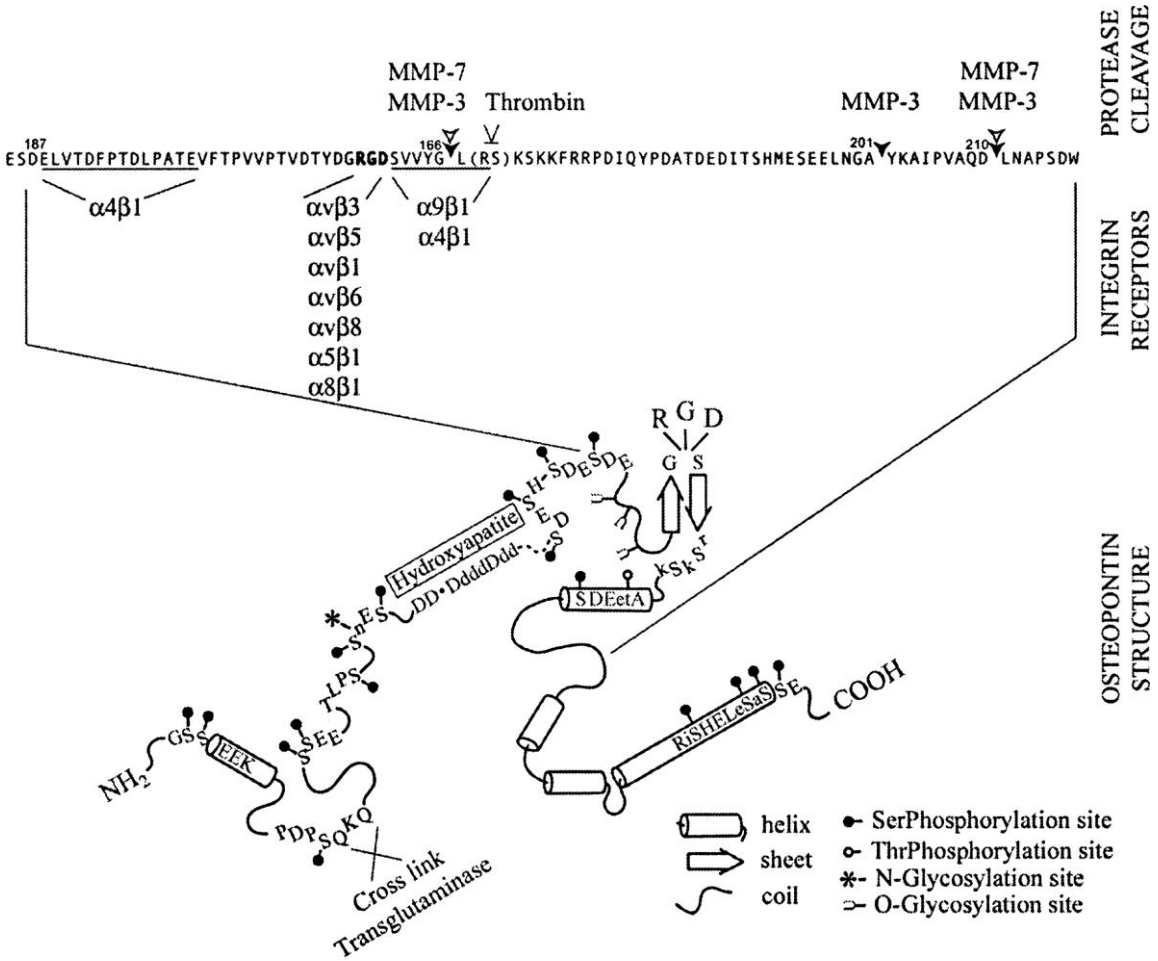

Figure 2-1. Structure of OPN (Johnson *et al*. 2011)

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(Tanaka *et al.* 1993, Zohar *et al.*1998, Katagiri *et al.*1999) and glutamines at positions 50 and 52 (Butler *et al* 1996). Differing from other species, both bovine and ovine OPN have a 22 amino acid deletion that would otherwise be inserted between residues 196 and 197 as well as a replacement of the RS with a KS thrombin cleavage site (Butler *et al* 1996).

As previously mentioned, OPN has the ability to go through numerous post-translational modifications that are believed to be important to its function. These modifications involve, proteolytic cleavage, phosphorylation on serine and threonine residues, glycosylation with primarily *O*-linked oligosaccharides, and cross-linking with self and other macromolecules through transglutaminase and sulfation (Fresno *et al.*1981, Nagata *et al.*1989, Prince *et al.*1987, Prince *et al.*1991, Weber *et al.*1996, Kaartinen *et al.*1999, Agnihotri *et al.*2001). OPN was first discovered in bone and has been found to be present in the epithelial cells and in secretions of the gastrointestinal tract, kidneys, thyroid, breast, uterus, placenta and testes (Franzen *et al.*1985, Senger *et al.*1989, Yound *et al.*1990, Tezuka *et al.*1992, Brown *et al.*1992, Kohri *et al.*1993, Daiter *et al.*1996, Johnson *et al.*1999, Luedtke *et al.* 2002). OPN is also expressed by leukocytes, smooth muscle cells and highly metastatic cancers (Ashkar *et al.*2000, Malyankar *et al.*2000, Hotte *et al.*2002). There are a variety of actions attributed to OPN including: 1) stimulation of cell-cell adhesion, 2) increasing cell-ECM communication, 3) promotion of migration of immune cells, 4) decreasing cell death by reducing reactive oxygen species and nitric oxide production by injured tissues, 5) stimulation of immunoglobulin production by B cells, 6) induction of changes in the phosphorylation state of FA kinase

and paxillin, 7) stimulation of phosphatidylinositol 3'-kinase activity, 8) altering intracellular calcium levels, and 9) affecting tissue mineralization and promotion of calcium phosphate deposition in bone (Leibson *et al.*1981, Nabel *et al.*1981, Flores *et al.*1989, Butler *et al.*1989, Singh *et al.*1990, Miyauchi *et al.*1991, Hwang *et al.*1994, Hruska *et al.*1995, McKee *et al.*1995, Goldberg *et al.*1995, Weber *et al.*1996). Expression of OPN has been shown to increase in response to interleukin (IL)-1α and 1β, transforming growth factor β1, fibroblast growth factor, tumor necrosis factor α, IFNγ, estrogen, progesterone, glucocorticoids, and 1,25-digydroxy vitamin D3 (Craig *et al.*1991, Kreiss *et al.*1993, Mukherjee *et al.*1995, Singh *et al.*1995, Omigbodun *et al.*1997, Rittling *et al.*1997, Safran *et al.*1998, Johnson *et al*. 2000, White *et al*. 2005).

Contained within OPN is a GRGDS amino acid sequence that allows OPN to bind with cell surface integrin receptors to mediate cell adhesion, migration, differentiation, survival and immune function (Hynes *et al.*1987, Ruoslahti *et al.*1987, Somerman *et.al.*1989, Sauk *et. al.*1990, Miyauchi *et al.* 1991, Ross *et al.*1993, Ruoslahti *et al.*1994, Bautista *et al.*1995). Integrins are integral membrane proteins that belong to a ubiquitous family of cation-dependent heterodimeric intrinsic transmembrane glycoprotein receptors composed of noncovalently bound α and β subunits that are known to be involved in cell-cell and cell-ECM interactions such as adhesion which can cause cytoskeletal reorganization which stabilizes adhesion and they can transduce cell signaling through a variety of signaling intermediates (Yoshinaga *et al.*1989, Burghardt *et al.*1997, Giancotti *et al.*1999). Integrin receptor αvβ3 has long since been considered the main binding partner of OPN through OPNs RGD integrin binding domain. Recent

work has shown that there are multiple different integrin receptors that can bind OPN through alternate binding sequences. The αvβ1, αvβ5 and α8β1 integrins have affinities for the RGD motif of OPN, similar to αvβ3 (Hu *et al.*1995, Denda *et al.*1998). There is also non-RGD-mediated integrin binding of OPN which is illustrated by the dual α4β1 binding sites through which OPN promotes leukocyte adhesion (Bayless *et al.*1998, Bayless *et al.*2001). Integrin receptor α9β1 has the ability to bind OPN through a cryptic binding site (Smith *et al.*1996). OPN can also serve as an extracellular ligand for certain splice variants of the cell surface proteoglycan CD44 (CD44v3-v6), where it can serve as a cytokine immobilized on the luminal surface of endothelial cells to recruit leukocytes and trigger conversion of integrin expression to an active adherence configuration (Weber *et al.*1990, Tanaka *et al.*1993, Weber *et al.*1996). These CD44 variants can require the cooperation of ITGB1 subunit to bind multiple unidentified domains in OPN which can then stimulate cell motility and chemotaxis (Katagiri *et al.*1999). Intracellular OPN is also part of a hyaluronan-CD44-ERM (ezrin/radixin/moesin) attachment complex involved in fibroblast, macrophage and tumor cell migration (Zohar *et al.*1998, Zohar *et al.*2000). This indicates that OPN stimulates or partially regulates cell motility.

The ability of OPN to elicit a plethora of responses is due to its multiple receptors and binding sites as well as it various molecular and structural forms. When OPN is frozen and then thawed or treated with a serine protease such as thrombin the native 70 kDa protein gives rise to 24 kDa and 45 kDa fragments that have been shown to bind antigen and to nonspecifically suppress T-helper lymphocytes, respectively (Fresno *et al.*1981,

Weber *et al.*1996). The 45 kDa fragment of OPN retains the RGD binding domain and has been shown to improve cell attachment and spreading due to easier access to the RGD motif by integrin receptors (Senger *et al.* 1996). Integrin receptors α9β1 and α4β1 can only mediate attachment and migration of cells through by binding to the 45 kDa form of OPN through non-RGD sites. OPN is also a substrate for cleavage by multiple matrix metalloproteinases (MMPs) through one novel site that is cleaved by both MMP-3 (stromelysin 1) and MMP-7 (matrilysin), and another site is cleaved by MMP-3 alone. The fragments that are formed by these cleavages bind cell surface integrins to initiate adhesion and migration (Agnihotri *et al.*2001). OPN can exist in a polymerized form cross-linked by tissue transglutaminase which alters the conformation of OPN to increase its ability to bind to collagen. In bone, polymerized OPN is hypothesized to participate in cell adhesion, matrix assembly and maturation and calcification by simultaneously binding multiple receptors on different cells (Kaartinen *et al.*1999). Homotypic OPN-OPN bonds have high tensile strength *in vitro* which supports the idea that self-assembly is involved in cell-cell and cell-matrix adhesion (Goldsmith *et al.*2002).

Osteopontin in Pregnancy

OPN was first observed in endometrial tissue in 1988 by Nomura *et al.* They performed *in situ* hybridization which localized OPN in mouse embryos, the endometrium from the gravid and non-gravid uterine horns of pregnant mice and the endometrium from mice exposed to an intrauterine injection of oil to induce deciduomas (Nomura *et al.*1988).

There were high levels of OPN mRNA present in the LE of the gravid uterine horn, no expression was observed in the GE. The epithelial expression of OPN appeared to be specific to pregnant mice as little to no OPN was observed in uterine LE of non-gravid or psuedopregnant mice (Nomura *et al.*1988). High levels of OPN were also observed in the granulated metrial gland cells of the decidua and deciduoma, fewer OPN positive cells were observed in the deciduomas (Nomura *et al.*1988). This was the first instance where OPN was observed to have a unique role outside of bone growth and development. The decidual cells that expressed OPN are uterine natural killer (uNK) cells (White *et al.*2005, Herington *et.al.*2007). In 1990 Young and colleagues, using immunocytochemical studies, localized OPN protein in the decidua of women and in uterine GE during the secretory phase of the menstrual cycle (Young *et al.*1990). Those results suggested that the absence of OPN in uterine GE during the proliferative phase and its presence during the secretory phase of the menstrual cycle was due to hormonal regulation of expression of OPN and that the functions of OPN in the endometrium might be associated with its ability to enhance cell attachment (Young *et al.*1990).

In 1992 Brown and coworkers localized OPN mRNA and protein to epithelial cells of a variety of organs including the hypersecretory endometrial GE associated with pregnancy in women (Brown *et al.*1992). In the epithelium of all organs studied, OPN was seen to associate with the apical domain of the cell. OPN protein was also present in secretory fluids found in the lumen of these organs. These investigators hypothesized that the OPN secreted by these epithelial cells, including the uterine epithelium, binds to integrin receptors on the apical surface of the luminal LE to affect communication

between those cells and the external environment (Brown *et al.*1992). Lessey and co-workers, between 1992 and 1996, established that the transient uterine expression of integrin receptors αvβ3 and α4β1 defines the window of implantation in women and that when this expression is altered it can lead to infertility in women (Lessey *et al.* 1994, Lessey *et al.*1996a and b). Knowing that integrin receptors αvβ3 and α4β1 present during the implantation window bind OPN, involvement of OPN and integrins at the uterine-placental interface during the initial attachment phase of implantation was suggested (Lessey *et al.* 1994).

The temporal and spatial expression and hormonal regulation of OPN mRNA and protein as well as integrin subunit protein has been investigated in uteri and placenta of pigs and sheep. Studies pertaining to sheep provided strong evidence that expression of OPN is regulated by progesterone in the endometrial glands and that this secreted OPN binds integrin receptors on the apical surface of uterine LE cells to mediate attachment of Tr of the implanting conceptus (Johnson *et al.*1999a and b, Johnson *et al.*2000, Johnson *et al.*2001). When the uterine glands are knocked out of ewes there is no OPN present in the uterine flushings and pregnancy is not maintained through the peri-implantation period (Gray *et al.*2002). A similar phenotype is observed when RGD peptides are utilized to functionally block the ability of OPN to bind its integrin receptor which leads to a reduction in the number of implantation sites in mice and rabbits (Illera *et al.*2000, Illera *et al.*2003). Progesterone treatment increased OPN in human endometrial adenocarcinoma Ishakawa cells and the endometrium of rabbits, further elucidating the role of sex steroids in the regulation of OPN expression (Apparao *et*

*al.*2001, Apparao *et al.*2003). In contrast to the progesterone regulation of OPN expression in the species noted above, i.m. injections of estrogen increase expression of OPN in the uterine LE of cycling gilts (White *et al.*2005). These results were the first to indicate that the conceptus itself could be regulating the expression of OPN at specific implantation sites in the uterus during the peri-implantation period of pregnancy (Garlow *et al.*2002, White *et al.*2005). Multiple reports, using microarray studies, have strongly implicated OPN as having a major role during implantation because OPN is the most highly up-regulated ECM molecule in the human uterus during the window of implantation (Carson *et al.*2002, Kao *et al.*2002, Mirkin *et al.*2005).

Current research regarding OPN in the female reproductive tract is focusing on its interaction with integrin receptors. Burghardt *et al.* reported the *in vivo* assembly of large FAs containing aggregates of ITGAV, ITGA4, ITGA5, ITGB1, ITGB5, alpha actinin, and focal adhesion kinase (FAK) at the uterine-placental interface of sheep (Burghardt *et al.*2009). OPN is present at this interface between the uterine LE and Tr cells where these aggregates expand as pregnancy progresses (Burghardt *et al.*2009). Similar FAs form in pigs during implantation (Erikson *et al.*2009, Massuto *et al.*2009). Affinity chromatography and immunoprecipitation experiments revealed direct *in vitro* binding of αvβ6 on porcine Tr to αvβ3 on uterine epithelial cells, and αvβ3 integrins on ovine Tr bind OPN (Erikson *et al.*2009, Kim *et al.*2010). These were the first functional demonstrations that OPN directly binds specific integrins to promote Tr cell migration and attachment to uterine LE that may be critical to conceptus elongation and implantation. Recently Aplin and co-workers employed three *in vitro* models of early

implantation with Ishakawa cells to demonstrate that OPN potentially interacts with the αvβ3 integrin receptor during implantation in humans (Kang *et al.*2014).

Implantation of the Conceptus in Pigs

Although early stages of cleavage to formation of the blastocyst are similar to that of many other mammalian species, the biological processes initiated for the establishment and maintenance of the porcine pregnancy are unique following blastocyst hatching on Day 8 of gestation. To establish and maintain pregnancy, pig embryos must first migrate within and between the long horns of the bicornuate uterus. The classical studies of Dziuk and others (Dziuk *et al.*1964; Dhindsa *et al.*1967; Polge and Dziuk 1970) established that intra- and inter-uterine embryo migration is initiated shortly after zona hatching and continues until the time of rapid trophoblast expansion on Days 11 to 12 of gestation. Uterine migration is stimulated through conceptus estrogen and prostaglandin synthesis quite possibly through the induction of histamine release from the underlying endometrium (Pope *et al.*1982, 1986), as well as actions of lysophosphatidic acid (Seo *et al.* 2012). Uterine migration and spacing of embryos serves to provide sufficient surface area for attachment of the diffuse, epitheliochorial placenta of individual conceptuses from D13 to 18 of gestation, and to provide sufficient interface of Tr with the uterine LE to prevent luteolysis. The lungs of pigs do not metabolize prostaglandin F2α (PGF2α) as efficiently as ruminants (Davis *et al.*1979) providing both a systemic and local uterine vascular pathway for induction of CL regression (Del Campo and Ginther 1973; Ginther *et al.*1981). Therefore, it is essential that estrogens secreted by conceptuses cover the

uterine surface area as completely as possible at this early stage of pregnancy since leaving greater than one-quarter of a uterine horn unoccupied results in greater loss of pregnancy in the pig (Dhindsa and Dziuk 1968). Moreover, maintenance of a unilateral pregnancy during the first three weeks of gestation is a very rare occurrence in the pig (du Mesnil du Buisson 1961). After the embryos have effectively spaced themselves in the uterine horns, there is apposition of the conceptuses to uterine LE. Pigs present true epitheliochorial placentation wherein two epithelial layers, uterine LE and Tr/chorion are maintained throughout pregnancy. Another hallmark of pregnancy in pigs is a protracted peri-implantation period. During this time, the conceptuses are free within the uterine lumen where they elongate from a spherical to a filamentous conceptus. Pig embryos make their way into the uterus from the oviduct between 60 and 72 hours after the onset of estrus. The embryos reach the blastocyst stage by Day 5 of pregnancy when they shed the zona pelucida and expand to 2-6 mm in diameter by Day 10. At this stage of development, pig blastocysts diverge from what occurs in rodent and primate pregnancy. Within a few hours the presumptive placental membranes (Tr and extra-embryonic endoderm) elongate at a rate of 30-45 mm/hr from a 10mm blastocyst to a 150-200 mm long filamentous form (Figure 2-2). Further elongation occurs until the conceptuses are 800-1000 mm in length by Day 16 of pregnancy (Bazer and Johnson 2014). During this period of rapid elongation the porcine conceptuses secrete estrogen, the maternal recognition of pregnancy signal, beginning on Days 11 or 12 of pregnancy (Bazer *et al.*1977). This estrogen acts on the uterine epithelia to redirect the secretion of PGF2α away from endocrine secretin into the uterine vasculature to

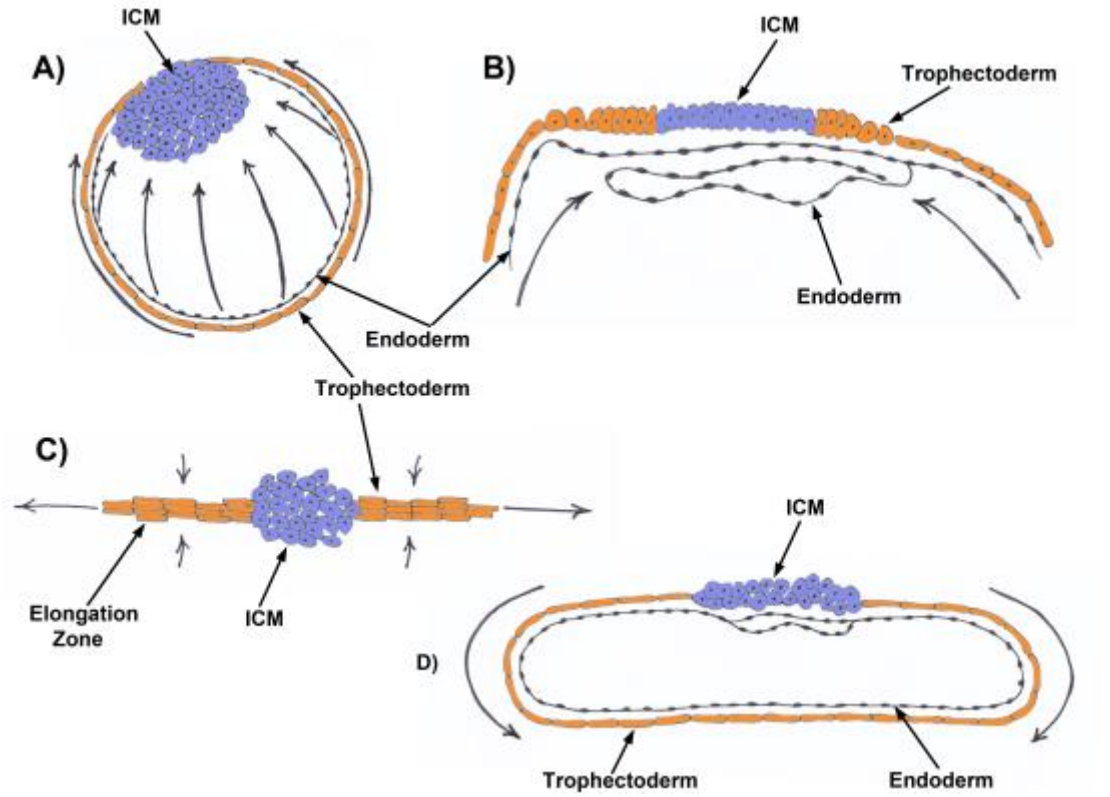

Fig 2-2. Elongation of conceptuses (Bazer and Johnson 2014). A) As the spherical pig blastocyst expands there are increases in proliferation and migration of trophectoderm and extra-embryonic endoderm cells toward the inner cell mass(ICM).Then, as depicted in Panels(B)and(C),this pattern of cell migration results in formation of an elongation zone due to migration of endoderm and trophectoderm cells into the plane of the ICM along the entire length of the blastocyst. There is also a change in morphology of trophectoderm cells to condensed columnar cells which results in a reduction in diameter of the blastocyst (cross-section).(D)After formation of the elongation zone, extra-embryonic endoderm and trophectoderm cells migrate towards the ends of the blastocyst (longitudinal section) which further reduces the diameter and increases the length of the blastocyst for formation of the filamentous conceptus that achieves maximum surface area of intimate contact with uterine luminal epithelial cells. This process of elongation allows for the central-type implantation initially and then the development of true epitheliochorial placentation during later stages of gestation.

the uterine lumen (exocrine secretion) so that PGF2α is not transported to the CL to cause luteolysis, but is metabolized within the uterine lumen of pregnant gilts (Bazer *et al.*1977, Frank *et al.*1977, Gross *et al.* 1990, Spencer *et al.*2004; Ziecik *et al.* 2011). By Day 13 of pregnancy the conceptuses begin an extended period of incremental attachment to the uterine LE (Johnson *et al.*2003) (Figure 2-3). The attachment at the uterine-placental interface, between the uterine LE and Tr, begins to develop microscopic folds around Day 35 of gestation. These folds increase the surface area of contact between maternal and fetal capillaries to maximize maternal-to-fetal exchange of nutrients and gases (Dantzer *et al.*1984).

In the pig, the OPN gene is a leading candidate to influence the complex intra-uterine environment that is present during pregnancy. The OPN gene is located on chromosome 8 under a quantitative trait loci (QTL) for prenatal survival and litter size (King *et al.*2003). The temporal and spatial expression of OPN in the porcine uterus and placenta is complex, with independent and overlapping expression by multiple cell types. Between Days 5 and 9 of the estrous cycle and pregnancy, OPN transcripts are detectable in a small percentage of cells in the sub-epithelial stratum compactum stroma of the endometrium (Garlow *et al.*2002). The morphology and distribution of OPN mRNA- and protein-positive cells in the stratum compactum stroma on Day 9 of the estrous cycle and pregnancy suggest that these are immune cells. OPN is an established component of the immune system that is secreted by activated T lymphocytes (Patarca *et al.*1989); therefore, it is reasonable to speculate that OPN expressing immune cells may protect against pathogens introduced during mating when there is intra-cervical/intra-

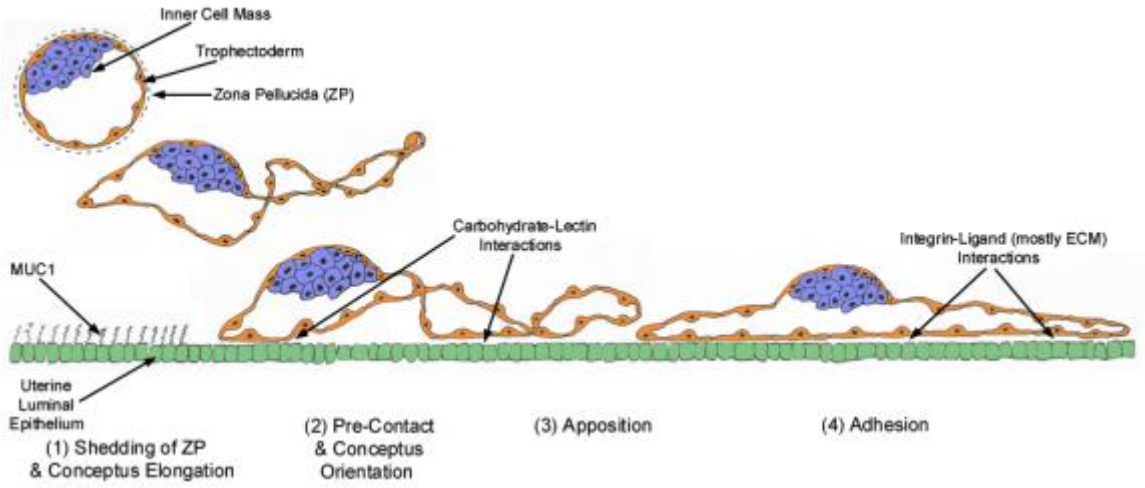

Figure 2-3. Implantation cascade. The phases of this adhesion cascade in pigs include: 1) elongation of the conceptus trophectoderm and shedding of the zona pellucida; 2) down-regulation of MUC1 at the apical surface of uterine LE to expose potential, but not yet identified, low affinity carbohydrate-lectin binding molecules to mediate pre-contact and conceptus trophectoderm orientation to the uterine LE; 3) low affinity contacts are then replaced by a more stable and extensive repertoire of adhesive interactions between integrins and maternal ECM to mediate apposition of trophectoderm to LE; 4) integrin receptors expressed at the apical surface of uterine LE cells bind to Arg-Gly-Asp (RGD) and non RGD amino acid sequence-containing ECM molecules and bridge to another complement of potential integrin receptors expressed at the apical surface of conceptus trophectoderm cells to mediate conceptus trophectoderm adhesion; and 5) development of interdigitating microvilli between LE and trophectoderm (not illustrated in figure).

uterine deposition of semen in pigs. A similar pattern of distribution of OPN-producing cells is evident in the allantois of the placenta beginning between Days 20 and 25 of pregnancy; the number of these cells increases as gestation progresses (White *et al.*2005). The identity of these cells remains to be determined.

OPN expressed by uterine LE increases during the peri-implantation period, but this same expression is not observed in normally cycling gilts (Garlow *et al.*2002). OPN mRNA is initially induced by estrogens secreted by implanting conceptuses in discrete regions of the uterine LE adjacent to the conceptuses prior to implantation on Day 13 of gestation (White *et al.*2005). Expression of OPN extends to the entire uterine LE by Day 20 of gestation when there is firm adhesion between the conceptus Tr and uterine LE (White *et al.*2005). OPN mRNA is not expressed by pig conceptuses (Garlow *et. al.*2002, White *et al.*2005); but OPN protein is present on the apical surface of uterine LE and Tr/chorion at sites of attachment (Garlow *et al.*2002, White *et al.*2005). Interestingly, OPN mRNA and protein are never detected in the areolae of the chorion of areolae or in uterine LE adjacent to areolae as chorion is not attached to uterine LE or GE in those regions. These areolae are pocket-like structures of columnar epithelial cells that take up and transport secretions of uterine GE into the placental vasculature by fluid phase pinocytosis (Renegar *et al.*1982). Levels of OPN remain high at the uterine-placental interface throughout pregnancy in pigs (Garlow *et al.*2002).

This uterine-placental interface is also characterized by expression of integrin subunits that potentially form heterodimeric receptors that bind OPN, including αvβ3, αvβ1,

αvβ5, αvβ6, α4β1, and α5β1 (Bowen *et al.*1996, Burghardt *et al.*1997). OPN directly binds to the αvβ6 integrin receptor on porcine Tr *in vitro*, and the αvβ3 integrin receptor on uterine LE cells *in vitro* (Erikson *et al.*2009). OPN binding promotes dose and integrin dependent binding of both Tr and uterine LE cells *in vitro* as well as stimulating haptotactic Tr cell migration (Erikson *et al.*2009). This shows that soluble OPN causes cells to migrate directionally along a physical gradient. OPN and ITGAV subunit protein have been localized to the uterine-placental interface on Day 20 of pregnancy in pigs (Erikson *et al.*2009). The ITGAV subunit forms large aggregates at this interface suggesting the formation of OPN-induced FA *in vivo* on the apical surfaces of both Tr and uterine LE. These FAs are hypothesized to facilitate attachment of the conceptus to the uterus for implantation. ITGB3 subunit forms large aggregates on the apical surface of uterine LE, but not Tr (Erikson *et al.*2009). The ITGAV subunit has also been co-localized with talin, a FA protein, in large aggregates at the apical surface of cells binding to OPN coated microspheres *in vitro* (Erikson *et al.*2009). It is hypothesized that OPN binds integrins to stimulate integrin-mediated FA assembly, attachment and cytoskeletal force-driven migration of Tr cells to promote conceptus development and implantation in pigs (Figure 2-4).

Besides expression of OPN by uterine LE during the peri-implantation period of pregnancy, there is a 20-fold increase in OPN mRNA between Days 25 and 85 of pregnancy in pigs (Garlow *et al.*2002). This increase in OPN mRNA can be accounted for due to its increased expression being induced in uterine GE (Garlow *et al.*2002). This increase of expression of OPN in the uterine GE begins on Day30 to 35 of gestation

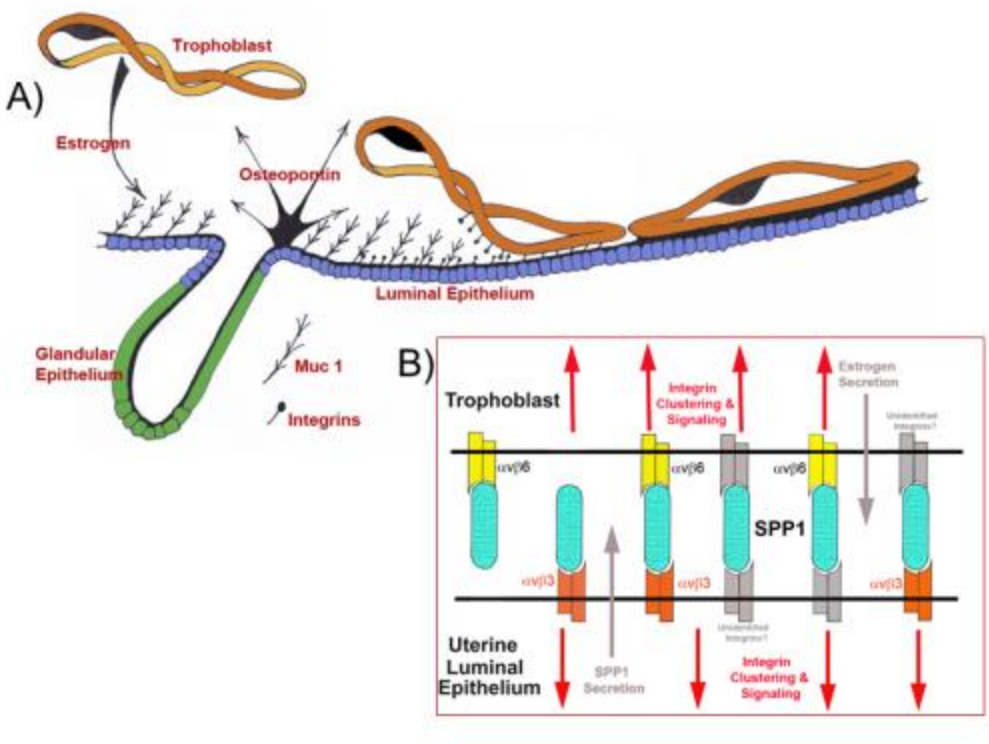

Fig 2-4 Expression, regulation and proposed function of OPN produced by the uterine LE of pregnant pigs (Johnson *et al.* 2014). A) As porcine conceptuses (Trophoblast) elongate they secrete estrogens for pregnancy recognition. These estrogens also induce the synthesis and secretion of OPN (osteopontin) from the uterine LE (luminal epithelium) directly adjacent to the conceptus undergoing implantation. The implantation cascade is initiated when progesterone from CL down-regulates Muc 1 on the surface of uterine LE. This exposes integrins on the LE and trophoblast surfaces for interaction with OPN, and likely other ECM proteins, to mediate adhesion of trophoblast to LE for implantation. B) *In vitro* experiments have identified the αvβ6 integrin receptor on Tr, and the αvβ3 integrin receptor on LE as binding partners for OPN. OPN may bind individually to these receptors to act as a bridging ligand between these receptors. Alternatively, OPN may serve as a bridging ligand between one of these receptors and an as yet unidentified integrin receptor expressed on the opposing tissue.

when placentation and placental growth are occurring in the pig (Sodek *et al.*2000). The later shift in expression of OPN in uterine GE also occurs during gestation in sheep (Johnson *et al.*2003). Microarray data has shown a 60 fold increase in OPN mRNA in the rat between Day 0 and 20 of pregnancy due to its increase in the decidua (Girotti *et al.*2003). OPN is expressed by uterine natural killer cells present in the mouse decidua (White *et al.*2005, Herington *et al.*2007). Normal secretions from uterine GE in farm species and from the decidua in rodents and primates are critical to support implantation, placentation and normal fetal growth and development (Roberts *et al.*1988, Irwin *et al.*1999). Working with late stage, Day 90, pseudopregnant pigs, it was shown that OPN is present in uterine GE which suggests that progesterone secreted by the corpus luteum is responsible for expression of OPN (White *et al.*2005). Progesterone regulates expression of OPN in uterine GE of sheep and rabbits, as well human Ishikawa cells (Johnson *et al.*2000, Apparao *et al.*2001, Illera *et al.*2003).

Recent work using long term progesterone-treated pigs has shown that progesterone alone does not induce expression of OPN in uterine GE (Bailey *et al.*2010). It is currently hypothesized that the hormonal milieu necessary for the production of individual components of histrotroph varies, and may require specific servomechanisms, similar to those found in sheep and rabbits. This may involve sequential exposure of the pregnant uterus to ovarian, conceptus and/or uterine factors that include progesterone, estrogens, and interferons (Chilton *et al.*1988, Young *et al.*1990, Spencer *et al.*1999). Recently OPN expression was compared in placental and uterine tissues associated with normal sized and small fetuses in hyperprolific Large White and Meishan gilts. Not only

were levels of OPN strikingly different between the two breeds of pigs, but OPN was higher in the LE and GE of uteri surrounding smaller fetuses suggesting that OPN may be associated with greater placental efficiency (Hernandez *et al.*2013).

Implantation of the Conceptus in Sheep

Following a surge of LH from the anterior pituitary the oocyte present in the dominant follicle is expelled from the ovary into the oviduct. The oocyte then migrates down the ampulla to the ampullary-isthmic junction where it will be fertilized. The newly fertilized zygote is a single cell embryo that is covered by a thick mucopolysaccaride zona pellucida. The embryo goes through multiple mitotic divisions as it descends the oviduct towards the uterus (Figure 2-5). These mitotic divisions yield a 32-64 cell embryo that enters the uterus at the morula stage approximately Day 4 post-fertilization (Chang *et al.*1965, Rowson *et al.*1966, Bindon *et al.*1971). The embryo continues dividing into the morula stage still surrounded by the zona pellucida, which promotes increased cell surface contact with neighboring cells, a process referred to as compaction which is accompanied by cell polarization that will ultimately determine the fate of cells present in the embryo. Compaction results in the formation and segregation of the inner cell mass, which will give rise to the fetus, from the trophoblast which will form the placenta. During this time, cells of the inner cell mass begin to form gap junctions which mediate cell-cell communication; while the cells that will be the trophoblast begin to form tight junctions. The formation of these tight junctions causes a change in permeability and polarity of this cell layer which leads to an influx of fluid. This fluid

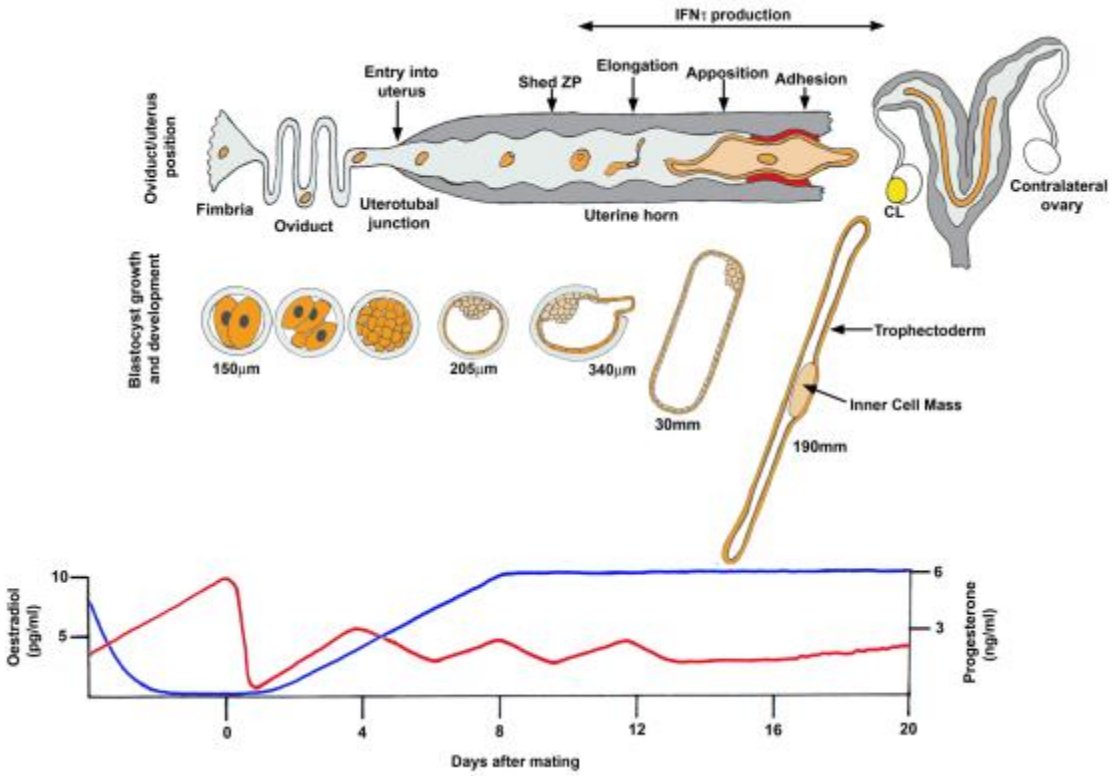

Fig 2-5. Early pregnancy events in sheep (Spencer *et al.* 2004). This schematic summarizes the relative changes in embryo/blastocyst/conceptus development after fertilization in relation to position in the female reproductive tract and circulating levels of ovarian steroid hormones. Fertilization occurs in the oviduct, and the morula stage embryo enters the uterus on day 4. The blastocyst is formed by day 7, and it hatches from the zona pellucida by day 9. The blastocyst develops from a spherical to a tubular form by days 12 to 13 and then elongates to a filamentous conceptus between days 13 and 19. Elongation of the conceptus marks the beginning of implantation, which involves apposition and transient attachment (days 12 to 16) and firm adhesion by day 16 and is concomitant with synthesis and secretions of interferon tau (IFNT) and prostaglandins (PG) by the trophectoderm. E2 = estrogen; P4 = progesterone.

causes the formation of the blastocoele, which is a fluid filled cavity within what is now the blastocyst (Chang *et al.*1965, Rowson *et al.*1966, Bindon *et al.*1971, Ziomek *et al.*1980). The trophoblast cells surround the blastocoele, while the inner cell mass is allocated to a single pole of the developing embryo. After its formtation the blastocyst hatches from the zona pellucida. The newly hatched blastocyst starts out as a sphere of roughly 200-400 μm in diameter that expands until Day 10. The blastocyst then undergoes a morphogenesis that transforms a spherical (<1mm) blastocyst to a tubular and then an elongated filamentous conceptus by Day 14 reaching 10 cm in length by Day 14 and 25cm or more by Day 17 (Fuillomot *et al.*1993). Elongation of the blastocyst is dependent on uterine secretions as evidenced by the fact that the blastocyst will not elongate *in vitro*, but will elongate when transferred into the uterus (Heyman *et al.*1984, Flechon *et al.*1986). Elongation of the blastocyst causes the developmentally regulated synthesis of interferon tau (IFNT), which is the maternal recognition of pregnancy signal in the sheep and other ruminants (Farin *et al.*1989, Guillomot *et al.*1990, Gray *et al.*2002). IFNT is a type I interferon that silences transcription of estrogen receptor alpha and oxytocin receptor to prevent oxytocin-induced pulsatile secretion of PGF2α (PGF) that would otherwise cause regression of the ovarian corpus luteum and its production of progesterone, the hormone of pregnancy (Thatcher *et al.*1989, Spencer *et al.*2007). In addition to acting as the maternal recognition signal, IFNT induces or enhances the expression of a number of genes within the endometrium that are believed to further support pregnancy (Hansen *et al.*1999, Spencer *et al.*2007).

After shedding of the zona pellucida and prior to apposition of Tr there is loss of anti-adhesive molecules normally present on the apical surface of the uterine LE. One such molecule is MUC1, an intrinsic transmembrane mucin that is reduced during this period to allow the conceptus to adhere to the uterus (Johnson *et al.* 2001). Apposition of the elongating conceptus involves loose adherence to the uterine LE by interdigitation of microvilli present on both the trophoblast and uterine LE. These loose adhesions occur in proximity to the inner cell mass and then begin to spread to the poles of the elongated conceptus. During this phase of implantation trophoblast papillae are formed that penetrate into the mouths of uterine glands. These papillae are short lived, only lasting until about Day 20, and are believed to act to anchor the pre-attached conceptus to the uterus and absorb histotrophic secretions from uterine GE (Guillomot *et al.*1981). Apposition is followed by adhesion of Tr of the conceptus to the uterine LE which is mediated by integrin binding to extracellular matrix (ECM) bridging ligands. Implantation in sheep occurs between approximately Days 17 and 20 of pregnancy. As pregnancy progresses the ovine placenta organizes into discrete regions called placentomes that are composed of highly branched placental chorioallantoic villi termed cotyledons which grow rapidly and interdigitate with the maternal aglandular endometrial crypts termed caruncles. Approximately 90% of the blood from the uterine artery flows into the placentomes for the transfer of nutrient and exchange of gases between the maternal uterine circulation and the fetus (Caton *et al.*1983).

The temporal and spatial expression of OPN in the uterus and placenta of sheep is similar to that previously described for the pig with some key exceptions: 1) unlike in

the pig, OPN is not expressed by uterine LE; 2) induction of OPN in uterine GE occurs earlier during the peri-implantation period than in the pig , and expression in uterine GE is regulated by progesterone; 3) OPN is a prominent component of the stratum compactum stroma; and 4) although large FAs assemble during the peri-implantation period of pigs, they are not observed at the uterine-placental interface until later stages of pregnancy in sheep.

During the early stages of the estrous cycle and pregnancy in sheep OPN mRNA and protein are observed in a small population of cells scattered within the stratum compactum stroma immediately beneath the endometrial LE (Johnson *et al.*1999). A similar distribution of OPN producing cells is also observed in the allantois of the ovine placenta beginning between Days 20 and 25 of pregnancy and they increase in number as pregnancy progresses (Johnson *et al.*2003a). Similar to what is hypothesized in the pig; these cells are believed to be immune cells because OPN is a prominent player in the immune system (Patarca *et al.*1989). OPN expressing cells are also present in the stratum compactum stroma, but are harder to identify than those in the pig due to an increase in expression of OPN by stromal cells between Days 20 and 25 of pregnancy (Johnson *et al.* 2003b). In pregnant mice and primates, OPN is expressed in the decidua and is considered to be marker for decidualization (Waterhouse *et al.*1992, Fazleabas *et al.*1997). Decidualization involves transformation of spindle-like fibroblasts into polygonal epithelial-like cells that are hypothesized to limit conceptus trophoblast invasion through the uterine wall during invasive implantation (Irwin *et al.*1999). During pregnancy in the sheep endometrial stromal cells increase in size and become polyhedral

in shape following conceptus attachment. Classic decidualization markers, desmin, and α-smooth muscle actin, are expressed by the polyhedral cells found in the stratum compactum stroma (Johnson *et al.*2003a). Taken in conjunction with evidence for expression of OPN in the stromal compartment, this may be part of a uterine decidualization-like response to the conceptus during ovine pregnancy (Johnson *et al.*2003b). One of the primary roles of decidua in invasive implanting species is to restrain conceptus trophoblast invasion to a specific region of the endometrium. Although pigs and sheep exhibit a non-invasive form of implantation, the extent of conceptus invasion into the endometrium differs between these two species. Pigs exhibit a true epitheliochorial placentation in which the uterine LE remains intact throughout pregnancy and the apical surfaces of the conceptus Tr and uterine LE attach to each other without any contact with the uterine stroma (Bjorkman *et al.*1973). The synepitheliochorial placentation in sheep involves extensive erosion of uterine LE due to formation of syncytia with binucleate cells of the Tr. After Day 19 of pregnancy conceptus tissue is opposed to, but does not penetrate, the ovine uterine stroma (Boshier *et al.*1969). It is thus hypothesized that the differences in stromal expression of OPN between these two species is due to the degree conceptus invasion.

In the sheep OPN is not expressed in uterine LE like it is in the pig, but it is still a component of the histotroph as it is secreted from the endometrial GE as early as Day 13 of pregnancy (Johnson *et al.* 1999a). OPN is not seen to be expressed by the uterine GE of cyclic sheep (Johnson *et al.*1999a and b). OPN mRNA is detected in some uterine glands by Day 13 and is present in all glands by Day 19 of pregnancy (Johnson *et*

*al.*1999a). Progesterone induces production of OPN by uterine GE and the onset of expression of OPN is associated with the loss of progesterone receptors in the uterine LE and GE. Analysis of uterine flushing from pregnant ewes identified a 45 kDa fragment of OPN with greater binding affinity for αvβ3 integrin receptor than native 70 kDa OPN (Johnson *et al.*1999b, Johnson *et al.* 2000, Johnson *et al.* 2001, Senger *et al.*1996). OPN mRNA is present in uterine GE during the peri-implantation period of pregnancy, but not in uterine LE or conceptus Tr (Johnson *et al.*1999b). OPN protein is present at the apical surfaces of endometrial LE and GE as well as the apical surface of conceptus Tr (Johnson *et al.*1999a). Also present on these apical surfaces are integrin subunits ITGAV, ITGA4, ITGA5, ITGB1, ITGB3, and ITGB5 which could contribute to the assembly of several OPN receptors including αvβ3, αvβ1, αvβ5, α4β1, and α5β1 (Johnson *et al.*1999b, Johnson *et al.*2001). These integrin subunits are constitutively expressed on the apical surfaces of Tr and uterine LE (Johnson *et al.* 2001). These results suggest that OPN is a component of histotroph which is secreted from the uterine GE into the uterine lumen of pregnant ewes in response to progesterone and that OPN binds integrin receptors expressed on both the endometrial LE and conceptus Tr (Figure 2-6).

Integrin subunits ITGAV and ITGB3 on the surface of ovine Tr cells bind OPN indicating integrin receptor αvβ3 binds OPN *in vitro* (Kim *et al.* 2010). OPN binding to the αvβ3 integrin receptor induced the formation of FAs *in vitro* which are a prerequisite for adhesion and migration of Tr through the

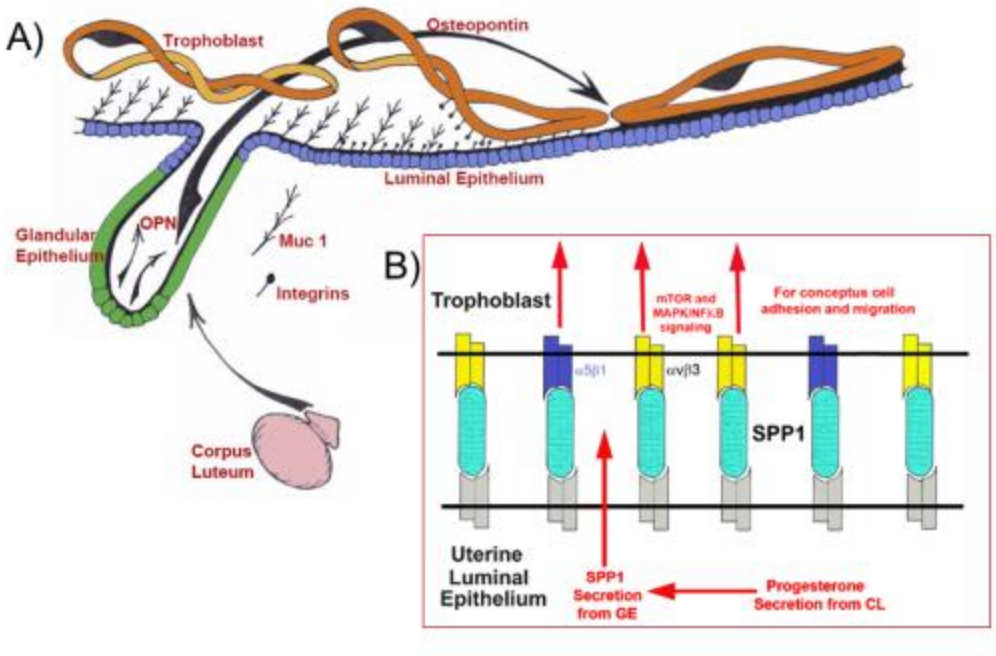

Figure 2-6. Expression, regulation and proposed function of OPN produced by the uterine GE of pregnant sheep (Johnson *et al.* 2014). A) As the lifespan of the CL is extended as the result of the actions of interferon tau secretion from elongating ovine conceptuses (Trophoblast) they secrete progesterone. Progesterone then induces the synthesis and secretion of OPN (Osteopontin) from the uterine GE (Glandular Epithelium). The implantation cascade is initiated with down-regulation Muc 1 (the regulatory mechanism remains to be identified) on the LE surface to expose integrins on the LE and trophoblast surfaces for interaction with OPN to mediate adhesion of trophoblast to LE for implantation. B) *In vitro* experiments have identified the αvβ3 integrin receptor on trophoblast as a binding partner for OPN. OPN then likely acts as a bridging ligand between αvβ3 on trophoblast and as yet unidentified integrin receptor(s) expressed on the opposing uterine LE. Note that the ITGA5 subunit was immunoprecipitated from membrane extracts of biotinylated oTr1 cells that were eluted from an OPN-Sepharose column, but the ITGB1 subunit, the only known binding partner for ITGA5, could not be immunoprecipitated. Therefore, while we cannot definitively state that OPN binds α5β1 integrin on oTr1, we are reticent to exclude this possibility.

activation of: 1) P70S6K via crosstalk between FRAP1/MTOR and MAPK pathways; 2) MTOR, PI3K, MAPK3/MAPK1 (Erk1/2) and MAPK14 (p38) signaling to stimulate Tr cell migration; and 3) FA assembly and myosin II motor activity to induce migration of Tr cells (Kim *et al.* 2010). These results indicate that OPN binding to αvβ3 integrin receptors activates cell signaling pathways that act in concert to mediate adhesion, migration and cytoskeletal remodeling of Tr cells essential for expansion and elongation of conceptuses as well as their attachment to uterine LE for implantation.

Focal adhesions are prominent structures of cells grown in culture, but they are rarely observed *in vivo*. Large aggregates of FA-associated protein at the uterine-placental interface are interpreted to be three dimensional FAs (Burghardt *et al.* 2009). By Day 40 of pregnancy in sheep punctate staining of integrin subunits on the apical surfaces of uterine LE and conceptus Tr is replaced by large scattered aggregates of ITGAV, ITGA4, ITGB1, and ITGB5 subunits in interplacentomal LE and Tr/chorion (Johnson *et al.*2001). These integrin aggregates are observed only in the gravid uterine horns of unilaterally pregnant sheep indicating a requirement for Tr attachment to the uterine LE (Burghardt *et al* 2009). Aggregates increase in size and number through Day 120 of pregnancy (Burghardt *et al.*2009). Although ITGB3 subunit is prominent at the uterine-placental interface during the peri-implantation period, there is no aggregation observed on Day 80 of pregnancy (Johnson *et al.* 2001, Burghardt *et al.* 2009). Placentomes, which provide hematotrophic support to the fetus and placenta, have diffuse expression of ITGAV, ITGA4, ITGB1 and ITGB5 integrin subunits

(Burghardt *et al.*2009). This diffuse staining could be due to the extensive folding present in the placentome. Taken together, these results suggest that FA assembly at the uterine-placental interface in the inter-placentomal region reflects a dynamic adaptation to increasing forces caused by the growing fetus. Cooperative binding of multiple integrin receptors to OPN at the uterine-placental interface may form an adhesive mosaic which maintains tight connections and increases tensile strength as well as signaling activity between uterine and placental surfaces in interplacentomal regions where uterine LE and conceptus Tr are maintained.

Steady-state levels of OPN mRNA in total ovine endometrium remain constant between Days 20 and 40, increase 40-fold between Days 40 and 100, and remain maximal thereafter (Johnson *et al.*1999a). The major source of this OPN is uterine GE which undergoes hyperplasia through Day 50 followed by hypertrophy and maximal production of histotroph after Day 60 (Johnson *et al.*2003). Additionally, immunofluorescence microscopy demonstrated that the secreted 45-kDa OPN cleavage fragment is exclusively, continuously, and abundantly present along the apical surface of uterine LE, on Tr, and along the entire uterine-placental interface of both interplacentomal and placentomal regions through Day 120 of the 147 day ovine pregnancy (Johnson *et al.*2003). OPN is a secretory product of the uterine GE where intimate contact between the conceptus Tr and the uterine LE occurs. In these areas OPN may influence fetal/placental development and growth as well as mediate communication between placental and uterine tissues to support pregnancy to term.

CHAPTER III

DIRECT EVIDENCE THAT TROPHECTODERM CELLS ENGAGE ITGAV (ALPHA V INTEGRIN) TO ADHERE TO OSTEOPONTIN: IMPLICATIONS FOR IMPLANTATION

Introduction

Early pregnancy is influenced by rapid conceptus [embryo (inner cell mass) and associated placental membranes] development, endometrial remodeling for implantation, and pregnancy recognition signaling. Due to the complexity of these overlapping events, there is an increased propensity for dysfunction that results in high rates of pregnancy loss across most mammalian species (Flint *et al.* 1982, Bazer *et al.* 1983, Jainudeen *et al.* 1993). Amongst these events, implantation requires exquisitely precise spatiotemporally regulated endocrine, paracrine, and autocrine mediation of cell-cell and cell-matrix interactions (Denker *et al.* 1993, Guillomot *et al.* 1993, Aplin *et al.* 1997, Carson *et al*. 2000, Burghardt *et al*. 2002, Armant *et al*. 2005, Spencer *et al*. 2007, Johnson *et al*. 2009). Implantation is the process by which the conceptus initiates a close association with the maternal uterus leading to the establishment of a functional placenta and successful pregnancy. In all eutherian mammals, implantation begins when the apical surfaces of uterine luminal epithelium (LE) and conceptus trophectoderm (Tr) adhere to each other within a restricted period of the uterine cycle termed the "window of receptivity" (Denker *et al*. 1993, Lessey *et al*. 1994, Lessey *et al*. 1996, Aplin *et al*. 1997, Burghardt *et al*. 2002). Our laboratory has had a long-term interest in the potential

function of osteopontin [OPN, also known as secreted phosphoprotein 1 (SPP1)] and its integrin receptors to adhere placental tissues to uterine tissues during implantation (Johnson *et al*. 2014)

OPN is a secreted extracellular matrix (ECM) protein that binds integrins (Denhardt *et al*. 1993, Butler *et al*. 1996). Integrins are transmembrane glycoprotein receptors composed of non-covalently bound α and β subunits that promote cell-cell and cell-ECM adhesion, cause cytoskeletal reorganization to stabilize adhesion, and transduce signals through numerous signaling intermediates (Albelda *et al*. 1990, Giancotti *et al*. 1999). OPN binds multiple integrins through Arg-Gly-Asp (RGD)-mediated or alternative integrin recognition sequences. These integrins, include; αvβ3, αvβ1, αvβ5, αvβ6, α4β1, α9β1, and α8β1. Binding of OPN to integrins results in diverse effects, including cell-cell and cell-ECM adhesion, and cell migration (Singh *et al*. 1990, Senger *et al*. 1994, Hu *et al*. 1995, Liam *et al*. 1995, Senger *et al*. 1996, Bayless *et al*. 1998, Smith *et al*. 1998, Denda *et al*. 1998, Yokosaki *et al*. 2005).

OPN expression in uterine LE increases markedly during the peri-implantation period of pregnancy in pigs (Garlow *et al*. 2002, White *et al*. 2005) and other mammalian species, including sheep, goats, humans, rabbits, and mice (Johnson *et al*. 1999, Apparao *et al*. 2003, Johnson *et al*. 2003, Mirkin *et al*. 2005, Joyce *et al*. 2005, White *et al*. 2006). In pigs, OPN mRNA is initially induced by conceptus estrogens in discrete regions of the LE juxtaposed to the conceptus just prior to implantation on Day 13. By Day 20, when firm adhesion of conceptus Tr to uterine LE has occurred, OPN and multiple integrin

subunits that form heterodimer receptors that bind OPN are abundant along the entire LE (Bowen *et al*. 1996, Garlow *et al*. 2002, Johnson *et al*. 2003, White *et al*. 2005, Erikson *et al*. 2009). *OPN* mRNA levels remain high at this interface throughout pregnancy (Garlow *et al*. 2002), suggesting that LE-derived OPN binds integrins on uterine LE and adjacent trophectoderm/chorion to mediate adhesion between the conceptus and uterus that is essential for implantation and placentation. In support of this hypothesis, *in vitro* studies have shown that OPN directly binds the αvβ6 integrin heterodimer on porcine trophectoderm cells and the αvβ3 integrin on porcine uterine LE cells, and that OPN promotes dose- and integrin-dependent attachment of Tr and LE cells to culture wells, and stimulates directional haptotactic migration of Tr cells along a physical gradient of non-soluble OPN (Erikson *et al*. 2009). Finally, culturing porcine Tr cells with OPN-coated microspheres revealed co-localization of the ITGAV subunit and talin to FAs at the apical domain of these cells (Johnson *et al*. 2001, Erikson *et al*. 2009). These results indicate that OPN and various integrin receptors are present at the uterine-placental interface and may be involved in the attachment of the implanting conceptus to the uterus during the peri-implantation period.

Although the expression of OPN in uterine and placental tissues of pigs throughout pregnancy has been established, and the ability of OPN to support Tr cell adhesion and migration conducive to implantation of the conceptus has been demonstrated, similar information about the integrins hypothesized to mediate these events remains unknown. Therefore, the current study was performed to establish the temporal, cell-type specific expression, and hormonal regulation of the ITGAV, ITGB3 and ITGB6 subunits at the

uterine-placental interface of pigs throughout pregnancy, and to mechanistically demonstrate whether OPN binds ITGAV heterodimers to support Tr cell adhesion *in vitro*.

Materials and Methods

Animals and Tissue Collection

All experimental and surgical procedures complied with the Guide for Care and Use of Agriculture Animals and were approved by the Institutional Agricultural Animal Care and Use Committee of Texas A&M University. Sexually mature gilts were observed for signs of estrus [Day 0 (estrus/mating)] daily and exhibited at least two estrous cycles of normal duration (18-21 days) prior to being used in these studies.

To assess the effects of pregnancy on the temporal and spatial expression of integrin subunits, gilts were assigned randomly to either cyclic or pregnant status. Gilts in the cyclic group were hysterectomized on either Day 9, 12 or 15 of the estrous cycle. Gilts assigned to the pregnant group were naturally bred to reproductively competent boars and hysterectomized on either Day 9, 12, 13, 14, 15, 20, 24, 25, 30, 35, 40, 45, 50, 60, 70, 85, or 90 of pregnancy (n=4 pigs/day/status).

To assess the effects of estrogen on the expression of integrins by the uterine epithelium, a previously validated pseudopregnancy model was utilized (White *et al*. 1977). Gilts were randomly assigned to pseudopregnant or control status. Gilts for pseudopregnancy received intramuscular injections of estradiol benzoate (5 mg in 5 ml of corn oil/day) or

corn oil alone (n=4/treatment) on Days 11, 12, 13 and 14 of the estrous cycle. All gilts were then hysterectomized on Day 15 of the estrous cycle or pseudopregnancy.

To evaluate the effects of long-term progesterone on the expression of integrins by the uterine epithelium, gilts were treated with progesterone as previously described (Bailey *et al.* 2010). Gilts were ovariectomized on Day 12 of the estrous cycle and assigned randomly to receive daily injections (i.m.) of either 4 ml corn oil or 200 mg P4 in 4 ml corn oil on Days 12 through 39 post estrus (n=4/ treatment). All gilts were hysterectomized on Day 40 post-estrus.

To assess distribution of integrin subunit mRNAs, at hysterectomy, several cross sections (approximately 1-1.5 cm thick) from the middle of each uterine horn from all gilts were placed in fresh 4% paraformaldehyde fixative for 24 h and then embedded in Paraplast Plus (Oxford Labware, St Louis, MO). To assess the assembly of FAs at the uterine-placental interface that incorporate integrin subunit proteins, several 1-1.5 cm sections of uterine wall from the middle of each uterine horn of all pregnant gilts were snap frozen in Tissue-Tek OCT compound (Miles, Oneata, NY).

Cloning of Partial cDNAs for Porcine *ITGAV, ITGB3* and *ITGB6*

Partial cDNAs for *ITGAV, ITGB3,* and *ITGB6* were generated by RT-PCR as previously described using total RNA from endometrial tissue from Day 25 pregnant gilts (Joyce *et al*. 2008). Primers for each target gene (see Table 3-1) were designed from sequences of the respective porcine genes using Primer 3

Table 3-1. Integrin primers used for cloning

Gene	Sequence
ITGAV	FOR 5’ CTGGTCTTCGTTTCAGTGTGC REV 5’ GCCTTGCTGAATGAACTTGG
ITGB3	FOR 5’ AGATTGGAGACACGGTGAGC REV 5’ GTACTTGCCCGTCATCTTGC
ITGB6	FOR 5’ ATGAAGGTGGGAGACACAGC REV 5’ AGGAGAAATCATCGCATTGG

(Version 2.0-alpha; htpp://primer3.sourceforge.net/). PCR amplification was conducted as follows for porcine *ITGAV, ITGB3,* and *ITGB6* subunits: 1) 95°C for 5 min; 2) 95°C for 45 sec, 60.4°C (*ITGAV*), or 62.4°C (*ITGB3, ITGB6*); and 72°C for 1 min for 35 cycles; and 3) 72°C for 10 min. Amplified PCR products were subcloned into the pCRII cloning vector using a TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) and sequenced in both directions using an ABI PRISM Dye Terminator Cycle Sequencing kit and an ABI PRISM automated DNA sequencer (Applied Biosystems, Foster City, CA) to confirm sequence identity.

In situ Hybridization Analyses

ITGAV, ITGB3, and *ITGB6* mRNAs were localized in paraffin-embedded uterine and placental tissues as previously described (Johnson *et al*. 1999). Uterine cross sections from each animal (5 μm) were deparaffinized, rehydrated, and deproteinated, then hybridized with radiolabeled antisense and sense cRNA probes generated from linearized plasmid DNA templates. Radiolabeled antisense or sense cRNA probes were synthesized by *in vitro* transcription with [α-^{35}S] uridine 5-triphosphate (PerkinElmer Life Sciences, Wellesley, MA). After hybridization, washing and RNase A digestion were performed followed by autoradiography using NTB-2 liquid photographic emulsion (Eastman Kodak, Rochester, NY). Slides were exposed at 4°C for 8 days, developed in Kodak D-19 developer, counterstained with Harris modified hematoxylin (Fisher Scientific, Fairlawn, NJ), dehydrated, and protected with cover slips sealed with Permount (Fisher Scientific).

Quantification of *in situ* Hybridization

To quantify mRNA levels observed on *in situ* hybridization slides, three digital photomicrographs were taken per slide/day of pregnancy or cycle using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY). Photomicrographs were captured as Tagged Image File (TIF) and loaded into Adobe Photoshop (version9.0, Adobe Systems Inc., San Jose, CA) to measure the intensity of silver grains. Six sections across the uterine-placental interface were chosen at random to measure integrated density and area measured. These measurements were then analyzed and graphed as a ratio of density/area. A one-way ANOVA was utilized to determine statistical significance with $p<0.05$ considered significant.

Immunofluorescence Analyses of Uterine and Placental Tissues

ITGAV and ITGB3 subunits as well as OPN were localized in frozen uterine cross sections (Days 24, 25, 50, 70 and 90 of pregnancy, n=4/day) as previously described [Johnson *et al.* 2001]. Briefly, tissues were fixed in -20°C methanol, rinsed in PBS containing 3% (vol/vol) Tween 20, blocked in 10% (vol/vol) normal goat serum, and incubated overnight with 2 µg/ml of rabbit antiserum directed against either human anti-ITGAV subunit, human anti-ITGB3 subunit, or human anti-OPN (AB1930, AB1968, AB10910; EMD Millipore International, Billerica, MA). Tissue-bound primary antibody was detected with goat anti-rabbit IgG Alexa 488 (8 µg/ml). Slides were overlaid with Prolong Gold Antifade reagent with DAPI (Life Technologies, Grand Island NY) and a cover glass.

Table 3-2. Integrin primers used for characterization of porcine trophoblast cells

Gene	Sequence
ITGA5	FOR 5’ GAGCCTGTGGAGTACAAGTCC REV 5’ CCTTGCCAGAAATAGCTTCC
ITGAV	FOR 5’ CTGGTCTTCGTTTCAGTGTGC REV 5’ GCCTTGCTGAATGAACTTGG
ITGA2	FOR 5’ CATGCCAGATCCCTTCATCT REV 5’ CGCTTAAGGCTTGGAAACTG
ITGA4	FOR 5’ CAGATGGGATCTCGTCAACC REV 5’ TCTGCTGGACACCTGTATGC
ITGB1	FOR 5’ GACCTGCCTTGGTGTCTGTGC REV 5’ AGCAACCACACCAGCTACAAT
ITGB3	FOR 5’ AGATTGGAGACACGGTGAGC REV 5’ GTACTTGCCCGTCATCTTGC
ITGB6	FOR 5’ ATGAAGGTGGGAGACACAGC REV 5’ AGGAGAAATCATCGCATTGG
GAPDH	FOR 5’ GGTGAAGGTCGGAGTGAACG REV 5’ TGACTGTGCCGTGGAATTTG

Porcine Cell Culture

The porcine Tr cell line (pTr2) was developed and propagated as described previously (Ka *et al*. 2001) in Dulbecco modified Eagle medium-Ham F12 (DMEM-F12; Sigma-Aldrich, St. Louis, MO) with 10% (vol/vol) fetal bovine serum (FBS). Cells were maintained in a 5% CO_2 humidified environment at 37°C.

RNA Isolation and RT-PCR Analyses

Total cellular RNA was isolated from pTr2 cells using an RNeasy Mini Kit (Qiagen, Valencia CA) according to the manufacturer's instructions. cDNA was prepared using 3 µg total RNA with the SuperScript III First-Strand Synthesis System (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Following cDNA synthesis, PCR was performed using 50 ng/reaction of cDNA with a recombinant Taq DNA polymerase (Life Technologies) according to the manufacturer's instructions. Primers for integrin subunits *ITGA5*, *ITGAV*, *ITGA2*, *ITGA4*, *ITGB1*, *ITGB3*, *ITGB6*, and *GAPDH* (positive control; Table 3-2) were derived from conserved porcine sequences and designed using Primer3 (primer3.sourceforge.net/) and produced by Eurofins MWF Operon (Huntsville, AL). The PCR products were analyzed on 1% (wt/vol) agarose gels, and the gels were imaged using the PhotoDoc-It imaging system (UVP, Upland, CA).

Knockdown of ITGAV in pTr2 Cells

The pTr2 cells were grown to confluence in T75 flasks prior to transfection. The siRNAs were designed (Table 3-3) from established sequences for porcine *ITGAV* and *GAPDH* and produced by Ambion, Inc. (Austin, TX). Complexes with siRNA were assembled by combining 30 µl/reaction siPortAmine (Ambion) and 3.482 ml/reaction OPTI-MEM (Life Technologies). Cells were plated at 80% confluence and transfected with 50 nM siRNA in antibiotic-free DMEM containing 10% FBS. Treatment groups included: vehicle, *ITGAV* siRNA, or *GAPDH* siRNA. At 8 h, cells were supplemented with 15 ml DMEM-F12 with 10% FBS. The medium was changed at 24 h and cells were grown to confluence prior to running further assays. This assay was performed with 4 intra-assay replicates per treatment per siRNA and was performed three times to ensure statistical viability. Prior to utilizing knock-down cells for assays three different clones of each siRNA (provided by Ambion) were tested at concentrations of 10, 50 or 100 nM. The optimal clone (sequence noted in Table 3-3) was utilized at a concentration of 50 nM as it produced the optimal knock down efficiency.

Western Blot Analyses

Western blots were performed on protein extracts of pTr2 cells from each siRNA treatment group using RIPA lysis buffer (50nM Tris HCl, 150 mM Triton X100, 1.0% Sodium Deoxycholate w/v, 0.1% SDS w/v, 100 mM Na_3VO_4) containing Complete EDTA-free Protease Inhibitors (Roche Diagnostics, Indianapolis, IN). Cells were pelleted and flash frozen on dry ice and stored at -80°C until extraction was performed.

Table 3-3. siRNA sequences

Gene	Sequence
ITGAV siRNA	Sense 5’ CCAACUUCAUUAUAGAUUUTT Antisense 5’ AAAUCUAUAAUGAAGUUGGTA
GAPDH siRNA	Sense 5’ GCAUGAACCAUGAGAAGUATT Antisense 5’UACUUCUCAUGGUUCAUGCCC

For protein extraction, cells were re-suspended in RIPA lysis buffer and incubated on ice for 10 min. Cells were then sheared by pipetting multiple times. Protein concentrations were determined using a modified Lowry assay (Cat# 500-0116, Bio-Rad, Hercules, CA) on a NanoDrop 1000 (Thermo Scientific, Rockford, IL) spectrophotometer. SDS sample buffer/dye was added to protein samples to a 1X final concentration (50 mM Tris-Cl, 2% wt/vol SDS, 0.1% wt/vol bromophenol blue, and 10% vol/vol glycerol) and samples were heated at 95°C for 5 min. Proteins were separated by 10% SDS-polyacrylamide gels (100 μg/lane) and transferred to nitrocellulose membranes (0.45 μm, GE Healthcare, Piscataway, NJ). Immunoreactive proteins were detected as described previously (Joyce *et al*. 2005). Briefly, membranes were washed with Tris-buffered saline-Tween-20 (TBST; 20 mM Tris base, 137 mM NaCl, and 0.1% Tween 20; pH 7.6) and blocked with 5% milk protein in TBST for 1h at room temp. Rabbit anti-human ITGAV serum (1 mg/ml), mouse anti-rabbit GAPDH (1 mg/ml, ab8245, Abcam, Cambridge, MA) or mouse anti-human cytokeratin (1 mg/ml, Sigma-Aldrich) were diluted 1:1000, 1:1000, and 1:3000 in blocking solution, respectively, and incubated on blots with constant agitation overnight at 4°C. Blots were then washed again and probed with either goat anti-rabbit HRP or goat anti-mouse HRP (1 mg/ml, 474-1506 and 474-1806, KPL, Gaithersburg, MD) diluted 1:5000 in 5% milk protein in TBST and incubated for 1 h at room temperature followed by washing with TBST. Blots were developed with the Pierce SuperSignal West Pico chemiluminescence substrate detection system (Thermo Scientific) according to the manufacturer's instructions. Multiple exposures were generated to ensure the linearity of film exposures. Blots were

stripped as needed with Restore Western blot stripping solution between probes. Blots were first probed with rabbit anti-human ITGAV antibody or mouse anti-rabbit GAPDH antibody, then stripped and re-probed with mouse anti-human cytokeratin.

Cell Adhesion Analyses

Cell adhesion assays were conducted as previously described (Erikson *et al*. 2009). Polystyrene microwells (Corning-Costar, Sigma Aldrich) were coated overnight at 4°C with 50 µl (20 µg/ml diluted in PBS) of bovine milk OPN, bovine fibronectin, bovine serum albumin (BSA; negative control) and type I collagen (positive control; n=4 replicates/treatment). After blocking each well in 10 mg/ml BSA in PBS (100 µl), 50,000 pTr2 cells were added per well and allowed to attach for 1 h (37°C, 5% CO_2). In all cell attachment experiments, nonadherent cells were removed by washing in isotonic saline and wells were fixed in 4% vol/vol formalin in PBS. Plates were stained with 0.1% wt/vol amido black in 30% methanol and 10% acetic acid for 15 min, rinsed, and solubilized with 2N NaOH (50ul per well) to obtain an absorbance reading at 595 nm, which directly correlated with the number of cells stained in each well. A two-way ANOVA with a Bonferroni post-test was utilized to determine statistical significance with $p<0.05$ considered significant.

Immunofluorescence Analysis of Cultured Cells

The 15 mm coverslips used in this analysis were coated with bOPN (20 µg/ml) overnight at 4°C. pTr2 cells ($1.5X10^5$) were seeded onto the coverslips and allowed to attach for 2

h (37°C, 5% CO_2) in DMEM-F12 plus 10% FBS. Cells were then fixed with paraformaldehyde for 10 min and washed 2 times with glutaraldehyde. Immunofluorescence staining was performed as previously described (Johnson *et al*. 2001). After washing with PBS containing 0.3% vol/vol Tween 20, cells were blocked with 10% vol/vol goat serum and incubated overnight at 4°C with a monoclonal antibody to human ITGAV (1 mg/ml, AB1930). Cell-bound primary antibody was then detected with goat anti-rabbit IgG Alexa 488 (1 mg/ml, Life Technologies). Rabbit IgG at the same concentration as the primary monoclonal antibodies was used as a negative control. Coverslides were adhered to normal slides via Prolong Antifade Mounting Reagent with DAPI.

Photomicrography

Digital photomicrographs of *in situ* hybridization (brightfield and darkfield images) and immunofluorescence staining were evaluated using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) interfaced with an Axioplan HR digital camera and Axiovision 4.3 software. Individual fluorophore images (Alexa 488 and DAPI) were recorded sequentially with AxioVision 4.3 software and evaluated in multiple fluorophore overlay images recorded in Zeiss Vision Image (ZVI) file format, which were subsequently converted to Tagged Image File (TIF) format. Photographic plates were assembled using Adobe Photoshop (version 6.0, Adobe Systems Inc., San Jose, CA). All sections from each day/treatment were assessed as a group and sections exhibiting the most

representative hybridization/immunostaining pattern for each day/treatment were selected for inclusion in photographic plates.

Results

Temporal and Spatial Expression of ITGAV, ITGB3, ITGB6 and OPN mRNA and Protein

In situ hybridization analyses localized *ITGAV, ITGB3* and *ITGB6* mRNAs to uterine LE or the uterine-placental interface in cyclic and pregnant animals, respectively. Integrin mRNA is observed at the uterine-placental interface during the peri-implantation period of pregnancy on Days 20 and 25 (Figures 3-1A, 3-2A and 3-3A). Relative mRNA levels were quantified by measuring density of silver grains in the uterine LE or at the uterine-placental interface (Figures 3-1B, 3-2B and 3-3B). Quantification revealed no change in relative mRNA levels for the three integrin subunits between cyclic and pregnant gilts or between days of pregnancy. *ITGAV, ITGB3,* and *ITGB6* mRNAs are available for translation in conceptus Tr and uterine LE while the conceptus is undergoing implantation.

Estrogen was used to induce pseudopregnancy and effects on integrin *ITGAV, ITGB3,* and *ITGB6* mRNA expression were examined. *In situ* hybridization analyses revealed that uterine *ITGAV, ITGB3,* and *ITGB6* mRNA localization was not different between control and pseudopregnant gilts (Figure 3-4). Progesterone was injected into ovariectomized gilts, and effects of progesterone treatment in the absence of estrogen on

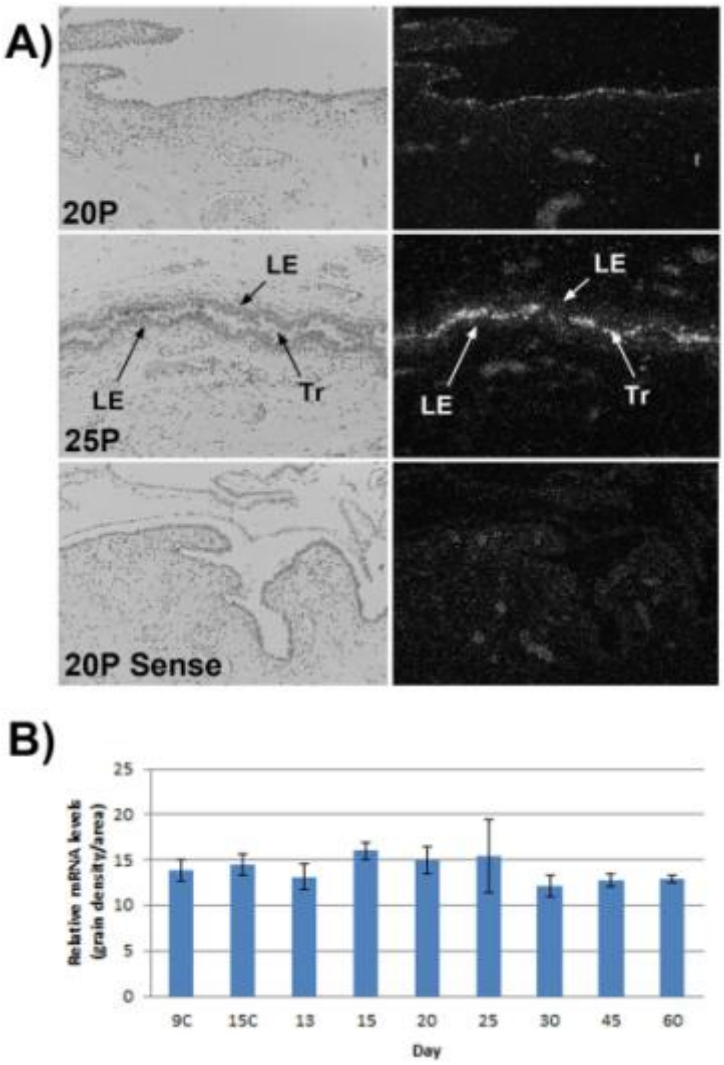

Fig 3-1. ***ITGAV*** **subunit mRNA in pig endometrium and placenta. (A)** *In situ* hybridization revealed *ITGAV* mRNA in uterine luminal epithelial (LE) and conceptus trophectoderm (Tr) during pregnancy. Corresponding brightfield and darkfield images from different days of the estrous cycle (C) and pregnancy (P) hybridized with radiolabeled antisense RNA probe are shown. A representative section hybridized with radiolabeled sense RNA probe (sense) serves as a negative control. Width of field for all microsopic images is 940 μm. **(B)** Relative density of silver grains per area measured was graphed and analyzed by one-way ANOVA. Hybridization for *ITGAV* mRNA did not change over Days of pregnancy ($P > 0.05$).

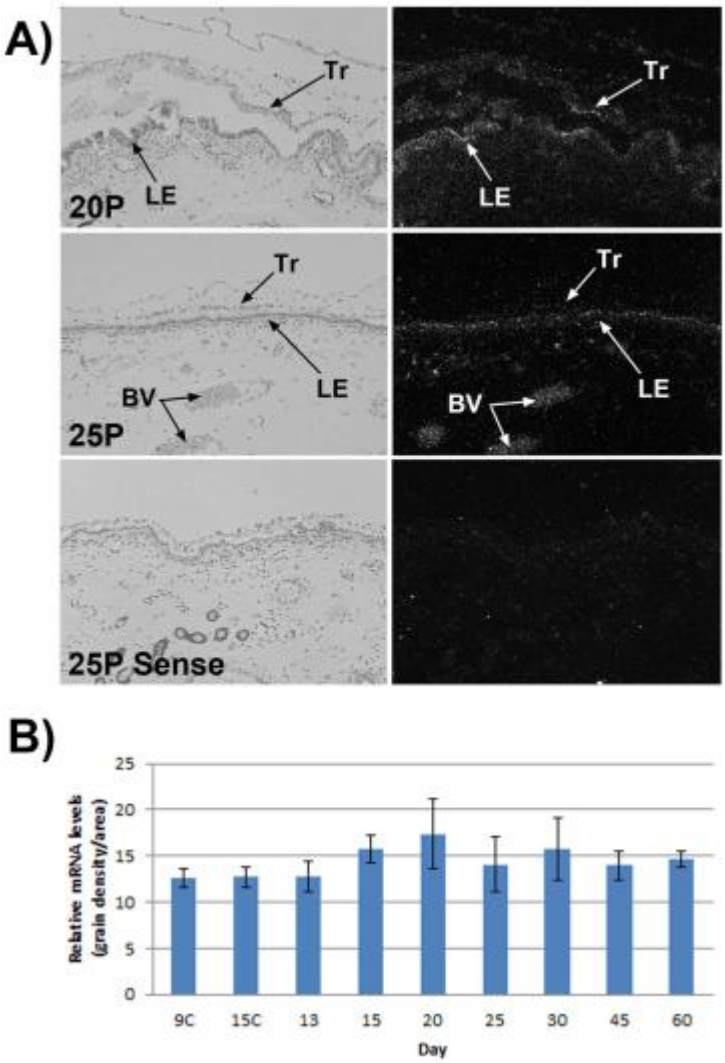

Fig 3-2. *ITGB3* subunit mRNA in pig endometrium and placenta. (A) *In situ* hybridization reveals *ITGB3* mRNA in uterine luminal epithelial (LE) and conceptus trophectoderm (Tr) during pregnancy. Corresponding brightfield and darkfield images from different days of the estrous cycle (C) and pregnancy (P) hybridized with radiolabeled antisense RNA probe are shown. A representative section hybridized with radiolabeled sense RNA probe (sense) serves as a negative control. Width of field for all microsopic images is 940 μm. (B) Relative density of silver grains per area measured was graphed and analyzed by one-way ANOVA. Hybridization for *ITGB3* mRNA did not change over Days of pregnancy ($P > 0.05$).

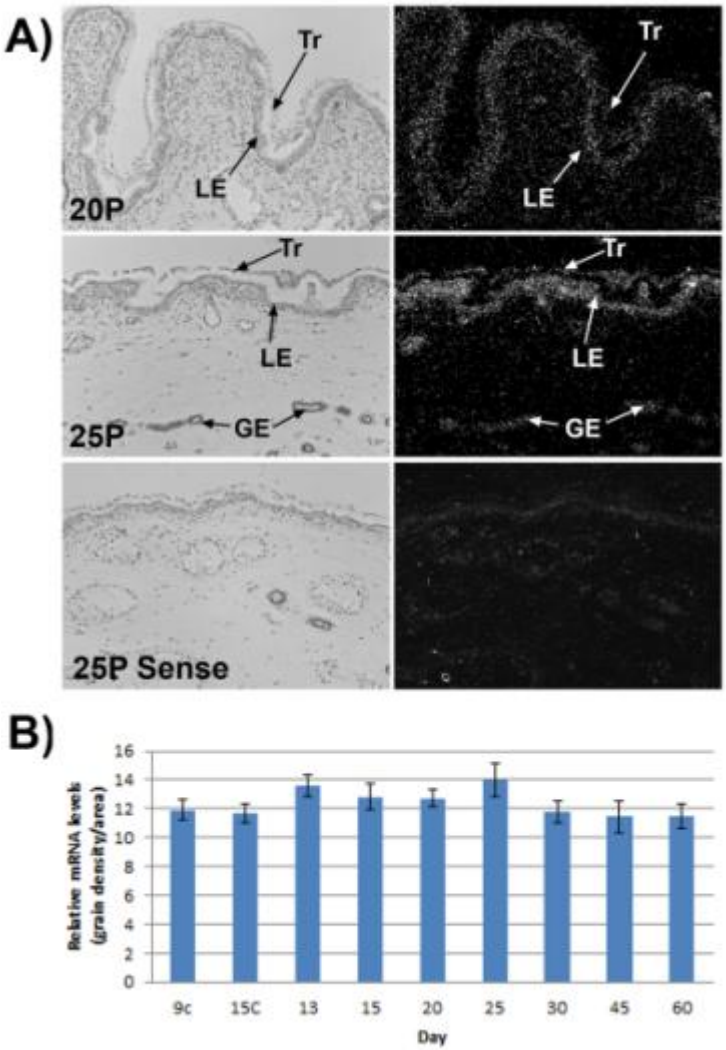

Fig 3-3. *ITGB6* subunit mRNA in pig endometrium and placenta. (A) *In situ* hybridization reveals *ITGB6* mRNA in uterine luminal epithelial (LE) and conceptus trophectoderm (Tr) during pregnancy. Corresponding brightfield and darkfield images from different days of the estrous cycle (C) and pregnancy (P) hybridized with radiolabeled antisense RNA probe are shown. A representative section hybridized with radiolabeled sense RNA probe (sense) serves as a negative control. Width of field for all microsopic images is 940 μm. (B) Relative density of silver grains per area measured was graphed and analyzed by one-way ANOVA. Hybridization for *ITGB6* mRNA did not change over Days of pregnancy ($P > 0.05$).

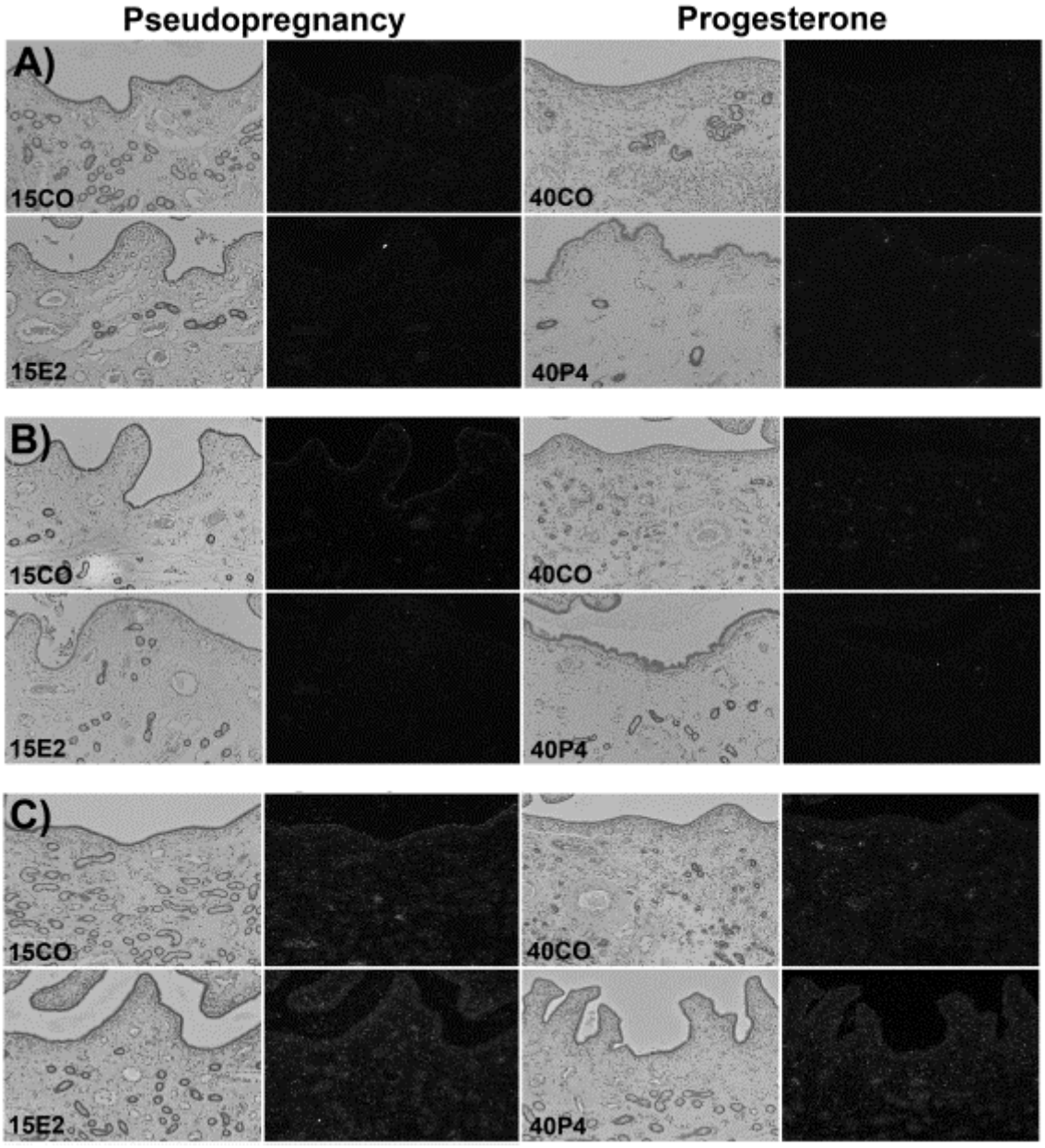

Fig. 3-4. Hormonal regulation of *ITGAV, ITGB3* and *ITGB6* mRNAs in pig endometria. Corresponding brightfield and darkfield images from pseudopregnant and progesterone treated animals were hybridized with radiolabeled antisense RNA probe are shown. *ITGAV, ITGB3* and *ITGB6* mRNAs were not increased in uterine LE of Day 15 pseudopregnant pigs, and did not increase in uterine LE in response to exogenous progesterone (A, B and C respectively). Width of field for all microscopic images is 940 μm. Legend: CO, corn oil; E2, pseudopregnant; P4, progesterone-treated.

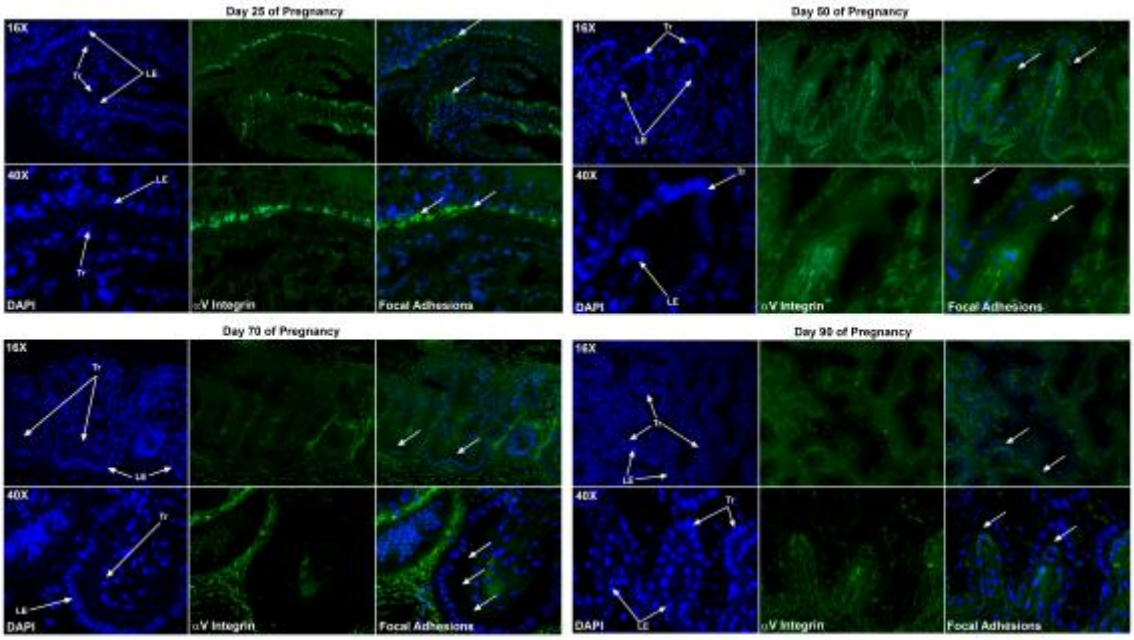

Fig 3-5. Immunofluorescence detection of ITGAV subunit protein at the uterine-placental interface on Days 25, 50, 70, and 90 of pregnancy. Large aggregates of ITGAV, suggestive of FA assembly were detected at the interface between uterine luminal epithelial (LE) and conceptus trophectoderm/chorionic epithelial (Tr) cells on Day 25, but were not detected on Days 50, 70 or 90 of gestation. Nuclei are stained blue with DAPI. Width of field for the 16X magnification is 540 μm, and for the 40X magnification is 230 μm. Legend: arrows, interface between LE and Tr. The antibody used to detect ITGAV was AB1930 from Millipore.

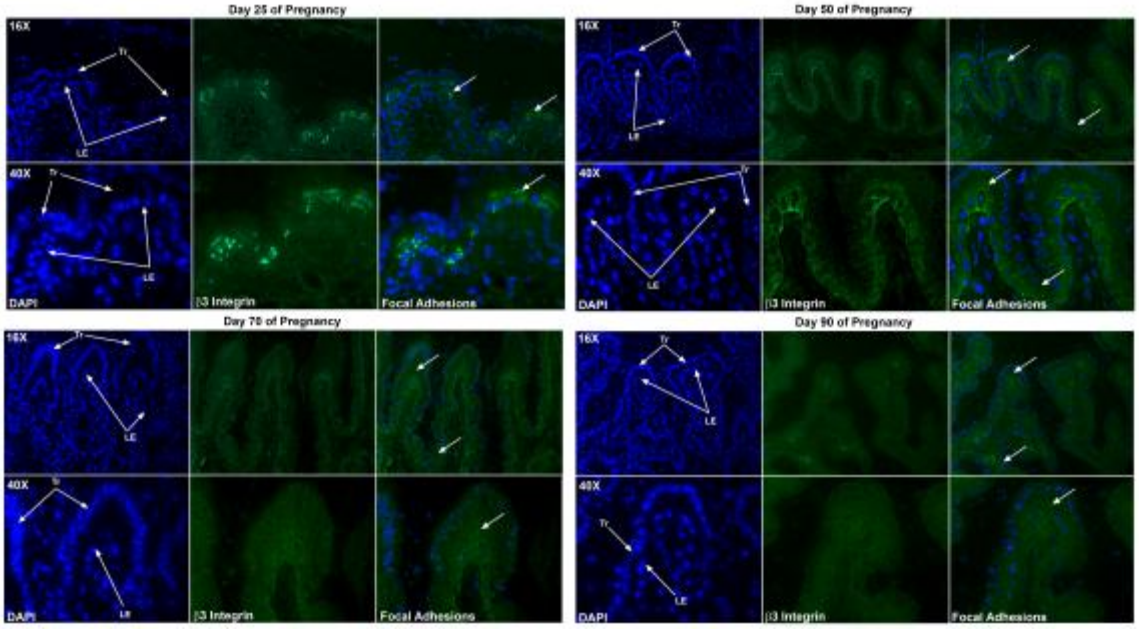

Fig 3-6. Immunofluorescence detection of ITGB3 subunit protein at the uterine-placental interface on Days 25, 50, 70, and 90 of pregnancy. (A) Large aggregates of ITGB3 suggestive of FA assembly were detected at the interface between uterine luminal epithelial (LE) and conceptus trophectoderm/chorionic epithelial (Tr) cells on Day 25, but were not detected on Days 50, 70 or 90 of gestation. The antibody used to detect ITGB3 was AB1968 from Millipore.

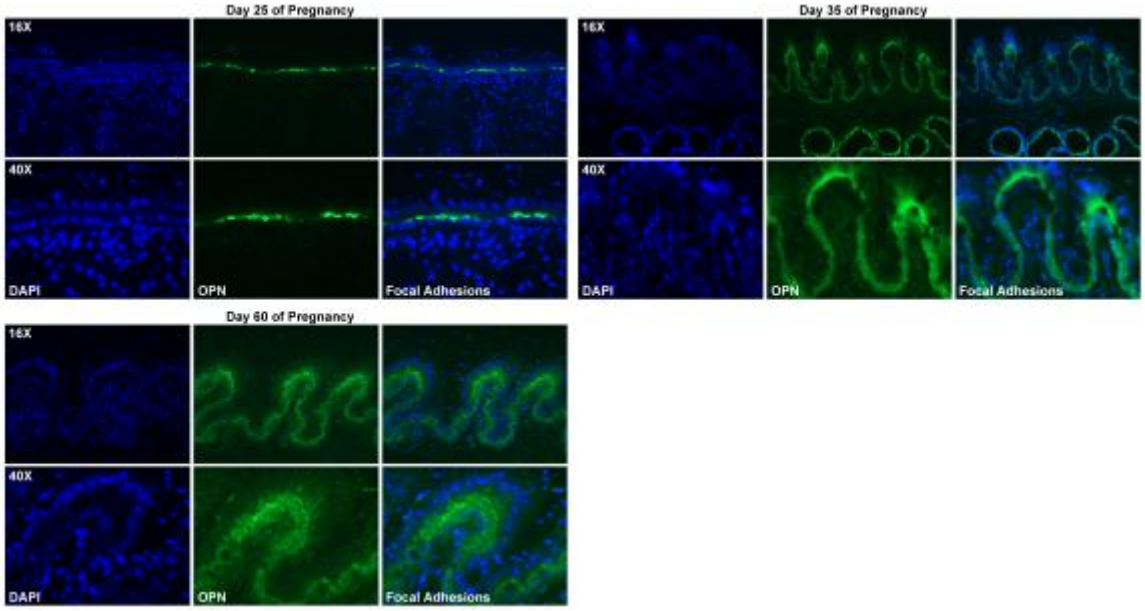

Fig 3-7. Immunofluorescence detection of OPN protein at the uterine-placental interface on Days 25, 35 and 60 of pregnancy. (A) Large aggregates of OPN suggestive of FA assembly were detected at the interface between uterine luminal epithelial (LE) and conceptus trophectoderm/chorionic epithelial (Tr) cells on Day 25, but were not detected on Days 35 and 60 of gestation. The antibody used to detect OPN was AB10910 from Millipore.

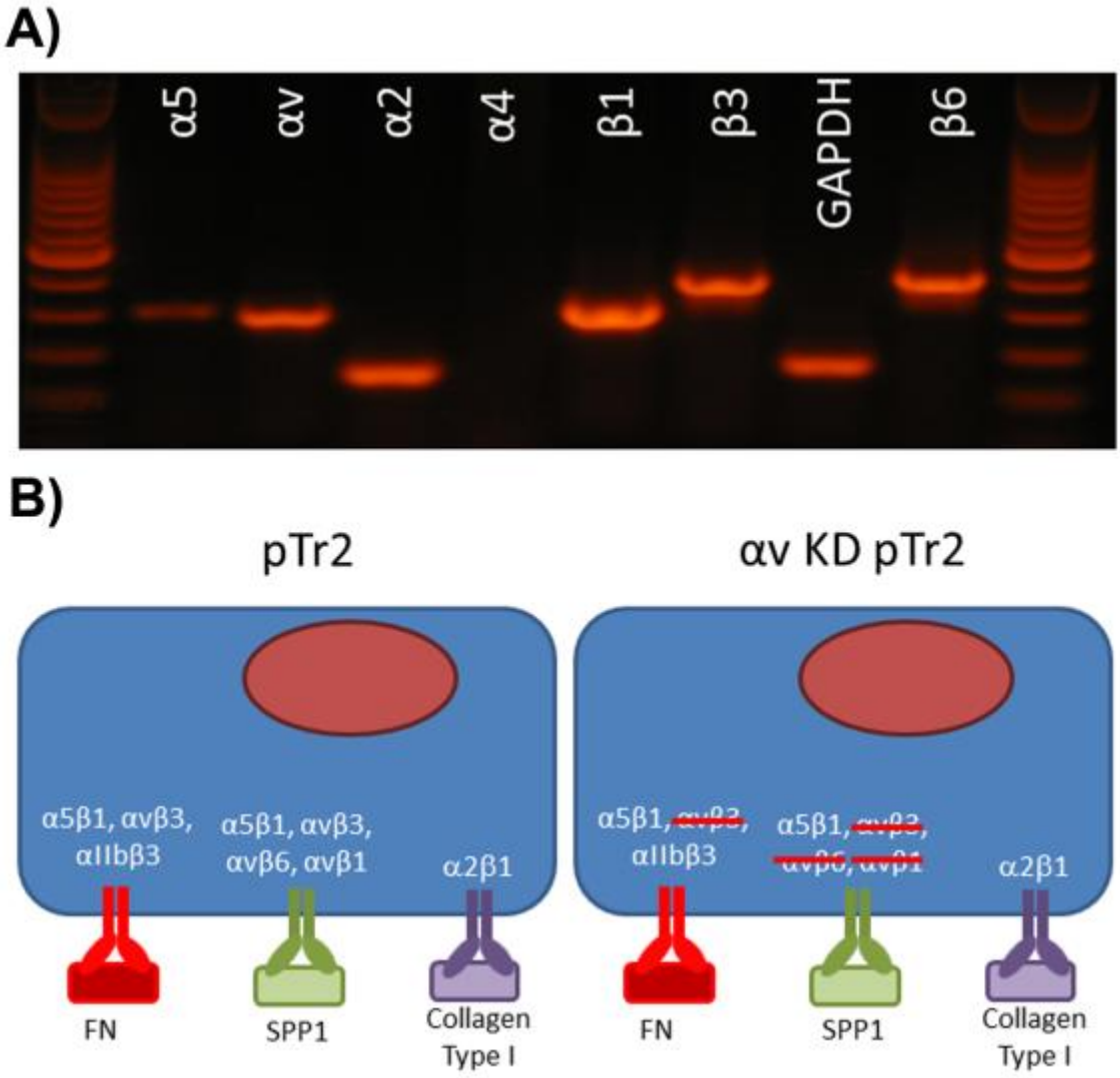

Fig 3-8. Expression of integrin subunit mRNAs by the porcine trophectoderm (pTr2) cell line. (A) PCR profile of integrin subunits expressed by pTr2 cells. **(B)** Cartoon depicting potential integrin heterodimeric receptors present on pTr2 cells based upon PCR analysis. Schematics show integrin expression in wild-type pTr2 cells (left panel) and pTr2 cells treated with siRNA directed to the ITGAV subunit (right panel). Legend: KD, knockdown; FN, fibronectin.

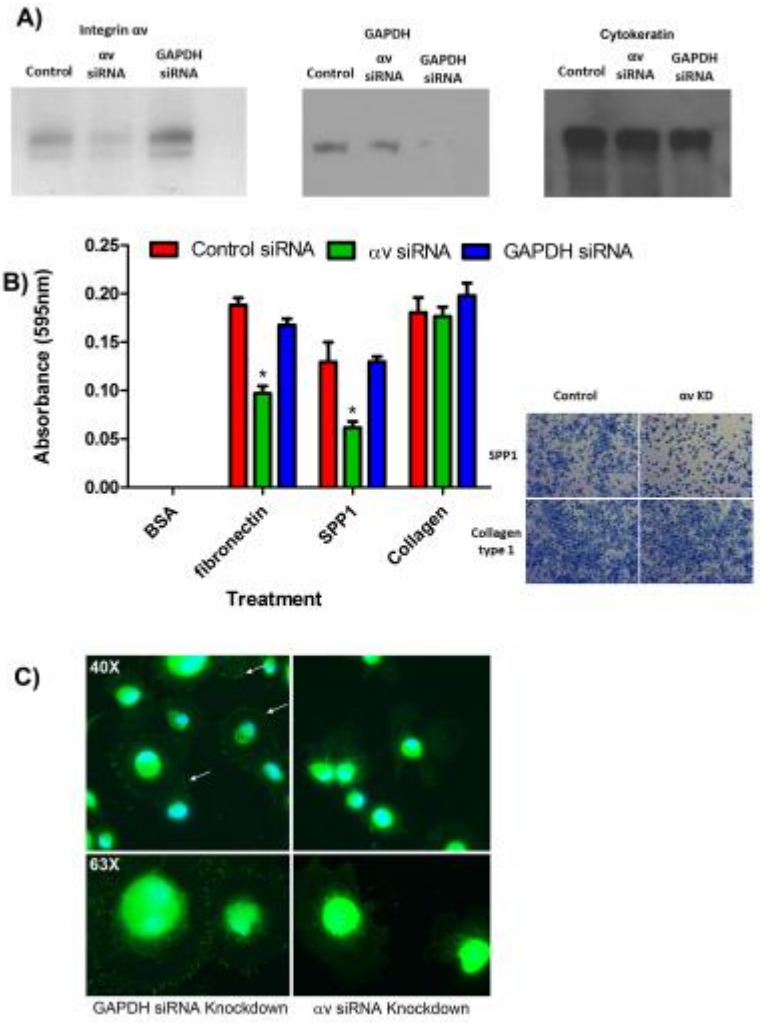

Fig. 3-9. siRNA knockdown of ITGAV decreases adhesion of pTr2 cells to OPN. (A) Western Blot showing decreased ITGAV protein in cells treated with *ITGAV* siRNA, decreased GAPDH protein in cells treated with *GAPDH* siRNA, and equal loading of gels using anti-cytokeratin IgG to detect protein. **(B)** Adhesion of cells to extracellular matrix molecules. pTr2 cells were added to multiwell plates that were coated with 20 µg/ml BSA (negative control), bovine fibronectin (fibronectin), bovine OPN (OPN), or collagen type 1 (collagen) for 1 h prior to fixing and staining with Amido Black and quantification of absorption at 595 nm. Error bars represent the standard error of the mean $p \leq .05$. **(C)** Immunofluorescence localization of ITGAV on pTr2 cells bound to OPN. Immunofluorescence analysis confirmed that ITGAV is a component of FAs (white arrows) that assemble at the base of pTr2 cells. However, siRNA knockdown of ITGAV protein decreased the assembly of FAs in response to binding OPN. Images are 230 and 140 um wide for 40X and 63X. Antibody used to detect ITGAV (AB1930; Millipore).

ITGAV, ITGB3, and *ITGB6* mRNA expression were examined. *In situ* hybridization analyses revealed that uterine *ITGAV, ITGB3,* and *ITGB6* mRNA localization was not different between control and progesterone-treated gilts (Figure 3-4).

Immunofluorescence was performed to determine whether integrin ITGAV and ITGB3, and OPN proteins (antibody that cross-reacted with the pig ITGB6 protein was not available) assemble into FAs that serve to attach LE to Tr during implantation and placentation. ITGAV, ITGB3, and OPN proteins were present in large aggregates at the uterine-placental interface on Day 25 of pregnancy (Figures 3-5, 3-6 and 3-7), similar to what was reported previously for pigs (Erikson *et al*. 2009, Massuto *et al*. 2009), but different from what has been reported for sheep during the peri-implantation period of pregnancy (Burghardt *et al*. 2009). These large protein aggregates were no longer observed at the uterine-placental interface in pigs by Day 35 of pregnancy and remained absent at the interface thereafter (Figures 3-5, 3-6 and 3-7). This is in contrast to what was previously reported for the interplacentomal regions of pregnant uteri from sheep where FAs progressively increased as pregnancy proceeded (Burghardt *et al*. 2009). It is noteworthy that OPN protein was detected along the uterine-placental interface throughout pregnancy in pigs which was expected due to previously reported expression of *OPN* mRNA at this interface (Garlow *et al*. 2002). However, aggregates of OPN protein were not observed after Day 25 of pregnancy (Figure 3-7). The ITGAV subunit was present in the endometrial stroma on Days 50 and 70 of pregnancy in pigs (Figure 3-5).

These results demonstrate that mRNAs for *ITGAV, ITGB3,* and *ITGB6* are present in uterine LE and conceptus Tr. Additionally, ITGAV, ITGB3, and OPN proteins appear to incorporate into large aggregates, which are interpreted to be FAs at the uterine-placental interface.

Characterization of Integrin Subunits Present in pTr2 Cells

A porcine Tr (pTr2) cell line (Ka *et al*. 2001, Erikson *et al*. 2009) was utilized for *in vitro* analysis of functional integrin receptors. First, characterization of integrin subunits expressed by pTr2 cells using PCR analyses revealed the presence of integrin subunits *ITGAV, ITGA2, ITGA5, ITGB1, ITGB3* and *ITGB6,* but not *ITGA4* (Figure 3-8A). Detection of *GAPDH* mRNA was used as a positive control for the PCR reaction. The cartoon in Figure 3-8B depicts the integrin heterodimeric protein receptors that are potentially present on pTr2 cells. There are four potential receptors that bind OPN (α5β1, αvβ3, αvβ6, αvβ1), three potential receptors that bind fibronectin (α5β1, αvβ3, αIIbβ3; Rout *et al*. 2004) and one potential receptor that binds type 1 collagen (α2β1). Because ITGAV is a component of three out of four of the integrin receptors that bind OPN, we chose to knockdown expression of this integrin subunit protein in pTr2 cells using siRNA. This knockdown results in the loss of three integrin receptors reported to bind OPN (αvβ3, αvβ6, αvβ1), and one integrin receptor that binds fibronectin (αvβ3), but ITGAV knockdown does not affect the ability of pTr2 cells to bind type 1 collagen through its integrin receptors (Figure 3-8B).

Effects of ITGAV Subunit Knockdown in pTr2 Cells

Western blotting was utilized to determine the ability of siRNAs to knock down ITGAV protein in pTr2 cells. Cells were treated with no siRNA (control) or siRNAs targeting either integrin ITGAV or GAPDH. The *GAPDH* siRNA was utilized to determine if the siRNA itself affects the function of cells. Cells transfected with ITGAV siRNA had a reduction in ITGAV protein in cell lysates when compared to the control and *GAPDH* siRNA transfected cells (Figure 3-8A). Cells treated with *GAPDH* siRNA exhibited a decrease in GAPDH protein in cell lysates compared to control and *ITGAV* siRNA treated cells (Figure 3-9A). Treatment with *ITGAV* or *GAPDH* siRNAs did not affect expression of cytokeratin, an epithelial cell intermediate filament in pTr2 cells. An adhesion assay was utilized to determine the effects of ITGAV subunit knockdown on the ability of pTr2 cells to bind OPN, collagen type 1 or BSA. There was a significant decrease in the number of cells that bound fibronectin and OPN in the *ITGAV* siRNA treated group when compared to treatment with Control or *GAPDH* siRNA (Figure 3-9B). Therefore, the ITGAV subunit has a significant role in attachment of pTr2 cells to OPN. As expected, there was no difference in attachment of pTr2 cells among treatment groups to collagen type 1, because collagen does not bind to ITGAV containing integrin heterodimers (Figure 3-9B). Immunofluorescence analysis was utilized to determine whether ITGAV knockdown affects the ability of pTr2 cells to bind OPN and form FAs at their basal surface. Large aggregates of ITGAV subunit were observed in the *GAPDH* siRNA treatment group at the periphery of the cells attached to OPN coated coverslips (Figure 3-8C). In contrast, there were few FAs observed when pTr2 cells were

transfected with *ITGAV* siRNA as compared to the other treatment groups (Figure 3-9C). In addition, cells treated with ITGAV siRNA did not flatten and spread on the OPN when compared with the *GAPDH* siRNA treated cells (Figure 3-9C).

Discussion

Integrins are heterodimeric transmembrane receptors that are formed from 18 α- and 8 β-subunits capable of dimerizing to form 24 different heterodimer combinations that can bind to numerous extracellular ligands including a variety of ECM proteins, along with a smaller number of adhesion receptors on adjacent cells (Albelda 1990, Gallant *et al*. 2005, Humphries *et al*. 2006). Integrin-mediated adhesion is focused within a primary mechanotransduction unit of dynamic structure and composition known as a FA whose size, composition, cell signaling activity and adhesion strength are force-dependent (Hynes *et al*. 1987, Vogel *et al*. 2006). The intrinsic properties of the ECM in different niches and tissue-level compartments affects the composition and size of FAs that, in turn, modulate cell behavior including gene expression, protein synthesis, secretion, adhesion, migration, proliferation, viability, and/or apoptosis (Geiger *et al*. 2009). Integrins are dominant glycoproteins in many cell adhesion cascades, including well defined roles in leukocyte adhesion to the apical surface of polarized endothelia for extravasation of leukocytes from the vasculature into tissues (Kling *et al*. 1992).

A similar adhesion cascade involving apically expressed integrin receptors on uterine LE is proposed as a mechanism for initial attachment of Tr to uterine LE for implantation (Aplin *et al*. 1994). The general consensus is that the integrins involved in this

attachment are those of the ITGAV family (Aplin *et al*. 1997). Lessey and co-workers established that transient expression of αvβ3 is during the menstrual cycle and early pregnancy is a marker of the "window of implantation" in women (Lessey *et al*. 1994, Lessey *et al*. 1996) and that ITGB3 is absent in endometria of women with luteal phase deficiency (Lessey *et al*. 1992). Further, the pattern of expression of ITGB3 is altered in endometria of women with mild endometriosis and unexplained infertility (Lessey *et al*. 1994, Lessey *et al*. 1995). Null mutation of the *ITGAV* gene in mice leads to peri-implantation lethality (Hynes *et al*. 1996), while functional blockade of ITGAV using neutralizing antibody reduces the number of implantation sites in mice and rabbits (Illera *et al*. 2000, Illera *et al*. 2003). The ITGAV integrin subunit has also been localized to the apical surface of uterine LE of species that have non-invasive implantation, including pigs, sheep, goats, and cattle (Bowen *et al*. 1996, Guillomot *et al*. 1999, Kimmins *et al*.1999, Johnson *et al*. 2001). Indeed, in pigs, co-localization of ITGAV and talin at the apical domains of Tr and uterine LE cells of the uterine-placental interface during the peri-implantation period of pregnancy strongly suggests an active role for ITGAV in the formation of FA-mediated attachments (Erikson *et al*. 2009). Further, ITGAV expressed by porcine and ovine Tr cells was shown to bind to OPN-coated beads in affinity chromatography experiments, and immunoprecipitation of the eluted fractions from these experiments showed this ITGAV was assembled into integrin receptors (pigs αvβ6; sheep αvβ3, α5β1) (Erikson *et al*. 2009; Kim *et al*. 2010). Because OPN, an ECM ligand with prominent expression at the uterine-placental interface, supports FA assembly, cell adhesion, and migration of pTR2 cells *in vitro*, it is hypothesized that the

αvβ6 integrin heterodimer mediates these events during implantation in the pig (Erikson *et al*. 2009, Kim *et al*. 2010).

Although ITGAV aggregates at the apical surface of sheep Tr cells along the periphery of OPN-coated polystyrene beads and is present in FAs at the base of porcine and ovine Tr cells cultured on OPN, it is not known whether ITGAV is mechanistically involved in Tr cell adhesion, an event central to implantation. Therefore, a primary focus of the present study was to directly test whether ITGAV-containing integrin heterodimers support Tr cell adhesion to OPN. An established porcine Tr cell line was utilized for *in vitro* assays. The pTr2 cells were first characterized to determine their integrin expression profile to determine the validity of utilizing this cell line. PCR analysis showed the presence of integrin subunits *ITGAV, ITGA2, ITGA5, ITGB1, ITGB3* and *ITGB6,* but not *ITGA4*. These same subunits have been previously observed on pig conceptus Tr *in vivo* (Bowen *et al*. 1996). To determine the physiological relevance of integrin ITGAV in these cells, siRNA was utilized to knock down this integrin subunit *in vitro*. Treated cells were analyzed to determine their ability to bind different ECM proteins. Loss of ITGAV significantly reduced the ability of pTr2 cells to bind OPN and form FAs when grown on OPN coated dishes. These results indicate that the decrease in the ability of these cells to bind OPN is due to the reduced ability of the cells to form ITGAV containing FAs, and are a strong indication that ITGAV plays a role in the ability of porcine Tr cells to bind to OPN. Results of these functional studies support the long held hypothesis that ITGAV plays a role in integrin binding to OPN to mediate adhesion of conceptus Tr to uterine LE during implantation in pigs.

Another focus of this study was to determine the expression of select integrin subunits by uterine and placental tissues during pregnancy in pigs. Integrin expression within these tissues had previously been determined through Day 20 of gestation (Bowen *et al*. 1996; Erikson *et al*. 2009). In a previous study, we demonstrated that porcine Tr and uterine LE cells express αvβ6 and αvβ3 integrins, respectively, in their plasma membranes, and that these integrins may be involved in attachment of pTr2 cells to OPN (Erikson et. al. 2009). Immunofluorescence screening for ITGAV, ITGB3 and OPN identified areas of intense apical punctate staining that appeared to aggregate into dense deposits in single cells or small groups of cells at the uterine-placental interface on Day 25 of pregnancy. Immunostaining detected ITGAV protein in both Tr and uterine LE cells, whereas integrin ITGB3 immunostaining was limited to uterine LE. The aggregation of OPN at this interface indicates that transmembrane ITGAV and ITGB3 and OPN are incorporated into FAs that span from the cytoplasmic plasma membrane surface (intracellular integrin subunit tails) to the intercellular space where OPN resides. We hypothesize that aggregates of ITGAV, ITGB3 and OPN represent functionally distinct FAs that are formed in response to tensile or shear forces at the uterine-placental interface imposed during attachment of Tr of long filamentous porcine conceptuses to uterine LE.

Similar FAs have been described at the interplacentomal uterine-placental interface in sheep (Burghardt et. al. 2009), and at the uterine-placental interface of pigs on Days 20 and 24 of pregnancy (Erikson *et al*. 2009, Massuto *et al*. 2009). However, of particular interest in the present study, is that ITGAV and ITGB3 immunostaining at the uterine-

placental interface in pigs was no longer detectable on Days 50, 70 and 90 of pregnancy. This temporal change in distribution of FAs contrasts with what has been observed for sheep where FAs progressively increase as pregnancy progresses (Burghardt *et al*. 2009). It has been hypothesized, for sheep, that the development of large FAs reflects adaptation of the Tr/chorion to maintain a tight connection with uterine LE along regions of true epitheliochorial placentation in response to dynamic increases in tensile, compression, and shear loads imposed by expanding fetal and placental mass and dynamic responses to changes in maternal posture, locomotion, and/or position of the fetus (Hynes *et al*. 1987). A similar increase in FAs at the uterine-placental interface was predicted for pigs; however, this prediction did not take into account the dramatic increase in folding that is observed between the uterine endometrium and chorioallantois in pigs as compared to the interplacentomal regions of sheep. The attached chorion-endometrial epithelial bilayer develops microscopic folds, beginning about Day 35 of gestation, and these folds increase the area of contact between maternal and fetal capillaries to maximize maternal-to-fetal exchange of nutrients in pigs (Dantzer *et al*.1984). These folds may also serve to structurally redistribute and disperse forces generated by increasing tensile, compression, and shear loads, removing the requirement for expansive FAs to maintain firm attachment between the uterus and placenta.

In conclusion, results of the present study are the first to directly demonstrate that Tr cells engage ITGAV to adhere to OPN, and suggest that attachment of the conceptus Tr to the uterine LE for implantation in pigs is mediated by ITGAV-containing integrin receptors that interact with ECM proteins. OPN is the leading candidate ligand for

which expression is induced by estrogen secreted by pig conceptuses at sites of implantation. These cell-ECM interactions respond to tension and shear forces generated by the act of tethering the elongating filamentous conceptuses to the uterine LE to form FAs. As implantation transitions into stable placentation, the chorion-endometrial epithelial bilayer develops folds to increase interdigitation of uterine and placental tissues, thereby providing a 3-dimensional structure that disperses shear stress at the uterine-placental interface. Without these physical perturbations to drive FA assemble there is an eventual loss of FAs at the uterine-placental interface.

CHAPTER IV

OSTEOPONTIN AND MULTIPLE INTEGRINS CO-DISTRIBUTE WITHIN LARGE PROTEIN AGGREGATES AS CONSTITUENTS OF FOCAL ADHESIONS AT THE UTERINE-PLACENTAL INTERFACE

Introduction

Osteopontin (OPN; also known as secreted phosphoprotein 1, SPP1) is a secreted extracellular matrix (ECM) protein that binds to a variety of cell surface integrins (Hynes *et al*. 1987, Denhardt *et al.*1993, Senger *et al.*1994, Butler *et al.*1996, Sodek *et al.* 2000). Integrins are transmembrane glycoprotein receptors composed of non-covalently bound α and β subunits that promote cell-cell and cell-ECM adhesion, cause cytoskeletal reorganization to stabilize adhesion, and transduce cell signaling through numerous signaling intermediates (Giancotti *et al.*1999, Burghardt *et al.*2002). Integrins are dominant glycoproteins in many cell adhesion cascades, including well defined roles in leukocyte adhesion to the apical surface of polarized endothelial cells for extravasation of leukocytes from the vasculature into tissues (Kling *et al.*1992). A similar adhesion cascade involving interactions between the extracellular matrix (ECM) and apically expressed integrin receptors on the uterine luminal epithelium (LE) and conceptus (embryo and placental membranes) trophectoderm (Tr) is proposed as a mechanism for attachment of the conceptus to the uterus for implantation; the initial step for the extensive tissue remodeling that occurs during placentation (Aplin *et al.*1994).

OPN is a leading candidate adhesion molecule for implantation and placentation in pigs and sheep (Johnson *et al.*2003; Johnson *et al.*2014).

Focal adhesions (FA) 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 and Burridge 2000, Wozniak *et al.*2004, Larsen *et al.*2006). Studies in cultured cells indicate that the location, size and specific protein components of FAs are dependent upon the composition and rigidity of the ECM and the application of external or generated internal mechanical forces to integrin-ECM complexes (Katz *et al.*2000; Galbraith *et al.*2002). Indeed, the intrinsic properties of the ECM in different niches and tissue-level compartments affect the composition and size of FAs that, in turn, modulate cell behavior including gene expression, protein synthesis, secretion, adhesion, migration, proliferation, viability, and/or apoptosis (Geiger *et al.*2009). Although great variations in histoarchitecture are observed among different species, the uterine-placental interface of mammals is a unique physiological tissue niche in which the apical surfaces of uterine LE attach to the apical surfaces of placental Tr/chorion during the initial stages of implantation, and intimate interactions between uterine and placental tissues that are maintained throughout pregnancy. This interface is subjected to changing mechanical forces that appear to drive the temporal and spatial assembly of large FAs through the aggregation of integrins between uterine LE and Tr (Burghardt *et al.*2009; Erikson *et al.*2009).

Research regarding OPN has begun to focus on its interactions with integrin receptors in the female reproductive tract. Affinity chromatography and immunoprecipitation experiments revealed *in vitro* binding of ovine Tr αvβ3 integrin receptor, porcine Tr αvβ6, and porcine uterine LE αvβ3, to OPN (Erikson *et al.*2009, Kim *et al.*2010). Further, OPN supports Tr cell migration and attachment to uterine LE that may be critical to conceptus elongation and implantation. Aplin and co-workers (Kang *et al.*2014) employed three *in vitro* models of early implantation with Ishakawa cells to demonstrate that OPN potentially interacts with the αvβ3 integrin receptor during implantation in humans. Studies by Kim *et al*. found that OPN binding to the αvβ3 integrin receptor induced *in vitro* FA assembly, a prerequisite for adhesion and migration of Tr, through activation of: 1) P70S6K via crosstalk between FRAP1/MTOR and MAPK pathways; 2) MTOR, PI3K, MAPK3/MAPK1 (Erk1/2) and MAPK14 (p38) signaling to stimulate Tr cell migration; and 3) FA assembly and myosin II motor activity to induce migration of Tr cells (Kim *et al.*2010). These *in vitro* data strongly implicate OPN as a binding partner for integrin receptors to induce FA formation at the uterine-placental interface.

Sheep, and other domestic animal models for *in vivo* studies offer unique characteristics of pregnancy: 1) elongation of the blastocyst into a filamentous conceptus (embryo/fetus and associated placental membranes); 2) a protracted and incremental attachment cascade of trophoblast to endometrial LE during implantation; and 3) development of regions of true epitheliochorial placentation that maintain connections of apical surfaces of endometrial LE to Tr. Examination of the temporal and spatial expression and

hormonal regulation of uterine OPN mRNA and protein, and integrin subunit mRNAs and proteins in the uteri and placentae of sheep from 1999 through 2002 provide valuable insights into the possible roles of OPN and integrins during placentation. The consensus is that OPN is a progesterone-induced secretory product of endometrial glandular epithelium (GE) that binds integrins on the apical surfaces of endometrial LE and conceptus Tr to mediate attachment of uterus to Tr during the peri-implantation period of sheep (Johnson *et al.*1999; Johnson *et al.*1999; Johnson *et al.*2000; Johnson *et al.*2001). In 2009, Burghardt and colleagues (Burghardt *et al.*2009) reported the *in vivo* assembly of large FAs containing aggregates of ITGAV, ITGA4, ITGA5, ITGB1, ITGB5, alpha-actinin, and focal adhesion kinase (FAK) at the uterine-placental interface of sheep; however, OPN was not shown to aggregate in the same manner (Burghardt *et al.*2009).

We hypothesize that OPN binds integrins to attach uterine LE to conceptus Tr during implantation in sheep; and as fetal, placental, and fetal fluids grow they exert increasing tension, compression, and/or shear forces against this interface that drive OPN and integrins to initiate the assembly of FAs to adapt to the changing microenvironment. However key gaps in our knowledge about the dynamics of OPN and integrin expression during pregnancy severely hinder our understanding of their respective roles in implantation and placentation in sheep. OPN is present at the uterine-placental interface of sheep (Johnson *et al.*2003), but it has never been shown that OPN redistributes into discreet aggregates representing FAs. Further multiple integrin subunits are expressed evenly along the entire uterine-placental interface on Day 15 of pregnancy (during

attachment for implantation). The pattern of expression suggests adhesion of Tr to uterine LE without generating FAs; however, on Day 80 of gestation in sheep those integrins are found in large intermittent focal adhesion aggregates along the uterine-placental interface (Burghardt *et al.*2009). Presently there has been no comprehensive study to determine when these integrins begin to redistribute into FAs during pregnancy in sheep. In the current study we used immunohistochemistry to illustrate dynamic changes in the distribution of integrin subunits that are initiated between Days 15 and 20 along the interplacentomal regions of the uterine-placental interface. This is a period of instability in the integrity of the endometrial LE that developmentally demarks the transition from implantation to placentation. Further, for the first time in any species, we used immunofluorescence microscopy to demonstrate the precise co-localization of OPN with multiple integrin subunits to form large FAs at the uterine-placental interface of sheep. These results are the strongest indication, to date, that OPN and integrins interact at the uterine-placental interface of sheep and that these dynamic interactions change as the tissue compartment adapts to internal and external forces generated with advancing stages of pregnancy. These results from studies of pregnant sheep have significant implications for our understanding of the role of OPN across other species, including humans.

Materials and Methods

Animal Model

Mature Rambouillet ewes were observed daily for estrus (Day 0 is the day of onset of estrus) in the presence of vasectomized rams and used in experiments after they had exhibited at least two estrous cycles of normal duration (16-18 days). All experimental and surgical procedures were in compliance with the Guide for the Care and Use of Agriculture Animals in Research Teaching and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Ewes were mated to intact rams three times at 12 h intervals beginning at estrus. Pregnant ewes were ovariohysterectomized on either Day 9, 11, 13, 15, 16, 20, 25, 30, 35, 40, 50, 55, 60 or 80 (n=4 ewes/day). Uteri from Days 9, 11, 13, 15 and 16 were flushed with 0.9% NaCl, and pregnancy was verified by the recovery of an apparently normal conceptus in uterine flushes. Several sections (1-1.5 cm) of uterine wall from the middle of each uterine horn ipsilateral to the CL were fixed in 4% paraformaldehyde for 24 h and then embedded in Paraplast Plus (Oxford Labware, St Louis, MO) or snap frozen in Tissue-Tek OCT compound (Fisher Scientific, Pittsburgh, PA).

Immunohistochemical Analyses of Integrins at the Uterine-placental Interface

Immunohistochemical localization of integrin proteins in ovine uterine and conceptus tissues (Paraffin-embedded thin sections of 10 μm) was performed as previously described (Joyce *et al.*2005) with six rabbit anti-integrin (human) antibodies (5 μg/ml,

Millipore, Billerica, MA) including anti-ITGAV (AB1930), anti-ITGA4 (AB1924), anti-ITGA5 (AB1928), anti-ATGB1 (AB1952) and anti-ITGB3 (AB1968), anti-ITGB5 (AB1926). Antigen retrieval was performed using protease (0.5 mg/ml in PBS, Sigma-Aldrich) for ITGA4, ITGAV and ITGA5 antibodies, and boiling citrate (0.01M sodium citrate buffer, pH 6.0) for ITGB1 antibody. Purified rabbit IgG was used as a negative control. Immunoreactive proteins were visualized in sections using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) following the manufacturer's instructions. 3, 3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) was utilized as the color substrate. Sections were prepared using hematoxylin as a counterstain. OPN was localized in ovine uterine and conceptus tissues using an antibody to bovine OPN (25μg/ml; Erikson *et al*. 2007).

Immunofluorescence Co-localization of OPN and Integrins at the Uterine-placental Interface

To co-localize OPN and integrins ITGAV, ITGA4, ITGA5, ITGB1, ITGB3 and ITGB5 in tissue at the interplacentomal uterine-placental interface, frozen tissues were serially sectioned at 10 μm thickness. The serial sections were fixed with methanol at -20°C for 20 min, and then washed three times for 5 min in PBS at room temperature. These sections were then blocked in 10% normal goat serum diluted in antibody dilution buffer for 1 h at room temperature. Rabbit anti-OPN (1 μg/ml, AB10910, Millipore) or anti-integrin polyclonal antibody (the same antibodies described in the precious section were used at 1μg/ml), was added and incubated overnight at 4°C in a humidified chamber.

Normal rabbit IgG was substituted for primary antibody and served as a negative control. Tissue sections were washed three times for 5 min in PBS. The goat anti-rabbit IgG Alexa 594 (4 µg/ml; Chemicon, Temecula, CA) was added and incubated for 1 h at room temperature. Tissue sections were then washed three times for 5 min in PBS and slides were counterstained with Prolong Gold Antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies, Grand Island NY) and covered with glass coverslips (Johnson *et al.*2001).

Photomicrography

Digital photomicrographs of immunohistochemistry and immunofluorescence staining were evaluated using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) interfaced with an Axioplan HR digital camera and Axiovision 4.3 software. Individual fluorophore images (Alexa 488 and DAPI) were recorded sequentially with AxioVision 4.3 software and evaluated in multiple fluorophore overlay images recorded in Zeiss Vision Image (ZVI) file format, which were subsequently converted to Tagged Image File (TIF) format. Photographic plates were assembled using Adobe Photoshop (version 6.0, Adobe Systems Inc., San Jose, CA). All sections from each day of pregnancy were assessed as a group; and sections exhibiting the most representative immunostaining pattern for each day of pregnancy were selected for inclusion in photographic plates.

Results

Immunolocalization of Integrins during Pregnancy

To determine the temporal and spatial expression of integrin subunits at the interplacentomal uterine-placental interface during pregnancy in sheep, immunohistochemistry was conducted on paraffin-embedded thin sections from Days 9 through 80. ITGAV, ITGA4, ITGA5, ITGB1, ITGB3 and ITGB5 proteins were detected in uterine and placental tissues (Figures 4-1 through 4-7), and followed a general pattern of immunostaining: 1) subunits were not detectable through Day 9; 2) they presented as a continuous thin layer of proteins at the apical surface of uterine LE from Days 11 through 16; 3) large, infrequent, intermittent and disorganized integrin aggregates at the interface between uterine LE and placental Tr/chorion from Days 20 through 55; and 4) precisely distributed expression at the apical surfaces of apposed LE and Tr/chorion along extensive expanses of the uterine-placental interface on Day 60. A representative example of the immunostaining characteristic of the peri-implantation period, Days 11-16, is evident for ITGB3 (Figure 4-5). On Day 15 of gestation, ITGB3 is present only at the apical surface of LE cells and extends along the entire luminal surface, presumably to serve as a platform for attachment to Tr via ECM molecules. A representative example of the immunostaining characteristic of mid- pregnancy, Days 20-55, is evident for ITGB1 (Figure 4-4). On Days 20-35 of gestation, ITGB1 forms impressively large aggregates that often localize near the mouths of uterine glands and may represent FA assembly in response to regions of unstable placental contact with the uterine LE. A

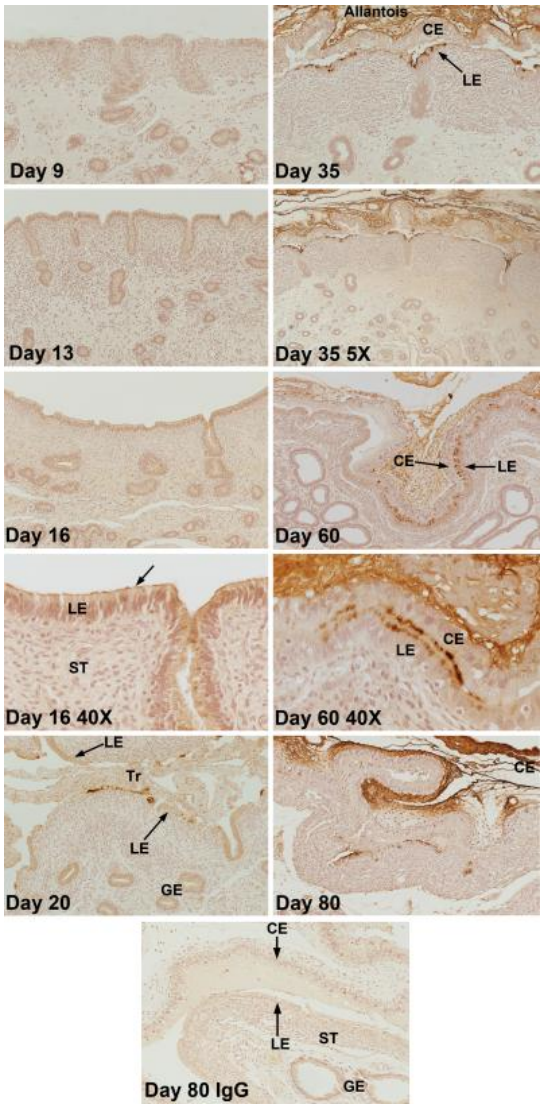

Figure 4-1. Immunohistochemical detection of integrin subunit ITGAV in uterine and placental tissues of sheep from Days 9 to 80 of gestation. ITGAV expression begins on Day 16 of pregnancy on the apical surface of uterine LE. Large aggregates that likely represent *in vivo* FAs form at the uterine-placental interface beginning on Day 20 of pregnancy and continue to grow in size and become more organized as pregnancy progresses. By Day 60 aggregates are observed across from each other in a "railroad" pattern on conceptus CE and uterine LE (40X) which continues to Day 80 of pregnancy. On Day 35 it is interesting to note that FAs are observed around the mouths of uterine glands (5X). A rabbit IgG was used as a negative control. Legend: Tr, trophectoderm; LE, luminal epithelium; ST, stroma; GE, glandular epithelium; CE, chorionic epithelium. Antibody used to detect ITGAV (AB 1930, Millipore).

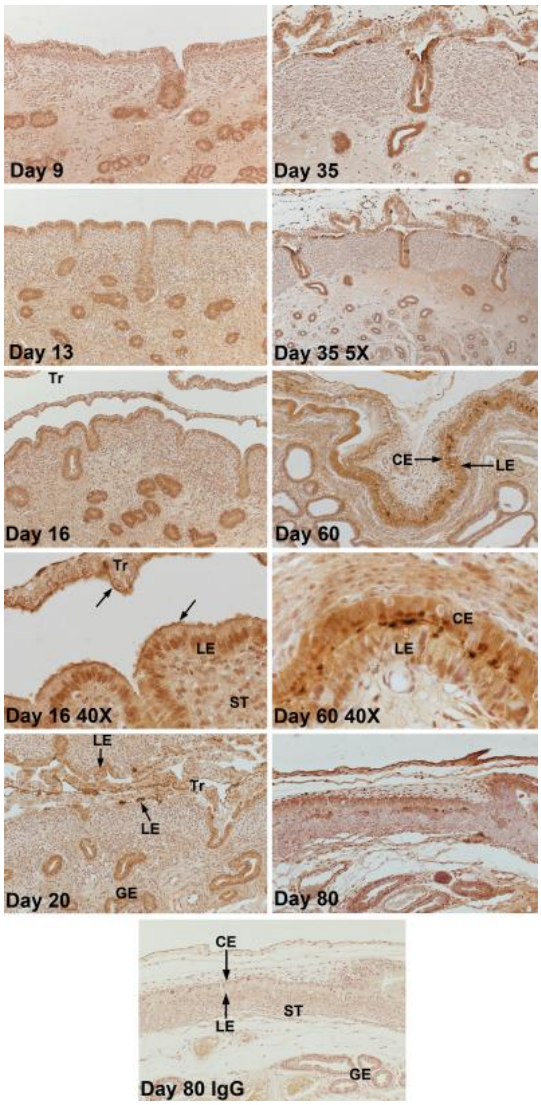

Figure 4 -2. Immunohistochemical detection of integrin subunit ITGA4 in uterine and placental tissues of sheep from Days 9 to 80 of gestation. ITGA4 expression begins on Day 16 of pregnancy on the apical surface of uterine LE. Large aggregates that likely represent *in vivo* FAs form at the uterine-placental interface beginning on Day 20 of pregnancy and continue to grow in size and become more organized as pregnancy progresses. By Day 60 aggregates are observed across from each other in a "railroad" pattern on conceptus CE and uterine LE (40X) which continue to Day 80 of pregnancy. On Day 35 it is interesting to note that FAs are observed around the mouths of glands (5X). A rabbit IgG was used as a negative control. Legend: Tr, trophectoderm; LE, luminal epithelium; ST, stroma; GE, glandular epithelium; CE, chorionic epithelium. Antibody used to detect ITGA4 (AB 1924, Millipore).

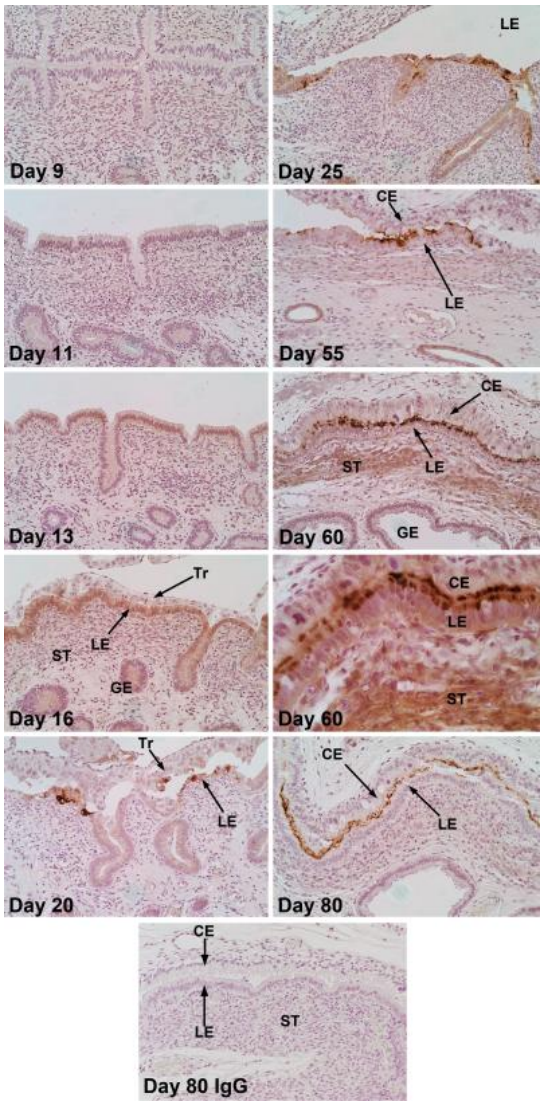

Figure 4-3. Immunohistochemical detection of integrin subunit ITGA5 in uterine and placental tissues of sheep from Days 9 to 80 of gestation. ITGA5 expression begins on Day 16 of pregnancy at the uterine-placental interface. Large aggregates that likely represent *in vivo* FAs form at the uterine-placental interface on the apical surfaces of conceptus Tr and uterine LE beginning on Day 20 of pregnancy and continue to grow in size and become more organized as pregnancy progresses. By Day 60 aggregates are observed across from each other in a "railroad" pattern on the conceptus CE and uterine LE (40X) which continue to Day 80 of pregnancy. A rabbit IgG was used as a negative control. Legend: Tr, trophectoderm; LE, luminal epithelium; ST, stroma; GE, glandular epithelium; CE, chorionic epithelium. Antibody used to detect ITGA5 (AB 1928, Millipore).

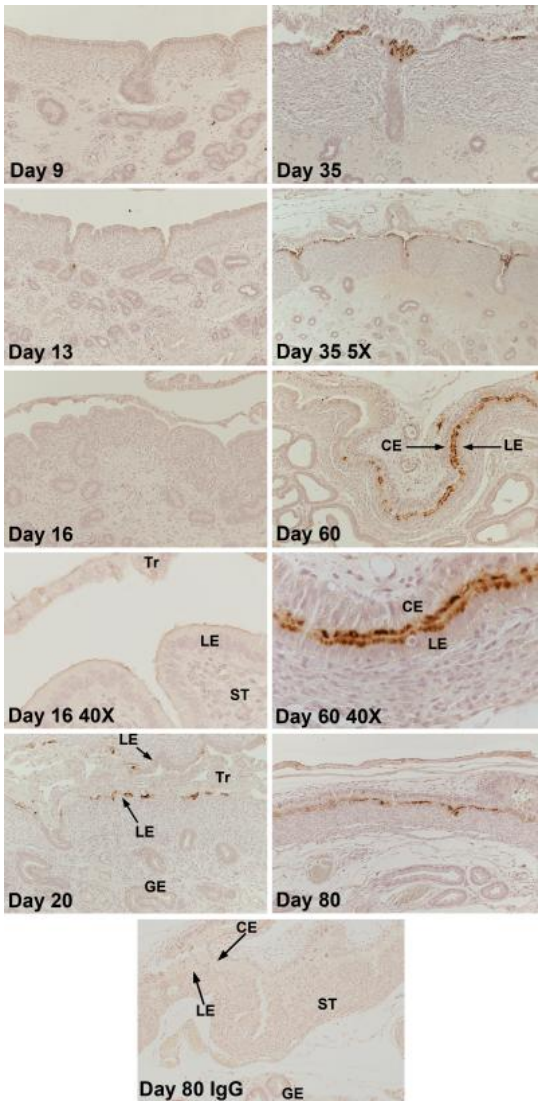

Figure 4-4. Immunohistochemical detection of integrin subunit ITGB1 in uterine and placental tissues of sheep from Days 9 to 80 of gestation. ITGB1 expression begins on Day 16 of pregnancy on the apical surface of uterine LE (40X). Large aggregates that likely represent *in vivo* FAs form at the uterine-placental interface beginning on Day 20 of pregnancy and continue to grow in size and become more organized as pregnancy progresses. By Day 60 aggregates are observed across from each other in a "railroad" pattern on the conceptus CE and uterine LE (40X) which continue to Day 80 of pregnancy. On Day 35 it is interesting to note that FAs are observed around the mouths of glands (5X). A rabbit IgG was used as a negative control. Legend: Tr, trophectoderm; LE, luminal epithelium; ST, stroma; GE, glandular epithelium; CE, chorionic epithelium. Antibody used to detect ITGB1 (AB 1952, Millipore).

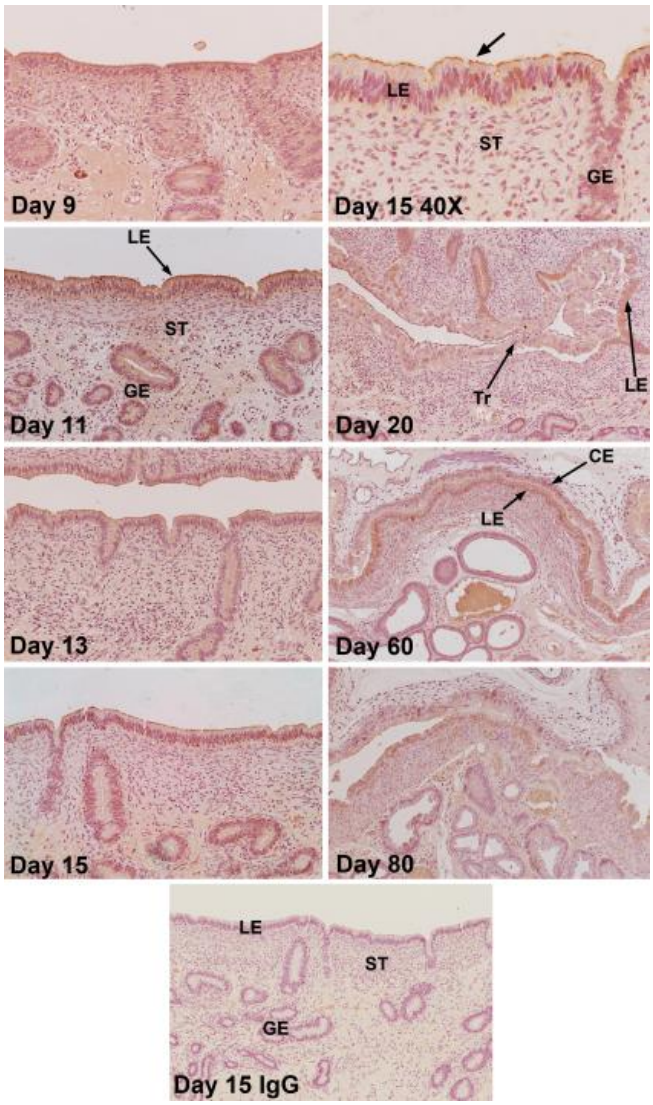

Figure 4-5. Immunohistochemical detection of integrin subunit ITGB3 in uterine and placental tissues of sheep from Days 9 to 80 of gestation. ITGB3 is observed on the apical surface of uterine LE cells on Day 15 of pregnancy (40X). Expression was not detected on Day 20 or thereafter during pregnancy. ITGB3 containing FA formation was not detected during pregnancy. A rabbit IgG was used as a negative control. Legend: Tr, trophectoderm; LE, luminal epithelium; ST, stroma; GE, glandular epithelium; CE, chorionic epithelium. Antibody used to detect ITGB3 (AB 1968, Millipore).

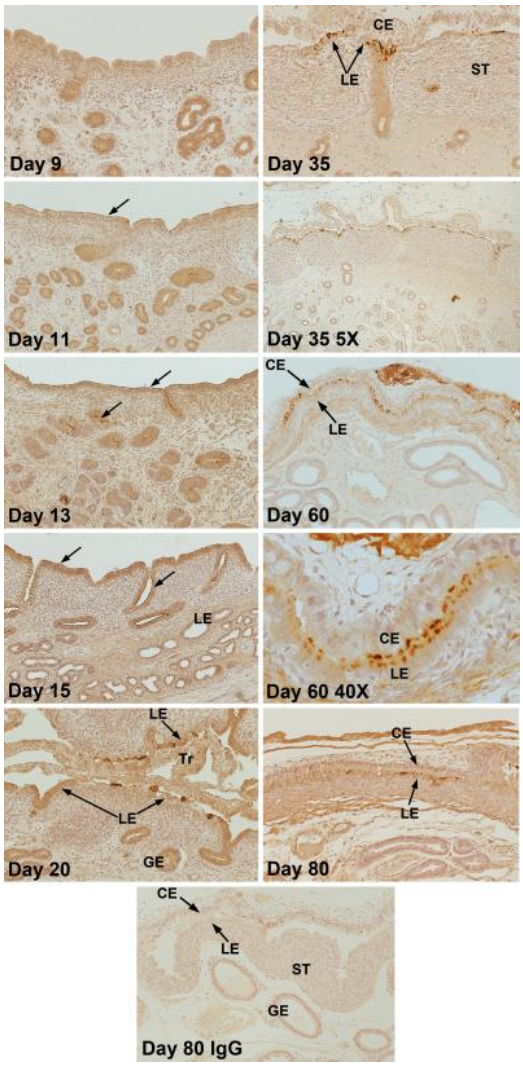

Figure 4-6. Immunohistochemical detection of integrin subunit ITGB5 in uterine and placental tissues of sheep from Days 9 to 80 of gestation. ITGB5 is first expressed on the apical surface of uterine LE on Day 11 of pregnancy, this spreads to the superficial GE on Day 13. This expression in uterine LE and GE continues to Day 15 and by Day 20 large aggregates that likely represent *in vivo* FAs, form at the uterine-placental interface and GE expression has ceased. These aggregates continue to grow in size and become more organized as pregnancy progresses. By Day 60 aggregates are observed across from each other in a "railroad" pattern on conceptus CE and uterine LE (40X) which continue to Day 80 of pregnancy. On Day 35 it is interesting to note that FAs are observed around the mouths of uterine glands (5X). A rabbit IgG was used as a negative control. Legend: Tr, trophectoderm; LE, luminal epithelium; ST, stroma; GE, glandular epithelium; CE, chorionic epithelium. Antibody used to detect ITGB5 (AB 1926, Millipore).

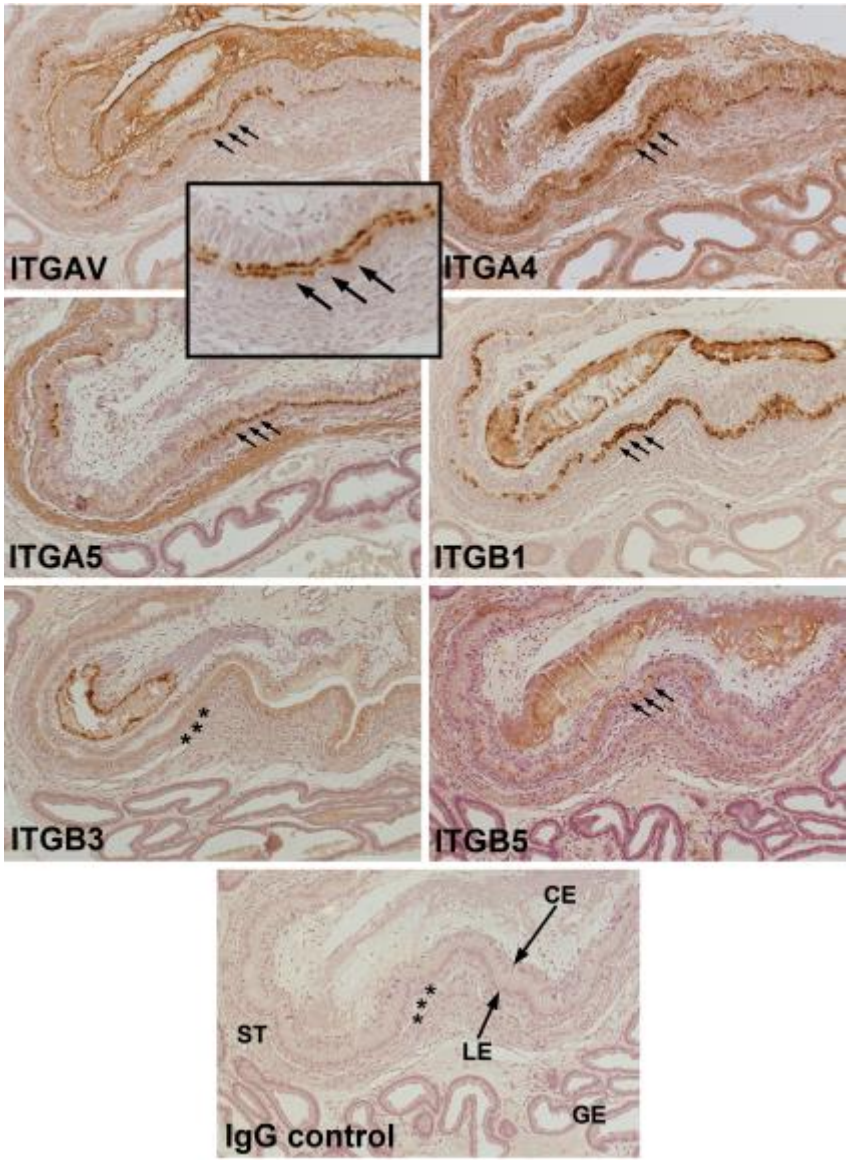

Figure 4-7. Co-distribution of integrin subunits at the uterine-placental interface on Day 60 of gestation. ITGAV, ITGA4, ITGA5, ITGB1, and ITGB5 integrin subunits are co-distributed into aggregates, presumably FAs, at the apical surfaces of apposed uterine LE and chorionic epithelium in a "train track" pattern in the same area of the uterine-placental interface (arrows). ITGB3 containing FAs were not detected (asterisks). A rabbit IgG was used as a negative control. Legend: Tr, trophectoderm; LE, luminal epithelium; ST, stroma; GE, glandular epithelium; CE, chorionic epithelium. Antibodies used to detect integrin subunits (AB 1930, 1924, 1928, 1952, 1968 and 1926).

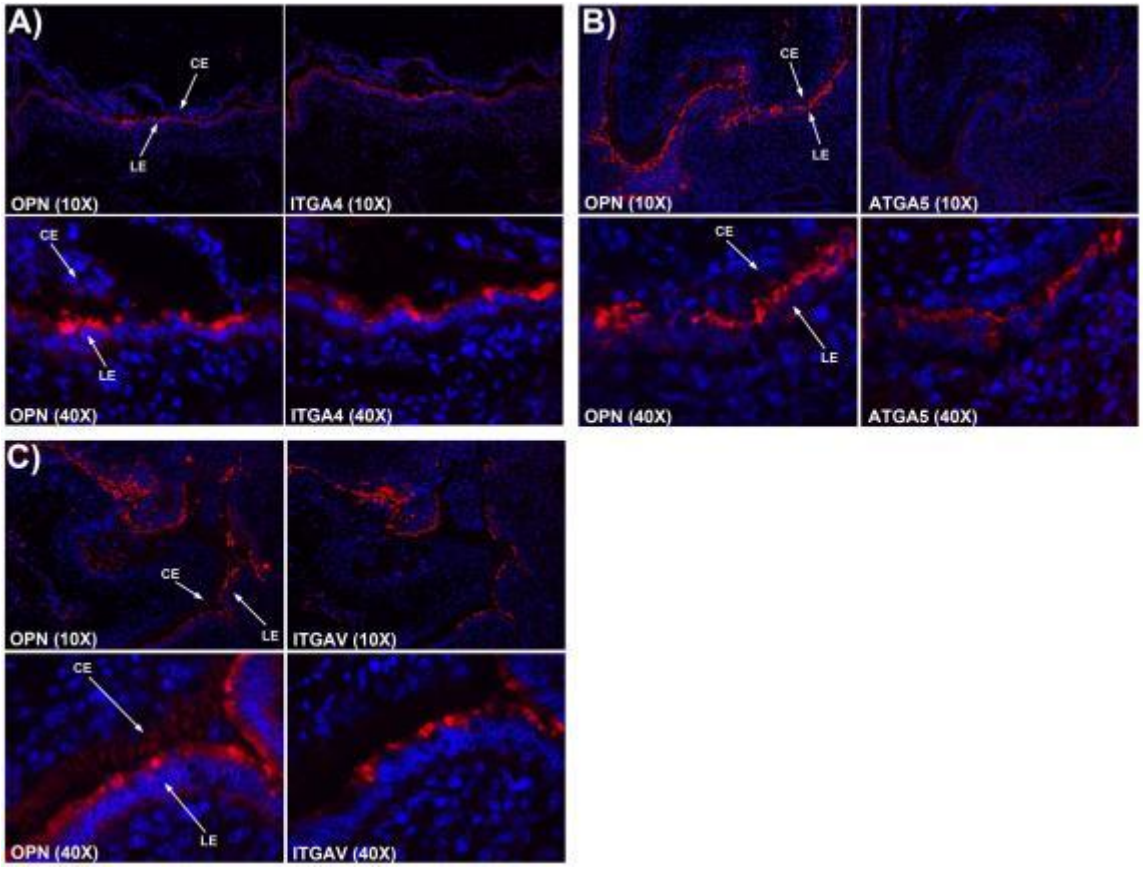

Figure 4-8. Co-distribution of integrin subunits ITGA4, ITGA5 and ITGAV with OPN by immunofluorescence microscopy. Serial sections on Day 60 of pregnancy were utilized to determine if integrin subunits were colocalized with OPN at the uterine-placental interface. Large aggregates of ITGA4, ITGA5, ITGAV and OPN are detectable on the apical surface of both LE and CE. This suggests that these integrin subunits are forming FAs that sequester OPN which is acting as a bridging ligand between these two epithelial surfaces. A rabbit IgG was used as a negative control and is represented in Figure 4-9. Legend: LE, luminal epithelium; CE, chorionic epithelium. Antibodies used to detect (AB 1930, 1924 and 1928). Antibody to detect OPN (AB 10910).

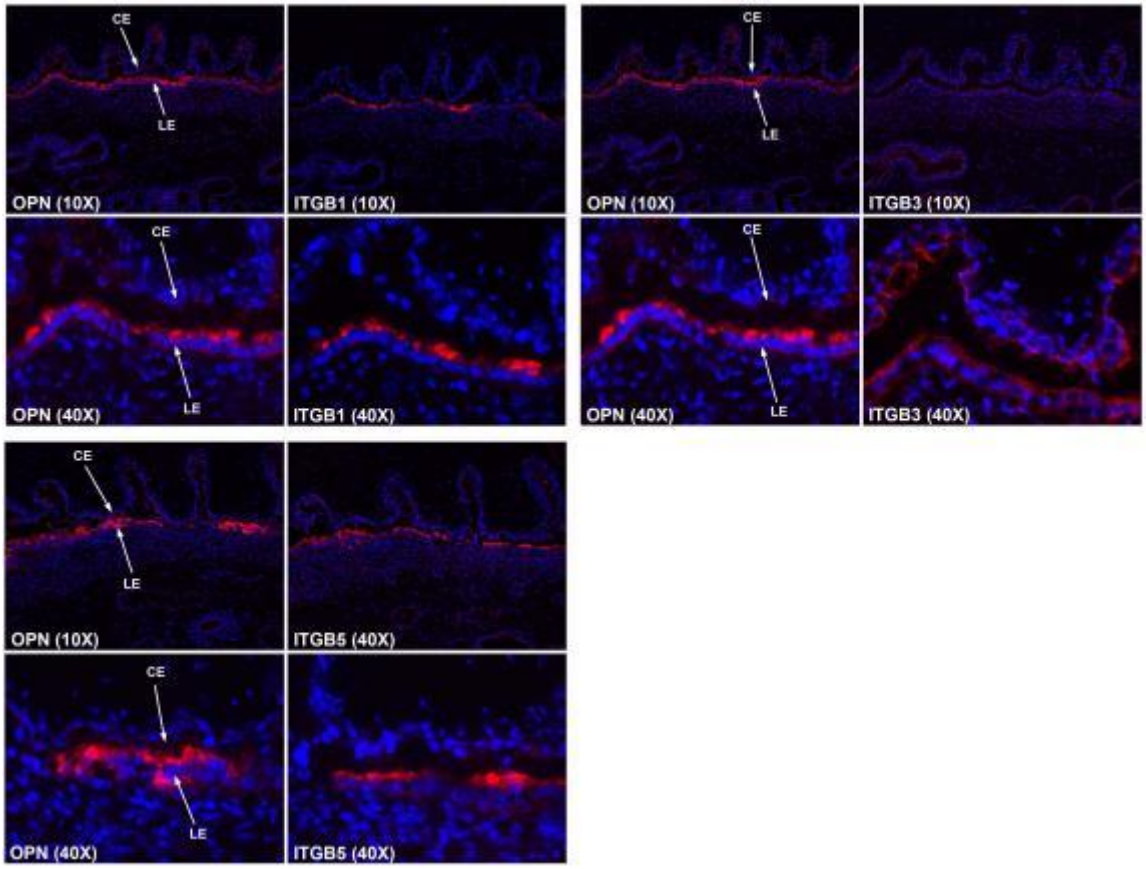

Figure 4-9. Co-distribution of integrin subunits ITGB1, ITGB3 and ITGB5 with OPN by immunofluorescence microscopy. Serial sections on Day 60 of pregnancy were utilized to determine if integrin subunits co-distributed with OPN at the uterine-placental interface. Large aggregates of integrin subunits ITGB1, ITGB5 and OPN are detectable on the apical surface of both LE and CE. Integrin subunit ITGB3 is not detectable in large aggregates at this interface. This suggests that integrin subunits ITGB1and ITGB5, but not ITGB3, form FAs that sequester OPN which acts as a bridging ligand between these two epithelial surfaces. A rabbit IgG was used as a negative control. Legend: LE, luminal epithelium; CE, chorionic epithelium. Antibodies used to detect ITGB1, ITGB3, ITGB5 (AB 1952, 1968 and 1926). Antibody to detect OPN (AB 10910).

representative example of the immunostaining characteristic of later stages of pregnancy, Days 60-80, is evident for ITGB5 (Figures 4-6 and 4-7). On Day 60 of gestation, ITGB5 shows a very organized pattern of expression in which the integrin subunit aggregates at the apical surfaces of both LE and chorionic epithelia, resulting in a gap between the apposed surfaces where adhesive ECM molecules could reside. ITGAV, ITGA4, ITGA5, ITGB1, ITGB3 and ITGB5 can be sorted into four distinct temporal and spatial patterns of expression. ITGAV, ITGA4 and ITGB1 exhibit expression as described in the previous paragraph (Figures 4-1, 4-2 and 4-4). ITGB5 and ITGA5 show similar patterns of expression to ITGAV, ITGA4 and ITGB1; however, during the peri-implantation period ITGA5 is present in the cytoplasm and minimal protein is evident at the apical surface of uterine LE (Figure 4-3), whereas ITGB5 is detectable on the apical surfaces of uterine LE and superficial GE (Figure 4-6). Further, immunostaining for ITGA5 is evident in the uterine stroma (Figure 4-3). Interestingly, ITGB3 exhibits a pattern of expression that contrasts from the other integrin subunits. There is prominent immunostaining for ITGB3 at the apical surface of uterine LE during the peri-implantation period, highlighting its potential role in initial adhesion of the conceptus Tr to uterine LE for implantation, but expression of ITGB3 was not detected at the uterine-placental interface after Day 16 of gestation (Figure 4-5). Therefore a major integrin receptor for ECM ligands, $\alpha v\beta 3$, does not appear to have a role in sheep placentation.

Co-localization of Integrin Subunits and OPN

The aggregation of integrins at the interplacentomal uterine-placental interface from Days 20 through 80 of gestation in sheep strongly suggests the presence of FAs. It has been hypothesized that ECM molecules such as OPN engage these integrins to induce FA assembly, but no ECM molecule has been demonstrated to localize to these presumed FAs. To determine if OPN is the ECM protein that binds to the integrin subunits at the uterine-placental interface, immunofluorescence microscopy was performed to co-localize OPN with integrin subunits in frozen Day 60 uterine-placental tissues. Because both the OPN and the integrin antibodies were generated in rabbits, co-localization was demonstrated by individual protein staining in serial thin sections. OPN co-localized with ITGAV, ITGA4, ITGA5, ITGB1 and ITGB5 in large aggregates at the uterine-placental interface (Figures 4-8 and 4-9). Indeed, the pattern of immunostaining in serial sections was nearly identical for OPN and each of the integrin subunits. Consistent with results from immunohistochemistry using paraffin-embedded tissues, large aggregates of ITGB3 were not detected at the uterine-placental interface of Day 60 of pregnancy by immunofluorescence microscopy (Figure 4-9).

Discussion

The expression of OPN and integrins at the uterine-placental interface of sheep reflects dynamic changes in the histoarchitecture of placentation as uterine and placental tissues remodel to adapt to changing physiological requirements. The initial interactions between apical surfaces of uterine LE and conceptus Tr begin with sequential phases i.e.,

non-adhesive or pre-contact, apposition, and adhesion, and conclude with formation of a placenta that supports fetal-placental development throughout pregnancy (Cross *et al.*1994, Carson *et al.*2000, Bazer *et al.*2005). In the sheep implantation begins on approximately Day 16 of pregnancy as Tr attaches to uterine LE (Godkin *et al.*1984, Spencer *et al.*2004). Attachment of this implanting conceptus first requires loss of anti-adhesive molecules in the glycocalyx of uterine LE, comprised largely of mucins that sterically inhibit attachment (Aplin *et al.*1995, Bowen *et al.*1996, Johnson *et al.*2001). This results in "unmasking" of molecules, including selectins and galectins, which contribute to initial attachment of conceptus Tr to uterine LE (Kimber *et al.*1995, Kimber *et al.*2000, Spencer *et al.*1994). These low affinity contacts are then replaced by a repertoire of adhesive interactions between integrins and maternal ECM which appear to be the dominant contributors to stable adhesion at implantation (Hynes *et al.*1987, Ruoslahti *et al.*1987, Burghardt *et al.*1997, Johnson *et al.*2001, Lessey *et al.*2002, Burghardt *et al.*2002). The conceptus continues the process of implantation until approximately Day 20 of pregnancy. At the culmination of this phase in pregnancy the uterine LE is absent in regions along the uterine-placental interface between Days 19 to 30 of pregnancy. In these regions the conceptus Tr binds directly to the basement membrane of the uterine stroma. We have noted that uterine LE at the mouths of glands never degrades and could serve as a stable reserve of epithelial stem cells to replaced uterine LE that is lost during this period of placentation. By approximately Day 30 of gestation, placentomes begin to form when highly branched placental chorioallantoic villi termed cotyledons grow rapidly and interdigitate with maternal aglandular

endometrial crypts termed caruncles. Approximately 90% of the blood from the uterine artery flows into the placentomes for transfer of nutrients and exchange of gases from the maternal circulation to the fetal-placental circulation (Caton *et al.*1983). During this period of placentome development, the interplacentomal expanses of the uterine-placental interface re-epithelialize and the attachment of LE to Tr becomes stabilized to provide a functional seal that limits the efficient delivery of histotroph from the uterine glands to the placental areolae for transport to the placental vasculature. It is important to note that the interplacentomal interface does not develop folding as observed with true epitheliochorial placentation of pigs, and is therefore subject to significant shear and tensional forces delivered by increasing mass of fetal-placental tissues and volumes of fetal fluids. Results of the present study suggest that three integrin receptors potentially assemble at the apical surface of uterine LE to mediate conceptus attachment during the peri-implantation period of sheep. These receptors are αvβ3, αvβ5 and α4β1. During the peri-implantation period of pregnancy, integrin subunits ITGAV, ITGA4, ITGA5, ITGB1, ITGB3 and ITGB5 are expressed by uterine LE. ITGA5 is present in the cytoplasm, but minimal ITGA5 protein is detectable on the apical surface of uterine LE (Figure 4-3). However, ITGAV, ITGA4, ITGB1, ITGB3 and ITGB5 are all present on the apical surface of LE cells during the critical period of conceptus attachment for implantation. Interestingly, unlike the other integrins, ITGB3 is limited exclusively to the apical surface of uterine LE during the peri-implantation period of pregnancy, and is not expressed thereafter, suggesting that ITGB3 may play an important implantation-specific role prior to placentation (Figure 4-5). In addition, ITGB5 expression extends

from uterine LE into the superficial GE where it potentially interacts with Tr papillae that extend into the mouths of uterine glands where they are thought to serve as tethers against which forces necessary to generate elongation are applied, and to serve as sites of maximal uptake of histotroph (Figure 4-6). We hypothesize that the continuous thin layer of immunostaining for ITGAV, ITGA4, ITGB1, ITGB3 and ITGB5 at the apical surface of uterine LE from Days 11 through 16 represents a role for αvβ3, αvβ5 and α4β1during the adhesion cascade of implantation. Ovine conceptuses elongate, appose and then tentatively attach to uterine LE through carbohydrate-lectin interactions. Integrins then engage ECM at the interface between Tr and uterine LE to adhere the conceptus to uterine LE. The filamentous sheep conceptus is already attached, if tenuously, and lacks the mass to generate shear forces necessary to drive the assembly of large FAs. The temporal and spatial exclusivity of ITGB3 to the apical surface of uterine LE during implantation highlights a possible role(s) for αvβ3 during implantation. The presence of ITGB5 in the mouths of uterine glands suggests the ability of αvβ5 to interact with Tr papillae. Previous work has shown that OPN is present at this interface during this time period (Johnson *et al.* 1999a). *OPN* mRNA is detected as early as Day 13 in some uterine glands and is present in all uterine glands by Day 19 (Johnson *et al.* 1999a). OPN expression by uterine GE is induced by progesterone (Johnson *et al.* 2000). Immunoreactive OPN protein is present on the apical surfaces of endometrial LE and GE, and on Tr where it potentially interacts with the integrin receptors αvβ3, αvβ5 and α4β1. Indeed, αvβ3 at the surface of cultured ovine Tr cells has been shown to bind OPN (Kim *et. al.* 2010). The diffuse aggregates begin to

organize into discrete aggregates that are present on the apical surface on CE and uterine LE by Day 60 of pregnancy (Fig 4-7).

After implantation and during the initial period of placentation when uterine LE begins to erode, formation of FA is first detectable. Large, infrequent, intermittent and disorganized aggregates of ITGAV, ITGA4, ITGA5, ITGB1, ITGB3 and ITGB5 assemble at the interface between the uterus and conceptus from Days 20 through 55. Some FAs are found at the apical interface between attached epithelial cells, but others form in regions where the conceptus Tr contacts the uterine stroma, and still others form at the base of uterine LE. As erosion of the uterine LE continues, FAs assemble with high frequency in regions around the mouths of uterine glands where uterine LE is maintained and from which epithelial stem cells may divide and migrate to re-epithelialize the uterine surface (Figures 4-1, 4-2, 4-3, 4-4, and 4-6). Clearly this is a histologically complex and unstable period of placentation in the sheep. There is extensive tissue remodeling at the uterine-placental interface while increasing shear and tensional forces are applied by the growing mass of fetal-placental tissues and volumes of fetal fluids. The size, composition, cell signaling activity and adhesion strength of FAs are force-dependent (Hynes *et al*. 1987, Vogel *et al*. 2006). These FA complexes act to transmit force placed on the cell by the ECM at sites of adhesion, or vice versa, and serve as signaling centers where cell signaling pathways involved in cell growth, proliferation, survival, gene expression, development, tissue repair, migration and invasion are activated. Focal adhesions play a major role in migrating cells. In this capacity, the FA interacts with the actin cytoskeleton to give the cells traction as they

migrate along an ECM. At the leading edge of the migrating cells, there are nascent, immature, focal complexes formed that mature into FAs as the cells become stably attached to the ECM and more force is exerted on the focal complex (Thievessen *et al.*2013). We hypothesize that the FAs present in these regions are crucial to maintaining contact between the uterus and developing conceptus and could be utilized to initiate the migration and proliferation of epithelial cells out of the uterine glands to repopulate the uterine LE during this unstable period. We further hypothesize that the FAs that are present in these regions are crucial to maintaining contact between the uterus and developing conceptus and could be utilized to initiate the migration and proliferation of epithelial cells out of the glands to repopulate the uterine LE during this unstable period.

By Day 60 of pregnancy, the interplacentomal uterine-placental interface stabilizes into a continuous seal between uterine LE and chorion except at the openings of uterine glands. Here the chorion never fuses with uterine LE, rather it forms a pocket referred to as an areola wherein secretions from uterine GE are absorbed and transported across the chorioallantoic placenta by fluid phase pinocytosis for release into the fetal circulation (Renegar *et. al.* 1982, Cantzer and Leiser 1993). The tall columnar cells of the areolar chorion are specialized to transport large macromolecules across the placenta and it is essential that all histotroph be directed to these structures. Therefore, tight attachment between chorion and uterine LE in non-areolar regions of the interface must be present even though this interface is exposed to exponential increases in mechanical forces generated from growing fetal-placental tissues and fetal fluids. We observed that immunostaining for ITGAV, ITGA4, ITGA5, ITGB1, ITGB3, and ITGB5 is distributed

precisely at the apical surfaces of apposed uterine LE and chorion along extensive expanses of the uterine-placental interface on Day 60 and Day 80 of gestation (Figures 4-1, 4-2, 4-3, 4-4, 4-6, and 4-7). Each integrin subunit shows a very organized pattern of expression in which the integrin subunit aggregates at the apical surfaces of both uterine LE and chorionic epithelia, resulting in a gap between the apposed surfaces where adhesive ECM molecules could reside. Further aggregates for each integrin co-localize to the same regions of uterine-placental interface (Figure 4-7). We hypothesize that the temporal and spatial formation of these mature FAs represents engagement of these integrins with the ECM to stabilize adhesion between uterine LE and chorionic epithelium in response to the increasing mechanical stress being placed on this interface by the ever increasing fetal size and fetal fluid volumes. An important advancement of the present studies is the clear co-distribution of OPN and these multiple integrins within these large protein aggregates as constituents of FAs at the uterine-placental interface of sheep (Figures 4-8 and 4-9). This is the first study to definitively co-localize OPN with large integrin subunit containing FAs, indicating that OPN is acting as a bridging ligand between these FAs to maintain contact between these two epithelial surfaces; uterine LE and Tr/chorion. The comprehensive nature of these results over the course of pregnancy is the best evidence thus far that integrins interacting with OPN play a major role in implantation and placentation in sheep and provides insights into implantation and placentation in other species that also exhibit prominent expression of OPN and integrins within uterine and placental tissues.

CHAPTER V

FUNCTIONAL LOSS OF ITGB3 (BETA 3 INTEGRIN) IN OVINE CONCEPTUSES DECREASES CONCEPTUS EXPRESSION OF NITRIC OXIDE SYNTHASE 3 AND SECRETED PHOSPHOPROTEIN 1: IMPLICATIONS FOR THE DEVELOPING PLACENTAL VASCULATURE

Introduction

The period of implantation in sheep is characterized by three distinct processes; apposition, attachment and adhesion of the uterine luminal epithelium (LE) and conceptus (embryo and associated placental membranes) trophectoderm Tr. In some species the adhesion phase is followed by invasion of the blastocyst into the endometrium where placental development will occur. In the sheep, however, there is no true invasion of the blastocyst. Rather, there is a period of loose apposition of the elongating conceptus to uterine LE followed by adhesion of conceptus Tr to uterine LE which is hypothesized to be mediated by integrin receptors binding to extra-cellular matrix (ECM) bridging ligands (Burghardt *et al.*2002, Johnson *et al.*2003, Spencer *et al.*2004). Integrins are integral membrane proteins composed of an alpha and beta subunit that are non-covalently bound to one-another. The N-terminal domains of these receptors are present on the outside of the cell and are involved in binding of integrins to ECM proteins primarily via their GRGDS amino acid motif. The cytoplasmic tail of the beta subunit allows the integrin receptor to bind to the actin cytoskeleton to induce a number of different cell signaling pathways involved in differentiation, motility, survival

and adhesion (Miyamoto *et al.*1995). Multiple integrin receptors can associate together to form large aggregates known as focal adhesions (FA) that associate with the actin cytoskeleton and a plethora of signaling molecules in response to tensional forces placed on the cell. Functional blockage of integrin receptors decreases the number of implantation sites in both mice and rabbits (Illera *et al.*2000, Illera *et al.*2003). Available evidence suggests that integrin receptors on conceptus Tr and uterine LE interact with ECM proteins to mediate implantation (Burghardt *et al.*2002).

Secreted phosphoprotein 1[(SPP1) also known as osteopontin] is a soluble ECM protein that is present at the uterine-placental interface during the peri-implantation period of pregnancy in all species studied to date (Johnson *et al.*1999, Apparao *et al.*2001, Von Wolff *et al.*2001, Garlow *et al.*2002, Apparao *et al.*2003, Joyce *et al.*2005). SPP1 is a 75 kDa matricellular protein that contains a GRGDS (glycine, arginine, glycine, aspartic acid, and serine) integrin binding domain. In the sheep, SPP1 expression is induced in uterine glandular epithelium (GE) by progesterone, the hormone of pregnancy (Johnson *et al.*1999; Johnson *et al.*2000). This glandular SPP1 is then secreted into the uterine lumen where it is hypothesized to bind to integrin receptors on conceptus Tr and uterine LE and act as a bridging ligand to mediate attachment, migration, and survival of the elongating conceptus as it apposes and adheres to the uterine LE (Johnson *et al.*2000; Johnson *et al.*2003; Kim *et al.*2010; Johnson *et al.*2014). During the period of implantation in sheep, integrin subunits ITGAV, ITGA4, ITGA5, ITGB1, ITGB3, and ITGB5 are expressed on the apical surface of conceptus Tr and uterine LE *in vivo* (Johnson *et al.*2001). Previous work with ovine Tr cells identified specific integrins that

bind SPP1 as integrins ITGAV, ITGB3 and perhaps ITGA5 (Kim *et al.*2010). Since ITGA5 can only heterodimerize with ITGB1, it was concluded that αvβ3 and α5β1 receptors on ovine Tr cells bind SPP1 to mediate attachment and implantation of the conceptus (Kim *et al.*2010). It was also found that SPP1 increased adhesion and migration of ovine Tr cells in a dose dependent manner *in vitro* (Kim *et al.*2010). SPP1 is abundant at the uterine-placental interface during the peri-implantation period of pregnancy when the conceptus is elongating and attaching to the uterine LE; the αvβ3 heterodimer is generally agreed to be the predominant receptor for SPP1. We therefore hypothesized that SPP1 is secreted by GE and binds to the αvβ3 integrin receptor on conceptus Tr to aid in elongation of the conceptus and mediate attachment of the conceptus to the uterine LE in sheep. This study investigated the effects of loss of function of Tr-expressed ITGB3 on the ability of the conceptus to elongate and attach to the uterine LE *in vivo*.

Materials and Methods

Morpholino Design

Morpholino antisense oligonucleotides (MOAs) were designed and synthesized by Gene Tools (Philomath, OR) to directly inhibit translational initiation of *ITGB3* mRNA and a standard control oligo served as the MAO control. The sequence used for the ITGB3 MAO was GCGCTCGCATCTCGTCCACCCGCT and the sequence used for the control MAO was

5’-GCGATCGAATATCGTCCAACCGAT-3’. All morpholinos were synthesized with a 3’-lissamine modification to allow confirmation of uptake of each MAO by conceptus Tr.

Animal Model

Mature Rambouillet ewes were observed daily for estrus (Day 0 is the day of onset of estrus) in the presence of vasectomized rams and used in experiments after they had exhibited at least two estrous cycles of normal duration (16-18 days). All experimental and surgical procedures were in compliance with the Guide for the Care and Use of Agriculture Animals in Research Teaching and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Experimental Design and Tissue Collection

Study 1: Expression of ITGB3 and SPP1 in uterine and placental tissue

Ewes were mated to intact rams three times at 12 h intervals beginning at estrus. Pregnant ewes were ovariohysterectomized on either Day 9, 11, 13, 15, 16, 20, 25, 30, 35, 40, 50, 55, 60, 80 or 120 of pregnancy (n=4 ewes/day). Uteri from Days 9, 11, 13, 15 and 16 were flushed with 0.9% NaCl, and pregnancy was verified by the recovery of an apparently normal conceptus in the uterine flushing. Several sections (1-1.5 cm) of uterine wall from the middle of each uterine horn were fixed in 4% paraformaldehyde for 24 h and then embedded in Paraplast Plus (Oxford Labware, St Louis, MO).

Study 2: Effects of loss of ITGB3 on conceptus elongation *in vivo*

At estrus ewes were mated to an intact rams of proven fertility. On Day 8 post-mating, each ewe was assigned randomly to receive either the control MAO (MAO control) or the ITGB3 MAO (MAO-ITGB3; n=6 for each MAO type). At surgery the base of the uterine horn ipsilateral to the corpus luteum (CL) was double ligated to prevent migration of the conceptus into the contralateral uterine horn. This procedure does not affect development or implantation of ovine conceptuses (Dunlap *et al.*2006). MAO-ITGB3 or MAO control (100 nmol) complexed with Gene Tools Endo Porter delivery reagent (100 µl) was brought to 1 ml of final volume with OPTI-MEM (Life Technologies, Grand Island, NY) and injected into the uterine lumen of the ipsilateral uterine horn, within which it was rapidly and uniformly distributed by gentle massage. On D16 ewes were ovariohysterectomized to allow collection and processing of conceptuses and uterine tissue from the MAO-ITGB3 and MAO control ewes. Each uterine horn was flushed with 10ml of sterile PBS to obtain the conceptus(s). Pregnancy rates were recorded based on the presence or absence of a functional CL and a conceptus at the time of recovery. Each conceptus was photographed using a digital camera to record morphology. A portion of the conceptus was snap frozen in Tissue-Tek OCT compound. Another portion of the conceptus was fixed in freshly prepared 4% (w/v) paraformaldehyde for 24 h and then embedded in Paraplast Plus. The remaining conceptus tissue was snap frozen and stored at -80°C for RNA extraction.

Study 3: Effects of loss of ITGB3 protein on conceptus attachment *in vivo.*

At estrus ewes were mated to an intact rams of proven fertility. On Day 12 post-mating each ewe was assigned randomly to receive either control MAO (MAO control) or the ITGB3 MAO (MAO-ITGB3; n=7 for each MAO type). At surgery on Day 24, the base of the uterine horn ipsilateral to the CL was double ligated to prevent migration of the conceptus into the contralateral uterine horn. Then MAO-ITGB3 or control MAO (100 nmol) complexed with Gene Tools Endo-Porter delivery reagent (100 µl) was brought to 1 ml final volume with OPTI-MEM and injected into the lumen of the uterine horn ipsilateral to the CL, within which it was rapidly and uniformly distributed by gentle massage. On Day 24 post-estrus ewes were ovariohysterectomized to collect and process uterine and conceptus tissues. Several sections (approximately 1-1.5 cm thick) from the middle of the uterine horn were placed in fresh 4% paraformaldehyde fixative for 24 h and then embedded in Paraplast Plus. Other sections of similar size were snap frozen in Tissue-Tek OCT compound. The remaining uterine tissue was dissected into endometrium and myometrium and snap frozen in tubes. Fetuses were dissected from placental tissue and fetal crown rump length was measured. Fetuses and placental tissue were snap frozen and stored at -80°C for RNA extraction.

Immunofluorescence Analyses of Conceptuses

The ITGB3 subunit was localized in frozen conceptuses from Study 1 as previously described (Johnson *et al.*2001). Briefly, tissues were fixed in -20°C methanol rinsed in PBS containing 3% (vol/vol) Tween 20, blocked in 10% (vol/vol) normal goat serum,

and incubated overnight with 2 µg/ml rabbit antiserum directed against human ITGB3 subunit (AB1968; EMD Millipore International , Billerica, MA). Tissue-bound primary antibody was then detected with goat anti-rabbit IgG Alexa 488 (8µg/ml). Slides were overlaid with Prolong Gold Antifade reagent with DAPI (Life Technologies) and a glass coverslip.

Histological Analysis of Conceptus Morphology

The uterine/placental tissues embedded in paraffin from Study 2 were sectioned (~5 µm) and mounted on glass slides for staining with hematoxylin and eosin. Uterine cross sections were deparaffinized, rehydrated, stained with hematoxylin and eosin, dehydrated and overlaid with a cover glass fixed with Permount (Fisher Scientific, Fairlawn, NJ).

Immunohistochemical Analyses

Immunohistochemical localization of ITGB3, cytokeratin, interferon stimulated gene 15 (ISG15), vimentin, and SPP1 proteins in ovine uterine/placental cross-sections from Studies 2 and 3 was performed as previously described (Joyce et al 2005) with rabbit anti-ITGB3 (human) IgG (5 µg/ml, AB1968, Millipore, Billerica, MA), mouse anti-cytokeratin and vimentin (human) IgG (C6909, V6389; Sigma Aldrich, St Louis, MO), rabbit anti-recombinant bovine ISG15 IgG (Pru *et al.*2000) and rabbit anti-bovine SPP1 IgG (5mg/ml, Erikson *et al.*2007) at a dilution of 1:200. No antigen retrieval of tissues was performed for any of the immunoglobulins. Purified rabbit or mouse IgG was used

as a negative control (Sigma-Aldrich, St Louis, MO). Immunoreactive protein was visualized in sections using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) following the manufacturer's instructions and 3, 3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) was utilized as the color substrate. Sections were prepared using hematoxylin as a counterstain.

Cloning of a Partial cDNA for Ovine *SPP1*

A partial cDNA for *SPP1* was generated by RT-PCR as previously described using total RNA from Day 15 pregnant ovine endometrium (Johnson *et al.*1999). Primers for the target gene were 5'-TGATGATAACAGCCAGGACGA-3' and 5'GTGAAGTCCTCCTCTGTGGC-3'. PCR amplification was conducted as follows for ovine SPP1: 1) 95°C for 5 min; 2) 95°C for 45 sec, 60.4°C, and 72°C for 1 min for 35 cycles; and 3) 72°C for 10 min. The amplified PCR product was sub-cloned into the pCRII cloning vector using a TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) and sequenced in both directions using an ABI PRISM Dye Terminator Cycle Sequencing kit and an ABI PRISM automated DNA sequencer (Applied Biosystems, Foster City, CA) to confirm sequence identity.

In situ Hybridization Analysis

SPP1 mRNA was localized in paraffin-embedded uterine and placental tissues as previously described (Johnson *et al.*1999). Uterine cross-sections from each animal (5 µm) were deparaffinized, rehydrated, and deproteinated and then hybridized with

radiolabeled antisense and sense cRNA probes generated from linearized plasmid DNA templates. Radiolabeled antisense or sense cRNA probes were synthesized by *in vitro* transcription with [α-^{35}S] uridine 5-triphosphate (PerkinElmer Life Sciences, Wellesley, MA). After hybridization, washing and RNase A digestion were performed followed by autoradiography using NTB-2 liquid photographic emulsion (Eastman Kodak, Rochester, NY). Slides were exposed at 4ºC for 8 days, developed in Kodak D-19 developer, counterstained with Harris modified hematoxylin (Fisher Scientific), dehydrated, and protected with cover slips sealed with Permount (Fisher Scientific).

RNA Extraction, cDNA Synthesis, & qPCR

Total RNA from conceptus extra-embryonic tissue was isolated using Trizol reagent (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. RNA yield and purity were determined using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). First strand cDNA was synthesized from 5µg of isolated RNA using Superscript III First Strand Kit (Life Technologies) following manufacturer's instructions. The cDNA was subjected to a 1:5 dilution with nuclease-free water and stored at -20°C.

Six genes were selected for examination in extra-embryonic tissues of conceptuses: *Solute Carrier Family 1 Member 4* (*SLC1A4*), *Solute Carrier 7 Member 5* (*SLC7A5*), *Solute Carrier 2 Member 1* (*SLC2A1*), *Nitric Oxide Synthase 3* (*NOS3*), *Ornithine Decarboxylase 1* (*ODC1*) and *SPP1* based on evidence that these genes are up-regulated in the conceptus during the peri-implantation period and involved in either nutrient

transport or angiogenesis (Gao *et al.*2003 a-d; Johnson *et al.* 2003). Primers were developed using NCBI Primer-BLAST and tested for specificity against the known ovine genome. Primer specificity was verified by single bands of expected length on a 2% agarose electrophoresis gel and dissociation curves were run in all qPCR replicates. Primer sequences and product lengths are presented in Table 5-1. The qPCR reactions were performed in triplicate, in 10µl reactions using PerfeCta SYBR Green Mastermix (Quanta Biosciences, Gaithersburg, MD) and 2.5 mM of each specific primer, on a Roche 480 Lightcycler (Roche, San Francisco, CA) with approximately 75 ng of cDNA per reaction. The PCR program began with 5 min at 95ºC followed by 40 cycles of 95ºC denaturation for 10 sec and 60ºC annealing/extension for 30 sec. Raw qPCR data were normalized to GAPDH via the Delta Delta Ct method (Livak and Schmittgen, 2001). Statistical analysis was performed using a Student's paired t-test and results are reported as mean±SEM with $p<0.05$ being significant.

Table 5-1. Primer sequences for qPCR.

Gene Name	***Genbank Reference Sequence***	***Sequence***	***Product Length***
GAPDH	NM_001190390.1	5'-GGTGAAGGTCGGAGTGAACG-3' 5'-TGACTGTGCCGTGGAATTTG-3'	173
NOS3	NM_001129901.1	5'-CCCCTACACTACGGGGTCTG-3' 5'-GCAGCCTCTCCTGCCAAAAT-3'	181
ODC1	XM_004005681.1	5'-CTGCAGAAGAGACCCAAACCA-3' 5'-CAAAGAGCATCCAATCGCCC-3'	133
OPN	NM_001009224.1	5'-AGCACTGCATCAGCATCACA-3' 5'-TCGGTTTAACTGGAAGGGCG-3'	134
SLC1A4	XM_004005836.1	5'-ATCAGCCGGTTCATTCTCCC-3' 5'-GTGGCGGTCACTAGAATGGT-3'	146
SLC2A1	XM_004001865.1	5'-CCAGCCAAAAGCTGACGGG-3' 5'-AACTCTTCAATCACCTTCTGGGG-3'	118
SLC7A5	NM_174613.2	5'-CCTGCTCTATGCCTTCTCCAA-3' 5'-GCCAGATGAACCTTGATGGG-3'	150

Results

Study 1: Expression of ITGB3 in Uterine and Placental Tissues

To determine the temporal and spatial expression of ITGB3 at the uterine-placental interface during pregnancy in sheep, immunohistochemistry was conducted on paraffin-embedded thin sections from Days 9 through 80 of gestation. ITGB3 was detected in uterine and placental tissues (Figure 5-1). Immunostaining for ITGB3 was not observed in endometrium on Day 9 of pregnancy; however, abundant ITGB3 protein was detected at the apical surface of uterine LE on Days 11, 13 and 15 of pregnancy. On Day 15 of gestation, ITGB3 was present only at the apical surface of uterine LE along the entire surface of the uterine lumen where it is well placed to serve as a platform for attachment of Tr to uterine LE via SPP1 (Figure 5-1).

Study 2: Effects of Loss of ITGB3 Subunit on Conceptus Elongation

To address whether ITGB3 is required for conceptus elongation, we performed loss of function by blocking translation of Tr-expressed ITGB3 mRNA by infusing MAO-ITGB3 into the uterine lumen of one uterine horn of ewes on Day 8, and assessed conceptus development on Day 16. No pregnancy loss was observed, 6 conceptuses were recovered from each the control MAO and ITGB3 MAO groups. The red colored 3′-lissamine tag in MAOs confirmed MAO uptake by conceptus Tr (Figure 5-2). Conceptuses exposed to the MAO-ITGB3 elongated similarly to conceptuses exposed to MAO control (Figure 5-2). Figure 5-3 shows that conceptuses treated with control

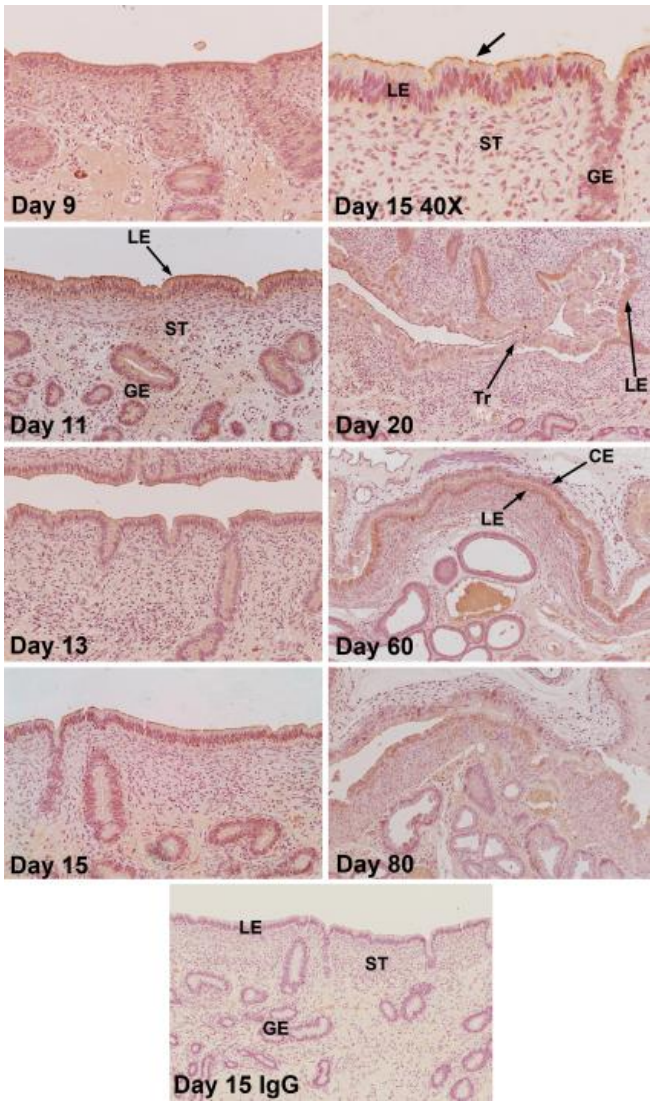

Figure 5-1. Immunohistochemical detection of integrin subunit ITGB3 in uterine and placental tissues of sheep from Days 9 to 80 of gestation. ITGB3 is observed on the apical surface of uterine LE cells on Day 15 of pregnancy (40X). Expression is no longer observed on Day 20 nor for the remainder of pregnancy. ITGB3 containing FA formation is not observed throughout pregnancy. A rabbit IgG was used as a negative control. Legend: Tr, trophectoderm; LE, luminal epithelium; ST, stroma; GE, glandular epithelium; CE, chorionic epithelium. Antibody used to detect ITGB3 (AB 1968, Millipore).

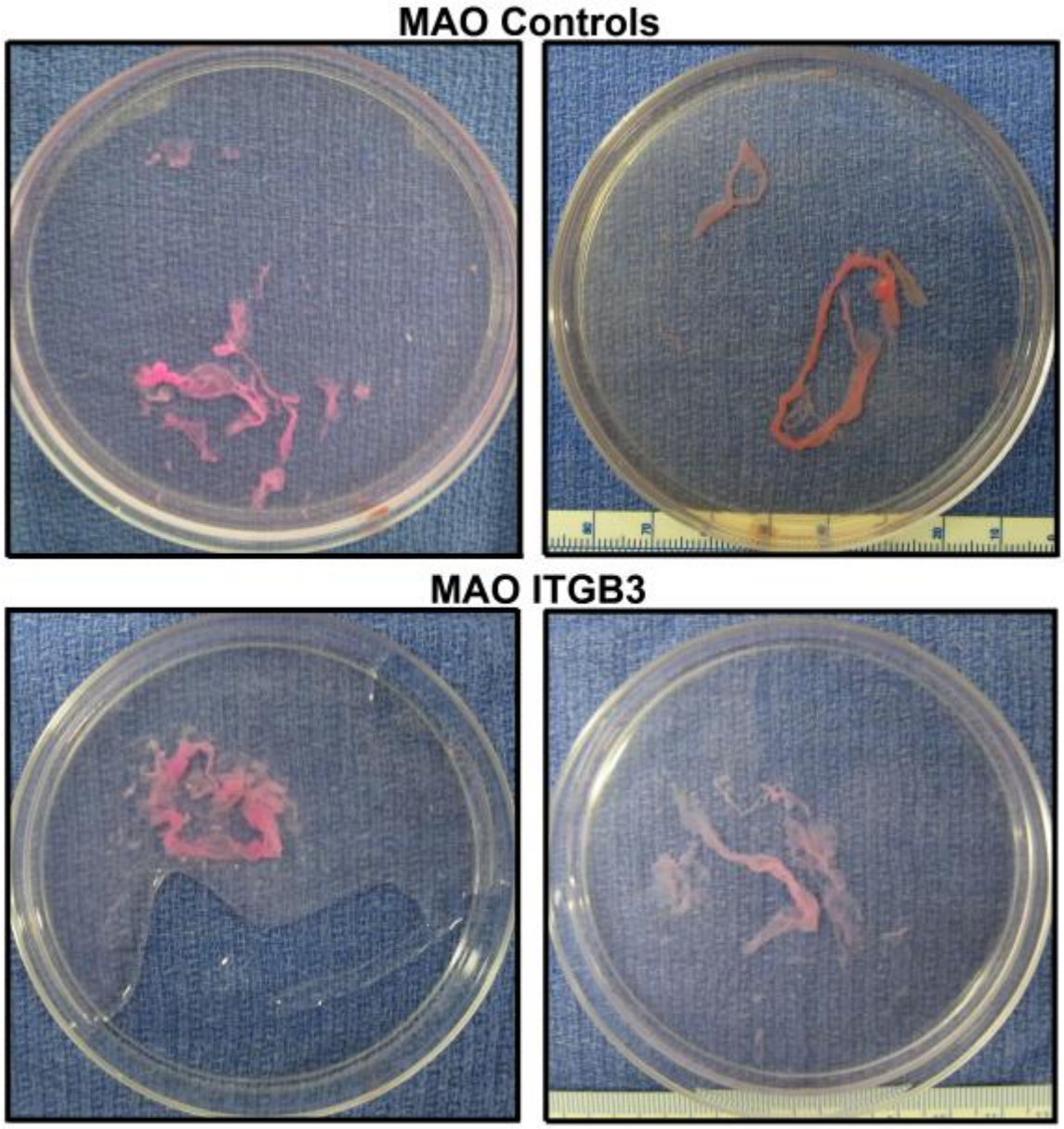

Figure 5-2. Effects of loss of ITGB3 on conceptus elongation. Shown are representative conceptuses that were flushed from the uterine lumen and observed grossly for uptake of the red colored 3′-lissamine tag in MAOs, and morphological elongation. All conceptuses contained MAOs, however, conceptuses exposed to MAO-ITGB3 elongated similarly to conceptuses exposed to MAO control, indicating that ITGB3, alone, is not critical for conceptus elongation in sheep. MAO, Morpholino antisense oligonucleotide.

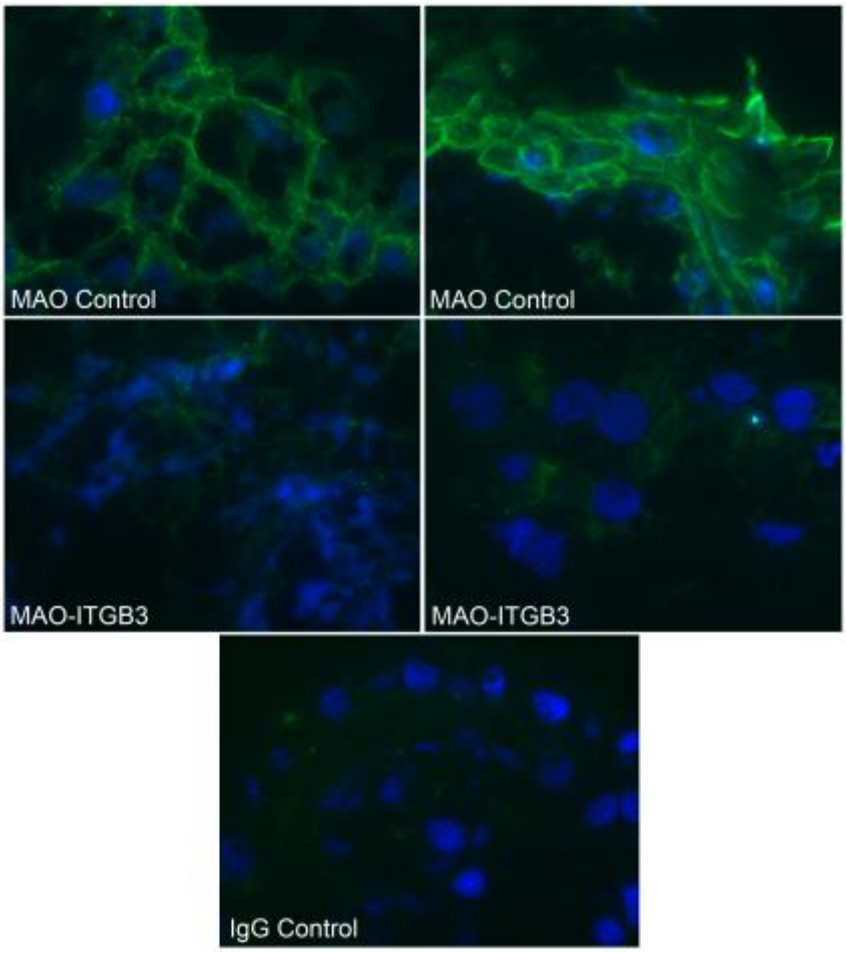

Figure 5-3. Confirmation of ITGB3 knock down in Day 16 conceptuses. Immunofluorescence analysis shows expression of ITGB3 (green color) by conceptuses exposed to MAO control whereas ITGB3 was not detectable in conceptuses exposed to MAO-ITGB3. A non-relevant rabbit IgG (rIgG) was utilized as a negative control.

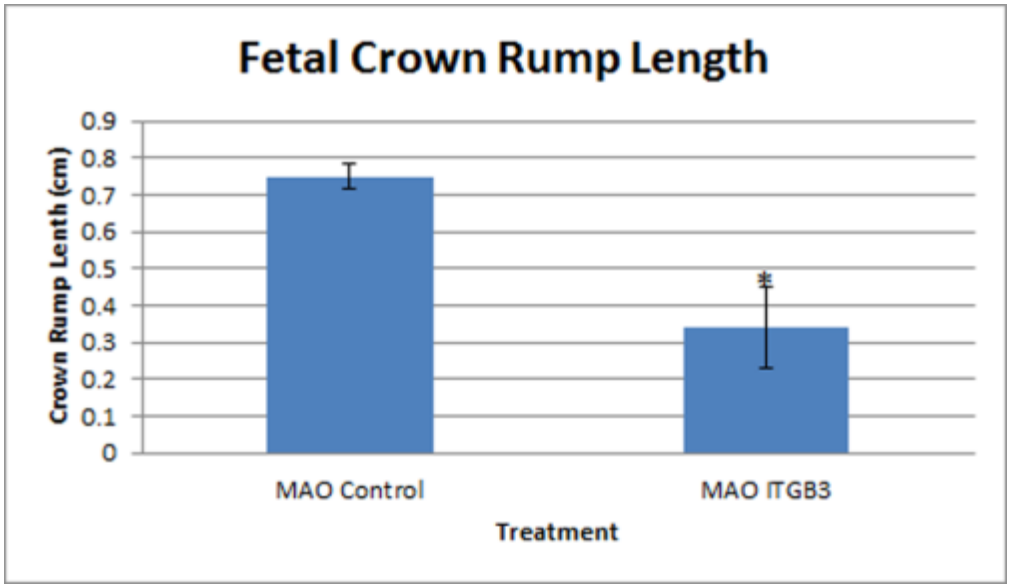

Figure 5-4. Effects of loss of function of ITGB3 on fetal growth. Fetal crown rump length was measured in fetuses from both the MAO control (n=3) and ITGB3-MAO (n=5) groups. Fetuses from pregnancies exposed to ITGB3-MAO were significantly smaller when compared to the pregnancies exposed to MAO control (P=0.0099).

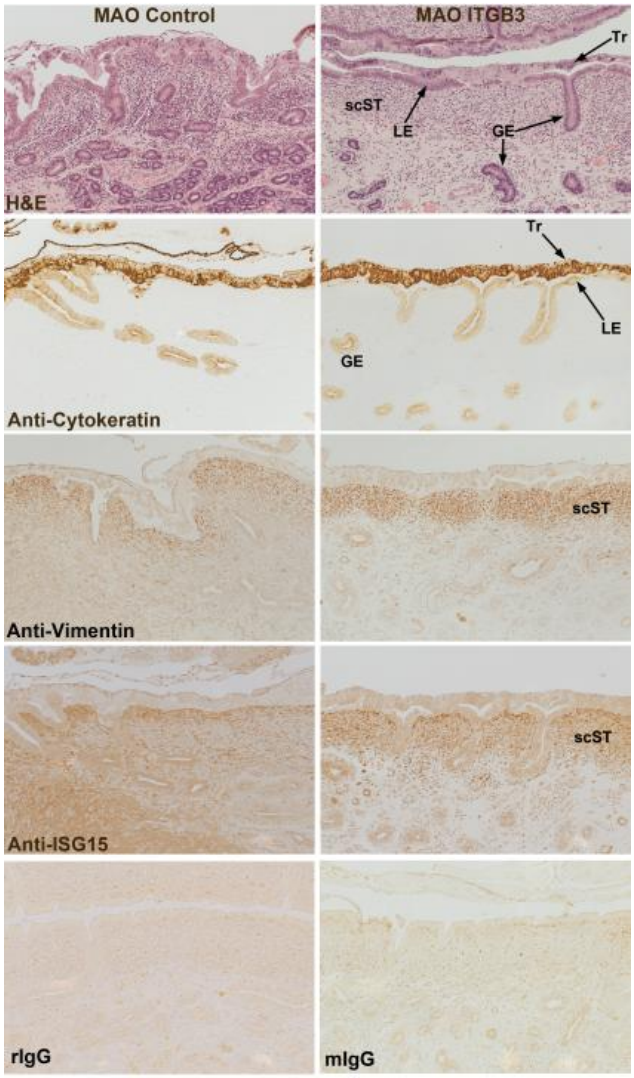

Figure 5-5. Histological examination of Day 24 implantation sites. Top Panels: Hematoxylin and Eosin staining was utilized to observe the histological morphology of implantation sites form pregnancies exposed to MAO-ITGBs3 and MAO control. Conceptuses from both groups implanted normally with no morphological differences observed. Second and Third Panels from Top: Immunohistochemistry confirmed normal distribution of the intermediate filaments cytokeratin and vimentin within the conceptus and uterine epithelia and the uterine stroma respectfully with no differences between treatment groups. Fourth Panel from Top: The uterine endometrium responded to conceptus secretion of interferon tau to express ISG15 in both treatment groups. Bottom Panel: A rabbit IgG was utilized as a negative control. Antibodies used to detect Cytokeratin (C6909, Sigma), Vimentin (V6389, Sigma) and ISG15 (Pru *et al.*2000). Legend: scST, stratum compactum stroma; LE, luminal epithelium; GE, glandular epithelium; Tr, trophectoderm; ISG15, interferon stimulated gene 15.

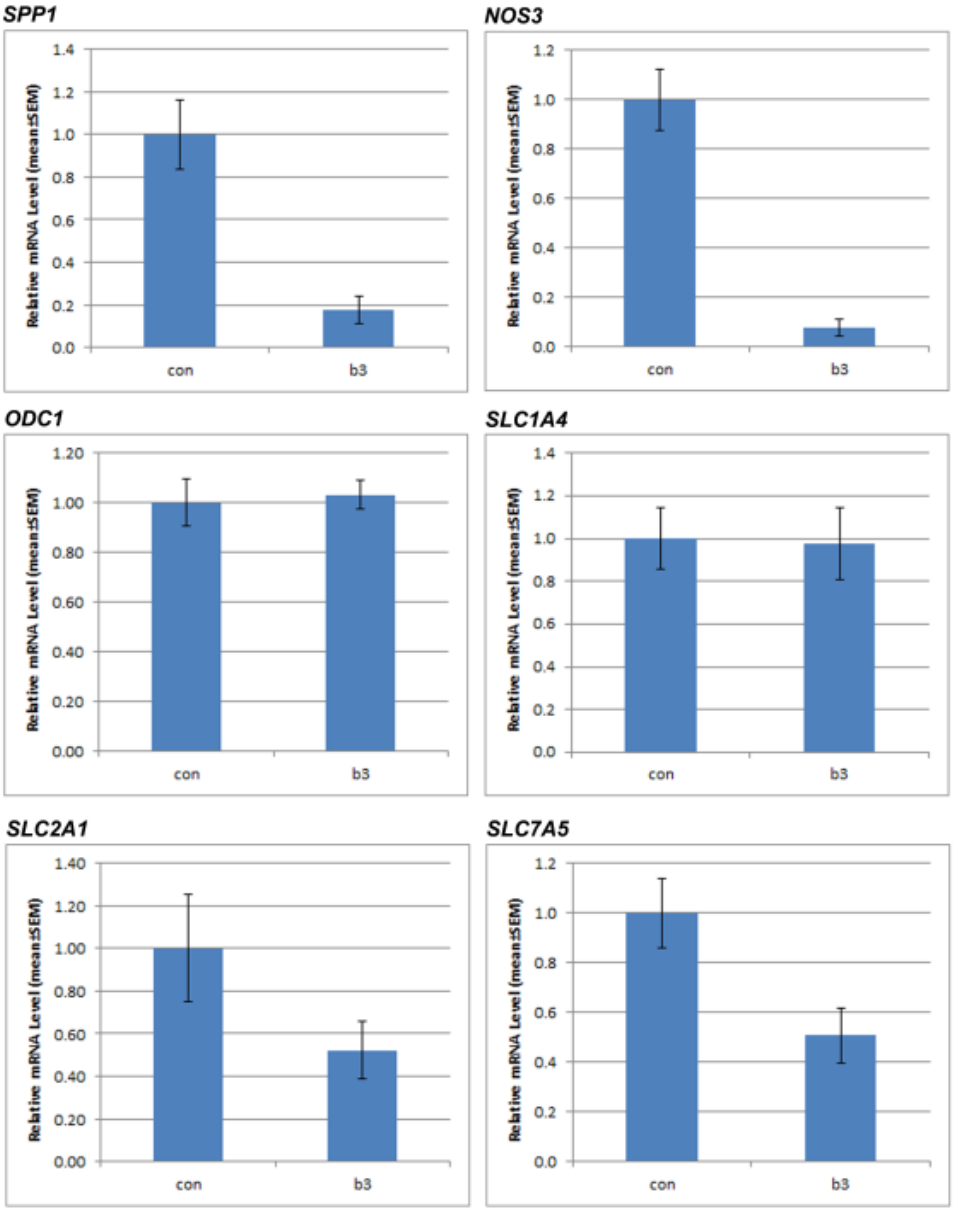

Figure 5-6. Gene expression in the placentae of Day 24 implantation sites. Real-time quantitative PCR was utilized to determine the placental expression of *SPP1*, *NOS3*, *ODC1*, *SLC1A4*, *SLC2A1* and *SLC7S5*, all previously described to be expressed by sheep conceptuses on Day 24 of gestation. No difference was observed in the magnitude of mRNA expression between conceptuses exposed to MAO-ITGB3 and MAO control for *ODC1*, *SLC1A4*, *SLC2A1* and *SLC7S5*; However, *SPP1* and *NOS3* mRNAs were decreased in the placentae of the MAO-ITGB3 treated ewes as compared to controls ($P<0.05$).

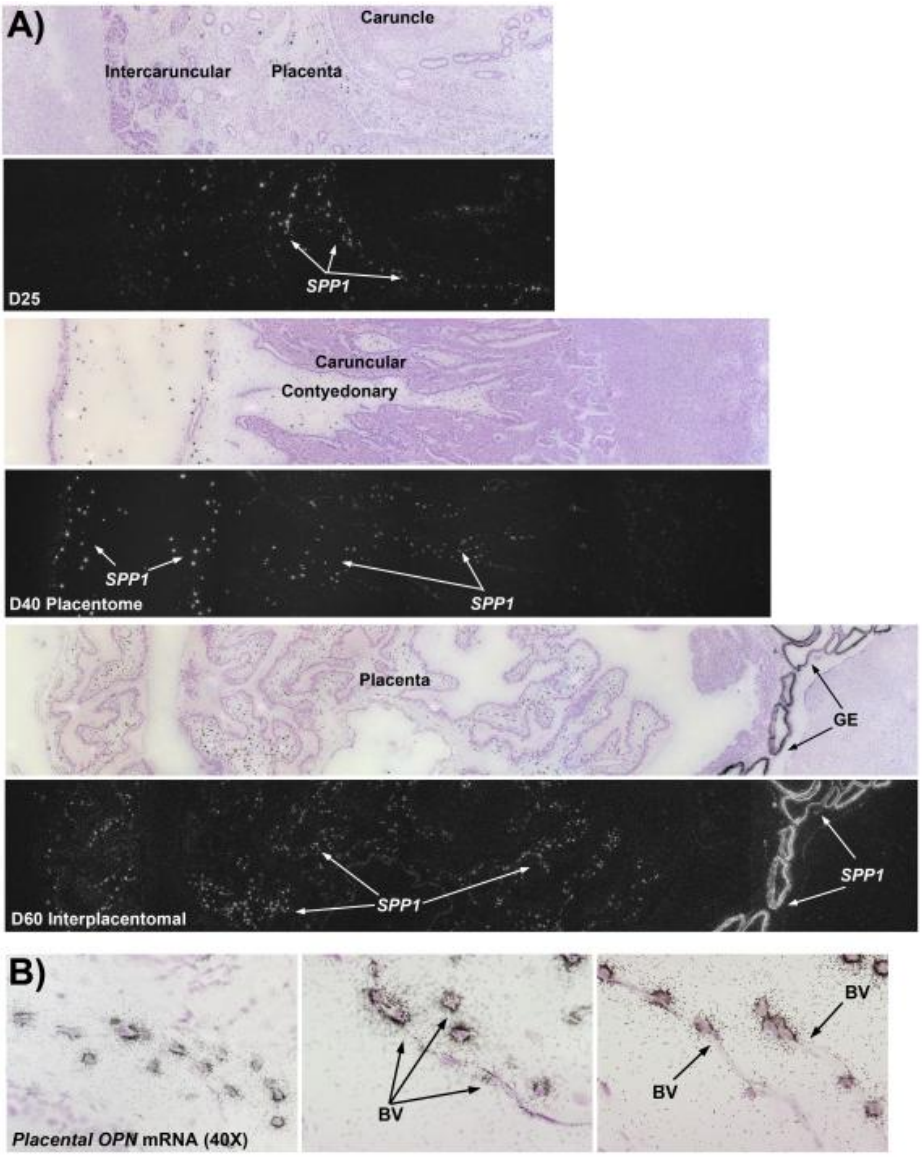

Figure 5-7. *SPP1* mRNA in the sheep allantois. *In situ* hybridization was utilized to localize *SPP1* mRNA localize in the placenta. A) *SPP1* mRNA is observed scattered cells within the allantois on Day 25, expression is observed in the cotyledonary connective tissue of placentomes on Day 40, and allantoic SPP1 mRNA increases through Day 60 of pregnancy. Corresponding brightfield and darkfield images from different Days (D) of pregnancy hybridized with radiolabeled antisense RNA probe are shown. A representative section hybridized with radiolabeled sense RNA probe (sense) serves as a negative control. Width of field for D25 is 2400 μm, for Day 40 is 3300 μm and for Day 60 is 4000 μm. B) Magnified images of Day 60 allantois shows cells containing *SPP1* mRNA directly adjacent to placental blood vessels. Width of fields is 230 μm. BV, blood vessel.

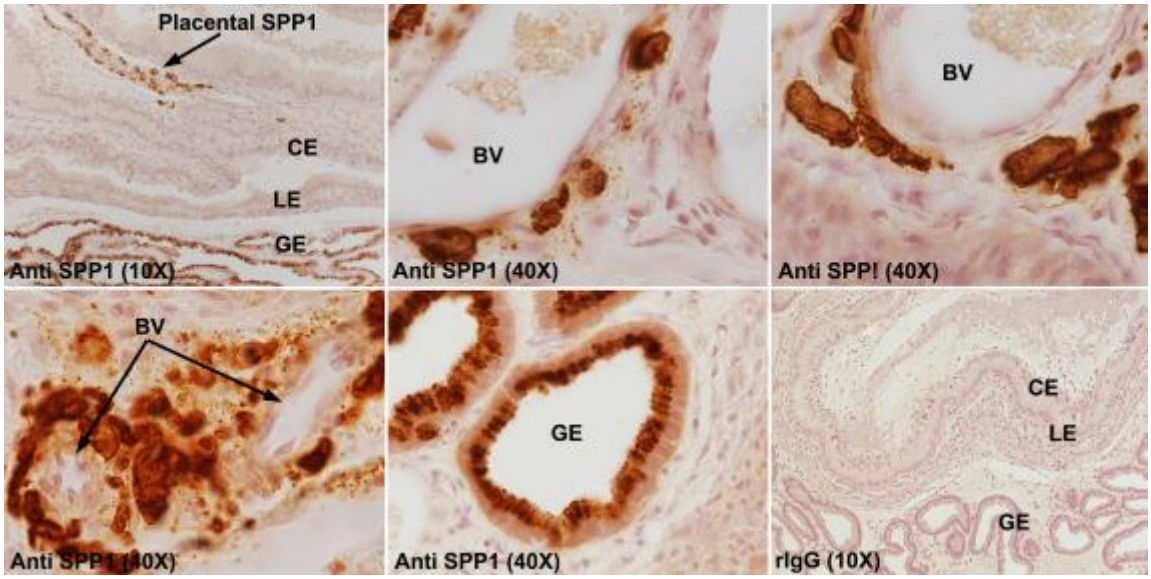

Figure 5-8. SPP1 protein in the sheep allantois. Immunohistochemistry was utilized to localize SPP1 protein in the placenta of a Day 120 pregnant sheep. Localization of protein was similar on all Days examined. The 10X magnified image shows overall tissue distribution of SPP1 protein within he allantois while images magnified 40X illustrate deposition of SPP1 protein directly adjacent to allantoic blood vessels. The Middle Panel of the bottom row is an example of SPP1 protein being synthesized and secreted as histotroph from the uterine GE. A non-relevant rabbit IgG was used as a negative control. The antibody used was bovine anti-SPP1 (Erikson *et al.*2007). Legend: CE, chorionic epithelium; LE, luminal epithelium; GE, glandular epithelium; BV, blood vessel.

MAO express ITGB3 protein on the surface of Tr cells, whereas conceptuses treated with MAO-ITGB3 do not express ITGB3 which confirmed successful knockdown of the protein. These results indicate that knockdown of the ITGB3 subunit was effective, but loss of function of ITGB3 in conceptuses did not, alone, alter conceptus elongation in the ewes.

Study 3: Effects of Loss of ITGB3 Subunit on Conceptus Implantation

To address whether ITGB3 is required for implantation of the conceptus, we performed loss of function by blocking translation for Tr-expressed ITGB3 mRNA by infusing MAO-ITGB3 into the lumen of one uterine horn of ewes on Day 12, and assessed conceptus development on Day 24 of pregnancy. Pregnancy loss was observed in four MAO-control ewes and two ewes in the ITGB3 MAO group. Three embryos were recovered from the Control MAO group and five were recovered from the ITGB3 MAO group. Embryos from pregnancies exposed to MAO-ITGB3 were smaller than embryos from pregnancies exposed to MAO-control. The crown rump lengths for control embryos were 0.80 cm, 0.70 cm and 0.75 cm whereas the crown rump lengths for treated embryos were 0.55 cm, 0.30 cm, 0.45 cm 0.20 cm and 0.20 cm (Figure 5-4).

H&E staining was utilized to determine the effects of MAO on morphological development of implantation sites. No differences in morphology of the uterine-placental interface were observed in conceptuses treated with MAO-ITGB3 compared to MAO-control treated conceptguses (Figure 5-5). The Day 24 uterine-placental interface is histologically complex in sheep. For all implantation sites examined, regions of true

epitheliochorial juxtaposition of intact uterine LE and conceptus Tr were observed next to intermittent regions of interface in which the LE was absent and Tr was in direct contact with the stromal compartment of the endometrium. Uterine LE was always maintained at the mouths of uterine glands, and trophoblast papillae extended into these glands.

Immunohistochemistry for cytokeratin, an epithelial cell intermediate filament, vimentin, a fibroblast cell intermediate filament, and interferon stimulated gene 15 (ISG15), a ubiquitin-like protein that is upregulated in the stroma of sheep in response to interferon tau (Johnson *et al.*2000), showed no difference in the patterns of expression between ewes exposed to MAO-ITGB3 and MAO control (Figure 5-5). Cytokeratine was expressed by conceptus Tr and uterine LE and GE, with conceptus immunostaining for cytokeratin being more intense than immunostaining in the endometrium. Vimentin and ISG15 immunostaining were observed in the stromal compartment as previously described (Johnson *et al.*1999; Joyce *et al.*2005).

Due to the decrease in crown-rump length of conceptuses in the MAO-ITGB3 group, real-time quantitative PCR was utilized to compare expression of selected genes, proposed to be involved in either nutrient transport or angiogenesis within the implanting placenta of conceptuses exposed to MAO-ITGB3 or MAO-control. There was no significant difference in magnitude of expression of *SLC1A4, SLC7A5, SLC2A1* or *ODC1* due to treatment (Figure 5-6). In contrast, there was a a significant decrease in

expression of *SPP1* and *NOS3* in conceptuses exposed to MAO-ITGB3 compared to conceptuses exposed to MAO control (Figure 5-6).

Placental Expression of *SPP1* during Pregnancy

Results from Study 2 indicate that loss of function of Tr-expressed ITGB3 adversely effects conceptus growth, and decreased transcription of *NOS3* which suggests a relationship between growth of the embryo and placental vasculature. Because placental expression of *SPP1* also decreased with loss of ITGB3 function, and SPP1 is expressed in the Day 25 sheep allantois (Johnson *et al.*2003), we examined expression of SPP1 mRNA and protein in the placentae of ewes during pregnancy utilizing *in situ* hybridization and immunohistochemistry. As previously reported (Johnson *et al.*2003), *SPP1* mRNA was present in a population of cells within the mesenchymal tissue between the chorion/Tr and allantois on Day 25 of pregnancy (Figure 5-7A). By Day 40, *SPP1* positive cells were observed within the allantois of interplacentomal placenta as well as within cotyledonary tissues of placentomes (Figure 5-7A). By Day 60 of gestation, the *SPP1* positive cells were detectable throughout the majority of the allantois where they were localized to cells adjacent to placental blood vessels (Figure 5-7A and 5-7B). Figure 5-8 shows the large accumulation of SPP1 protein around placental blood vessels. It is noteworthy that the magnitude of SPP1 expression near blood vessels of the allantois is comparable to the accumulation of SPP1 on the apical domain of uterine GE (Figure 5-8) which synthesize and secrete large quantities of SPP1 as a major component of histotroph in sheep (Johnson *et al.*2003)

Discussion

These results suggest that, although the ITGB3 subunit is present at the uterine-placental interface during the peri-implantation period of pregnancy, loss of ITGB3 alone does not affect the ability of conceptus Tr to attach to uterine LE. It does, however, lead to fetal growth retardation. The decrease in size of the fetus could be explained by decreases in expression of *SPP1* and *NOS3* mRNA in the placenta that occurred in response to knock down of ITGB3 in Tr. In a normal pregnancy, SPP1 mRNA and protein are present in cells adjacent to placental blood vessels. Taken together, this is the first study to indicate that ITGB3 influences expression of genes in conceptus Tr associated with vascular functions required for fetal development.

ITGB3 is present at the uterine-placental interface during the peri-implantation period of pregnancy. Immunohistochemistry confirmed prominent expression of ITGB3 at the apical surface of uterine LE from Days 11 through 15 when the initial attachment phase of implantation occurs in sheep. Interestingly, ITGB3 was no longer detectable at the uterine-placental interface by Day 20 and thereafter, highlighting the precise temporal and spatial localization of ITGB3 to initial sites of implantation. ITGB3 is also expressed by uterine LE during implantation in pigs, mice, rabbits and sheep (Bowen *et al.*1996, Aplin *et al.*1996, Illera *et al.*2003, Johnson *et al.*2001). Lessey and co-workers established that transient uterine expression of αvβ3 is stage of menstrual cycle-dependent and defines the implantation window in women (Lessey *et al.*1994, Lessey *et al.*1996). Further, the ITGB3 subunit is absent in endometria of women with luteal

phase deficiency (Lessey *et al.*1992), and expression is altered in endometria of women with mild endometriosis and unexplained infertility (Lessey *et al.*1994, Lessey *et al.*1995). Functional blockade of integrin ITGB3 using a neutralizing antibody reduces the number of implantation sites in mice and rabbits (Illera *et al.*2000, Illera *et al.*2003).

Elongation of the ovine conceptus is a prerequisite for central implantation involving apposition and adhesion between ovine Tr and uterine LE. The mechanism for elongation in sheep conceptuses is likely similar to that for conceptuses of pigs wherein alterations in microfilaments, junctional complexes, and the distribution of the actin cytoskeleton within Tr cells allow the conceptus to develop from spherical to tubular and filamentous forms (Geisert *et al.*1982; Albertini *et al.*1987 and Mattson *et al.*1990; Bazer and Johnson 2014). Association of the actin cytoskeleton with myosin II is essential to generate forces necessary to constrict regions along the length of filamentous conceptus as the cells migrate during elongation. Focal adhesions are macromolecular signaling centers comprised of integrin receptors, such as αvβ3, that transmit diverse signals between the ECM, such as SPP1, and the actin cytoskeleton to regulate cell growth, migration and morphology (Burghardt *et al.*2009). To investigate whether ITGB3 plays a role in elongation of the implanting conceptus, knockdown of ITGB3 protein was performed to reveal no change in conceptus morphology when compared to control conceptuses. Twin conceptuses either exposed to or not exposed to ITGB3-MAO were also observed to have similar morphologies (data not shown). These results suggest that ITGB3 does not have a crucial role in mediating conceptus elongation; however, the results do not completely rule out a role for ITGB3 during conceptus

elongation, as there may be redundancy in function for different integrin-ECM interactions to support morphological changes in the developing ovine conceptus. For example, SPP1 binds α5β1 integrin receptor *in vitro*, and this interaction could be involved in elongation of the conceptus (Kim *et al.*2010). Further, ITGAV, ITGA4, ITGA5, ITGB1, and ITGB5 subunits expressed by Tr of elongating conceptuses which can potentially form receptors to bind SPP1 that could be having a compensatory effect to overcome the loss of ITGB3 subunit to mediate elongation of the conceptus (Johnson *et al.*2001).

The presence of ITGB3 on the apical surface of uterine LE and conceptus Tr during the peri-implantation period places it in an excellent position to influence attachment for implantation. In pigs, integrin subunits ITGAV and ITGB3 localize to FAs during the peri-implantation period of pregnancy and siRNA knockdown of ITGV in porcine Tr cells reduces the ability of SPP1 to support Tr cell adhesion suggesting that these integrins have a role in mediating attachment of the implanting conceptus. To determine if ITGB3 has a similar role during implantation of the sheep conceptus, MAOs were utilized to knockdown ITGB3 in conceptuses during the peri-implantation period of pregnancy. Histological examination showed that loss of ITGB3 did not affect the ability of the conceptus to adhere to the uterine LE. Cross-sections of the uterine-placental interface were not different morphologically between ITGB3-MAO and control-MAO conceptuses. No differences in the distribution of cytokeratin, vimentin, or ISG15 were detected in uteri or placentae of implantation sites from ITGB3 knockdown compared to control ewes indicating, at a superficial level, integrity of both the epithelial

and stromal tissues of these organs, and the ability of conceptus interferon tau to increase gene expression in the endometrium (Johnson *et al.*2000). Integrin receptors that have the ability to bind SPP1at the uterine-placental interface during the peri-implantation period of pregnancy are: αvβ1, αvβ5, α4β1, and α5β1. We hypothesize that implantation is not being affected by loss of ITGB3 due to the redundancy of potential integrin receptors that bind SPP1.

Although ovine implantation sites appeared normal in pregnancies of the MAO-ITGB3 treated concepuses, there was a significant reduction in fetal crown rump length due to knockdown of ITGB3 in Tr. To begin to investigate the possible mechanism(s) by which growth in the developing embryos may be reduced, expression of selected genes was examined in the placentae of conceptuses. *SLC1A4, SLC7A5, SLC2A1, NOS3, ODC1* and *SPP1* are genes that have been shown to be up-regulated in the conceptus during the peri-implantation period of pregnancy and are involved in either nutrient transport, angiogenesis or adhesion. Two of these genes, *NOS3* and *SPP1*, were significantly decreased in the placentae of conceptuses exposed to MAO-ITGB3.

The reduction in *SPP1* and *NOS3* mRNAs in the placenta and the presence of *SPP1* mRNA and protein adjacent to blood vessels in mesenchymal cells of the chorioallantois led us to hypothesize that angiogenesis is impaired in extra-embryonic tissues following ITGB3 knockdown in Tr, resulting in a decreased supply of nutrients to the developing fetus and, therefore, a decrease in fetal growth. NOS3 plays an important role in pregnancy (Gouge *et al.* 1998, Stuehr 1999, Zheng *et al.*2000) and Wang *et al.*

determined that SPP1 binds integrin αvβ3 to activate NOS3 and nitric oxide production in endothelial progenitor cells (2011). Additionally, SPP1 enhances angiogenesis in different tumor types (Chakraborty *et al.* 2008, Dai *et al.* 2009). In the conceptuses with ITGB3 knockdown, loss of the signaling pathway for SPP1 could impact NOS3 mRNA expression, as well as overall angiogenesis to impair fetal development. Integrin αvβ3 has also been found to interact with VEGRF2 to induce migration and proliferation of endothelial cells leading to an independent role for ITGB3 from SPP1 (Lakshmikanthan *et al.* 2011). While the regulators of SPP1 are numerous, there is the possibility that knockdown of ITGB3 interrupts a positive feedback loop that leads to up-regulation of expression of SPP1 around the placental blood vessels as detected in normal pregnancy in ewes. The actions of ITGB3 through binding of SPP1 could influence multiple pro-angiogenic signaling pathways in the placenta of sheep.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Summary

Interactions between the apical surfaces of conceptus trophectoderm (Tr)/chorionic epithelium and uterine LE are crucial for the establishment and maintenance of pregnancy in pigs and sheep. In a progesterone dominated uterine environment, establishment and maintenance of pregnancy in pigs and sheep requires; (i) secretion of estrogens or interferon tau, respectively, from the conceptus to signal pregnancy recognition (Frank *et al.*1977, Bazer *et al.*1977, Godkin *et al.*1984, Vallet *et al.*1988), (ii) secretions from uterine LE and GE, i.e., histotroph, to support attachment, development and growth of the conceptus (Ashworth *et al.*1989, Gray *et al.*2001, Burton *et al.*2002), and (iii) cellular remodeling at the uterine LE-conceptus Tr interface to allow for attachment during implantation (Glasser *et al.*1993, Denker *et al.*1993,Burghardt *et al.*2002). These events are orchestrated through endocrine, paracrine, autocrine, and juxtracrine communication between the conceptus and uterus, and the complexity of these events likely underlies the high rates of conceptus mortality during the peri-implantation period of pregnancy (Bazer *et al.*1983, Johnson *et al.*2009). The studies described in this Dissertation focused on the juxtacrine interactions between OPN and integrins in the conceptus and uterus during implantation and placentation.

Implantation and placentation are critical events in pregnancy. Implantation failure during the first three weeks of pregnancy is a major cause of infertility in all mammals (Flint *et al.*1982, Bazer *et al.*1983, Johnson *et al.*2009). The process of implantation is highly synchronized, requiring reciprocal secretory and physical interactions between a developmentally competent conceptus and the uterine endometrium during a restricted period of the uterine cycle termed the "window of receptivity." These initial interactions between apical surfaces of uterine LE and conceptus Tr begin with sequential phases i.e., non-adhesive or pre-contact, apposition, and adhesion, and conclude with formation of a placenta that supports fetal-placental development throughout pregnancy (Cross *et al.*1994, Carson *et al.*2000, Bazer *et al.*2005). Conceptus attachment first requires loss of anti-adhesive molecules in the glycocalyx of uterine LE, comprised largely of mucins that sterically inhibit attachment (Bowen *et al.*1996, Aplin *et al.*2001, Johnson *et al.*2001). This results in "unmasking" of molecules, including integrins, selectins and galectins which contribute to initial attachment of conceptus Tr to uterine LE (Kimber *et al.*1995, Kimber *et al.*2000, Spencer *et al.*2004). These low affinity contacts are then replaced by a repertoire of adhesive interactions between integrins and maternal ECM which appear to be the dominant contributors to stable adhesion at implantation sites Hynes *et al.*1987, Ruoslahti *et al.*1987, Burghardt *et al.*1997 Johnson *et al.*2001, Burghardt *et al.*2002, Lessey *et al.*2002). OPN is expressed abundantly within the conceptus-maternal environment in numerous species, including pigs and sheep (Johnson *et al.*1999b, Garlow *et al.*2002 Apparao *et al.*2003, Johnson *et al.*2003, Mirkin *et al.*2005, Joyce *et al.*2005, White *et al.*2006).

This dissertation describes the interactions between the apical surfaces of conceptus Tr/chorionic epithelium and the uterine LE during pregnancy in pigs and sheep, specifically focusing on the interactions between integrin receptors and OPN that are present at this interface.

Questions Addressed in Resarch for the Dissertation and Relevant Findings

What are the temporal, cell-type specific expression, and hormonal regulation of the ITGAV, ITGB3 and ITGB6 subunits at the uterine-placental interface of pigs throughout pregnancy, and does OPN bind ITGAV heterodimers to support Tr cell adhesion *in vitro*?

Temporal and spatial expression of mRNAs and proteins demonstrated that although expression of mRNAs did not change at the uterine-placental interface, ITGAV and ITGB3 proteins aggregate *in vivo* into FAs during the peri-implantation period of pregnancy in pigs. However, as the maternal-conceptus interface becomes more folded with advancing placentation those FAs are no longer observed. We hypothesize that these FAs are no longer necessary because folding of the utero-placental tissues disperses forces, including tension, compression, and/or shear exerted against this interface so that there is no longer a need for FAs to maintain integrity of attachment between uterus and placenta. Therefore, FA assembly at the uterine-placental interface is dynamic and specific for the tissue compartment niche being observed. These studies also determined that knockdown of expression of ITGAV decreases the ability of pTr2 cells to bind OPN *in vitro*. These data are the first to directly confirm, in any species,

that ITGAV mediates adhesion of Tr cells to OPN and suggests that ITGAV plays a critical role in binding OPN to mediate attachment of Tr to uterine LE for implantation in pigs and perhaps other species.

When do integrin subunits redistribute into FA at the uterine-placental interface in sheep?

Results of the present studies revealed that multiple integrin subunits are expressed uniformly along the entire uterine-placental interface through Day 15, during attachment for implantation, in a pattern that suggests adhesion, but a lack of assembly into large FAs; however, by Day 20, a period of instability in the integrity of the endometrial LE that developmentally demarks implantation from placentation, these integrins are found in large intermittent aggregates along the uterine-placental interface. The FAs containing integrin subunits ITGAV, ITGA4, ITGA5, ITGB1, and ITGB5 increase in abundance and become more organized at the maternal-conceptus interface as pregnancy progresses; however, ITGB3 is only present during the peri-implantation period of pregnancy suggesting that it is playing a role in initial attachment of conceptus Tr to the uterine LE. Further, these studies were the first to co-localize OPN with ITGAV, ITGA4, ITGA5, ITGB1, and ITGB5 in large FAs that form at the uterine-placental interface of sheep. These results are the strongest indication to date that OPN and integrins interact at the uterine-placental interface of sheep and that these interactions are changing in a dynamic way as the tissue compartment adapts to internal and external

forces generated as the contents of the uterus increase in mass and volume during the course of pregnancy.

Does OPN secreted by uterine GE bind to the αvβ3 integrin receptor on conceptus Tr to aid in elongation of the conceptus and mediate attachment of conceptus Tr to uterine LE in sheep?

Sheep conceptuses elongate and implant on uterine LE in the absence of ITGB3 protein on the Tr. Loss of ITGB3 did, however, cause a reduction in fetal growth when compared to fetal growth in control ewes. Further, expression of both OPN and nitric oxide synthase 3 (NOS3) was significantly decreased in placentae of growth retarded fetuses, suggesting that delayed growth is related to impaired vascular function. Temporal and spatial examination of the expression of OPN mRNA and protein in the placentae of pregnant sheep revealed localization of OPN near vascular networks. Collectively, these results suggest that delayed embryonic growth is related to impaired vascular function and/or angiogenesis as noted by the decrease in NOS3, and that activation of integrin receptors containing the ITGB3 subunit decreases placental production of OPN that may be involved in the development of a healthy placental vasculature, leading to fetal growth retardation due to decreased nutrient intake by the growing fetus.

Conclusions

The results of studies for this dissertation indicate that integrin mediated FAs that bind OPN play a crucial role in maintaining contact between conceptus Tr and uterine LE in regions exposed to mechanical stress during pregnancy. These results were the first to: 1) directly confirm, in any species, that the ITGAV mediates adhesion of Tr cells to OPN; 2) co-localize OPN with ITGAV, ITGA4, ITGA5, ITGB1, and ITGB5 in large FAs at the uterine-placental interface of sheep and; 3) implicate ITGB3, through interactions with OPN, in vascular development in the developing placenta of sheep. Future experiments will need to determine the mechanism(s) by which force contributes to the formation of FAs at the uterine-placental interface and how ITGB3 affects vascular development.

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