

**INTEGRATED FUNCTIONS OF TRANSFORMING GROWTH FACTOR
BETA, LATENCY ASSOCIATED PEPTIDE, AND INTEGRINS
DURING EARLY PORCINE PREGNANCY**

A Dissertation

by

DANA A. MASSUTO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2009

Major Subject: Veterinary Anatomy

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ABSTRACT

Integrated Functions of Transforming Growth Factor Beta, Latency Associated Peptide,
and Integrins During Early Porcine Pregnancy. (December 2009)

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In pigs and other mammals, embryonic losses often occur during implantation when the conceptus (embryo plus its extra-embryonic membranes) attaches to the maternal uterine epithelium. Mechanisms controlling this process are not completely understood. Integrins and growth factors are among many molecules likely involved in controlling implantation. Numerous integrins (ITG), including subunits ITGAV (alpha v), ITGB1 (beta 1), ITGB3 (beta 3), and ITGB5 (beta 5), and transforming growth factor betas (TGFβs), in both latent and active forms, are present at the porcine conceptus-maternal interface. TGFβs are released as latent precursors which cannot interact with TGFβRs prior to their activation. Latency associated peptide (LAP), part of the TGFβ latent complex, contains an amino acid sequence Arg-Gly-Asp (RGD) that is found in other extracellular matrix molecules and may interact with and signal through integrins. We hypothesize that LAP will bind to and activate ITGAV-containing heterodimers at the conceptus-maternal interface and that these interactions are a functional component of implantation. We also hypothesize that TGFβ acting via TGFβRs has critical roles

during peri-implantation, and such roles may include promoting conceptus development, survival, and adhesion.

Immunofluorescence was used to colocalize TGFB, LAP, and integrins in porcine peri-implantation uterus and conceptus; immunohistochemistry of phosphorylated SMAD2/3 provided evidence of TGFB activity. Affinity chromatography identified cell surface integrins on porcine trophectoderm that are capable of binding LAP. In vivo, intrauterine infusions of LAP with its native RGD site (LAP-RGD) resulted in inhibition of conceptus elongation; LAP-RGE infusions yielded normal-appearing filamentous conceptuses at d13 of pregnancy. At d24, allantois length and fetal weights were greater in gilts which received LAP-RGE infusions compared to controls which received vehicle only.

Results provide evidence for 1) active and latent TGFB in porcine conceptus and uterus; 2) receptor-ligand interactions of integrins and LAP; 3) integrin aggregation and potential focal adhesion formation at the conceptus-maternal interface; and 4) TGFB- and/or integrin-associated mechanisms which regulate conceptus elongation and placental and fetal size. Collectively, results suggest that TGFB and integrins are extensively involved in communication at the porcine conceptus-maternal interface, particularly regulating conceptus development, adhesion, and placental and fetal development.

DEDICATION

This is dedicated to my parents, Daniel and Nancy Massuto, and my sisters who have supported me no matter what decision I have made and no matter how far away that decision took me. They have never let me forget the importance of family.

This is also dedicated to my husband, Eric Kneese. He has put so much of his time and energy into this project along every step of the way. There was no task too big that he and I could not tackle as a team, and in the end, we came out stronger.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Laurie Jaeger for all of her guidance, patience, and support over the years. I am grateful I was able to work under someone who had confidence in me. I also thank Eric Kneese, who was a member of our lab and helped in every aspect of this project. I would also like to thank Dr. Gregory Johnson for all of his help and guidance especially in regards to writing manuscripts. I would like to thank Dr. R. Neil Hooper for performing all of our surgeries and helping me learn some of the clinical aspects of the field. I would like to thank Dr. Nancy Ing for her molecular expertise help and Cindy Balog-Alverz for all of her help with pig surgeries. I would also like to thank Dr. Robert Burghardt for all of his support and guidance to help me become a better scientist. In addition, I acknowledge Dr. Robert Burghardt's Image Analysis Laboratory. I also acknowledge Vet Med Park for providing use of their facilities and the United States Department of Agriculture for funding this project.

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CHAPTER I

INTRODUCTION

Embryonic loss in domestic swine presents a major reproductive problem resulting in fewer total piglets per sow per year. Discovering ways to improve reproductive success is beneficial for improving porcine livestock production, and such improvements can be relevant to pregnancy loss in other mammals including humans. A significant proportion of pregnancy loss in pigs occurs during the first 25-30 days of gestation (Flint *et al.* 1982, Jainudeen & Hafez 1987). During this period, one of the critical events influencing pregnancy success is conceptus development and attachment to the uterine epithelium (Cross *et al.* 1994).

Implantation, a critical period during pregnancy, is initiated when the conceptus comes in close proximity to the uterine luminal epithelium (LE), comprising what may be referred to as the conceptus-maternal interface. The implantation process varies among species. Unlike that in mice and humans, which exhibit an invasive type of implantation, implantation in pigs is superficial, during which the blastocyst does not invade maternal tissues. Placental types also vary among species and are divided into three main categories: hemochorial, epitheliochorial, and endotheliochorial. Epitheliochorial implantation in pigs results in interdigitation of the microvilli of the trophoctoderm and the uterine LE along with further interdigitation of the chorion and maternal tissue (Keys & King 1990). This interdigitation of conceptus and maternal tissues provides increased

This dissertation follows the style of *Reproduction*.

surface area for contact and communication in this non-invasive type of implantation. Cells of the uterus and conceptus must communicate to establish successful implantation. Autocrine, paracrine, and juxtacrine communication present at the interface involves a variety of molecules necessary for implantation including, but not limited to, cytokines, growth factors, ECM proteins, proteases, protease inhibitors, interferons, integrins, and steroid hormones (Bazer 1992, Bowen *et al.* 1996, Lessey & Arnold 1998, Bowen & Hunt 2000, Carson *et al.* 2000, Jaeger *et al.* 2001, Burghardt *et al.* 2002, Lessey 2002, Dey *et al.* 2004, Armant 2005, Lea & Sandra 2007, Guzeloglu-Kayisli *et al.* 2009). Synchrony between conceptus and uterine communication is essential for promoting a uterine environment that is receptive to the attaching conceptus. In the absence of synchrony, defects in implantation leading to the loss of pregnancy may result (Pope *et al.* 1982, Geisert *et al.* 2006, Ross *et al.* 2007). Mechanisms supporting porcine conceptus development and attachment are not well defined, and alterations in autocrine, paracrine, and juxtacrine signaling during the peri-implantation period likely contribute to reproductive failure. Therefore, the mechanisms involved in conceptus development and attachment are the focus of this project, with a particular emphasis on specific integrins and growth factors.

The beta transforming growth factors (TGFβs) and their type I and type II receptors (TGFβR1, TGFβR2) are among the growth factors that have been identified at the conceptus-maternal interface (Gupta *et al.* 1996, Gupta *et al.* 1998a, Gupta *et al.* 1998b). Active TGFβs are functional when they bind to TGFβ receptors; however, functional ligand-receptor interactions of TGFβs at the porcine conceptus-maternal

interface have not been fully defined. In addition to TGFβs, numerous integrin heterodimers, which have the ability to bind to many extracellular matrix (ECM) proteins, have also been identified at this interface (Bowen *et al.* 1996, Burghardt *et al.* 2002, Erikson *et al.* 2009). Evidence suggests that TGFβs and integrins may be key players during porcine implantation, and further understanding their specific roles, interactions, and the signals they transmit during porcine implantation are the major focuses of this project.

CHAPTER II

LITERATURE REVIEW

Porcine Blastocyst Development

Pigs are classified as a polyestrus, spontaneous ovulators with an approximately 21-day estrus cycle. At release of the ova, progression of the oocyte within the oviduct is slow and continuous, which is similar to that in other litter bearing species. The uterine tube, commonly referred to oviduct, is approximately 25 cm in length and is divided into three main segments: the infundibulum, the ampulla, and the isthmus, with distinguishable ampulla-isthmic and utero-tubal junctions (Brüssow 1985). The uterine tube supports preparation of the gametes, fertilization, and development of the zygote. Within the uterine tube, the fertilized ovum undergoes its first cleavage by 26 h after fertilization (Brüssow *et al.* 2008). The embryo undergoes further cleavages, and during the 8 to 16 cell stage, activation of the porcine conceptus genome must occur as a prerequisite for successful pregnancy (Dey *et al.* 2004).

The embryo is encased within an anti-adhesive barrier, the zona pellucida (ZP), which helps facilitate migration through the oviduct by resisting adhesion to the uterine tube wall (Carson *et al.* 2000). Pioneering work involving *in vitro* co-culture of sheep embryos with oviductal cells determined that oviductal secretions support early embryo development (Gandolfi & Moor 1987). The porcine oviduct secretes proteins such as porcine oviductal secretory proteins (POST) 1-3, which are up-regulated during estrus, can bind and remain associated to the ZP, and may help facilitate oocyte maturation and

early embryo development (Buhi *et al.* 1990, Buhi *et al.* 1993). Other classes of proteins identified in the oviductal environment include protease inhibitors, growth factors, cytokines, binding proteins, enzymes, and immunoglobulins (Buhi *et al.* 1997, Buhi *et al.* 2000, Buhi & Alvarez 2003). Of these, the more abundantly synthesized proteins include protease inhibitors such as tissue inhibitor of metalloproteinase 1 (TIMP1) (Buhi *et al.* 1997) and plasminogen activator inhibitor 1 (PAI1) (Kouba *et al.* 2000).

The developing embryo enters the uterus at the 4 to 8 cell stage, between 48 to 56 h after fertilization, where it continues to develop into a blastocyst. Differentiation of two different cell populations occurs, comprising a peripheral cuboidal cell layer termed the trophectoderm and an inner cell mass (ICM). The trophectoderm contains dense microvilli, functions in selective nutrient uptake, and gives rise to the chorion of the placenta. The ICM will give rise to the embryo proper. When pig blastocysts reach 0.5 to 1 mm diameter in size, they shed the zona pellucida, and this typically occurs by day 6-7 of pregnancy. The blastocyst continues to develop and differentiate into a conceptus (embryo and associated extra-embryonic membranes).

Porcine Gastrulation

Gastrulation or formation of the three distinct germ layers, ectoderm, mesoderm, and endoderm, occurs after the blastocyst hatches from the ZP. In an estrogen stimulated uterine environment, enzymes secreted by the trophectoderm and prostaglandins aid in the digestion of the ZP and subsequent hatching of the blastocyst (Bazer *et al.* 1987). In species such as the pig, in which the conceptus undergoes elongation, the polar

trophectoderm (Rauber's layer) directly overlaying the ICM degenerates, exposing the ICM. The ICM rapidly undergoes proliferation and delamination of cells forming a hypoblast, which extends on the inner surface of both the ICM and trophoblast, and an epiblast, which is the source of all three germ layers, which are all collectively referred to as the embryonic disc. The first morphological sign of cranial and caudal polarity in the embryonic disc occurs by day 9 in the pig, and by day 11, the mesoderm and endoderm begin to form from the embryonic disc, and are positioned under the epiblast. Epiblast cells have an autonomous tendency to develop into neural tissue; therefore, for the remainder of the epiblast to develop into the ectoderm, an active cascade involving bone morphogenetic proteins (BMPs) and receptor regulated SMADs (homologous of mothers against decapentaplegic in *Drosophila* and *sma-2*, *-3*, and *-4* in *C. elegans*) proteins are required (Blomberg *et al.* 2008).

The mesoderm layer extends caudally and laterally in between the trophoctoderm and endoderm. The portion of mesoderm that lies under the embryonic disk is intra-embryonic, and the portion underlying the trophoctoderm is extra-embryonic. The intra-embryonic coelom gives rise to the body cavities, and the extra-embryonic mesoderm will give rise to the allantois. In addition, the extra-embryonic mesoderm will later fuse with the primitive endoderm, the ectoderm, and trophoctoderm to give rise to the yolk sac, amnion, and chorion, respectively. Further fusion of the chorion and allantoic membranes gives rise to the chorio-allantoic placenta (Blomberg *et al.* 2006, Vejlsted *et al.* 2006, Blomberg *et al.* 2008).

Conceptus Elongation

Gastrulation in the pig is concomitant with conceptus elongation. Spherical day 10 porcine blastocysts (2-6 mm) undergo a morphological transition to larger spheres (10 to 15 mm) then to tubular forms (15 mm by 50 mm) and finally to filamentous forms (1 mm by 100-200 mm) (Geisert *et al.* 1982b). During the transition from spherical to filamentous forms between days 10 to 12 of pregnancy, extra-embryonic tissues elongate at a rate of 30 to 45 mm/h, which is the most exaggerated of all species that undergo conceptus elongation (Geisert *et al.* 1982b, Bennett & Leymaster 1989). The elongated conceptus provides increased surface area for exchange of nutrients between the conceptus and uterus, and is essential for successful implantation (Stroband & Van der Lende 1990). Initially, porcine conceptus elongation was hypothesized to occur primarily through reorganization and remodeling of the cells of the extra-embryonic tissues rather than hyperplasia (Geisert *et al.* 1982a, Pusateri *et al.* 1990). More recently, hyperplasia has been recognized as a very active component of porcine elongation as it is in other ungulates (Bazer *et al.* 1987, Blomberg *et al.* 2006, Blomberg *et al.* 2008) (Bazer *et al.* 1993).

Uterine Histoarchitecture and Placentation

Uterine endometrium consists of simple columnar luminal epithelium (LE) and an underlying lamina propria consisting of an upper stratum compactum and lower stratum spongiosum. Cells of the lamina propria include stromal cells, immune cells, and vasculature. The LE invaginates downward forming tubular compartments with distinct

lumens called endometrial glands, and glandular epithelium (GE) lines these lumens. The myometrium of the uterus contains inner circular and outer longitudinal layers of smooth muscle cells.

Porcine implantation is epitheliochorial, characterized by being superficial, non-invasive, and with a uterine LE that remains intact. Porcine conceptuses undergo transuterine migration, and myometrial contractions induced by estrogen and prostaglandins may aid in the spacing of conceptuses (King *et al.* 1982). By day 13 of porcine pregnancy, conceptuses begin to make initial attachments to the uterine LE (Keys & King 1990). Each filamentous conceptus occupies its own segment of the uterine horn, and attachment is initiated at the embryonic disk and progresses toward the trophoblastic tips (Dantzer 1985, Keys & King 1990). Attaching conceptuses do not overlap, and trophoblastic tips that are exposed to each other undergo necrosis called necrotic tips. By day 24 of pregnancy, complete interdigitation of the LE and trophectoderm provides increased surface area of contact between conceptus and maternal tissues (Keys & King 1990). In areas where the trophectoderm overlays openings of endometrial glands at the luminal surface, specialized absorptive structures called areolae are formed. Areolae begin to appear by day 17 of pregnancy, and specialize in uptake of histotrophic nutrition secreted from endometrial glands. Histotrophic secretions support the growing embryo; following establishment of the placenta, the embryo is also supported by the maternal and fetal vasculature network termed hematotrophic support. By day 24 of pregnancy, a network of blood vessels arising from the caudal end of the fetal aorta and feeding into the umbilical vein is responsible for hematotrophic support (King *et al.* 1982).

The placenta is a transient organ that consists of both fetal and maternal components, functions in metabolic exchange, and serves as an endocrine organ. Interdigitation of conceptus and maternal tissues maximizes the surface area for metabolic exchange in the pig, and the chorion intimately associates with the uterus by interdigitating with the endometrium. Chorionic villi, created by interdigitating chorion and endometrium, are uniform in distribution, which characterizes the diffuse porcine placenta. The allantois is another placental compartment that serves as a storage site for nutrients but also produces factors such as growth factors. The amnion directly surrounding the fetus supports a uniformly growing fetus.

The placenta continues to grow until about day 60 to 70 of porcine pregnancy to support the growing fetus. By day 25 of pregnancy, the average number of live embryos is 11. In addition, average for day 25 fetal crown-rump length is 1.8 cm and for fetal wet weight is 0.53 g (Knight *et al.* 1977).

Pregnancy Recognition

The conceptus is responsible for secreting the signal for pregnancy, and in the pig, estrogens produced by the conceptus are believed to be a signal for maternal recognition signal of pregnancy (Geisert *et al.* 1982b). Exogenous administration of estrogen, in the absence of embryos, will induce a false pregnant state called pseudopregnancy. Because the CL is responsible for producing progesterone during pregnancy, inhibition of corpora lutea (CL) demise on the ovary is required to maintain a pregnant or pseudopregnant state. Progesterone is essential for regulation of endometrial functions to support events

of embryonic development, implantation, and placentation (Bazer 1992). Progesterone, along with estrogens, are the primary effectors responsible for the uterus becoming receptive to the conceptus for implantation.

In the non-pregnant gilt, pulsatile release of prostaglandin $F_{2\alpha}$ (PGF) from the endometrial LE is stimulated by pulsatile release of oxytocin binding to oxytocin receptors (Gross *et al.* 1988, Mirando *et al.* 1995, Edgerton *et al.* 1996). PGF is released into the uterine venous system beginning on Day 15 or 16 of the estrous cycle and causes regression of the CL (Bazer *et al.* 1989).

In the pregnant gilt, conceptuses secrete estrogen as a signal for pregnancy when they are at least 5 mm in size (Geisert *et al.* 1990). Maintenance of pregnancy requires biphasic estradiol secretions occurring on days 11-12 of pregnancy and again between days 15 and 20 to 25. Both surges are required to maintain pregnancy (Bazer 1992, Geisert & Yelich 1997). Administration of biphasic exogenous estradiol on day 11 through 15 of the estrus cycle can maintain the CL for the duration of a normal pregnancy of 114 days (Geisert & Yelich 1997). Inducing asynchrony in the uterine environment during this time results in conceptus demise. This is evident by administration of estradiol before day 11 of pregnancy, which results in embryonic loss (Geisert *et al.* 2006, Ross *et al.* 2007).

The initial hypothesis that steroid hormones are involved in inhibiting regression of the CL resulted from the finding that cultured porcine blastocysts synthesize estrogens and the detection of estrogens in the pregnant pig by day 16 of pregnancy (Perry *et al.* 1973, Robertson & King 1974, Perry *et al.* 1976). The endocrine-exocrine theory

illustrates how conceptus secretion of estrogen prevents the actions of PGF's on the CL since PGF is secreted in both pregnant and cyclic pigs. In the cyclic gilt, PGF is secreted in an endocrine manner and released towards the utero-ovarian vein where it can reach the ovary and lyse the CL. In the pregnant pig, PGF is secreted in an exocrine manner towards the uterine lumen where it cannot reach the utero-ovarian vasculature (Bazer & Thatcher 1977). Estrogen is hypothesized to induce the endocrine-exocrine switch during pregnancy or pseudopregnancy by indirectly inducing calcium cycling across the epithelia to facilitate exocrine secretion of PGF into the uterine lumen (Bazer 1992). In addition, estrogen increases the ratio of prostaglandin E2 (PGE) to PGF. PGE aids in protecting the CL from luteolytic effects of PGF. Finally, estrogen also maintains LH (luteinizing hormone) receptors in both CL and uterus, which is necessary for steroid hormone production (Spencer *et al.* 2004).

Factors Mediating Conceptus Elongation

Prior to their attachment to the uterine LE, porcine conceptuses elongate from spherical to filamentous forms. Conceptus loss during this time can approach 20% in pigs (Bennett & Leymaster 1989, Blomberg *et al.* 2008). Investigating which factors mediate conceptus elongation is difficult, in part due to the failure of conceptuses to elongate *in vitro* (Stroband & Van der Lende 1990). Therefore, the signals responsible for initiating conceptus elongation are largely unknown.

Initially, porcine conceptus elongation was hypothesized to occur primarily through reorganization and remodeling of the cells of the extra-embryonic tissues rather

than hyperplasia (Geisert *et al.* 1982a, Pusateri *et al.* 1990). However, it was recently reported that hyperplasia may be a very active component of porcine elongation as it appears to be in other ungulates (Bazer *et al.* 1987, Blomberg *et al.* 2006, Blomberg *et al.* 2008). The expression of Ki67 in both the embryonic disc and extraembryonic tissues provides evidence of proliferation within these anatomical regions. Ki67 is a protein ubiquitously expressed during the G1, S, and G2 phases of the cell cycle and not during the G0 phase, which indicates that cell division is active within the trophectoderm and embryonic disc during the time conceptuses elongate between day 11 and day 12 of pregnancy (Blomberg *et al.* 2006).

Studies involving techniques such as gene analysis were utilized to identify abundantly and differentially expressed proteins during the time of conceptus elongation. Expression of genes that encode proteins known to be involved in cellular differentiation, immune modulation, maternal recognition, protein synthesis, protein trafficking, RNA post-transcriptional modification, molecular transport, cell growth /proliferation, cellular assembly organization, and cellular morphology have been identified during conceptus elongation. In the tubular stage of conceptus elongation, more than 200 genes associated with cell growth /proliferation were identified further supporting that hyperplasia occurs during elongation (Blomberg *et al.* 2008).

Genes involved in intracellular signaling pathways were also identified during conceptus elongation. Pathways involving integrin, TGFB, and WNT were well represented, suggesting mechanisms for maternal growth factor signaling and cytoskeletal rearrangement/cell motility during conceptus elongation. Other genes

identified during conceptus elongation included steroidogenic acute regulatory protein (StAR), TGF β , epidermal growth factor receptor (EGFR), interferon-gamma, retinol binding protein, interleukin 1-beta (IL1B), 17-beta hydroxysteroid dehydrogenase, estradiol (E2), cytokeratin 8 and 18, stratifin, ribosomal proteins, and less abundant trans retinoic acid, all which may have roles in supporting conceptus elongation (Blomberg *et al.* 2008). IL1B may be responsible for maternal immune suppression to prevent conceptus rejection and modulate estradiol (E2) synthesis via upregulation of aromatase, while 17-beta hydroxysteroid dehydrogenase is an important enzyme needed for steroid hormone synthesis. Cytokeratin 8 and 18 may be important for embryonic differentiation, and stratifin may be important for cell survival, growth, and migration. Finally, ribosomal proteins may be important for protein processing during conceptus elongation (Blomberg *et al.* 2006, Blomberg *et al.* 2008). From these studies, it was hypothesized that E2 has a central role in initiating the transition between ovoid to tubular stage.

Tolerance of Pregnancy and Other Immunoendocrine Functions

A large number of spontaneous conceptus loss is thought to occur because of dysregulation of the immune system (Wessels *et al.* 2007). The conceptus is considered semi-allograft because it contains paternally inherited antigens that may be presented on apical surfaces by MHC class I (MHC1) glycoproteins. MHC1 are receptors that are on the surface of all nucleated cells and present self or non-self antigens to immune cells such as B and T cells. Successful implantation and pregnancy relies on the ability of the

conceptus to circumvent the maternal immune system, and both mother and fetus contribute to an immunotolerant uterine environment. The trophoblast and uterine endometrial cells may play an important role in suppressing the immune system by producing immunosuppressive and immunotolerant factors such as PGs and TGF β . In addition, progesterone, secreted by the CL in the pig, is immunosuppressive (Hunt 2006). Other events critical for conceptus tolerance include down regulation of MHC1 on the surface of porcine trophoblast cells (Joyce *et al.* 2008) and shifting from a T helper 1 (Th1) to a T helper 2 (Th2) immune state (Croy 2001).

Cells of the immune system may have other functions in addition to immune regulation, which include, but are not limited to, angiogenesis and oxygen sensing. Recruitment of immune cells occurs during implantation, and porcine conceptuses recruit uterine natural killer lymphocytes (uNK) between days 15 and 28 of pregnancy. uNK cells aggregate around uterine glands and blood vessels, and may aid in angiogenesis via up regulating vascular endothelia growth factor (VEGF) and placental growth factor (PlGF) (Wessels *et al.* 2007). The conceptus also secretes many cytokines such as interferons and interleukins that participate in many biological functions during implantation.

Window of Implantation

Apposition and adhesion are two events that comprise porcine implantation (King *et al.* 1982). Initiation and completion of these phases are dependent upon many factors supporting the blastocyst's ability to achieve morphological transformation and adhere to

the uterine endometrium (Albertini *et al.* 1987, Mattson *et al.* 1990). The conceptus-maternal interface, created at the beginning of apposition, is a focal point for communication between the conceptus and uterus. Bi-directional communication present at the conceptus-maternal interface appears to be crucial for establishing and maintaining pregnancy. The autocrine, paracrine, and juxtacrine interactions at the conceptus-maternal interface are complex and involve many adhesion molecules and other factors.

Steroid hormones estrogen and progesterone mediate events critical for creating a receptive environment for successful conceptus attachment. Some marked events include down regulation of progesterone receptors in the LE and GE, down regulation of anti-adhesive mucin-1 (MUC1), and an increase in adhesive molecules (Burghardt *et al.* 1997, Burghardt *et al.* 2002). Progesterone receptors (PR) are down regulated in the LE and GE after day 5 of pregnancy; however, PRs are still evident in the underlying stroma and myometrium (Bazer *et al.* 2009). The stroma maintains hormonal responsiveness and secretory function of the uterine epithelium, and progesterone acting via progesterone receptors in the stroma may regulate the decrease of MUC1 on epithelial surfaces and increase adhesive molecules such as integrin subunits (ITG) alpha 4 (ITGA4), alpha 5 (ITGA5), and beta 1 (ITGB1) as a prerequisite for implantation (Burghardt *et al.* 1997, Lessey 2002, Armant 2005). In addition, progesterone also regulates growth factors such as VEGF, fibroblastic growth factor 2 (FGF-2), and FGF receptors 1 and 2 (FGFR1 and 2) (Jaeger *et al.* 2001).

Estrogen, in addition to serving as the porcine maternal recognition signal, modulates uterine gene expression that supports uterine histotroph. Estrogen receptor

(ER) alpha is present in LE, trophoblast, and stroma during the peri-implantation period, and ER beta is located in day 12 conceptuses. Estrogen acting via ERs function to increase expression of many factors such as retinol binding protein, spermidine/spermine N1-acetyltransferase, ITGB5, F-actin, and osteopontin (SPP1) (Spencer *et al.* 2004).

The Porcine Conceptus-Maternal Interface

Prostaglandins, interferons, growth factors, integrins, and ECM proteins are some of the many factors that are found on the apical surfaces or within histotroph at the conceptus-maternal interface.

Prostaglandins are among the abundantly detected proteins identified during porcine pregnancy, and secretion of prostaglandins is necessary for successful porcine pregnancy. Both conceptuses and endometrium secrete PGE₂, and PGF is also present in the uterine lumen (Geisert *et al.* 1982b). The ratio of PGE₂:PGF may have a role in regulating the uterine environment. For example, a higher ratio for PGE₂ may aid in stabilizing the ECM in addition to regulating immune functions.

Type I interferons (IFN) such as IFN delta (IFND), and type II IFNs, such as IFN gamma (IFNG), are secreted between day 12 and 20 of porcine pregnancy. In contrast to sheep, in which a type I IFN (interferon tau) is the signal for maternal recognition of pregnancy, IFNs produced by pig conceptuses do not appear to be antiluteolytic (Spencer *et al.* 2004). IFN receptors are detected on the uterine LE (Lefevre *et al.* 1998), and IFNs secreted by conceptuses may act via IFN receptors found on the uterine LE, which is an

example of the many kinds of paracrine communication detected at the conceptus-maternal interface. IFNs may function during porcine pregnancy by increasing secretion of prostaglandins, (Harney & Bazer 1989), modulating uterine stromal and GE gene expression, and stimulating loss of polarity of the uterine LE for implantation (Joyce *et al.* 2007a, Joyce *et al.* 2007b, Joyce *et al.* 2008). Finally, IFNG also induces MHCII, which is detected in the stroma in vascular elements from day 15 on of porcine pregnancy.

Adhesion molecules present during porcine implantation include, but are not limited to, integrins (Bowen *et al.* 1997, Burghardt *et al.* 2002), secreted phosphoprotein (SPP1) (Garlow *et al.* 2002), fibronectin (FN) (Tuo & Bazer 1996), oncofetal FN (oFN), and vitronectin (VN), and heparin sulfate proteoglycans. SPP1 is uterine derived and present in the LE and trophoctoderm during implantation. Unlike in the sheep, porcine SPP1 is not detected in the GE until day 35 of pregnancy (Garlow *et al.* 2002). SPP1 is induced by estrogens secreted by conceptuses and can bind to integrin receptors which may promote adhesion of the conceptus to the uterine LE during porcine implantation (Erikson *et al.* 2009). Fibronectin (FN) is a ubiquitous homodimeric ECM protein that has at least 20 different isoforms as a result of alternative splicing. FN plays a role in cellular adhesion, migration, implantation, embryogenesis and oncogenic transformation (Rashev *et al.* 2005). Abundant FN was detected during porcine pregnancy on the apical surfaces of the uterine LE and trophoctoderm (Rashev *et al.* 2005). Oncofetal FN, a variant of FN, has also been detected in trophoctoderm and uterine LE throughout porcine pregnancy (Tuo & Bazer 1996). In addition, vitronectin (VN), was detected on the apical

surfaces of trophectoderm and uterine LE during the porcine implantation period (Bowen *et al.* 1996).

Integrins, another class of adhesion molecules, are heterodimeric transmembrane glycoprotein receptors and consist of 18 alpha and 8 beta subunits. 24 different combinations of alpha and beta subunits forming integrin heterodimers function as receptors for specific ECM proteins (Hynes 1992, Yamada & Miyamoto 1995, Giancotti 1997, Aplin *et al.* 1998). Many integrin heterodimers recognize and interact with an arginine-glycine-aspartate (RGD) sequence on its ECM protein ligand. Such ECM proteins that contain an RGD sequence include SPP1, FN, VN, and LAP. Integrins along with several potential integrin ligands have been identified at the porcine conceptus-maternal interface, and these interactions play a major role in supporting conceptus adhesions to the uterine LE.

Heparin sulfate proteoglycans are adhesion molecules that can complex with growth factors to modulate cellular action by storing growth factors in basement membranes and the ECM. Growth factors that bind heparin sulfate include FGF-2, FGF-7, connective tissue growth factor (CTGF), and VEGF, all of which have been identified at the porcine conceptus-maternal interface. FGFs have autocrine and paracrine effects and participate in proliferation, migration, angiogenesis, embryonic development, and cellular differentiation. CTGF stimulates mitosis, migration, adhesion and ECM production in fibroblasts. CTGFs may participate in cell migration along with increasing expression of adhesion molecules such as FN. VEGF is expressed in the uterus and conceptus and increasingly progresses from day 44-112 of porcine pregnancy. VEGF

receptor 1 (VEGFR1) is primarily expressed in maternal tissues while VEGFR2 is primarily expressed in trophoblast. VEGF correlates with fetal weight and placental efficiency, and may have roles in the development of the placental vasculature. Other growth factors found at the porcine conceptus maternal interface include insulin-like growth factor (IGF-1), epidermal growth factor (EGF), and of particular interest TGF β (Jaeger *et al.* 2001).

Non- Invasive Porcine Implantation

Like those of all species, porcine conceptuses are inherently invasive. In artificial or ectopic sites, porcine conceptuses are invasive, and sites of invasion accumulate collagen deposition (Samuel & Perry 1972). The stroma of the uterus modulates invasion in both invasive and non-invasive species. Proteases such as plasminogen and plasminogen activator (PA), and plasminogen activator inhibitor (PAI), MMPs and their inhibitors, TIMPs, are hypothesized to modulate invasiveness of porcine conceptus. Plasmin and MMP9 and MMP2 aid in conceptus invasion by degrading the ECM, while PAI and TIMPs inhibit the actions of the proteases.

Plasminogen, the inactive form of plasmin, was detected in early pregnant porcine uterine flushes; PA, which converts plasminogen to plasmin, is produced by porcine blastocysts during days 10 to 16 in culture (Mullins *et al.* 1980, Fazleabas *et al.* 1983). Therefore, plasmin is potentially produced within the uterine lumen of pregnant pigs. After day 12 of pregnancy, PA could no longer be detected in porcine uterine flushes (Mullins *et al.* 1980, Fazleabas *et al.* 1983); however, plasminogen activator inhibitor

(PAI) is detected in uterine tissues. The presence of PAI during porcine implantation may be important to protect uterine LE from conceptus invasion by inhibiting the conversion of plasminogen to active plasmin. The conversion of plasminogen to plasmin may be inhibited in the majority of the uterine environment to prevent degradation of the uterine LE and subsequent conceptus invasion.

MMPs are also detected at the porcine conceptus-maternal interface. MMP2 was detected, in increasing amounts between days 10 to 14, in uterine flushes of pregnant sows (Foxcroft *et al.* 2000, Kayser *et al.* 2006). In addition, MMP2 transcripts were detected in blastocysts and uterine tissues from days 11 to 16 (Menino *et al.* 1997, Kayser *et al.* 2006). MMP9 transcripts were identified in both embryo and uteri between days 15 and 16 (Menino *et al.* 1997); however, in other reports, MMP9 was undetectable (Foxcroft *et al.* 2000). TIMPs were also identified in porcine uterine flushes (Kayser *et al.* 2006) and localized in the trophectoderm and endometrial stroma (Menino *et al.* 1997), however, were not detected in the uterine LE or GE. TIMP expression in the trophectoderm may inhibit degradative actions of MMP9 on the ECM.

Many factors, including but not limited to those mentioned above, appear to play a role in the complex communication network at the porcine conceptus-maternal interface. Communication between the conceptus and uterus is essential for successful implantation and pregnancy, and appears to modulate phenomena such as pregnancy recognition, tolerance of pregnancy, conceptus attachment, and the control of conceptus invasion. Growth factors, such as TGFB, and adhesion molecules, such as integrins, appear to play an intricate role in this communication network at the conceptus-maternal

interface. The project focus is of the roles that TGFβs and integrins play in promoting successful conceptus development and attachment.

Transforming Growth Factor Beta

TGFβ belongs to the TGFβ superfamily, which includes BMPs, activins, and inhibin alpha (Massague 1990). Mammalian TGFβs consist of three different isoforms (TGFβ 1, 2, and 3), each of which is encoded on a separate chromosome (Munger *et al.* 1997). Unless otherwise noted, reference to TGFβ in this document refers to the TGFβ1 isoform. TGFβ is a disulfide-linked dimer of two identical chains of 112 amino acids. Each chain is synthesized as the C-terminal domain of a 390 amino acid precursor. The 5' region of the TGFβ gene contains 2 transcriptional start sites, and expression of TGFβs can be regulated by a number of factors including estrogen, progesterone, glucocorticoids, integrins, and TGFβs themselves (Kim *et al.* 1990, Kanzaki *et al.* 1995, Arici *et al.* 1996, Ortega-Velazquez *et al.* 2003).

TGFβs are synthesized as homodimeric propeptides, and the pre-propeptide latency associated peptide (LAP) is cleaved from the mature TGFβ dimer by furin-type enzymes. LAP remains non-covalently associated with TGFβ comprising the small latency complex (SLC) (Munger *et al.* 1997, Rifkin *et al.* 1999). The SLC is usually associated with one of several TGFβ binding proteins (latent TGFβ binding proteins; LTβPs) forming the large latency complex (LLC) (Munger *et al.* 1997).

LTβPs belong to the fibrillin-like ECM family and have 4 different isoforms. LTβPs range in sizes from 124-210 kDa, and most have two protease sensitive regions.

LTBP1 and LTBP3 associate with the SLC of TGF β 1, 2, and 3 and LTBP4 can only associate with TGF β 1. LTBPs covalently bind LAP and serve to link the SLC to the extracellular matrix by covalently cross-linking extracellular matrix proteins via transglutaminase (Oklu & Hesketh 2000). LTBPs sequester inactive growth factors and serve to target TGF β s to the ECMs by binding to extracellular matrix proteins such as fibronectin and fibrillin-1. LTBPs contain an RGD sequence, but there is no current evidence that they bind to integrins via this sequence (Hyytiainen *et al.* 2004). LTBPs, however, do bind integrins α 3 β 1 and α 6 β 1 for cell adhesion in melanoma cells, but this is not via the RGD sequence. Cells secrete latent forms of TGF β s typically as the LLC however, some cases such as in lymphocytes the SLC is secreted.

LAP is part of the SLC and LLC and enhances proper folding and secretion of TGF β . LAP is a homodimer and formed within the secretory pathway after the removal of a signal sequence and cleavage from the mature TGF β peptide sequence at a processing site containing basic AA. LAP contains mannose-6-phosphate and can associate with insulin-like growth factor II/mannose-6-phosphate receptors, which may be a mechanism to sequester latent TGF β to cell surfaces and aid in TGF β activation. LAP 1 and 3 monomers contain an RGD sequence that is recognized by and binds to integrin receptors in multiple tissues (Derynck & Rhee 1987, Munger *et al.* 1998). LAP 1 also contains a DLXXL sequence that directly follows the RGD sequence that may be responsible for LAP's higher affinity binding to integrin α v β 6 (Kraft *et al.* 1999, Munger *et al.* 1999).

Transforming Growth Factor Beta Activation via Proteases

After latent TGFβs are secreted, activation of the latent forms is required before TGFβs can bind their receptors (Rifkin *et al.* 1999). Post-translationally, TGFβs become available mostly through multiple extracellular activation mechanisms that include proteases, thrombospondin-1, and integrins (Lawrence 1996, Munger *et al.* 1999, Murphy-Ullrich & Poczatek 2000, Annes *et al.* 2002, Mu *et al.* 2002, Annes *et al.* 2003). Other mechanisms of TGFβ activation include reactive oxygen species causing side group modifications, and changes in pH (pH 4.5 or lower) causing protein denaturation.

Plasmin, a well defined activator of TGFβ, is generated by proteolysis of plasminogen via tissue type plasminogen activator (tPA) or urokinase type plasminogen activator (uPA). Localized activation of TGFβ via plasmin is facilitated by thrombospondin-1 (TSP-1), a cell surface associated protein, which can bind both plasminogen and LAP (Murphy-Ullrich & Poczatek 2000). Proteolysis of LAP can then occur at the cell surface, thus liberating active TGFβ. Other potential targets for plasmin include a protease sensitive hinge region of the LTBP, which can liberate LTBP from the LLC (Annes *et al.* 2003). Similar to plasmin, thrombin can activate TGFβ via proteolytic cleavage of LAP (Jenkins 2008).

Another class of proteases, matrix metalloproteinases (MMPs), including MMP2 and MMP9, proteolytically activate TGFβ. *In vivo*, proteases may not be the primary mechanism for TGFβ activation, as protease deficient mice are not completely devoid of TGFβ activation. For example, plasminogen null mice share no similarities to TGFβ null mice while MMP9 null mice exhibit only partial reduction in TGFβ (Bugge *et al.*

1995, Lee *et al.* 2001). These data suggests that proteolytic cleavage of LAP may that multiple mechanisms may compensate for TGFB activation.

Transforming Growth Factor Beta Activation via Integrins

Based on in vivo studies in mice, activation via TSP1 and integrins may play a very important role in non-proteolytic activation of TGFB (Jenkins 2008). Previous studies involving integrin null mice resulted in similar phenotypes to TGFB null mice. ITGAV, ITGB6, and ITGB8 knockout mice share similar phenotypes with TGFB1 knockout mice such as lung and skin inflammation (Huang *et al.* 1996, Bader *et al.* 1998, Zhu *et al.* 2002). More recently, transgenic mice containing a mutant RGE sequence in LAP were shown to exhibit a similar phenotype to that of TGFB1 null mice, supporting that integrins via the RGD recognition site play a significant role in TGFB activation (Yang *et al.* 2007). LAP, like many ECM proteins such as fibronectin, osteopontin, and vitronectin, contains an RGD site and binds to integrin heterodimers (Derynck & Rhee 1987, Ruoslahti 1996, Munger *et al.* 1998, Lu *et al.* 2002, Ludbrook *et al.* 2003). The RGD sequence in LAP is conserved in pigs, mice, and humans (Derynck & Rhee 1987). To date, LAP has been shown to bind to integrins $\alpha\beta1$, $\alpha\beta3$, $\alpha\beta5$, $\alpha\beta6$, $\alpha\beta8$, $\alpha5\beta1$, $\alpha8\beta1$, and platelet integrin $\alpha\text{IIb}\beta3$, several of which are implicated in TGFB activation (Wipff & Hinz 2008).

TGFB activation via integrins involves mechanisms such as traction forces in the presence and absence of proteases (Keski-Oja *et al.* 2004). Integrins $\alpha\beta3$, $\alpha\beta5$, $\alpha\beta6$, and possibly $\alpha\beta1$ have been determined to activate latent TGFB by mechanical forces

such as cellular contraction and external stretching (Jenkins *et al.* 2006, Wipff *et al.* 2007). Both of these mechanisms require: a) latent TGFB of the LLC tethered to an ECM that resists traction forces exerted on the LLC, b) LAP bound to integrin receptors, and c) presence of the actin cytoskeleton to generate force or provide resistance to extracellular forces (Wipff *et al.* 2007, Wipff & Hinz 2008). LAP associated with the LLC binds via the RGD site to integrins that are linked to the actin cytoskeleton. It is hypothesized that extracellular force, such as ECM rigidity, is applied to the LAP-integrin complex, causing external stretch on the LLC and subsequent conformational change in the latent complex (Keski-Oja *et al.* 2004, Larson *et al.* 2006). The conformational change in the latent complex liberates TGFB, and TGFB is therefore, available to interact with TGFBRs.

Intracellular forces such as cellular contraction may also play a role in activating TGFB. Cellular contraction mediated by the cross bridging of α -smooth muscle actin (ACTA2) and myosin is involved in balancing mechanical forces placed on the ECM. These contractile forces have previously been shown to activate TGFB in myofibroblast cultures (Wipff *et al.* 2007). Contraction of the actin filaments associated with integrins generates a force on the LAP-integrin complex, causing a conformational change in the LLC, and liberating TGFB from its latent complex.

Integrins can also aid in activating TGFB in the presence of proteases. TGFB activation via integrin $\alpha\beta 8$ utilizes the transmembrane matrix metalloproteinase-1 (MT1-MMP or MMP14). MT1-MMP and $\alpha\beta 8$ form a cell surface complex, and it is believed that $\alpha\beta 8$ concentrates latent TGFB, which is then activated by MT1-MMP.

Mechanism of activation includes $\alpha\beta 8$ binding to the RGD sequence on LAP, and MT1-MMP cleaving LAP, releasing TGFB (Mu *et al.* 2002).

$\alpha\beta 6$ can activate TGFB in the presence of thrombin. Thrombin creates intracellular force by binding to epithelial protease activated receptor 1 (PAR1), and initiating downstream effector Rho kinase induced actin polymerization. Intracellular forces generated by thrombin are exerted on the actin filaments tethered by ITGB6, and LAP bound to $\alpha\beta 6$ undergoes a subsequent conformational change thereby activating TGFB (Jenkins 2008).

$\alpha\beta 3$ may activate TGFB in the presence of MMP2 and MMP9. $\alpha\beta 3$ has docking points for MMP2 and MMP9, and it is hypothesized that integrins serve as a common docking port for latent TGFB and MMPs. MMP2 and MMP9 along with LAP interact with $\alpha\beta 3$, and MMPs liberate TGFB from LAP by proteolytically cleaving LAP (Wipff & Hinz 2008).

Transforming Growth Factor Beta SMAD Signaling

Active TGFBs signal through two known receptors, TGFB receptor types I and II (TGFBR1 and TGFBR2), which are transmembrane serine/threonine kinase receptors and present on the cell surface as homodimers (Lin *et al.* 1992, Franzen *et al.* 1993). TGFB1 and 3 bind to TGFBR2 initiating recruitment of TGFBR1 and subsequent formation of a heteromeric complex. TGFB sequential binding to TGFBR2 and TGFBR1 causes serine and threonine transphosphorylation of TGFBR1 by TGFBR2 (Wrana *et al.* 1994, Chen & Weinberg 1995). TGFBR1 then phosphorylates serine residues of downstream effector

SMAD proteins 2 and 3 (Macias-Silva *et al.* 1996, Zhang *et al.* 1996). Phosphorylation dependent conformational changes in SMAD2/3 enable heteromerization with SMAD4, and the resultant SMAD complex translocates into the nucleus (Nakao *et al.* 1997).

Within the nucleus, the combination of SMADs and cofactors within the transcriptional complex regulate transcriptional activity of target genes including but not limited to those encoding for ECM proteins (Neubauer *et al.* 1999, Okamoto *et al.* 2002, Xie *et al.* 2003, Bakin *et al.* 2004, Cho *et al.* 2006, Sinpitaksakul *et al.* 2008) and integrins (Zambruno *et al.* 1995, Kagami *et al.* 1996, Lai *et al.* 2000).

SMADs are direct substrates of the TGF β family receptor kinases, and in the basal state, SMADs exist as homo-oligomers within the cytoplasm. SMADs consist of an MH1 domain, a linker domain, and an MH2 domain. The MH1 domain contains the DNA binding domain and interacts with transcription factors. The MH2 domain interacts with receptors and transcriptional factors and is the site for SMAD oligomerization. There are 3 classes of SMADs: receptor activated SMADs (R-SMADs), common mediator SMADs (Co-SMADs), and inhibitory SMADs (I-SMADs). R-SMADs include SMAD1, 2, 3, 5, and 8. SMAD 4 is a Co-SMAD, and I-SMADs include SMAD6 and 7. Only R-SMADs contain a C-terminal SSXS motif in the MH2 domain that is phosphorylated by receptors. In the basal state, R-SMADs are localized in the cytoplasm, and I-SMADs are within the nucleus. R-SMADs associate with type I receptors and upon ligand activation, R-SMADs are serine phosphorylated (Shi & Massague 2003, Brown *et al.* 2007). Kinetics of TGF β phosphorylating R-SMADs is slow (t 0.5 ~ 5 min), and results in dissociation of SMADs from the receptors. TGF β

mostly induces SMAD3 with SMAD4; or SMAD2, SMAD3, and SMAD 4 heterotrimers (Shi & Massague 2003, Brown *et al.* 2007).

TGFBRs remain active for 3-4 hours after ligand binding and continuous receptor activation maintains SMADs complexes in nucleus. Inhibition of SMAD accumulation in the nucleus can occur via phosphorylation of MAP-kinase sites in the linker region. Agonists that activate ERK MAP Kinases are EGF and HGF. In addition, I-SMADs such as SMAD7 and SMAD6 can inhibit receptor activated signaling of TGFB. High concentrations of SMAD6 and SMAD7 are responsible for inhibiting TGFB signaling. I-SMADs can bind to TGFB receptors, interfering with phosphorylation of R-SMADs to prevent intracellular signaling (Shi & Massague 2003, Brown *et al.* 2007). In certain physiological cases such as shear stress in vascular endothelial cells, I-SMADs can be up-regulated. This response may be mediated by its TGFB acting in an autocrine fashion.

Transforming Growth Factor Beta SMAD-Independent Signaling

TGFB can also signal through non-SMAD pathways and can activate ERK, JNK, and p38 MAPK pathways. The ability of TGFB to activate SMAD and non-SMAD factors such as TAK1/MEKK1, Ras, RhoA, and PP2A have biological roles in addition to transcription (Rahimi & Leof 2007). TGFB activation of RhoA and p38MAPK, along with SMAD signaling may induce stress-fiber formation and epithelial to mesenchymal transdifferentiation, and TGFB signaling through PP2A has roles in cell cycle progression inhibiting proliferation (Rahimi & Leof 2007).

Transforming Growth Factor Beta in Reproduction

TGFβs have cell-type specific actions and contribute to normal growth and development in many different types of cells. Their broad range of functions includes regulating cell proliferation, stimulating ECM protein production, suppressing ECM protein degradation, and altering the expression of integrins. Furthermore, TGFβs are functional during diverse biological processes such as embryogenesis, tissue repair, immunosuppression, and in altered states such as oncogenesis and fibrosis. Specific roles for TGFβ during porcine implantation have yet to be fully defined, but TGFβ has been identified in the reproductive tissues in many species.

In mice, TGFβs are found in the inner cellular mass and trophoctoderm in preimplanting conceptuses. Preimplantation embryo development, haematopoiesis, and yolk sac vasculogenesis are defective in a percentage of TGFβ1 deficient conceptuses suggesting roles for TGFβ during these events (Ingman & Robertson 2002). In addition, TGFβ1^{-/-} mice exhibit defects in embryonic vascular development and a lethal autoimmune syndrome driven by activated CD4⁺ T cells (Shull *et al.* 1992). TGFβ2^{-/-} mice have defects in the skeletal, cardiac, and genitourinary systems (Sanford *et al.* 1997). TGFβ3^{-/-} mice have cleft palate and defects in lung development (Kaartinen *et al.* 1995, Proetzel *et al.* 1995).

In humans, VEGF production in first trimester trophoblast cells is up-regulated by TGFβ suggesting that TGFβ may have a role in promoting vascular development during placentation (Ingman & Robertson 2002). In addition to promoting vascular development, TGFβs are implicated in many other events important for pregnancy.

TGFB participates in creating an immunotolerant uterine environment by inhibiting T helper type 1 responses, regulating NK cell behavior, and down regulating IFN-gamma induced activation (Jones *et al.* 2006). In early embryo development, TGFB has been attributed with roles in gastrulation and organogenesis. TGFB may also have roles in preparing the endometrium for implantation by regulating hormone production, facilitating production of ECM proteins, inducing apoptosis of endometrial epithelial cells, and regulating conceptus invasion (Jones *et al.* 2006).

TGFB is also expressed in the uterine LE and is proposed to enhance conceptus attachment by up regulating production of oFN (Ingman & Robertson 2002). TGFB is also proposed to regulate conceptus invasion by down regulating MMPs and plasmin, while up regulating TIMPs and plasminogen activator inhibitor (**Foxcroft** *et al.* 2000). Plasmin and MMPs have been shown to aid in degradation of the ECM during conceptus invasion, and down regulation of these components protects the ECM. Trophoblast invasion can be inhibited *in vitro* with treatment of exogenous TGFB, and studies involving human explant placental cell cultures displayed increased extravillous trophoblast cell invasion in the presence of neutralizing TGFB antibodies, while addition of exogenous TGFB decreased the invasive capacity (Graham & Lala 1991, Karmakar & Das 2002).

Integrin Heterodimers

Integrins are cation dependent membrane bound heterodimers that can mediate signal transduction through inside-out and outside-in signaling. Affinity for integrin

ligands is dependent on their structure and inside-out signaling can regulate the conformation of integrin extracellular domains. Cytoskeletal adaptor proteins such as talin play a role in regulating ligand affinity. Binding of talin to the beta cytoplasmic tail of the integrin heterodimer causes a conformational change in the extracellular regions of the alpha and beta subunits and subsequently increases the affinity for ligands (Wegener & Campbell 2008). Integrins can also be activated intracellularly by signals from other membrane receptors such as G-protein-coupled receptors that lead to phosphorylation of the beta subunit (Takada *et al.* 2007).

Outside-in signaling occurs when extracellular ligands bind integrins, which induces a conformational change causing separation of the alpha and beta transmembrane domains leading to interactions of the cytoplasmic tails with intracellular signaling molecules (Takada *et al.* 2007). Receptor-ligand interactions between integrins and ECM proteins can activate signaling pathways, which ultimately result in changes in cytoskeletal organization, gene expression, proliferation, differentiation, and apoptosis (Giancotti & Ruoslahti 1999). In addition, the contacts made between integrins and the ECM is important in cell adhesion, migration, and invasion (Gilcrease 2007). Each heterodimer can serve as a receptor for more than one type of ECM protein and different heterodimers can function as receptors for the same ECM proteins. For example, fibronectin is a ligand for the integrin heterodimers $\alpha 4\beta 1$, $\alpha 5\beta 1$, $\alpha 8\beta 1$, $\alpha \nu\beta 1$, $\alpha \nu\beta 3$, $\alpha \nu\beta 5$, $\alpha \nu\beta 6$, and $\alpha \nu\beta 8$, while vitronectin is a ligand for $\alpha \nu\beta 3$ and $\alpha \nu\beta 5$ (Takada *et al.* 2007). Specificity and redundancy may be crucial for cellular processes such as cell adhesion

and migration, and binding of different ligands to the same type of integrin heterodimer can transmit different cellular signals depending on the cell type and context.

Integrins provide links to the extracellular and intracellular environments. Upon binding of ECM proteins, integrins aggregate to enhance signal transduction and form cytoplasmic focal adhesions (Wozniak *et al.* 2004). There are 4 different classifications of focal adhesions that are based on subcellular localization, size, and composition; these include focal complexes, focal adhesions, fibrillar adhesions, and 3-D matrix adhesions (Berrier & Yamada 2007). Focal complexes are small focal adhesions that are located at the periphery of migrating cells. Focal complexes typically precede focal adhesions, which are associated with stress fibers in cells on 2-D rigid matrices. Fibrillar adhesions are elongated focal adhesions that contain $\alpha 5\beta 1$, fibronectin, and tensin. Finally, 3-D matrix adhesions occur when fibroblasts adhere to 3-D collagen gels.

Formation of focal adhesions stabilizes cell attachments to the ECM by connecting ECM proteins to the actin cytoskeleton via actin filaments that can further assemble into contractile stress fibers (Bershadsky *et al.* 2006a). Cells subjected to a rigid matrix will reinforce their integrin-matrix adhesions by creating intracellular forces matching that of the force within the matrix, resulting in an isometric state. Initially, focal complexes are assembled that associate with the actin filaments (Galbraith *et al.* 2007). Focal complexes further develop into focal adhesions associated with contractile stress fibers (Bershadsky *et al.* 2006a, Bershadsky *et al.* 2006b).

In addition to stabilizing cell attachments, integrins also transform mechanical forces such as matrix rigidity and cellular contraction into intracellular signals that

activate many signaling pathways. Focal adhesions serve as signaling centers from which numerous intracellular pathways can regulate cellular behavior such as growth, migration, proliferation, gene expression, tissue repair, and invasion (Larson *et al.* 2006). Focal adhesions formed in cells culture *in vitro* cell cultures respond to intracellular forces generated by actin-myosin interaction or applied externally to the cell through the extracellular matrix by altering the size of focal adhesion and their composition (Galbraith *et al.* 2002). In addition, studies with stem cells demonstrate that matrix rigidity sensed via integrins directs stem cell lineage (Engler *et al.* 2006).

At least 156 components, including classes of receptor kinases, cytoskeletal proteins, and signaling molecules that involve up to 690 interactions, have been identified at integrin adhesion sites characterized by focal adhesions (Zaidel-Bar *et al.* 2007). The integrin adhesome consists of receptor kinases such as focal adhesion kinase (FAK), Src, integrin-linked kinase (ILK), and protein kinase B (PKB); cytoskeletal/linker molecules such as talin, vinculin, alpha actinin, and filamin; and signaling molecules such as Rho, Rac, and Ras. Assembly of these components at a focal adhesion may depend upon mechanical forces applied to integrin-ECM connections.

FAK, a tyrosine kinase, localizes at focal adhesions directly to the beta cytoplasmic integrin tail or through other components of the focal adhesion such as talin and paxillin. FAK is autophosphorylated at the tyrosine 397 site. Phosphorylation of FAK initiates a cascade of phosphorylation events and promotes interactions with Src-family kinases, which further phosphorylate FAK and other focal adhesion components including paxillin, tensin, and p130Cas. There are at least 6 tyrosines and a serine that

can be phosphorylated on FAK resulting in numerous intracellular cascades regulating cellular behaviors including cell adhesion, cell protrusion and migration, cytoskeletal reorganization, gene regulation (Hanks & Polte 1997).

Integrin and Growth Factor Receptor Cross-Talk

Integrins are capable of receptor cross talk with growth factor receptors.

Cytoplasmic links between integrins and growth factor receptors such as EGFR, PDGFR, and TGFBR are made via ILK. ILK binds directly to the ITGB1 and ITGB3 cytoplasmic tails, and ILK assembles with PINCH-1 and NCK2, bridging a link with growth factor receptors and integrins while providing a link to the cytoskeleton (Hehlgans *et al.* 2007). Links between integrins and growth factor receptors optimizes activation of growth factor receptors. For example, many growth factor receptors are optimally activated by their ligands only under appropriate cell attachments mediated by integrins (Giancotti & Ruoslahti 1999). Integrin aggregation with growth factor receptors has been demonstrated by immunoprecipitating $\alpha\beta3$ complexed with PDGFR, VEGFR, and, of particular interest, TGFBR2 (Scaffidi *et al.* 2004, Galliher & Schiemann 2006).

TGFB and integrin cross-talk has been identified during many biological processes, including fibroblast differentiation into myofibroblasts during wound healing. During wound healing, myofibroblasts are key players in the reconstructing of connective tissue, and fibroblast differentiation into myofibroblasts is largely controlled by mechanical and chemical environments (Hinz 2007). Changes in composition of the ECM causes increased stress within the tissue. Integrins are one of the multiple ways that

a cell can sense increasing stress, and this is mediated via their connections to the outside environment and actin cytoskeleton via focal adhesions. Increased ECM and incorporation of α -smooth muscle actin (ACTA2) into focal adhesion-linked stress fibers augments the contractile activity of fibroblast cells. TGFB aids in promoting myofibroblast development by inducing expression of ECM proteins and ACTA2. TGFB also activates proteins involved in focal adhesion such as FAK and protein kinase B via SMAD 3 and p38MAPK, respectively (Horowitz *et al.* 2007). Further, TGFB may stimulate focal adhesion assembly and size by activation of intracellular Rho and Rho kinase that increase myosin contraction thereby increasing endogenous force and size of focal adhesions (Wozniak *et al.* 2004).

Integrins and growth factor receptor cross-talk may regulate other biological events such as cell growth and proliferation via the Ras and ERK intracellular signals and activation of transcriptional factors such as SRF and cMyc (Gilcrease 2007). In addition, TGFB induces expression of ITGB3 and ITGB5 in adhered cell cultures via SMAD independent pathways involving c-Src kinase and MAPK phosphorylation and SMAD dependent pathways, respectively (Pechkovsky *et al.* 2008).

Transforming Growth Factor Beta and Integrins during Porcine Implantation

Relevant to our study, TGFBs and their receptors are present at the conceptus-maternal interface in many species including mice, pigs, and humans. Expression of TGFBs and TGFBRs on days 10, 11, 12, 13, and 14 of pregnancy in the porcine peri-implantation conceptus and endometrium were previously reported (Gupta *et al.* 1996,

Gupta *et al.* 1998a, Gupta *et al.* 1998b). Uterine flushes on days 10 and 11 contained mostly latent forms of TGF β s, while days 12 to 14 contained primarily active TGF β s. The conversion of latent TGF β to its active form appears to occur around days 12 to 14 of porcine pregnancy, which is concomitant with conceptus elongation and the beginning to attachment, suggesting that TGF β s may play a role in these processes. Once activated, TGF β s are able to bind to their receptors found on the cell surfaces of both trophoderm and uterine LE. TGF β Rs on apical surfaces of conceptus and uterine tissues may bind active TGF β s and increase expression of integrins and several ECM proteins including matrix molecules fibronectin, oncofetal fibronectin, and osteopontin. Studies that used a porcine trophoderm cell line (pTr2) provided evidence that TGF β s increase expression of *FN*; LAP and antibodies directed toward TGF β were able to inhibit increases in *FN*, further supporting hypothesized roles of TGF β during porcine implantation (Jaeger *et al.* 2005).

While integrins appear to be important in mammalian implantation, some of their specific functions during implantation are unclear. Several integrin heterodimers such as $\alpha\beta$ 1, $\alpha\beta$ 3, and $\alpha\beta$ 5 have been implicated in mediating implantation in multiple species. In mice, inhibition of $\alpha\beta$ 3 via intrauterine injection of neutralizing monoclonal antibodies towards $\alpha\beta$ 3 resulted in the reduction of implantation (Illera *et al.* 2000, Illera *et al.* 2003). Further, deletion of ITGA4, ITGA5, ITGAV, and ITGB1 in mice caused failure of chorioallantois fusion, placental defects, and embryonic deaths, respectively (Fassler & Meyer 1995, Bader *et al.* 1998, Chen & Sheppard 2007).

In pigs, ITA4, ITGA5, ITGAV, ITGB1, ITGB3, and ITGB5 have been identified

at the conceptus-maternal interface (Bowen *et al.* 1996, Burghardt *et al.* 2002). Expression of ITGAV and ITGB1 subunits on the trophoctoderm have been confirmed via immunoprecipitation of biotinylated pTr2 cells (Jaeger *et al.* 2005). Moreover, preliminary evidence suggests that ITGB6 and ITGB8 are present on days 9, 12, and 15 of porcine endometrium based on RT-PCR (Genbank accession # AY496298, AY496299). Integrin subunits identified at the porcine conceptus-maternal interface occur as heterodimers that serve as receptors for ECM proteins including VN, FN, oFN, and SPP1, which are also present at the maternal-conceptus interface during the peri-implantation period in pigs.

LAP, a known ligand for integrins, has not been directly identified at the porcine conceptus-maternal interface. However, *in situ* hybridization localization of TGFB at the conceptus-maternal interface suggests that LAP is present because the same gene produces TGFB and LAP, and TGFB is mostly secreted as the LLC. In addition, LAP was detected in porcine uterine flushes on day 12 of pregnancy via western blots (Jaeger, unpublished). If both LAP and ITGAV-containing heterodimers co-exist at the conceptus-maternal interface, they are potentially available to interact with each other. Interactions between TGFB, LAP, and integrins may be crucial during implantation.

Adhesion assays utilizing pTr2 cells demonstrated that LAP-integrin binding is mediated via the RGD sequence in LAP. In addition, culture dishes coated with LAP supported pTr2 adhesion, whereas, LAP in which the RGD sequence is mutated to RGE (LAP-RGE) failed to support adhesion. Similarly, polystyrene beads coated with LAP or FN induced focal adhesions containing α -actinin and talin when deposited on the apical

surfaces of trophectoderm and uterine LE cells *in vitro*. In support of this data, aggregation of α -actinin and talin were absent in the presence of control poly-l-lysine coated beads (Jaeger *et al.* 2005). To date, there are limited reports of data supporting focal adhesion formation *in vivo*; however, aggregation of integrins in response to mechanosensation and mechanotransduction at the ovine conceptus-maternal interface was recently reported (Burghardt *et al.* 2009).

Our hypothesis is that LAP binds to and activates ITGAV-containing heterodimers inducing integrin aggregation at the conceptus maternal interface and that these interactions are a functional component of implantation, particularly attachment of the conceptus to the uterine LE. In addition, LAP-integrin interactions may play an important role in activating TGF β in conceptus and uterine tissues. We also hypothesize that TGF β acting via TGFBRs have critical roles during preimplantation and implantation, and such roles may include promoting conceptus development and survival along with conceptus adhesion. We have formulated two objectives to be accomplished in the course of this project.

Objectives

Objective 1

Determine the temporal and spatial distribution of LAP, TGF β , and integrins during porcine peri-implantation. Identify TGF β induced TGFBR activation, and determine which integrins expressed at the porcine conceptus-maternal interface specifically bind LAP.

Objective 2

Determine effects of LAP and TGFB on conceptus development, conceptus survival, and implantation.

CHAPTER III

**PRESENCE OF TRANSFORMING GROWTH FACTOR BETA, LATENCY
ASSOCIATED PEPTIDE, AND INTEGRINS IMPLIES LIGAND-RECEPTOR
INTERACTIONS DURING PORCINE IMPLANTATION**

Introduction

Implantation is a critical period during pregnancy that begins when the conceptus comes in close proximity to the uterine luminal epithelium (LE) to initiate the development of the conceptus-maternal interface (King *et al.* 1982). Pigs are unique in that they have epitheliochorial implantation and their conceptuses undergo an exaggerated morphological transformation in which they elongate from 5 mm to 150 mm between days 10-12 of pregnancy (Geisert *et al.* 1982b). By day 13 of pregnancy, porcine filamentous conceptuses begin to make initial attachments to the uterine LE, and by day 24, implantation is essentially complete resulting in the interdigitation of LE and the trophoctoderm (Keys & King 1990). Autocrine, paracrine, and juxtacrine signaling during the peri-implantation period are evident by the presence of many signaling molecules at the conceptus-maternal interface. Alterations in these signaling processes can compromise implantation and contribute to reproductive failure. In pigs, a significant proportion of pregnancy loss occurs during the peri-implantation period (Flint *et al.* 1982, Jainudeen & Hafez 1987). Signaling molecules such as transforming growth factor betas (TGFBs) and integrins are among the various signaling molecules that have been reported

to be present in uterine and placental tissues prior to and during implantation in pigs (Bowen *et al.* 1996, Gupta *et al.* 1996, Gupta *et al.* 1998b, Burghardt *et al.* 2002).

The TGFB isoforms (TGFB1, 2 and 3) signal through TGFB receptor types I and II (TGFBR1 and TGFBR2). TGFBs bind TGFBR2 and recruit TGFBR1 to the receptor ligand complex, ultimately causing serine and threonine transphosphorylation of TGFBR1 by TGFBR2 (Lin *et al.* 1992, Franzen *et al.* 1993, Wrana *et al.* 1994, Chen & Weinberg 1995). TGFBR1 then phosphorylates the downstream effector SMAD (homologous of mothers against decapentaplegic in *Drosophila* and *sma-2,-3,* and *-4* in *C. elegans*) proteins 2 and 3 at serine residues (Macias-Silva *et al.* 1996, Zhang *et al.* 1996). Phosphorylation-dependent conformational changes in SMAD2 and 3 enable heteromerization with SMAD4; the resultant SMAD complex then translocates into the nucleus (Nakao *et al.* 1997) where the combination of SMADs and cofactors within the transcriptional complex regulate transcriptional activity of target genes (Macias-Silva *et al.* 1996, Nakao *et al.* 1997).

The cell secretes latent TGFB as a homodimer containing a non-covalent association with its prepropeptide homodimer, latency associated peptide (LAP), in the form of the small latency complex (SLC) (Lawrence *et al.* 1984, Gentry *et al.* 1988). The SLC further associates with latent TGFB binding proteins (LTBPs) to form a large latent complex (LLC) which is the predominant secreted form (Miyazono *et al.* 1988, Koli *et al.* 2001). LTBPs serve to link the SLC to ECM proteins such as fibronectin and fibrillin-1 via transglutaminase (Taipale *et al.* 1992, Nunes *et al.* 1997, Isogai *et al.* 2003). Conformational changes in the latent complex or dissociation of TGFBs from LAP

activate TGF β and allow it to become available for receptor interactions. This activation occurs through multiple extracellular mechanisms which may involve proteases (Lyons *et al.* 1988, Rifkin *et al.* 1999, Annes *et al.* 2003, Jenkins 2008), thrombospondin-1 (Crawford *et al.* 1998, Murphy-Ullrich & Poczatek 2000), and integrins (Munger *et al.* 1999, Annes *et al.* 2002, Mu *et al.* 2002, Annes *et al.* 2003, Sheppard 2005). Like many ECM proteins, LAP1 and 3 monomers, corresponding to respective TGF β 1 and 3 isoforms, contains an arginine-glycine-aspartate (RGD) site. Previous studies have demonstrated that LAP binds to integrin heterodimers via this RGD sequence (Munger *et al.* 1998, Munger *et al.* 1999, Mu *et al.* 2002, Ludbrook *et al.* 2003).

Integrins are a family of transmembrane glycoprotein receptors that form non-covalent alpha and beta heterodimers. The 18 alpha and 8 beta subunits combine to form 24 receptors that can sense external and internal cellular environments and function as receptors for specific ECM proteins (Hynes 1992) and cell surface proteins. Several of the integrin receptors recognize and interact with the Arg-Gly-Asp (RGD) sequence on various ECM protein ligands (Ruoslahti 1996). LAP binds to integrins $\alpha\beta$ 1, $\alpha\beta$ 3, $\alpha\beta$ 5, $\alpha\beta$ 6, $\alpha\beta$ 8 and α 8 β 1, most of which are implicated in TGF β activation (Munger *et al.* 1998, Munger *et al.* 1999, Annes *et al.* 2002, Lu *et al.* 2002, Mu *et al.* 2002, Asano *et al.* 2005b, Asano *et al.* 2005a). Recent evidence provides insight that ITGAV (alpha V)-containing integrin heterodimers mediate activation of TGF β and that this is of physiologic importance (Huang *et al.* 1996, Bader *et al.* 1998, Zhu *et al.* 2002, Yang *et al.* 2007).

TGFβs, TGFβRs, and integrin (ITG) subunits ITGAV, ITGB1 (beta 1), ITGB3 (beta 3), and ITGB5 (beta 5) were previously reported at the porcine conceptus-maternal interface (Bowen *et al.* 1996, Gupta *et al.* 1996, Gupta *et al.* 1998b, Burghardt *et al.* 2002). We hypothesize that active TGFβs and TGFβRs, along with LAP and integrins, functionally interact at the conceptus-maternal interface to mediate events essential to conceptus development and attachment in pigs. Although the presence of TGFβ and its receptors were separately reported during porcine implantation, evidence for their functional interactions was not determined. In addition, the probability that LAP serves as an integrin ligand that subsequently affects receptor-ligand actions during porcine implantation has not been investigated. The objective of this investigation was to investigate potential mechanistic roles for active and latent TGFβ and integrins during porcine implantation. Therefore, the present studies were designed to: 1) co-localize and identify the temporal and spatial distributions of TGFβ, LAP and integrins in porcine implantation sites; 2) demonstrate that LAP directly binds to integrins on the surface of porcine conceptuses; and 3) demonstrate activation of TGFβRs through the presence of phosphorylated downstream effector SMAD2/3 in porcine conceptus and endometrium.

Materials and Methods

Animals

Experimental procedures were approved by Texas A&M University's Animal Care and Use Committee (AUP # 2007-154). Crossbred gilts were checked daily for estrus, and bred approximately 12 and 24 hours after their second detected estrus.

Ovariohysterectomies were performed on days 10, 12, 16, 20, and 24 of pregnancy (n=3 gilts/day). Prior to surgery, anesthesia was induced with an intramuscular injection of tiletamine hydrochloride and zolazepam hydrochloride (6.6 mg/kg, Telazol, Fort Dodge Animal Health, Fort Dodge Iowa) and was maintained with isoflurane (2-3% in oxygen). After removal of the uterus, day 10 and 12 conceptuses were flushed from each horn with 10 mM Tris HCl pH 7.0 (25 ml/horn), and the uterine horns were opened along the anti-mesometrial border. Uterine and conceptus tissues were preserved by embedding in Tissue-Tek Optimal Cutting Temperature Compound (OCT, Miles, Inc, Onenta, NY) and freezing in liquid nitrogen. Remaining uterine and conceptus tissues were preserved in phosphate buffered 4% paraformaldehyde (PAF) and embedded in paraffin. After removal of the uterus on days 16, 20, and 24 of pregnancy, uterine horns were opened along the anti-mesometrial border and sections from attachment sites containing both uterine and conceptus tissues and non-attachment sites absent of conceptus tissues were dissected from the uterine horns. Attachment and non-attachment uterine sections were preserved using both methods stated above.

Immunofluorescence Staining

Antibodies used for immunofluorescence staining included: goat anti-LAP (#AB-246-NA) and chicken anti-TGFB (#AF-101-NA) from R&D Systems (Minneapolis, MN); rabbit anti-ITGAV (#AB1930), ITGB1 (#AB1952), ITGB3 (#AB1932) and ITGB5 (#AB1926) integrin subunits from Chemicon (Temecula, CA); and rabbit anti-ITGB6 (beta 6, #SC15329) and ITGB8 (beta 8, #SC25714) integrin subunits from Santa

Cruz (Santa Cruz, CA). Secondary antibodies used for immunofluorescence staining included FITC-conjugated rabbit anti-chicken, Texas Red-conjugated mouse anti-goat from Pierce Biotechnology Inc. (Rockford, IL) and FITC-conjugated donkey anti-rabbit from Southern Biotech (Birmingham, Alabama).

Discriminating between latent and active TGFB depends on specific antibodies, fixation methods, and tissue preparation (Barcellos-Hoff *et al.* 1994, Barcellos-Hoff *et al.* 1995). The double labeling protocol for latent TGFB (LAP) and active TGFB (TGFB, kindly provided by Barcellos-Hoff), was performed to ensure maximal discrimination of the antibodies for latent and active TGFB. Anti-LAP detected dimeric and monomeric LAP from TGFB isoform 1 in western blotting. In addition, anti-LAP was specific for latent TGFB1 immunostaining (Barcellos-Hoff *et al.* 1994, Ehrhardt *et al.* 1997). Anti-TGFB recognizes active TGFB1 not associated with LAP (Ehrhardt *et al.* 1997).

Frozen uterine and conceptus tissues were sectioned (8 μM), mounted on Superfrost Plus glass slides (Anapath, Cheyenne, WY), and immunofluorescent co-localization of LAP and TGFB were performed as described by (Ewan *et al.* 2002) with minor modifications. Sections were fixed with 2% PAF, washed with 0.1 M glycine in .02 M phosphate buffered saline (PBS), blocked with 0.5% casein in PBS, and incubated overnight at 4°C with TGFB and LAP primary antibodies at concentrations of 1.25 $\mu\text{g}/\text{ml}$ and 15 $\mu\text{g}/\text{ml}$, respectively. Controls included separate sections incubated with TGFB and irrelevant goat IgG; LAP and irrelevant chicken IgG; and with chicken IgG and goat IgG. Sections were incubated with appropriate FITC- or Texas Red- conjugated secondary antibodies for 1 h at room temperature at a concentration of 3.75 $\mu\text{g}/\text{ml}$ and 2.5

$\mu\text{g/ml}$, respectively. Slides were overlaid with Prolong antifade mounting reagent containing the nuclear counterstain DAPI (Invitrogen, Molecular Probes, Eugene, OR) and then coverslipped.

Integrins were localized with LAP in frozen sections by immunofluorescence staining. Sections were fixed and washed as described above then blocked in 10% normal goat and normal donkey sera. Sections were incubated overnight at 4°C with anti-LAP IgG (15 $\mu\text{g/ml}$) along with each integrin subunit ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, or ITGB8 primary antibody (5 $\mu\text{g/ml}$). Controls included separate sections incubated with each anti-integrin subunit IgG and irrelevant goat IgG; anti-LAP IgG with irrelevant rabbit IgG; and rabbit IgG with goat IgG. Sections were incubated with appropriate FITC- or Texas Red- conjugated secondary antibodies and coverslipped as described above.

Immunohistochemistry

Rabbit anti-phosphorylated SMAD2/3 (p-SMAD2/3) from Santa Cruz was used for immunohistochemical staining. Uterine and conceptus paraffin embedded tissues were sectioned (5 μM) and mounted on Superfrost plus slides. Tissues were then deparaffinized with xylene and rehydrated through a graded series of alcohol. Endogenous peroxidase was blocked with 0.3% H_2O_2 in dH_2O . Sections were subjected to antigen retrieval with citrate buffer (pH 6.2) at 80°C for 45 min. Sections were blocked with 10% normal goat serum at 25°C for 20 min, and incubated overnight at 4°C with primary antibody p-SMAD 2/3 (0.4 $\mu\text{g/ml}$) or control Rabbit IgG (0.4 $\mu\text{g/ml}$) in 2%

bovine serum albumin (BSA) in PBS (PBSA). Sections were rinsed with PBSA and PBSA containing 1% Triton and incubated with biotinylated-conjugated secondary antibody diluted in PBSA containing 5% normal goat serum for 1 h at 25°C. Sections were then rinsed as described above, incubated in Vectastain ABC (Vector Laboratories, Burlingame, CA) for 30 min at room temperature, and then rinsed again with PBS and 0.5 M Tris-HCl. Immunoreactions were detected with peroxidase solution (DAB/H₂O₂) and stopped by submerging in H₂O at 1.5 min after application of peroxidase solution. Sections were counterstained with Shandon Eosin-y aqueous (Thermo Scientific, Waltham, MA), rinsed with distilled water, dehydrated with the reverse series of graded alcohol, and covered slipped with Clarion Mounting Medium (Biomedica Corp, Foster City, CA).

Photomicrography

Representative immunofluorescence and immunohistochemistry images were collected and analyzed using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) interfaced with an Axioplan HR digital camera and Axiovision 4.6 software. Because of Axiovision's multidimensional acquisition with individual fluorophore channels, each fluorophore was captured as separate channels, and both single and double labeled sections were visualized. Images were converted to TIFF file format, and Adobe Photoshop CS2 (version 9.0, Adobe Systems Inc., San Jose, CA) was used to generate photographic composite images.

Biotinylation of apical proteins on cultured porcine trophectoderm cells

A porcine trophectoderm cell line (pTr2) was cultured as previously described (Ka *et al.* 2001) and kept in a 5% CO₂ humidified environment. Cells grown to confluence in 75 cm² culture flasks were biotinylated on their apical surfaces, as previously described (Jaeger *et al.* 2005). Cells were rinsed with Ca²⁺-Mg²⁺-free PBS following with a short incubation 5 mM EDTA for 60 seconds at room temperature and then rinsed with Ca²⁺-Mg²⁺-free PBS. The apical surfaces were biotinylated with 0.25 mg/ml of membrane impermeable biotin N-hydroxysulfosuccinimidobiotin, EZ-Link Sulfo-NHS-Biotin (Pierce Biotechnology, Inc) for 1 hr in the dark at room temperature. The reaction was stopped with 0.1 M glycine in PBS. Cells were then lysed with 50 mM OSGP lysis buffer (1 mM each Ca²⁺/Mg²⁺/Mn²⁺ and 3 mM PMSF), and the monolayer of cells was scraped and passed through a 25 gauge needle. Lysates were centrifuged (16,000 Xg) for 20 min at 4°C, and the supernatant was retained for subsequent affinity chromatography and immunoprecipitation.

Affinity Chromatography and Immunoprecipitations

Recombinant simian LAP, produced by Sf9 insect cells (Invitrogen, Carlsbad, CA) infected with a recombinant baculovirus (generously provided by Dr. J.S. Munger, New York University School of Medicine, New York, NY) and purified as previously described (Munger *et al.* 1998), was dialyzed using Slide-A-Lyzer dialysis cassette (Pierce Biotechnology, Inc) as per manufacturer's instructions. LAP (4.3 mg) was coupled to cyanogen bromide-activated sepharose (Sigma #c9142) as per manufacturer's

instructions. A small sample of LAP coupled with sepharose beads was subjected to 12% reducing and non-reducing SDS-PAGE to verify that sepharose beads were coupled with non-denatured LAP. In the presence of cations (Mg^{2+} and Mn^{2+}), surface biotinylated pTr2 cells were added to the column containing sepharose beads coupled with LAP and incubated for 2 h on ice. Columns were washed with TBS containing 3% OG, 1.5 mM Mg^{2+} , and 1.5 mM Mn^{2+} ; and TBS containing 1% OG, 1.5 mM Mg^{2+} , and 1.5 mM Mn^{2+} . Eight fractions (0.5 ml/fraction) were eluted with 1% OG in TBS and 10 mM EDTA. Eluate fractions were run on a 7.5% non-reducing SDS-PAGE, blotted to nitrocellulose, blocked with 5% BSA in TBS containing 0.03% Tween (TBST) for 30 min at room temperature, and incubated with ABC reagent for 30 min at room temperature to detect biotinylated proteins. Blots were visualized with chemiluminescent detection reagent, SuperSignal West Pico Substrate (Pierce Biotechnology, Inc.) and captured using a Fluorochem 8800 imager (AlphaInnotech; San Leandro, CA).

Eluate fractions containing biotinylated proteins were concentrated using Centriprep Centrifugal Filter Device (Millipore Corporation, Billerica, MA). Eluate fractions were immunoprecipitated with antibodies to integrin subunits (ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, or ITGB8) or normal rabbit serum control at a concentration of 2.5 μ g/ml and A-protein G agarose conjugated beads (Santa Cruz Biotechnology) as previously described (Jaeger *et al.* 2005). Briefly, eluates were pre-cleared with normal rabbit serum and A-protein G agarose and incubated with anti-integrin subunits or control IgG overnight with end over end rotation at 4°C. A-protein G agarose beads were washed with RIPA buffer (0.02M PBS containing 1% IGEPAL, 0.5% sodium

deoxycholate, and 0.1% SDS), were run on a 7.5% reducing SDS-PAGE, blotted, and visualized as described.

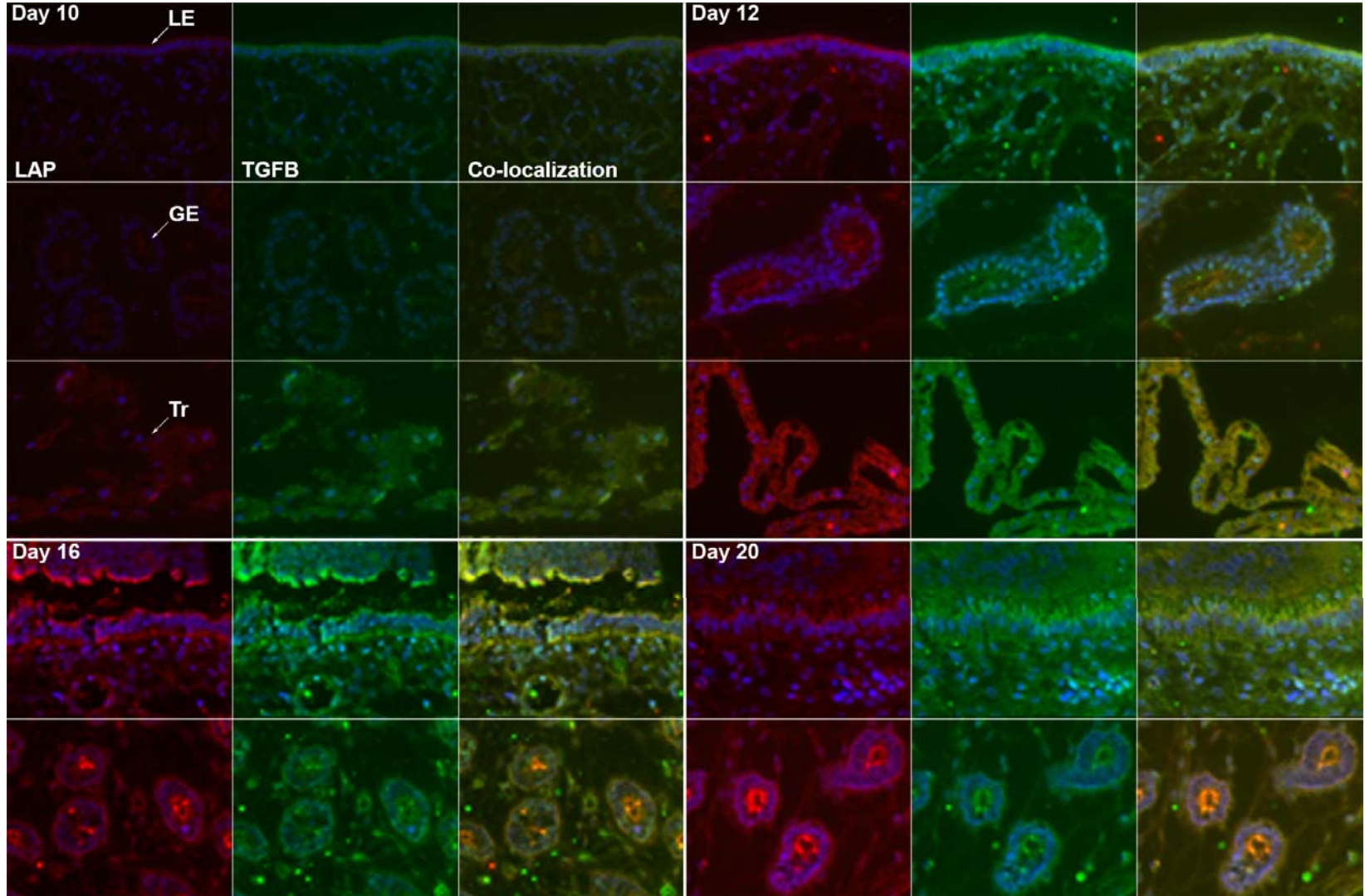
Results

When TGFB is associated with its prepropeptide LAP, it can not bind TGFB receptors and therefore is called latent TGFB. The antibody to LAP detects both LAP1 alone as well as latent TGFB1 (LAP). The TGFB antibody detects only TGFB1 that is not associated with LAP, which is termed active TGFB1 (TGFB). Co-localization ensures discrimination between latent and active TGFB (For detailed description see Materials and Methods).

Distribution of LAP and TGFB

On days 10 to 16 of pregnancy, LAP (Texas Red) and TGFB (FITC) were detected primarily on the surfaces of conceptus trophoctoderm, endometrial LE, and endometrial glandular epithelium (GE). Trophoctoderm, LE, and GE immunostaining gradually increased from days 10 through 16 and became more apical in distribution (**Figure 3.1**). By day 16, LAP and TGFB were also prominently expressed at the basal surface of endometrial epithelia. In addition, TGFB was detectable in endometrial stroma and blood vessels by day 10 and increased by day 16, whereas, LAP was first detected in these tissues by day 16 of pregnancy (**Figure 3.1**). Significant co-localization of LAP and TGFB, (appearing yellow in color in image overlays), was detected along all trophoctoderm and was greatest at apical surfaces of trophoctoderm and LE on day 16 of

Figure 3.1. Distribution of TGFB and LAP during Porcine Implantation. Immunofluorescence co-localization of TGFB with LAP in frozen cross-sections of day 10 and 12 pregnant pig endometrium and conceptuses, and day 16 and 20 conceptus attachment sites. TGFB immunoreactivity was detected using FITC-conjugated anti-chicken IgG (middle column of each quadrant; green fluorescence), whereas LAP immunoreactivity was detected using Texas Red-conjugated anti-goat IgG (left column of each quadrant; red fluorescence). Yellow immunofluorescence indicates co-localization of TGFB with LAP (right column of each quadrant). Compare the antibody staining with staining using chicken and goat IgG (Fig. 2; bottom row). LE, luminal epithelium; GE, glandular epithelium; Tr, trophoctoderm. Width of fields are 230 μm .

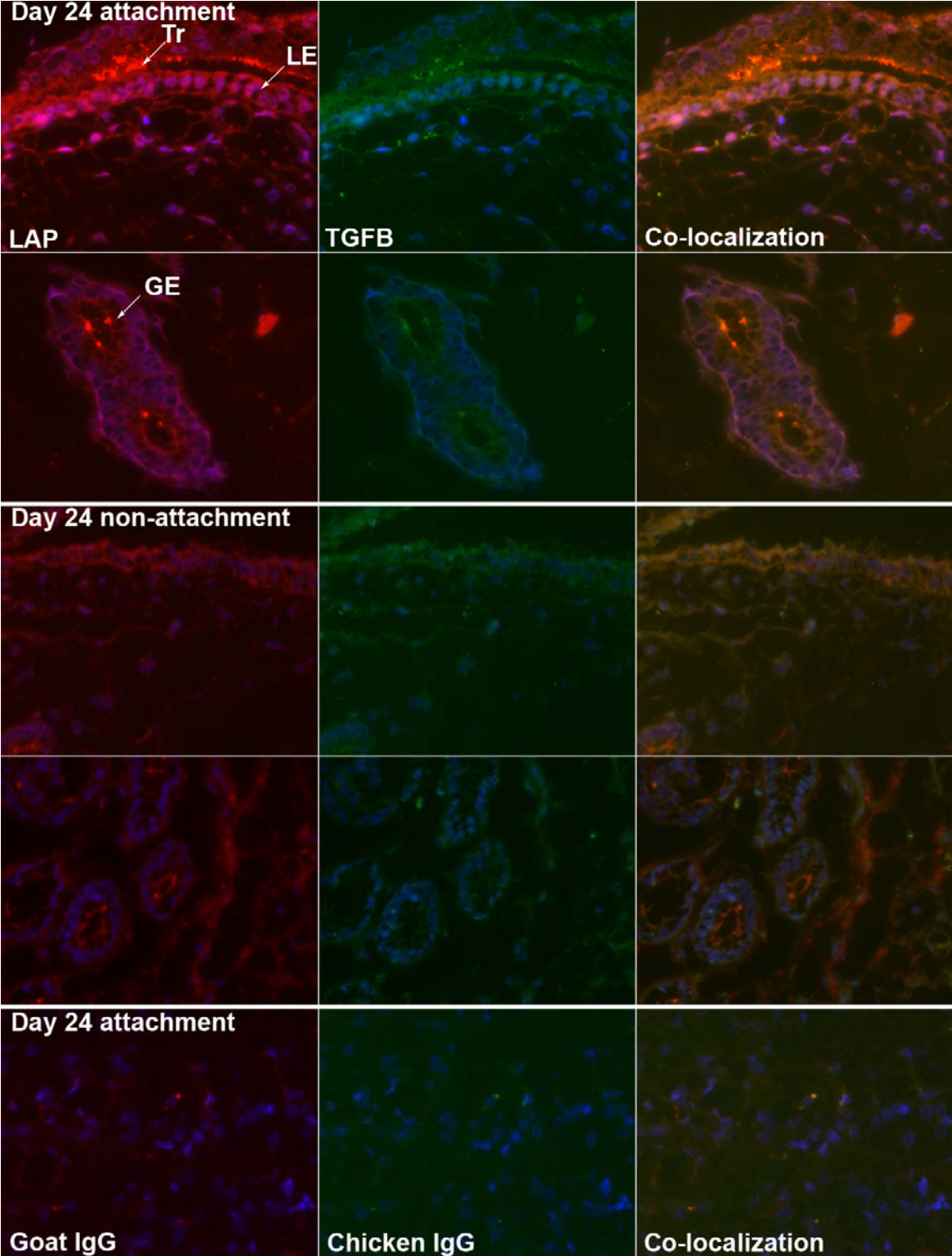


pregnancy (**Figure 3.1**). By day 20 of pregnancy, LAP immunostaining at the conceptus-maternal interface decreased; however, a striking punctate pattern for TGFB immunostaining was detected at the apical surfaces of LE and trophoctoderm (**Figure 3.1**). Further, intense immunostaining for LAP was observed at apical surfaces of GE, and this co-localized with lower levels of TGFB immunostaining (**Figure 3.1**). Uterine sections devoid of conceptus tissues (non-attachment sites) from day 16 and 20 of pregnancy displayed variable immunostaining for LAP and TGFB (data not shown).

During the later stages of implantation (day 24 of pregnancy), a dramatic shift in the presence of LAP and TGFB was observed. Large aggregates of LAP were prominent at the apical surfaces of LE and trophoctoderm at the conceptus-maternal interface. In contrast, TGFB immunostaining markedly decreased by day 24 of pregnancy (**Figure 3.2**). Similar to what was observed at day 20 attachment sites, intense LAP was detected at the apical surfaces of GE however, instead of uniform immunostaining, LAP was detected in aggregates (**Figure 3.2**). Low intensity immunostaining for TGFB was detected on the apical surfaces of GE, and low levels of LAP and TGFB co-localization were evident (**Figure 3.2**). The intensity of LAP and TGFB immunostaining was markedly lower on day 24 at non-attachment sites compared to attachment sites. At non-attachment sites on day 24, aggregates of LAP were no longer evident along the luminal surfaces; however, some aggregates were detected along the apical surfaces of GE (**Figure 3.2**).

Figure 3.2. Distribution of TGFB and LAP during Porcine Implantation.

Immunofluorescence co-localization of TGFB with LAP in day 24 frozen cross-sections of pig implantation (top row) and non implantation (middle row) sites. TGFB immunoreactivity was detected using FITC-conjugated anti-chicken IgG (middle column; green fluorescence), whereas LAP immunoreactivity was detected using Texas Red-conjugated anti-goat IgG (left column; red fluorescence). Yellow immunofluorescence indicates co-localization of TGFB with LAP (right column). Compare the antibody staining with staining using chicken and goat IgG (bottom row). LE, luminal epithelium; GE, glandular epithelium; Tr, trophoderm. Width of fields are 230 μm .



Detection of p-SMAD2/3 Implies Functional TGF β During Implantation

SMAD proteins 2 and 3 are phosphorylated in response to TGF β interacting with TGFBRs. Therefore, immunohistochemical analysis of p-SMAD2/3 was performed on conceptus and uterine tissues to evaluate functional interactions between TGF β s and TGFBRs during porcine implantation. On all days of the peri-implantation period (10 through 24) p-SMAD2/3 was detected in nearly all the nuclei of endometrial LE, GE, fibroblasts, and endothelia in both attachment and non-attachment sites (**Figure 3.3**). In conceptus tissues, p-SMAD2/3 was detected in the trophoctoderm of free floating spherical and filamentous conceptuses as well as at conceptus attachment sites. At all days, p-SMAD2/3 immunohistochemical staining appeared more intense in the trophoctoderm compared to in the uterine epithelium (**Figure 3.3**).

Integrin Subunits and LAP Are Available for Receptor Ligand Interaction During Implantation

Immunofluorescence analyses were used to co-localize integrin subunits ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, or ITGB8 (FITC) with LAP (Texas Red) on day 10 and 12 pre-implantation uterine tissues and on day 16, 20, and 24 conceptus attachment and non-attachment sites. On all days observed, immunostaining intensities for ITGB6 and ITGB8 were low and, in some cases, undetectable (**Figures 3.4-3.7**). On days 10 through 16, ITGB6 immunostaining was slightly higher in GE compared to LE; whereas, ITGB8 was slightly higher in LE as compared to GE. Both ITGB6 and ITGB8 were undetectable by day 20 of pregnancy (**Figures 3.4-3.7**).

Figure 3.3. Detection of p-SMAD2/3 Implies Functional TGFB during Implantation. Immunohistochemical localization of p-SMAD2/3 protein in paraffin-embedded uterine cross-sections from day 10 and 12 pregnant pig endometrium and conceptuses, and day 16 and 20 conceptus attachment sites, and day 24 conceptus attachment and non attachment sites. The *brown color* indicates positive immunostaining for p-SMAD2/3. All sections were counterstained with Shandon Eosin-y aqueous. Sections stained with nonimmune rabbit IgG serve as negative controls. LE, luminal epithelium; GE, glandular epithelium; Tr, trophoctoderm. Width of fields are 230 μ m.

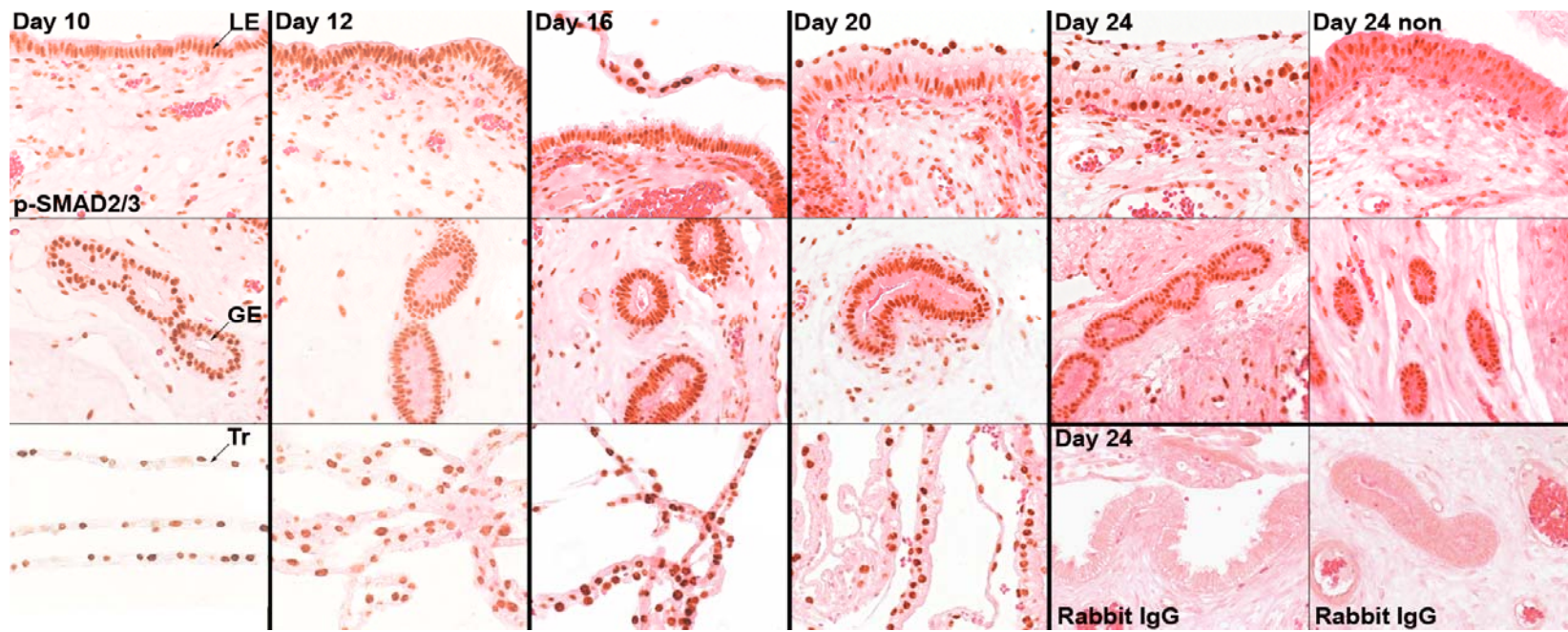
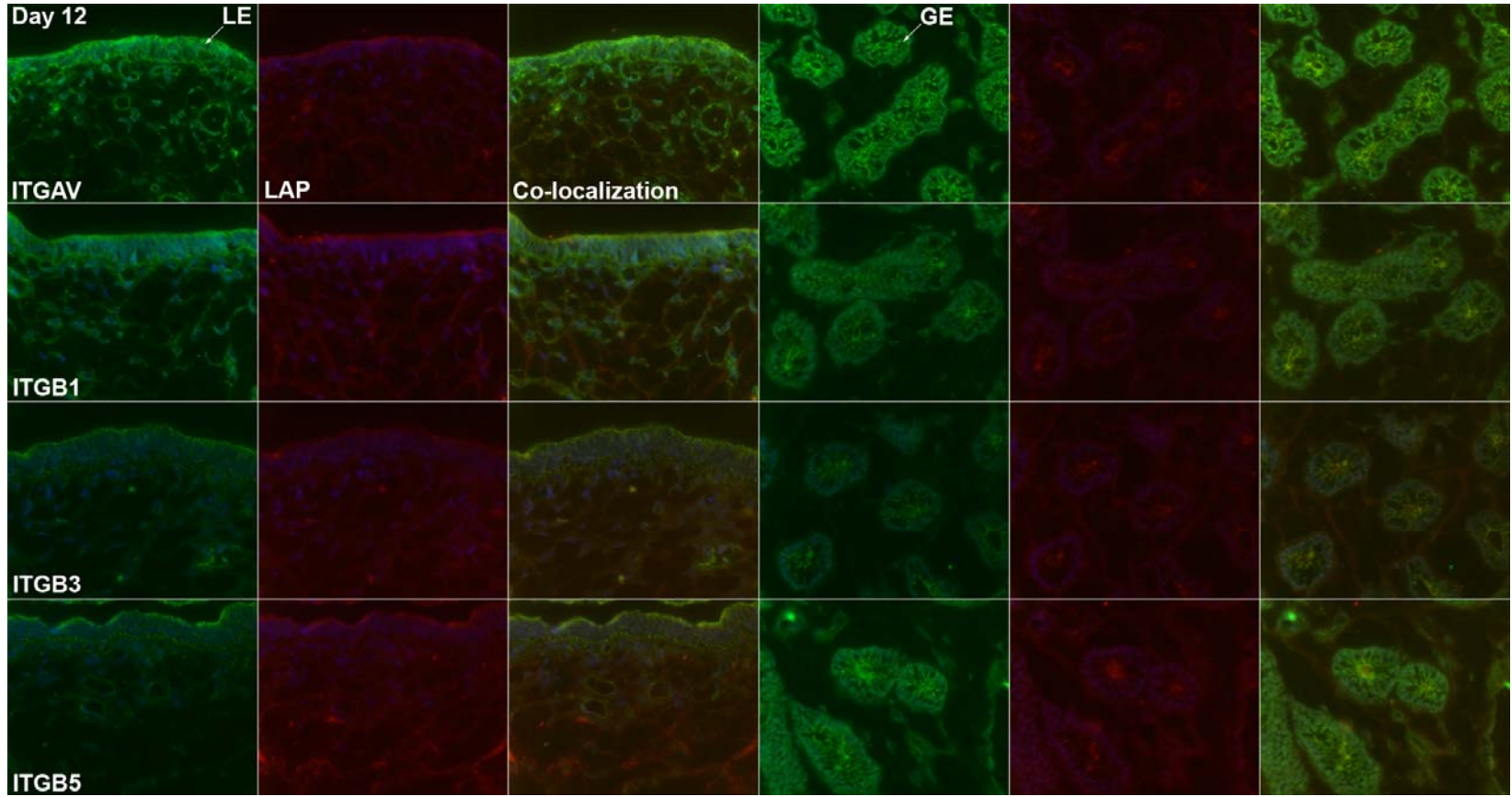


Figure 3.4. Integrin Subunits and LAP Are Available for Receptor Ligand Interaction on Day 12 of Pregnancy.

Immunofluorescence co-localization of integrin subunits (ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, ITGB8) with LAP in frozen cross-sections of day 12 pregnant pig endometrium. Integrin subunit immunoreactivity was detected using FITC-conjugated anti-rabbit IgG (columns 1 and 4; green fluorescence), whereas LAP immunoreactivity was detected using Texas Red-conjugated anti-goat IgG (columns 2 and 5; red fluorescence). Yellow immunofluorescence indicates co-localization of integrin subunit with LAP (columns 3 and 6). Compare the antibody staining with staining using rabbit and goat IgG (bottom row). LE, luminal epithelium; GE, glandular epithelium. Width of fields are 230 μm .



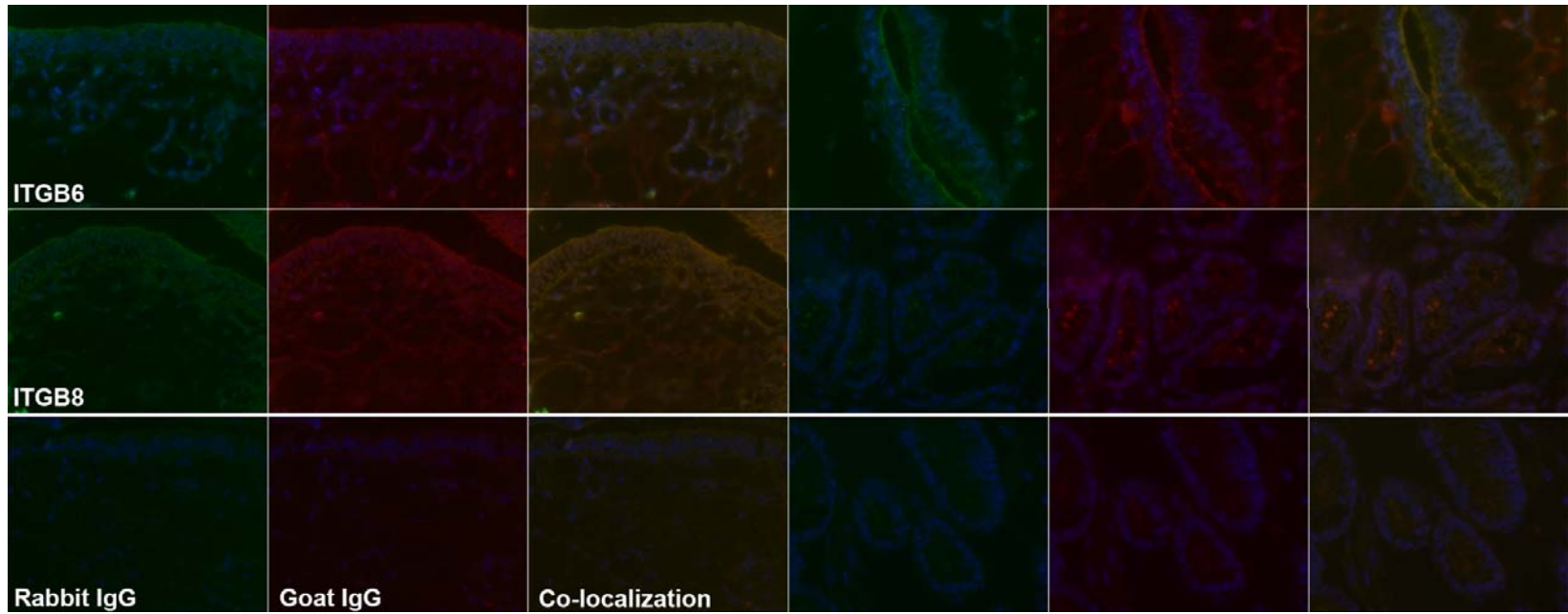
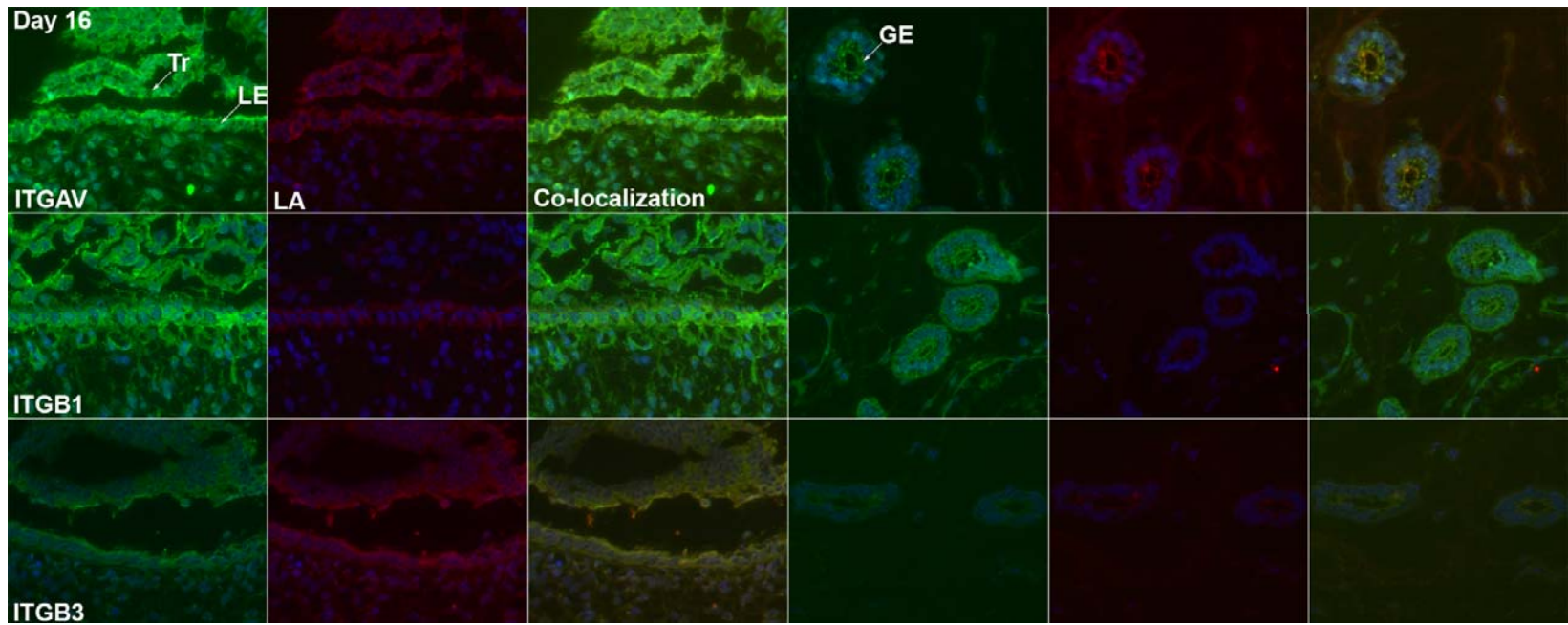


Figure 3.4. Continued

Figure 3.5. Integrin Subunits and LAP Are Available for Receptor Ligand Interaction at Conceptus Attachment Sites on Day 16 of Pregnancy. Immunofluorescence co-localization of integrin subunits (ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, ITGB8) with LAP in frozen cross-sections of day 16 pig conceptus attachment sites. Integrin subunit immunoreactivity was detected using FITC-conjugated anti-rabbit IgG (columns 1 and 4; green fluorescence), whereas LAP immunoreactivity was detected using Texas Red-conjugated anti-goat IgG (columns 2 and 5; red fluorescence). Yellow immunofluorescence indicates co-localization of integrin subunit with LAP (columns 3 and 6). Compare the antibody staining with staining using rabbit and goat IgG (Fig. 4; bottom row). LE, luminal epithelium; GE, glandular epithelium; Tr, trophoctoderm. Width of fields are 230 μm .



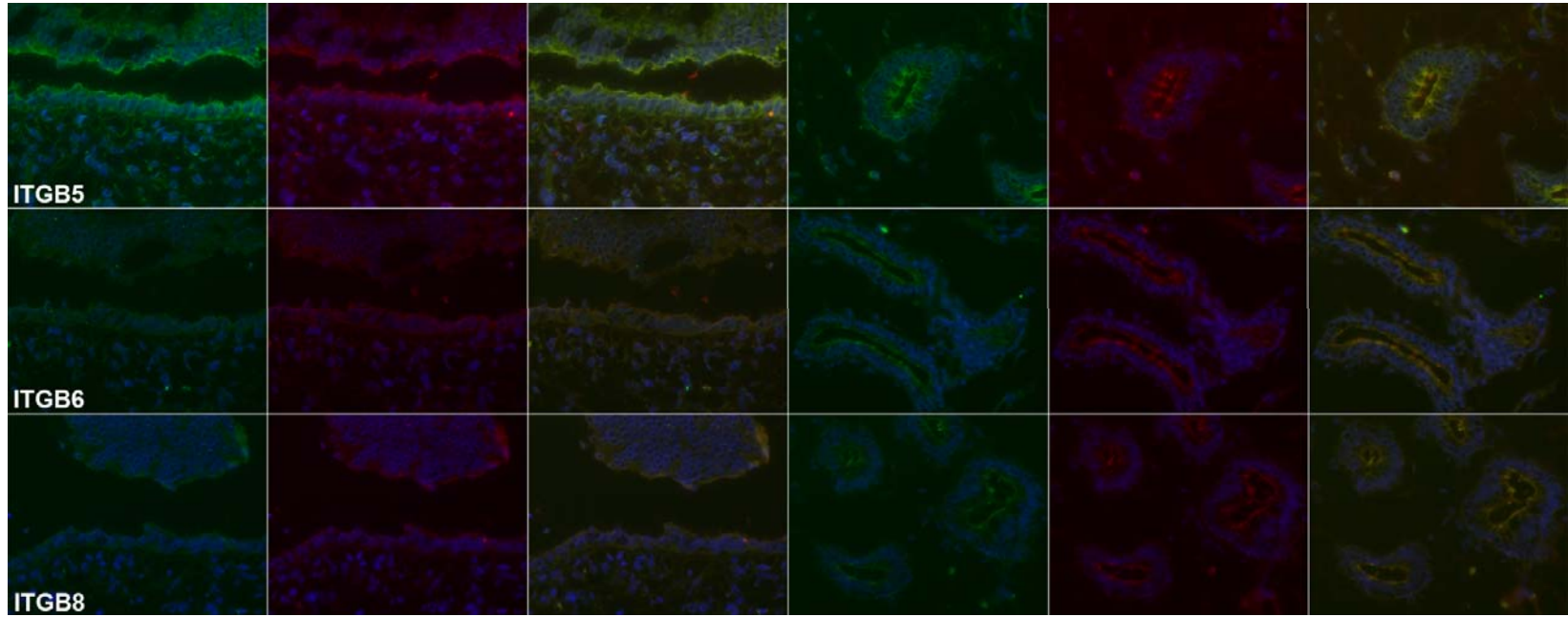
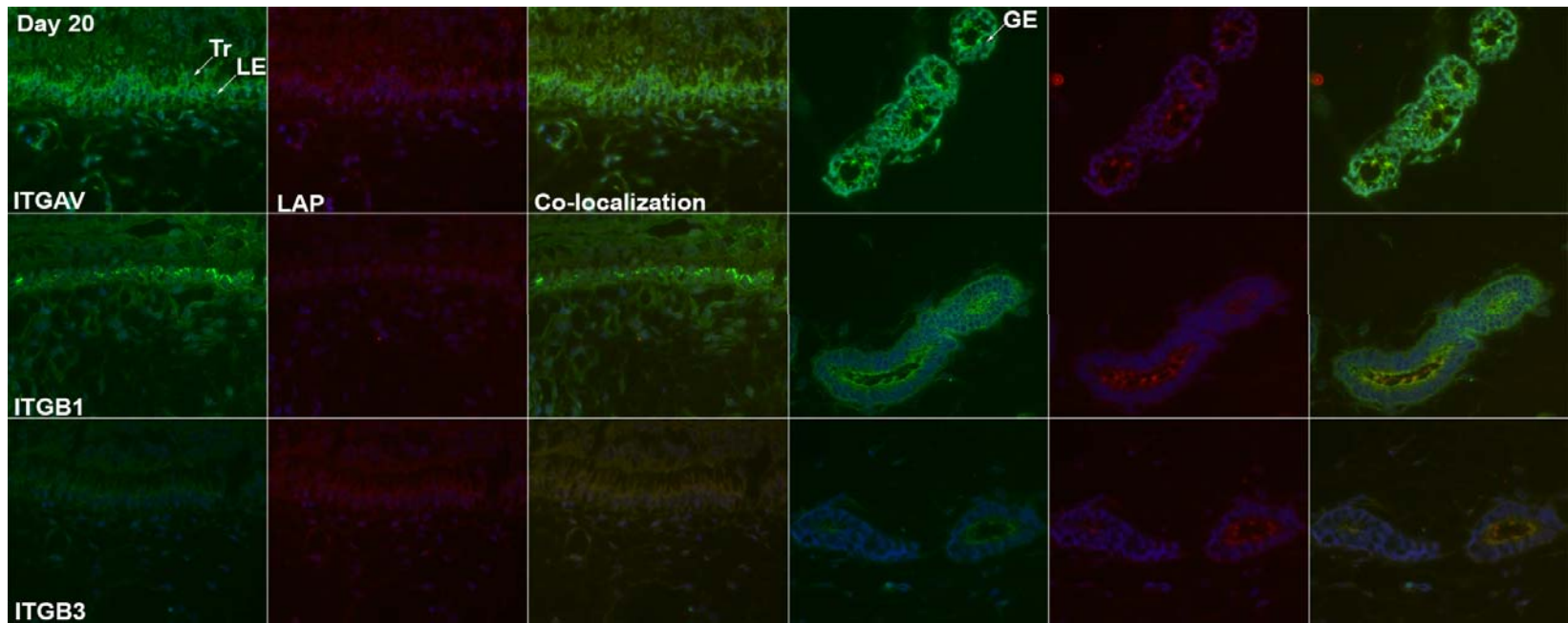


Figure 3.5. Continued

Figure 3.6. Integrin Subunits and LAP Are Available for Receptor Ligand Interaction at Conceptus Attachment Sites on Day 20 of Pregnancy. Immunofluorescence co-localization of integrin subunits (ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, ITGB8) with LAP in frozen cross-sections of day 20 pig conceptus attachment sites. Integrin subunit immunoreactivity was detected using FITC-conjugated anti-rabbit IgG (columns 1 and 4; green fluorescence), whereas LAP immunoreactivity was detected using Texas Red-conjugated anti-goat IgG (columns 2 and 5; red fluorescence). Yellow immunofluorescence indicates co-localization of integrin subunit with LAP (columns 3 and 6). Compare the antibody staining with staining using rabbit and goat IgG (Fig. 4; bottom row). LE, luminal epithelium; GE, glandular epithelium; Tr, trophoctoderm. Width of fields are 230 μm .



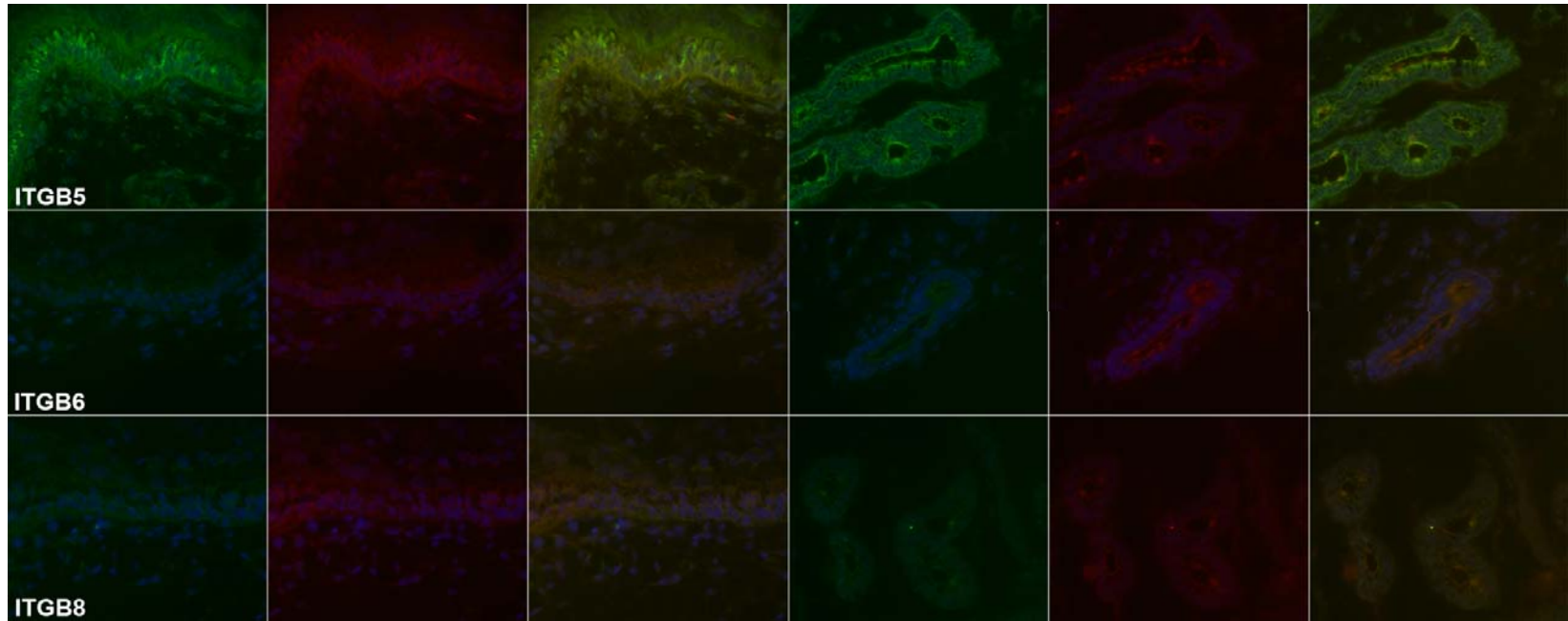
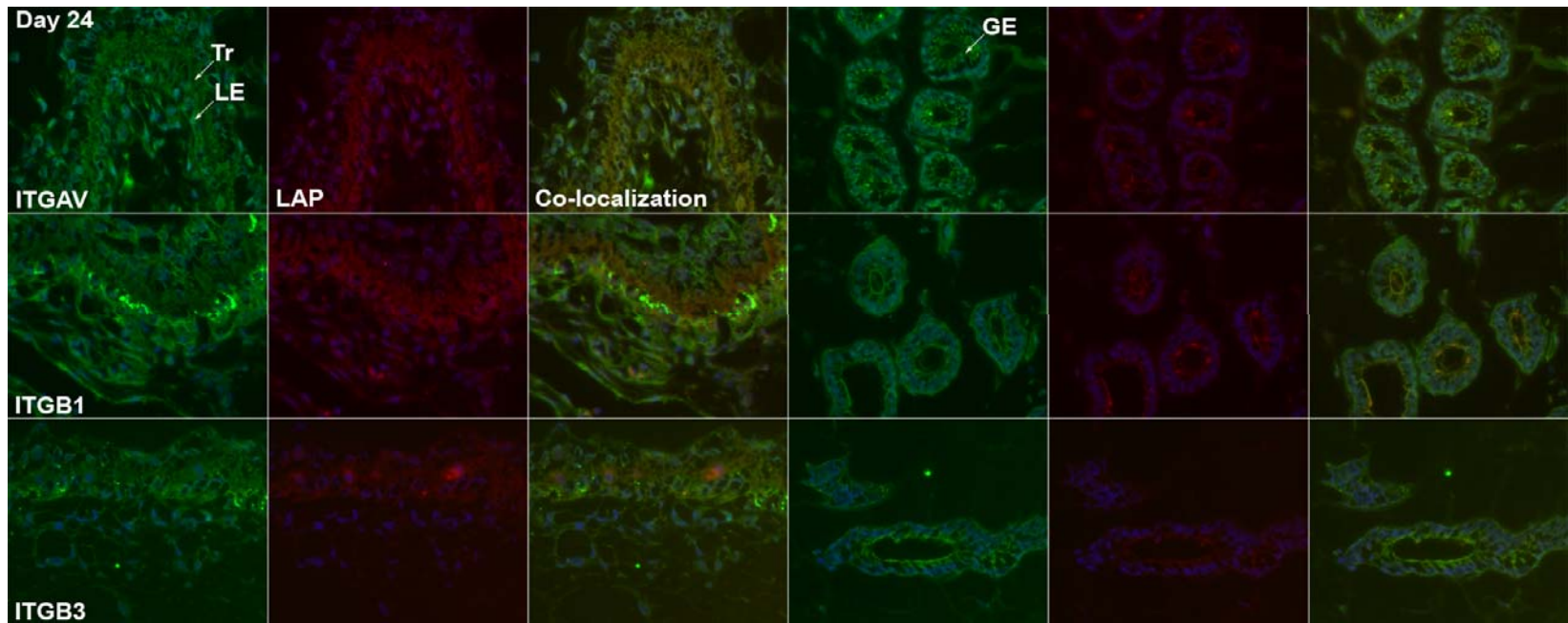


Figure 3.6. Continued

Figure 3.7. Integrin Subunits and LAP Are Available for Receptor Ligand Interaction at Conceptus Attachment Sites on Day 24 of Pregnancy. Immunofluorescence co-localization of integrin subunits (ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, ITGB8) with LAP in frozen cross-sections of day 24 pig conceptus attachment sites. Integrin subunit immunoreactivity was detected using FITC-conjugated anti-rabbit IgG (columns 1 and 4; green fluorescence), whereas LAP immunoreactivity was detected using Texas Red-conjugated anti-goat IgG (columns 2 and 5; red fluorescence). Yellow immunofluorescence indicates co-localization of integrin subunit with LAP (columns 3 and 6). Compare the antibody staining with staining using rabbit and goat IgG (Fig. 4; bottom row). LE, luminal epithelium; GE, glandular epithelium; Tr, trophoctoderm. Width of fields are 230 μm .



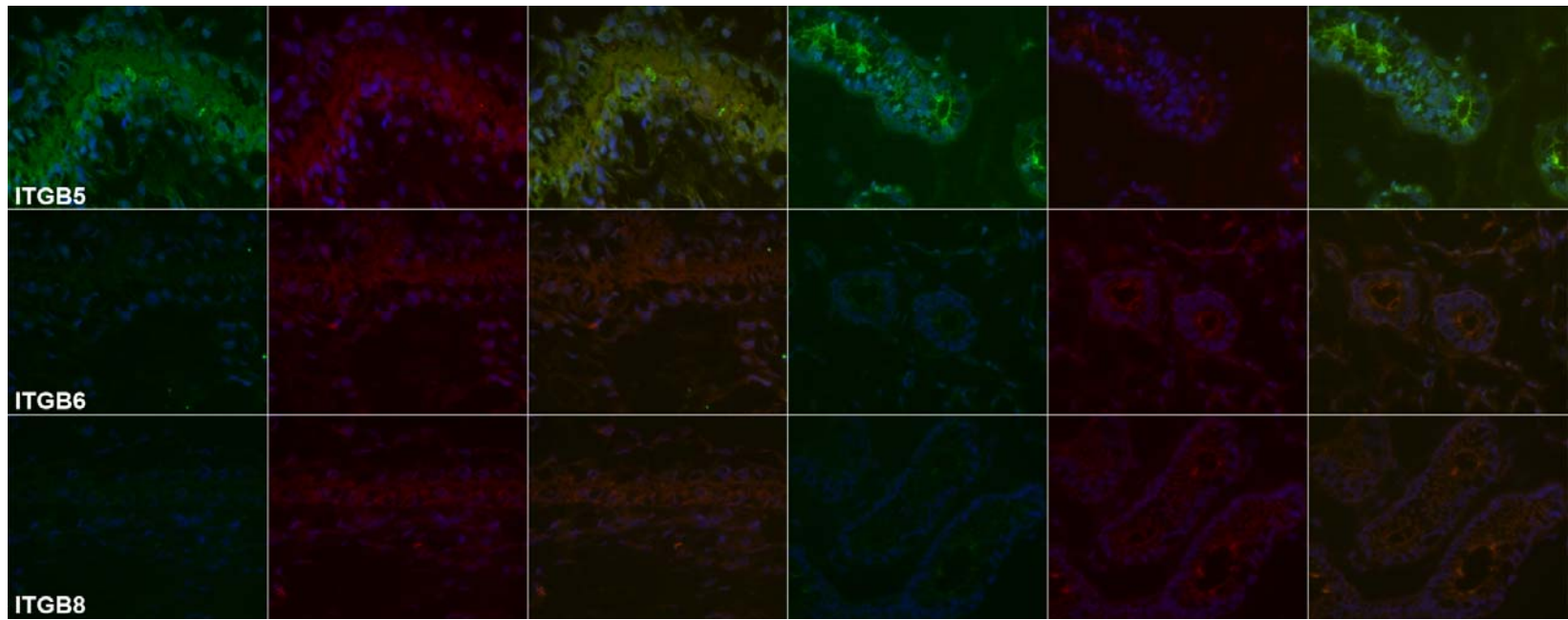


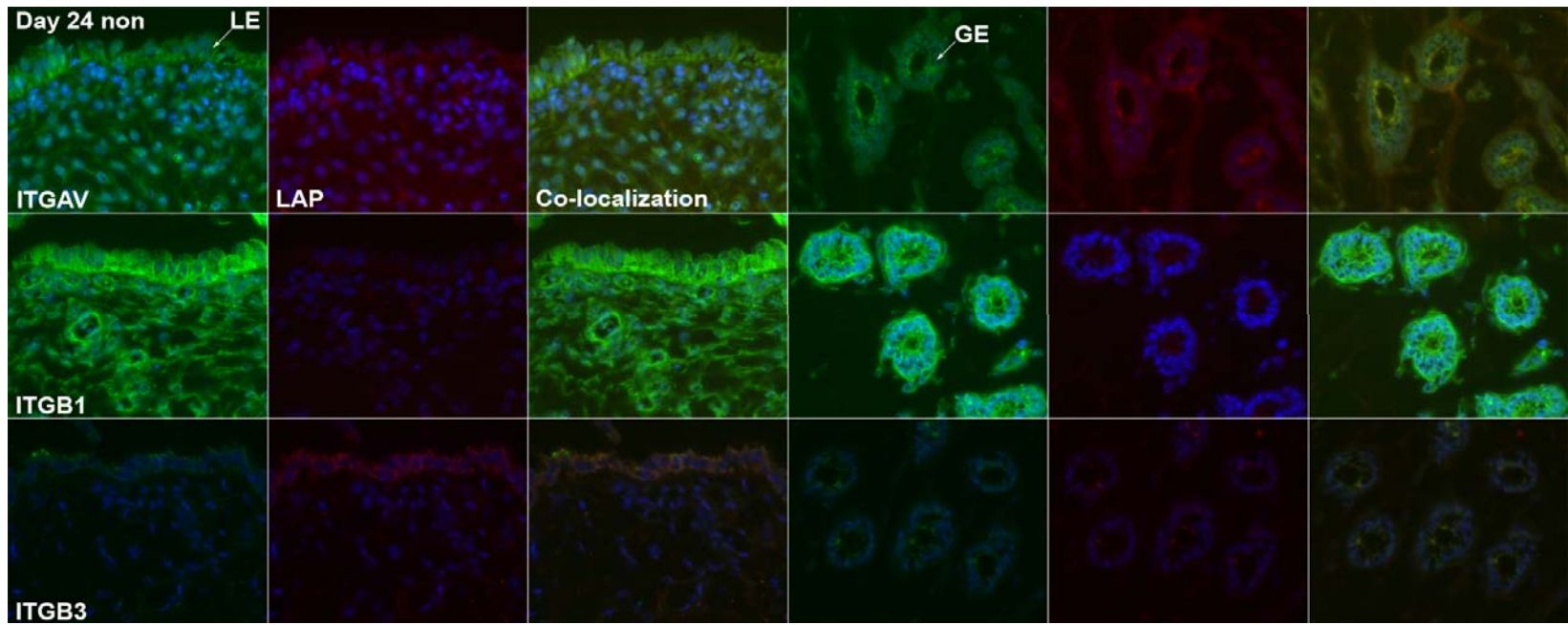
Figure 3.7. Continued

Immunostaining for ITGAV was detectable on all endometrial and conceptus tissues from days 10 through 24. Expression in LE and GE increased and became more apical in distribution by day 16, further increased at the conceptus-maternal interface by day 20, and these levels were maintained through day 24 (**Figures 3.4-3.7**). Immunostaining for ITGB3 was detectable, but low, in all endometrial and conceptus tissues from days 10 through 20. Interestingly, by day 24, ITGB3 became more apical in distribution in GE, but formed intermittent aggregates at the conceptus-maternal interface (**Figures 3.4-3.7**).

Immunostaining for ITGB1 and ITGB5 were similar in intensity to that of ITGAV on all conceptus and endometrial tissues on days 10 through 16 with the exception of ITGB5 which showed a more apical distribution than the other integrins. By day 20 of pregnancy, both ITGB1 and ITGB5 formed large distinct aggregates particularly in the LE at the conceptus-maternal interface which were maintained through day 24 (**Figures 3.4-3.7**). These aggregates likely represent a response to ligand binding and assembly of focal adhesions that serve to attach trophoctoderm to luminal epithelium for stable attachment. These are the first results to show evidence of ITGB1 and ITGB5 induced focal adhesions at the conceptus-maternal interface in pigs. **Figure 3.8** shows immunostaining for ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, and ITGB8 at day 24 non-attachment sites. Note the absence of integrin aggregation at the apical surface of LE, strongly suggesting that aggregates form in response to trophoctoderm attaching to luminal epithelium.

As noted previously, LAP was present in LE, GE, and trophoctoderm in all tissues observed, and LAP immunostaining increased at the conceptus-maternal interface by day

Figure 3.8. Integrin Subunits and LAP Are Available for Receptor Ligand Interaction at Non-Attachment Sites on Day 24 of Pregnancy. Immunofluorescence co-localization of integrin subunits (ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, ITGB8) with LAP in frozen cross-sections of day 24 pig non attachment sites. Integrin subunit immunoreactivity was detected using FITC-conjugated anti-rabbit IgG (columns 1 and 4; green fluorescence), whereas LAP immunoreactivity was detected using Texas Red-conjugated anti-goat IgG (columns 2 and 5; red fluorescence). Yellow immunofluorescence indicates co-localization of integrin subunit with LAP (columns 3 and 6). Compare the antibody staining with staining using rabbit and goat IgG (Fig. 4; bottom row). LE, luminal epithelium; GE, glandular epithelium. Width of fields are 230 μ m.



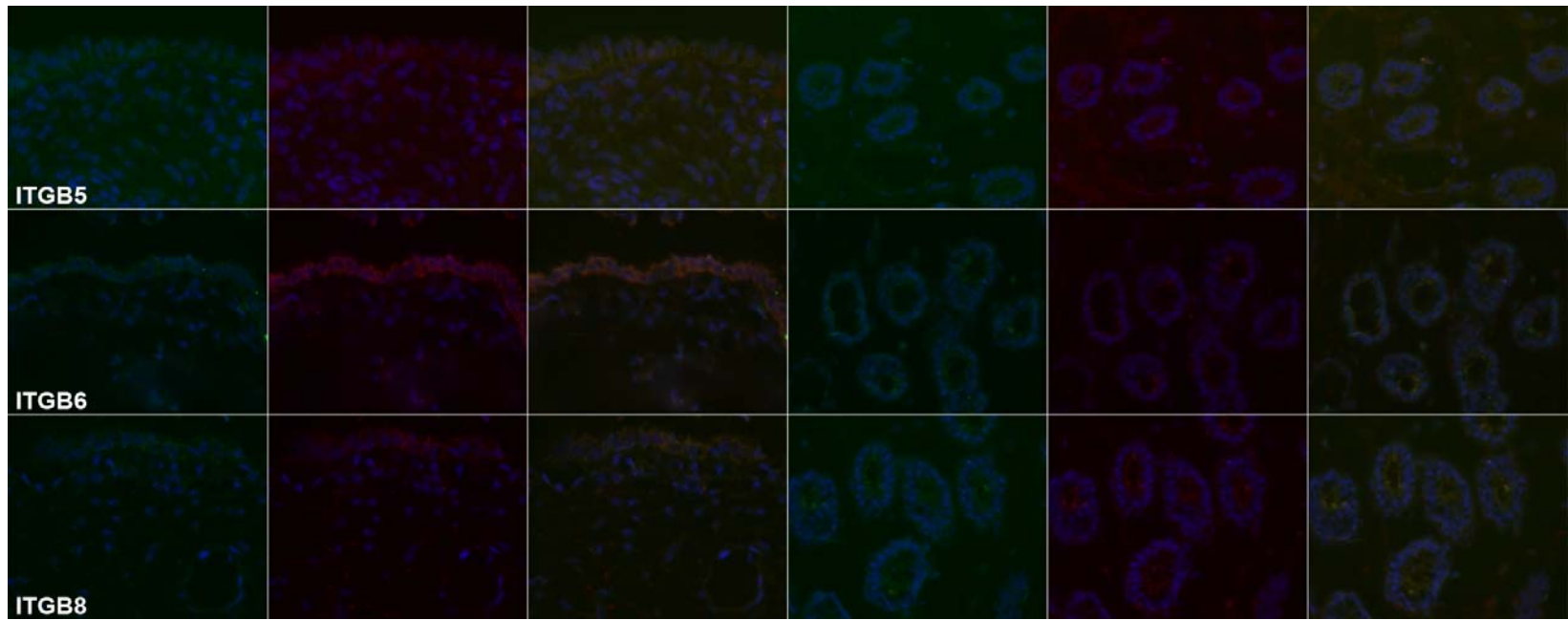


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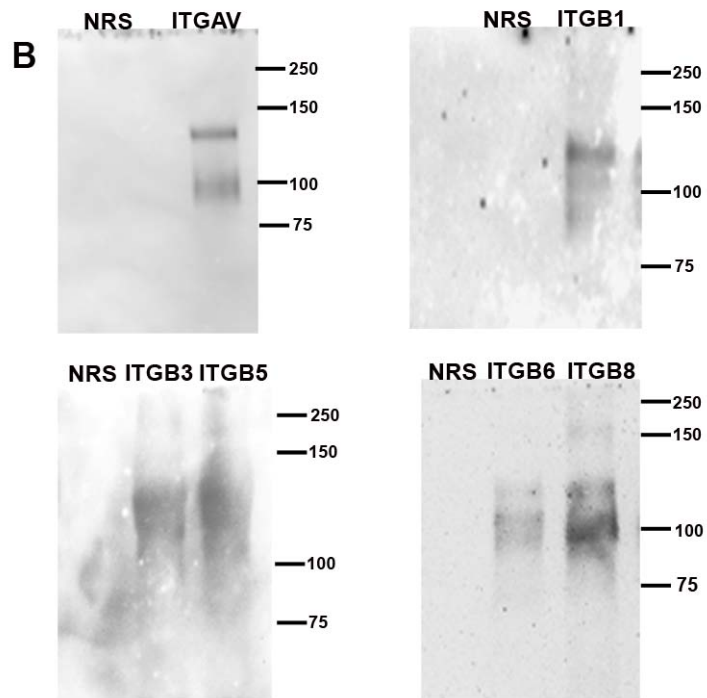
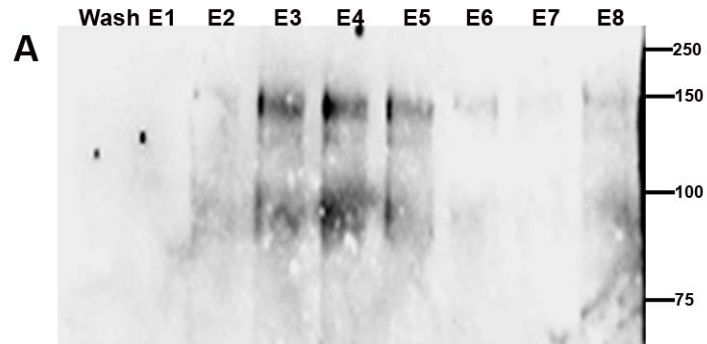
24 of pregnancy (**Figures 3.4-3.7**). In contrast to integrins, LAP appeared to be distributed in the matrix that resides between the trophoctoderm and LE, and did not precisely co-localize with aggregates of ITGB1 and ITGB5. Therefore, the presence of LAP in the matrix between trophoctoderm and LE perfectly places this protein to bind ITGB1, ITGB3, and ITGB5 integrins and stimulate the assembly of focal adhesions.

LAP binds to surface biotinylated integrins expressed on pTr2 cells via the RGD site

Biotinylated proteins from the apical surfaces of pTr2 cells were subjected to affinity chromatography with sepharose beads carrying LAP. Eluted proteins were separated on a non-reducing 7.5% SDS-PAGE gel. This resulted in biotinylated protein bands of approximately 150 kDa and 100 kDa. The molecular weight of 150 kDa corresponded with the known molecular weights of the non-reduced ITGAV subunit. The molecular weight of 100 kDa was within the range of multiple beta integrin subunits including ITGB1, ITGB3, ITGB5, ITGB6, and ITGB8 (**Figure 3.9A**).

Immunoprecipitations performed on these eluates using antibodies directed against integrin subunits ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, and ITGB8 successfully confirmed binding of each of these integrin subunits to LAP. ITGAV, ITGB3, ITGB5, ITGB6, and ITGB8 immunoprecipitates yielded a band at 125 kDa corresponding to the known reduced molecular weight of ITGAV (**Figure 3.9B**). ITGAV immunoprecipitates also yielded a broad band at approximately 100 kDa corresponding to the beta subunit of the heterodimer. ITGB1 has a reduced molecular weight of 130 kDa, and the ITGB1 immunoprecipitates yielded 2 bands in the range of

Figure 3.9. LAP Binds to Surface Biotinylated Integrins Expressed on pTr2 Cells via the RGD Site. **A**, pTr2 cells were surface labeled with biotin and detergent extracts of the cells were prepared and subjected to affinity chromatography as described in the Methods. EDTA eluate fractions (E1-E8) were separated by 7.5% SDS-PAGE under non reducing conditions, transferred to nitrocellulose, and probed for biotin using ABC. **B**, Eluate fractions containing biotinylated proteins were pooled. Proteins were immunoprecipitated with antibodies to integrin subunits (ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, or ITGB8) or rabbit IgG control and A-protein G agarose conjugated beads. Immunoprecipitates were separated by 7.5% SDS-PAGE under reducing conditions, transferred to nitrocellulose, and probed for biotin using ABC.



125 kDa to 130 kDa, which are likely both ITGAV (125 kDa) and ITGB1 (130 kDa) subunits. ITGB3 and ITGB5 immunoprecipitates yielded a diffuse band in the range of 110 kDa to 130 kDa. ITGB6 and ITGB8 immunoprecipitates yielded bands in the range of 95 kDa to 110 kDa (**Figure 3.9B**).

Discussion

Results of the present study in pigs provide evidence that: 1) latent and active TGF β are present in conceptus and endometrium during the porcine peri-implantation period; 2) TGF β is functional in both conceptus and endometrial tissues; and 3) LAP is a ligand for ITGAV-containing heterodimers in conceptus trophoctoderm during the peri-implantation period. Our detection of LAP in conceptus and endometrial tissues, along with earlier data (Gupta *et al.* 1996, Gupta *et al.* 1998b), support the finding that both the conceptus and uterus are sources of secreted TGF β . Further, abundant LAP immunostaining detected on the apical surfaces of GE on days 16 and 20 of pregnancy suggests that the GE secrete latent TGF β as a component of histotroph. Detection of abundant active TGF β at the apical surfaces of day 12 trophoctoderm and conceptus-maternal interface on days 16 and 20 suggests that TGF β is activated predominantly at the apical surfaces of filamentous conceptuses and at the conceptus-maternal interface. While activation of latent TGF β appears to occur primarily at sites of conceptus attachments, TGF β binds and activates TGFBRs at both attachment and non-attachment sites, as indicated by the detection of p-SMAD2/3 at both conceptus attachment and non-attachment sites during porcine implantation.

Large aggregates of LAP and low levels of TGFB immunostaining at the conceptus-maternal interface during the late stages of porcine implantation suggest sequestration of latent TGFB as a reservoir. Despite low levels of TGFB immunostaining, detection of abundant p-SMAD2/3 at the conceptus-maternal interface provides evidence for the maintenance of functionally active TGFB during the later stages of implantation. While large aggregates of LAP may still represent a reservoir for latent TGFB at the conceptus-maternal interface, particularly along apical surfaces of GE, it is also likely that LAP functions as an adhesion molecule promoting attachment of the conceptus to the uterine LE via binding to integrin receptors at the conceptus-maternal interface. Indeed, affinity chromatography results demonstrate that LAP binds to the integrin subunits ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, and ITGB8 on the surfaces of porcine trophectoderm cultured cells. These results, along with the detection of integrin aggregation at the conceptus-maternal interface, provide mechanistic and functional evidence for the hypothesis that LAP binding to ITGAV-containing integrin heterodimers serves as an adhesion complex that promotes and stabilizes conceptus attachment to the uterine LE during porcine implantation.

Integrin Aggregation at the Porcine Conceptus-Maternal Interface

Aggregation of integrins in response to mechanosensation and mechanotransduction at the conceptus-maternal interface in sheep was recently reported (Burghardt *et al.* 2009). However, this is the first report of ITGB1 and ITGB5 aggregation at the conceptus-maternal interface during porcine implantation. Upon

binding of ECM proteins, integrins aggregate to enhance signal transduction and form cytoplasmic focal adhesions that associate with the actin cytoskeleton (Wozniak *et al.* 2004). Formation of focal adhesions stabilizes cell attachments to the ECM by connecting ECM proteins to the actin cytoskeleton via actin filaments that can further assemble into contractile stress fibers (Bershadsky *et al.* 2006a). In addition to transmitting force at cell adhesion sites, focal adhesions serve as signaling centers from which numerous intracellular pathways can regulate cell growth, proliferation, survival, gene expression, development, tissue repair, migration and invasion. At least 156 components including classes of receptor kinases, cytoskeletal proteins, and signaling molecules that involve up to 690 interactions have been identified at integrin adhesion sites characterized by focal adhesions (Zaidel-Bar *et al.* 2007). Focal adhesions formed by *in vitro* cell cultures respond to intracellular forces generated by actin-myosin interaction or applied externally to the cell through the extracellular matrix by altering the size of focal adhesion and their composition (Galbraith *et al.* 2002). Integrin immunostaining at porcine conceptus attachment sites identified intense and intermittent immunoreactive aggregates of integrin subunits ITGB1, ITGB5 and later ITGB3. Detection of integrin aggregation at the conceptus-maternal interface beginning by day 20 of pregnancy implies that integrins bind to ECM proteins such as LAP to induce integrin aggregation and focal adhesion assembly that stabilizes adhesion of the conceptus to uterine LE during porcine implantation. Interestingly, ITGB3 aggregates were not detected until day 24 of pregnancy and, similar to ITGB1 and ITGB5, these aggregates were not found at non-attachment sites. Upregulation of ITGB3 integrin

subunit, possibly as the $\alpha\beta3$ heterodimer, on the apical surfaces at the conceptus-maternal interface may be important for further supporting conceptus adhesion. Changes in the integrin repertoire on LE, particularly with $\alpha\beta3$, have been detected during implantation in other species and are associated with stabilizing stronger conceptus adhesions (Johnson *et al.* 2001, Burghardt *et al.* 2002, Lessey 2002, Armant 2005, Burghardt *et al.* 2009). During porcine implantation, $\alpha\beta1$ and $\alpha\beta5$ may play important roles for stabilizing initial attachments of the conceptus to the LE, and as implantation progresses $\alpha\beta1$, $\alpha\beta5$, and $\alpha\beta3$ may be necessary to support stronger placental attachments.

TGFB Activation via Integrins During Porcine Implantation

The presence of active TGFB and p-SMAD2/3 provides strong evidence that TGFB is activated throughout the porcine peri-implantation period. Proteases such as plasmin and MMPs can activate TGFB; however, integrins also activate TGFB *in vivo* (Sato & Rifkin 1989, Lyons *et al.* 1990, Shull *et al.* 1992, Taipale *et al.* 1992, Huang *et al.* 1996, Bader *et al.* 1998, Yu & Stamenkovic 2000, Zhu *et al.* 2002, Jenkins 2008). More recently, transgenic mice, in which the RGD sequence in LAP is mutated to RGE, were shown to be phenotypically similar to TGFB1 null mice, further supporting the suggestion that integrin binding of LAP plays a significant role in activating TGFB *in vivo* (Yang *et al.* 2007). This process of TGFB activation via integrins can result from conformational changes in the latent complex. Activation of TGFB via $\alpha\beta3$, $\alpha\beta5$, $\alpha\beta6$, and an as yet unidentified ITGB1 heterodimer occurs if TGFB is part of the large

latent complex (LLC) (Annes *et al.* 2004, Wipff & Hinz 2008). LAP associated with the LLC binds integrins that are linked to the actin cytoskeleton; and since integrins can sense and respond to intracellular forces generated through the actin cytoskeleton and extracellular forces such as ECM rigidity, it is hypothesized that integrins can activate TGFB via traction forces (Keski-Oja *et al.* 2004, **Larson** *et al.* 2006).

Abundant active TGFB immunostaining was detected along the apical surfaces of filamentous conceptuses by day 12 of pregnancy and at the conceptus-maternal interface beginning by day 16 of pregnancy, suggesting that activation occurs along these surfaces. LAP and integrin subunits ITGAV, ITGB1, ITGB3, ITGB5, and low levels of ITGB6 and ITGB8 were detected along the uterine LE, and integrins $\alpha\beta3$, $\alpha\beta5$, $\alpha\beta6$, and possibly $\alpha\beta1$, have been determined to activate latent TGFB by mechanical forces such as cellular contraction and external stretching (Jenkins *et al.* 2006, Wipff & Hinz 2008). Both of these mechanisms require: a) latent TGFB of the LLC tethered to an ECM that resists traction forces exerted on the LLC, b) LAP bound to integrin receptors, and c) presence of the actin cytoskeleton to generate force or provide resistance to extracellular forces (Wipff & Hinz 2008). Such mechanisms of TGFB activation may occur at the porcine conceptus-maternal interface.

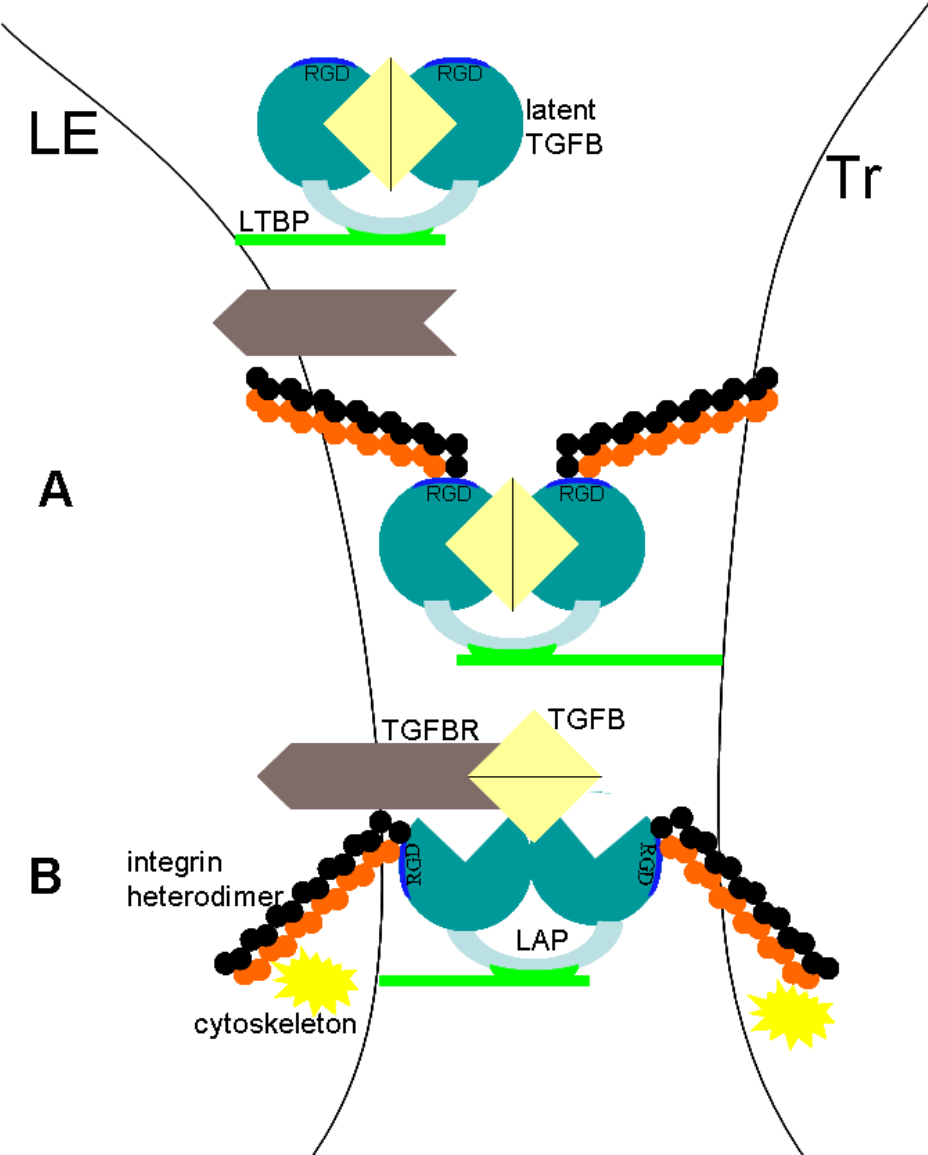
During porcine implantation, the conceptus migrates along the uterine epithelium that lines the uterine horns, and along these surfaces, the migrating conceptus may be a source of external force applied to the LAP-integrin adhesion complex. We hypothesize that the apically bound LAP-integrin adhesion complex, in addition to supporting conceptus attachments, can also activate TGFB by inducing conformational changes in

the latent complex. The conformational change generated by conceptus movement along the uterine LE may cause external stretch on the LLC and therefore liberate TGFB (Figure 3.10).

Intracellular forces may also play a role in activating TGFB during porcine implantation. It is noteworthy that the subepithelial stroma of pigs undergoes remodeling during pregnancy that is associated with the upregulation of α -smooth muscle actin (ACTA2) within myofibroblast-like stromal cells (Johnson *et al.* 2003). Increased ECM and incorporation of ACTA2 into stress fibers significantly augments the contractile activity of fibroblastic cells and are hallmarks of connective tissue remodeling (Hinz *et al.* 2001) which is expected to take place in a mechanically stressed environment such as the uterine wall during pregnancy. Cellular contraction mediated by the cross bridging ACTA2 and myosin is involved in balancing mechanical forces placed on the ECM. These contractile forces have previously been shown to activate TGFB in myofibroblast cultures (Wipff *et al.* 2007). It is possible that in the porcine uterus, contraction of the actin filaments associated with integrins generates a force on the LAP- integrin complex, causing a conformational change in the LLC, and liberating TGFB from its latent complex. As reported here, latent TGFB localized within the ECM of the stroma during porcine implantation may be subjected to activation by integrins transmitting intracellular mechanical forces.

We propose therefore that ITGAV-containing heterodimers participate in TGFB activation during porcine implantation via mechanisms including, but not limited to, traction forces. Forces generated by the conceptus may be a mechanism for activating

Figure 3.10. Proposed Model of External Forces Activate TGFB at the Conceptus-Maternal Interface. **A**, Integrin heterodimers on porcine trophectoderm (Tr) and uterine LE bind to LAP via its RGD sequence serving to support conceptus attachment to the LE during porcine implantation. **B**, LAP bound to integrins $\alpha\nu\beta3$, $\alpha\nu\beta5$, $\alpha\nu\beta6$, and possibly $\alpha\nu\beta1$ may serve to activate TGFB. External force, such as the conceptus migrating along the apical surfaces of the LE, is exerted on the LAP-integrin adhesion complex. LAP tethered to the ECM via LTBP along with integrins associated with the actin cytoskeleton both resists extracellular forces exerted on the complex, resulting in a conformational change in latent TGFB. TGFB is liberated from the latent complex and is available to bind to TGFBR on porcine Tr and uterine LE.



TGFB at sites of conceptus contact, and forces generated from within the cell may be a mechanism for activating TGFB within the endometrial stroma. Active TGFB can therefore, bind and activate TGFBRs at both conceptus attachment and non-attachment sites.

Potential Roles for TGFB During Porcine Implantation

In other species, TGFB has been linked to indirect role(s) in trophoblast attachment and invasion by enhancing production of ECM proteins such as oncofetal-fibronectin (oFN) (Feinberg *et al.* 1994), inducing expression of integrins (Zambruno *et al.* 1995, Kagami *et al.* 1996, Lai *et al.* 2000, Pechkovsky *et al.* 2008), and inhibiting trophoblast invasion by reducing production of proteases such as MMPs and plasmin (Graham & Lala 1991, Graham 1997, Kallapur *et al.* 1999, Karmakar & Das 2002). Similar actions of TGFB may also be involved in peri-implantation events in pigs. Based on p-SMAD2/3 localization in pigs, TGFB appears to initiate intracellular signaling within conceptus and uterine tissues, possibly regulating transcription of genes involved in porcine implantation.

In summary, *in vivo* results support the conclusion that latent TGFB is secreted and activated during the porcine peri-implantation period, LAP binds integrins and may aid in TGFB activation as well as promote conceptus attachment, and signaling through SMADs is present in both conceptus and uterine tissues. Our results provide evidence for functional TGFB and integrin activation during the porcine peri-implantation period. Furthermore, LAP binding to integrins at the porcine conceptus-maternal interface may induce integrin aggregation of ITGB1, ITGB3, and ITGB5 integrin subunits aiding in

strong conceptus attachment to the uterine LE. We are the first to report aggregation of integrin subunits ITGB1 and ITGB5 suggesting formation of focal adhesions at the conceptus-maternal interface in pigs. In addition the LAP-integrin adhesion complex may be subjected to, but not limited to, traction forces causing activation of latent TGFB at the conceptus-maternal interface during porcine implantation. TGFB activating TGFBR downstream signaling molecules may have roles in promoting conceptus attachment by upregulating pro-adhesive molecules such as integrins and oFN, and inhibiting conceptus invasion by decreasing production of proteases. The interrelationships between TGFB and integrins described here are likely an important part of the complex networking system of interplay between the conceptus and uterus during the porcine peri-implantation period.

CHAPTER IV
INTRAUTERINE INFUSION OF LATENCY ASSOCIATED PEPTIDE (LAP)
DURING EARLY PORCINE PREGNANCY AFFECTS CONCEPTUS
ELONGATION AND PLACENTAL SIZE

Introduction

Mechanisms supporting conceptus elongation and attachment during porcine pregnancy are largely unknown, but altering the uterine environment during the peri-implantation period may alter conceptus development and survival (Wilson *et al.* 2001). During this critical period, cross-talk between the conceptus and uterus mediates conceptus development and implantation, and crosstalk asynchrony may result in conceptus mortality (Pope *et al.* 1982, Pope *et al.* 1986, Geisert *et al.* 2006). Implantation in pigs is superficial, and prior to attachment, conceptuses undergo transformations from spherical (5 mm) to filamentous (150 mm) forms between days 10-12 of pregnancy (Geisert *et al.* 1982b). In a receptive uterine environment, filamentous conceptuses begin to make initial attachments to the uterine luminal epithelium (LE) by day 13 of pregnancy. Porcine implantation is essentially complete by day 24 of pregnancy, resulting in interdigitation of LE and the trophoctoderm (Keys & King 1990). Signals initiating conceptus elongation, and mechanisms supporting conceptus development and implantation in pigs, remain largely unknown.

Experimental intrauterine infusions with implantable osmotic pumps have been used to study development of the conceptus, physiology of the uterus, pathogenesis of

reproductive infections, and, to a limited extent, to deliver substances to uteri of domestic livestock (Pratt *et al.* 1979, Ayad *et al.* 1993, Vanderwall *et al.* 1994, Joyce *et al.* 2007a). Such experimental infusions have not been used to investigate conceptus development and attachment during the peri-implantation period in pregnant swine. Establishing a delivery system that remains contained within the abdominal cavity and enables controlled delivery of substances to the uterine lumen of pregnant gilts, while allowing maintenance of pregnancy, would be a novel way to alter *in vivo* basal levels of signaling proteins proposed to be involved in mediating porcine conceptus development and attachment.

Transforming growth factor beta (TGFB), TGFB receptors (TGFBRs), and integrins may contribute to the events of porcine implantation (Bowen *et al.* 1996, Gupta *et al.* 1996, Gupta *et al.* 1998a, Gupta *et al.* 1998b, Burghardt *et al.* 2002). TGFB homodimers are secreted by the cell in latent complexes, which include propeptide latency associate peptides (LAP), with which the homodimers are non-covalently associated. These complexes further associate with latent TGFB binding proteins (LTBPs), which can sequester the inactive growth factor to the extracellular matrix (ECM) (Lawrence *et al.* 1984, Gentry *et al.* 1988, Miyazono *et al.* 1988). Activation of latent TGFB must occur prior to TGFB signaling through TGFBRs. Activation of TGFBRs results in phosphorylation of downstream effector SMAD proteins 2 and 3 (p-SMAD2/3) and subsequent translocation of the phosphorylated SMADs into the nucleus with SMAD4. The combination of SMADs and cofactors included in the transcriptional

complex regulates transcriptional activity of target genes (Macias-Silva *et al.* 1996, Zhang *et al.* 1996, Nakao *et al.* 1997).

Extracellular activation of TGFB results from conformational changes in the latent complex or dissociation of TGFB from LAP (Annes *et al.* 2003, Jenkins 2008, Wipff & Hinz 2008). In addition to conferring latency to TGFB, the LAPs that associate with TGFBs 1 and 3 contain Arg-Gly-Asp (RGD) sequences that are recognized and bound by specific integrins (Ruoslahti 1996). Integrins are a family of transmembrane glycoprotein receptors that form non-covalent alpha and beta heterodimers, bind to multiple ECM proteins, aggregate, and form focal adhesions to stabilize cell attachments and serve as signaling centers (Hynes 1992, Wozniak *et al.* 2004, Bershadsky *et al.* 2006a, Zaidel-Bar *et al.* 2007). LAP via its RGD sequence can bind to integrin heterodimers on cell surfaces (Munger *et al.* 1998, Munger *et al.* 1999, Mu *et al.* 2002, Ludbrook *et al.* 2003), and these interactions, along with TGFB and TGFBR interactions, may function in supporting events of the peri-implantation period.

The consequences of altering or interfering with receptor-ligand interactions involving TGFB, LAP, and integrins within the uterus during porcine pregnancy have not yet been determined. We hypothesize that altering such interactions will affect conceptus development and attachment. Conduct of such investigations would require a specialized means for *in vivo* intrauterine infusion of exogenous proteins which is also compatible with maintenance of pregnancy. Therefore, the first objective of this study was to develop a system using interiorized catheters which would facilitate repeated delivery of agents, such as exogenous proteins at physiological concentrations, directly

into the uterus of pregnant gilts and require minimal post surgery manipulation and restraint.

The second objective of this study was to use the technique developed in *study 1* to infuse exogenous LAP directly into the uterus of pregnant gilts to alter receptor-ligand interactions of TGFB and TGFBRs, LAP and integrins. The use of this novel delivery system to infuse LAP containing its native RGD site (LAP-RGD) and a recombinant mutant of LAP (LAP-RGE) into the uterine lumen of pregnant gilts will aid in our understanding of TGFB and LAP-integrin mediated effects on conceptus development and attachment. LAP decreases *in vitro* TGFB activity by recombining with active TGFBs, rendering them latent, and thus inhibiting TGFB from binding to TGFBRs. Further *in vitro* evidence supports that LAP-RGD, but not LAP-RGE, can bind to integrin receptors and support cell adhesion (Jaeger *et al.* 2005). Exogenous LAP-RGD infused into the uterine lumen of pregnant gilts beginning on day 9 of pregnancy is expected to disrupt the balance of active TGFB and alter normal integrin signaling during the peri-implantation period, whereas exogenous LAP-RGE infusions will disrupt TGFB activity without altering integrin-ligand interactions at the conceptus-maternal interface.

We hypothesize that intrauterine infusion of LAP –RGD during the peri-implantation period will decrease downstream signaling of TGFB at the conceptus-maternal interface, while also increasing LAP-integrin interactions, and that the net effect will be enhanced conceptus survival and attachment early in the peri-implantation phase, but possibly abnormal placentation later in pregnancy. In contrast, infusion of

LAP-RGE will disrupt TGFB signals, but because of the RGD to RGE mutation, will not alter integrin signaling and thus the net result will be decreased conceptus survival and abnormal development. This design will aid in distinguishing between TGFB- and integrin-mediated effects on conceptus development and attachment.

Materials and Methods

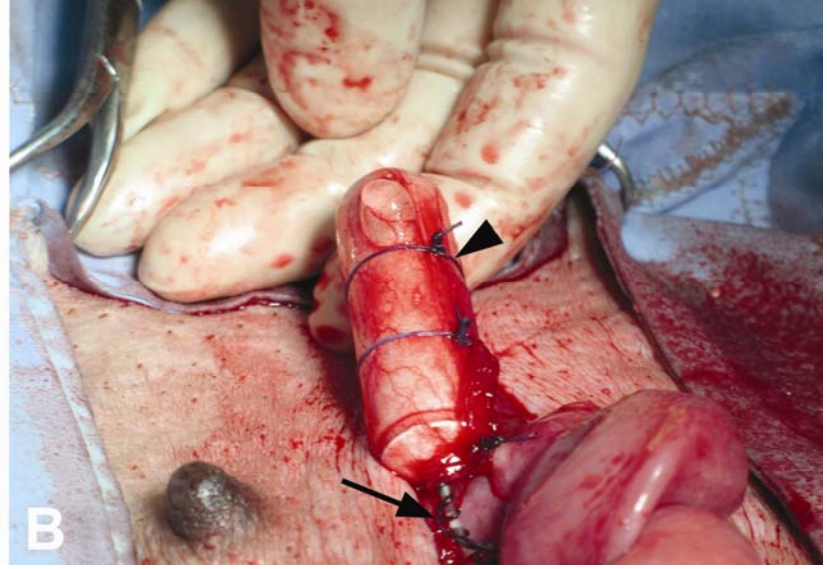
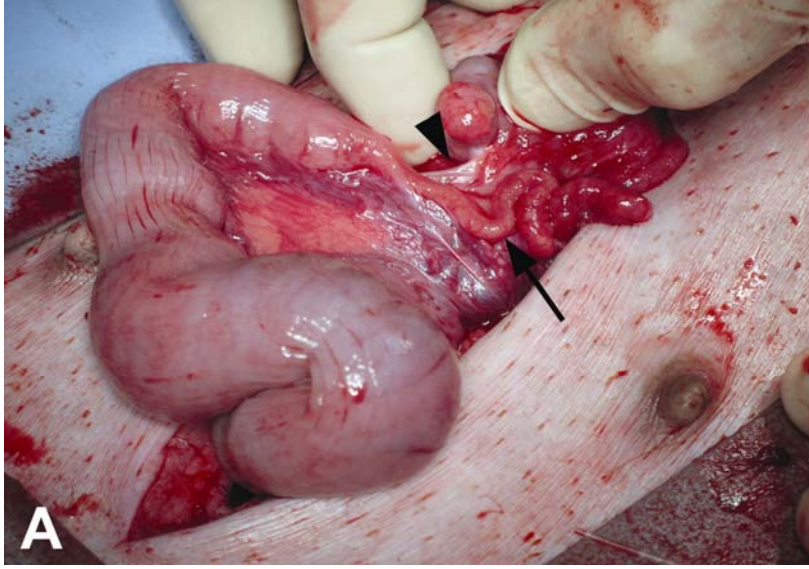
Animals and Tissue Collection

All experimental and surgical procedures complied with the Guide for Care and Use of Laboratory Animals and approved by the Texas A&M University Laboratory Animal Care and Use Committee. Gilts were observed daily for estrus (day 0) and mated with boars at 12 and 24 h after the onset of estrus.

Study One. A primary experimental need of our laboratory is to be able to deliver low concentrations of proteins to the pregnant uterus during the peri-implantation period without adverse effects on the endometrial mucosa, conceptus development, or conceptus attachment. Thus, three gilts underwent bilateral catheterization of uterine horns and received infusions of 0.1% PSA from days 9 to 13 of pregnancy. Because some studies may require maintenance of pregnancy beyond the pre-implantation period, additional three gilts were used to determine whether catheterization and infusion of 0.1% PSA would be compatible with the maintenance of pregnancy for the duration of the period of conceptus attachment, extending through day 24 of pregnancy. All gilts underwent an initial surgery on day 9 of pregnancy and were ovariectomized on either day 13 or 24 of pregnancy.

Anesthesia was induced in gilts on day 9 of pregnancy by intramuscular injection of a tiletamine hydrochloride and zolazepam hydrochloride (6.6 mg/kg), and was maintained with isoflurane (2-4% in oxygen). Sterile ALZET[®] osmotic pumps (Durect Corp., Cupertino, CA), previously prepared as per manufacturer's instructions, were installed into each uterine horn. To install the pumps, Metzenbaum scissors were used to transect the uterine tube 3-5 cm distal to the uterotubal junction. The polyethylene catheter (1.2 mm outside diameter) was inserted into the transected end of the uterine tube and threaded toward the uterine horn until 1-2 cm of the catheter resided in the uterine horn. In some cases, the mesosalpinx was cut to relieve tortuosity of the uterine tube and facilitate passage of the catheter. Two encircling ligatures of 2-0 polygalactin 910 were placed around the uterine tube containing the polyethylene catheter approximately 2-3 mm apart and beginning 2-3 mm from the transected end of the uterine tube. The tails of the suture were wrapped around the tubing in a crossing fashion that allowed the suture to tighten around the tube if the tube began to slide out of the uterine tube. The junction of the uterine tube and polyethylene catheter was dried with a gauze sponge and 1-2 drops of sterile cyanoacrylate glue were applied to the uterine tube, catheter, and suture. After the catheter was secured in the uterine tube, the pump was slipped into the ovarian bursa and the mesovarium was sutured closed, using 2-0 polygalactin 910. Two additional encircling sutures were placed through the mesovarium, around the pump, to ensure that the pump remained in the bursa adjacent to the ovary (**Figure 4.1**). Ovariohysterectomies were performed on days 13 and 24 of pregnant gilts. Immediately after removal, reproductive tissues were visually assessed to

Figure 4.1. Surgical Implantation of Osmotic Pumps. A) The uterine tube was transected (arrow) and the polyethylene catheter inserted and passed toward and then into the uterine horn. The mesosalpinx (arrowhead) was often transected to help relieve tortuosity of the uterine tube and ease passage of the catheter. B) Encircling ligatures were used to secure the polyethylene catheter within the uterine tube (arrow) and 1-2 drops of sterile cyanoacrylate glue were applied to the uterine tube, catheter and suture. The pump was slipped into the ovarian bursa and two encircling sutures (arrowhead) were placed through the mesovarium, around the pump.



assure placement and patency of catheters and document the conditions of the ovaries and any gross abnormalities of the reproductive tract. In addition, any remaining fluid was aspirated from the removed pumps and measured to assure that pumps had delivered the expected volume of fluid over the experimental period. On day 13 of pregnancy, 50 mLs of 10 mM Tris was used to flush conceptuses from each horn separately. On day 24 of pregnancy, uterine horns were not flushed but rather opened longitudinally along the anti-mesometrial border. The presence and gross normality of fetuses was visually assessed. Day 13 conceptuses and full thickness uterine tissue samples from days 13 and 24 of pregnancy were fixed in 4% paraformaldehyde (4% PAF) prepared in phosphate buffered saline (PBS), pH 7.2. Routine processing of uterine and conceptus tissues into paraffin followed. Sections were processed and stained with hematoxylin and eosin (H&E), and evaluated by a board-certified pathologist.

Study Two. To evaluate the effect of exogenous recombinant LAP-RGD or LAP-RGE at the conceptus-maternal interface during the peri-implantation period, gilts received infusions in both uterine horns of either LAP-RGD, LAP-RGE, or PSA control (9.6 µg LAP-RGD or LAP-RGE per horn per day delivered in PSA vehicle for a total protein concentration of 1 mg/ml; n=5 gilts/treatment group) beginning on day 9 of pregnancy. Surgical implantation of two indwelling osmotic pumps was performed on day 9 of pregnancy, as described in *study 1*. Pumps delivered either LAP-RGD, LAP-RGE, or PSA into each uterine horn at a rate of 10 ul/hour. On day 13 of pregnancy, ovariectomies were performed and assessment of placement and patency of catheters was noted, as described in *study 1*. Conceptuses from each uterine horn were

flushed with 10 mM Tris HCl on day 9 of pregnancy, and both uterine and conceptus tissues were preserved as described in *study 1*.

Treatments that resulted in grossly normal conceptus development and maintenance of pregnancy on day 13 were used for an additional experiment using another group of gilts, beginning on day 9 of pregnancy and concluding at the completion of implantation, on day 24 of pregnancy. Day 9 pregnant gilts underwent surgical implantation of two indwelling osmotic pumps as described above; pumps delivered either LAP-RGE or PSA control (9.6 µg LAP-RGE per horn per day delivered in PSA vehicle for a total protein concentration of 1 mg/ml; n=5 gilts/treatment group) for approximately 6.5 days, until the 2 ml volume within the pump was expended. At ovariectomy on day 24 of pregnancy, uterine horns were opened along the anti-mesometrial border, and placental and fetal parameters including allantois length (cm), allantoic fluid volume (mL), fetal length (crown to rump, cm), fetal weights (g), and number of fetuses per litter were recorded. Placental attachment sites and fetuses were preserved in 4% PAF as described, and placental attachment sites were routinely processed, embedded in paraffin, sectioned, stained with H&E, and evaluated histopathologically as described above.

Immunohistochemistry

Rabbit anti-phosphorylated SMAD2/3 (p-SMAD2/3) from Santa Cruz (Santa Cruz, CA) was used for immunohistochemical staining. Uterine and conceptus paraffin embedded tissues were sectioned (5 µM) and mounted on Superfrost plus slides. Tissues

were then deparaffinized with xylene and rehydrated through a graded series of alcohol. Endogenous peroxidase was blocked with 0.3% H₂O₂ in dH₂O. Sections were subjected to antigen retrieval with citrate buffer (pH 6.2) at 80°C for 45 min. Sections were blocked with 10% normal goat serum at 25°C for 20 min, and incubated overnight at 4°C with primary antibody p-SMAD 2/3 (0.4 µg/ml) or control Rabbit IgG (0.4 µg/ml) in 2% bovine serum albumin (BSA) in PBS (PBSA). Sections were rinsed with PBSA and PBSA containing 1% Triton and incubated with biotinylated-conjugated secondary antibody diluted in PBSA containing 5% normal goat serum for 1 h at 25°C. Sections were then rinsed as described, incubated in Vectastain ABC (Vector Laboratories, Burlingame, CA) for 30 min at room temperature, and then rinsed again with PBS and 0.5 M Tris-HCl. Immunoreactions were detected with peroxidase solution (DAB/H₂O₂) and stopped by submerging in H₂O at 1.5 min after application of peroxidase solution. Sections were counterstained with Shandon Eosin-y aqueous (Thermo Scientific, Waltham, MA), rinsed with distilled water, dehydrated with the reverse series of graded alcohol, and covered slipped with Clarion Mounting Medium (Biomedica Corp, Foster City, CA). Sections were evaluated by two independent investigators who were blinded to sample identity and treatment. Intensity of p-SMAD2/3 immunostaining for trophoderm, uterine LE, and glandular (GE) was graded as; (–) no immunostaining, (+) weak and/or intermittent immunostaining, (++) moderate immunostaining, or (+++) strong immunostaining.

Statistical Analysis

Quantitative data from day 24 infused gilts in *study 2* were subjected to least-squares analysis of variance (ANOVA) using the General Linear Models procedures of the Statistical Analysis System (SAS Institute, Cary, NC). Tests of significance were performed using the appropriate error terms according to the expectation of the means squares for error, and $P < 0.05$ was considered statistically significant.

Photomicrography

Digital photomicrographs of immunohistochemistry and H&E stained sections were evaluated using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) interfaced with an Axioplan HR digital camera and Axiovision 4.3 software. Photographic templates were assembled using Adobe Photoshop (version 6.0, Adobe Systems Inc., San Jose, CA).

Results

Effects of Surgical Implantation of Osmotic Pumps and Infusion of PSA on Endometrial Morphology, Conceptus Development, and Conceptus Attachment (Study 1)

At hysterectomy, uteri from all three gilts exhibited normal muscular tone and visible exterior striations as is commonly observed at this stage of pregnancy, and catheters and pumps remained in place (**Figure 4.2**). In gilts receiving PSA treatment until day 13 of pregnancy, conceptuses were recovered from both uterine horns. Some variability in developmental stage was observed between and within the recovered

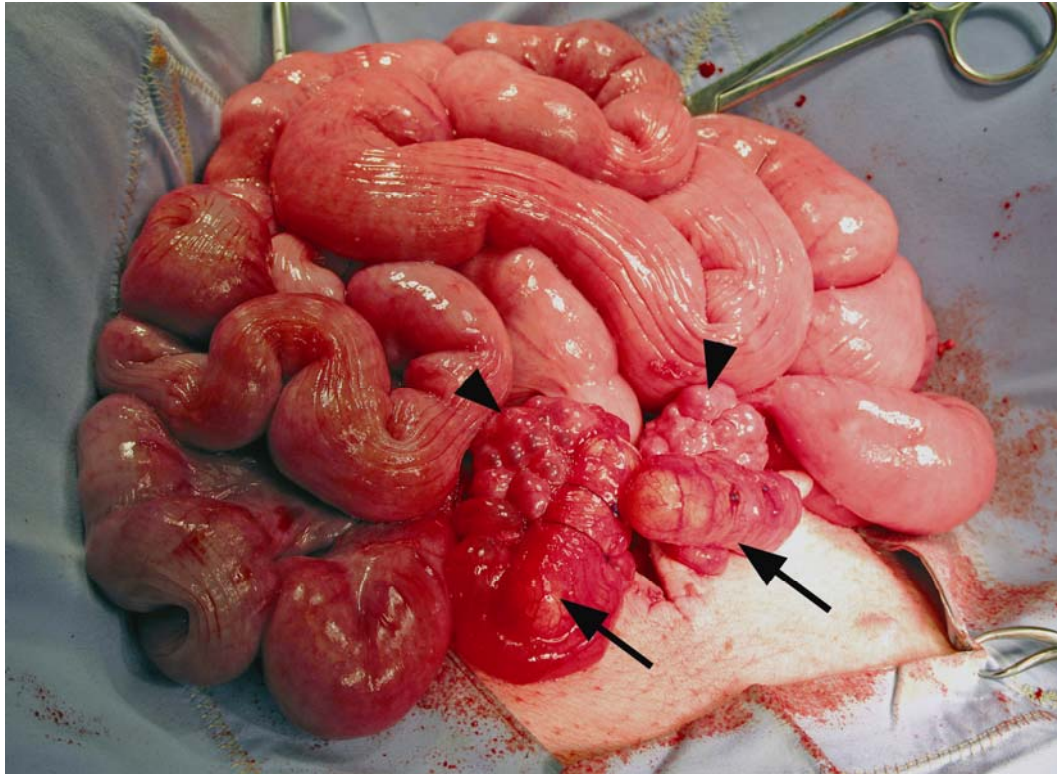


Figure 4.2. Exteriorized Uterus and Ovaries Just Prior to Ovariohysterectomy on Day 13 of Gestation. The muscular striations and uterine tone are usual for this day of gestation during which conceptuses secrete estrogens. Corpora lutea (arrowheads) are present on the ovaries. Pumps are enclosed within the ovarian bursa (arrows). Fluid accumulation within the bursa, as seen here, was observed occasionally.

litters, however, all conceptuses were in elongated, filamentous forms (**Figure 4.3a**). The mucosa of the endometrium, after opening the horns longitudinally, appeared grossly normal in all three gilts. In gilts receiving PSA treatments until day 24 of pregnancy, implantation sites were maintained, and development of fetuses appeared to be grossly normal (**Figure 4.4a**).

No significant histopathological abnormalities were noted in H&E stained sections of day 13 endometrial mucosae or conceptus tissue or day 24 implantation sites (**Figure 4.3b and 4.3c, Figure 4.4b**). All endometrial sections were characterized by mild submucosal edema with occasional dilated lymphatics, mild margination of neutrophils within submucosal veins, and rare submucosal eosinophils. Occasionally, a few necrotic or apoptotic cells were observed with day 13 conceptus trophoderm.

Intrauterine Infusion of Recombinant LAP-RGD Reduced Conceptus Elongation (Study 2)

Fifteen gilts underwent bilateral surgical implantation of osmotic pumps filled with LAP-RGD, LAP-RGE, or PSA control beginning on day 9 of pregnancy. On day 13 of pregnancy, infused gilts were ovariohysterectomized and morphology of their conceptuses were assessed. In the LAP-RGD infused gilts, reduction in conceptus elongation in both horns of 4 out 5 litters was grossly visible (**Figure 4.5**). In litter A, spherical and filamentous conceptuses with bulbous ends were present, and two spherical conceptuses had sizes 0.5 cm and 0.65 cm. In litter B, spherical and

Figure 4.3. Normal Filamentous Conceptuses Flushed from Uterine Horns of PSA Infused Pregnant Gilts on Day 13 of Pregnancy. A) Conceptuses flushed from the uterus of the PSA infused horns exhibited a range of filamentous morphology consistent with this stage of gestation. Bar = 1 cm. B) Filamentous conceptus showing normal morphologic development for this stage of gestation: embryonic ectoderm, arrow; trophoderm, arrowhead. Bar = 50 microns. C) Normal uterine tissue from PSA infused horns, day 13 of pregnancy, H&E stain. Endometrial mucosae were characterized by normal luminal epithelium (arrow) and slightly edematous underlying stromal tissue.

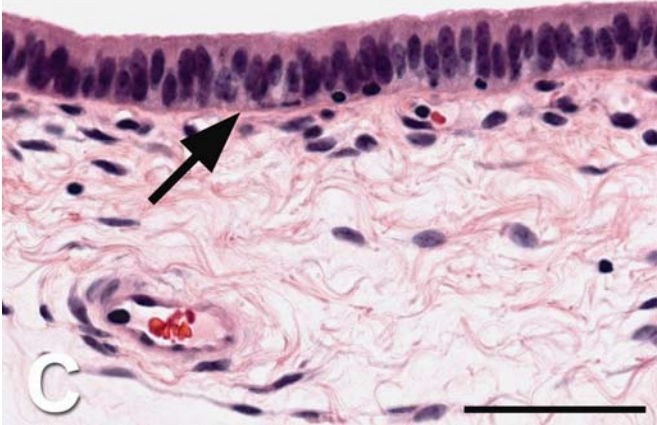
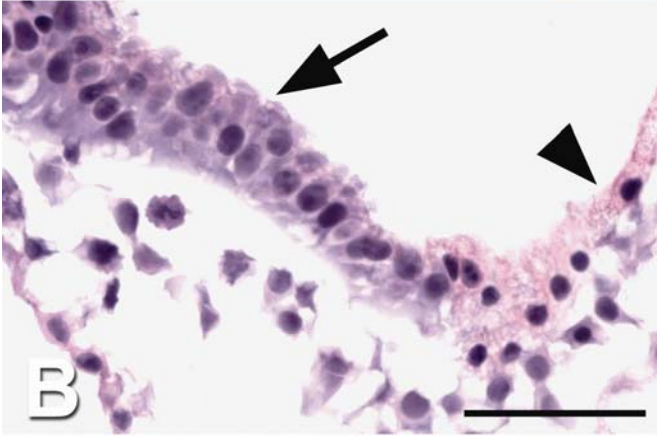
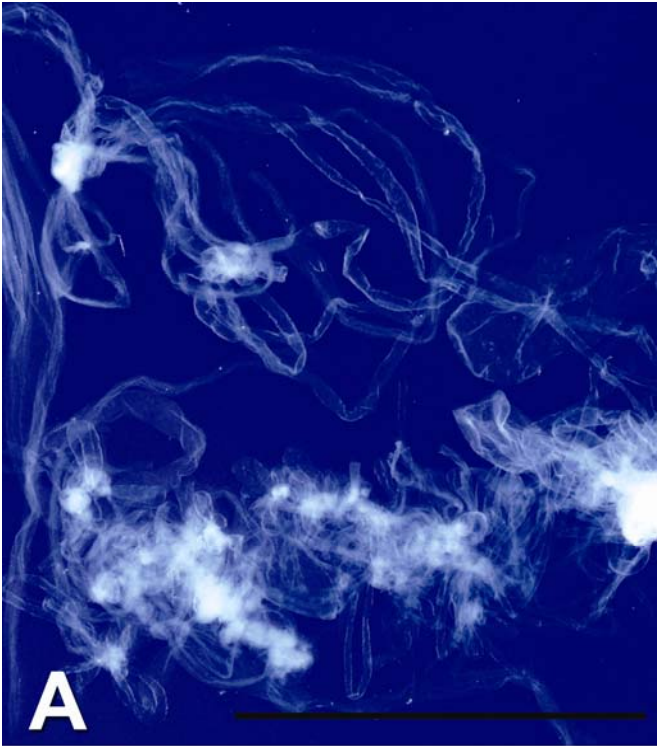


Figure 4.4. Normal Fetus and Placenta from PSA Infused Horn, Day 24 Gestation. A. Morphologically normal fetus (arrow) and placenta present in uterine horn infused with PSA beginning on day 9 gestation. Bar = 1 cm. B. The chorion of the conceptus is interdigitating with the underlying maternal endometrial epithelium (arrowhead); placentation appears to be proceeding in a normal manner. H&E stain; bar = 50 microns.

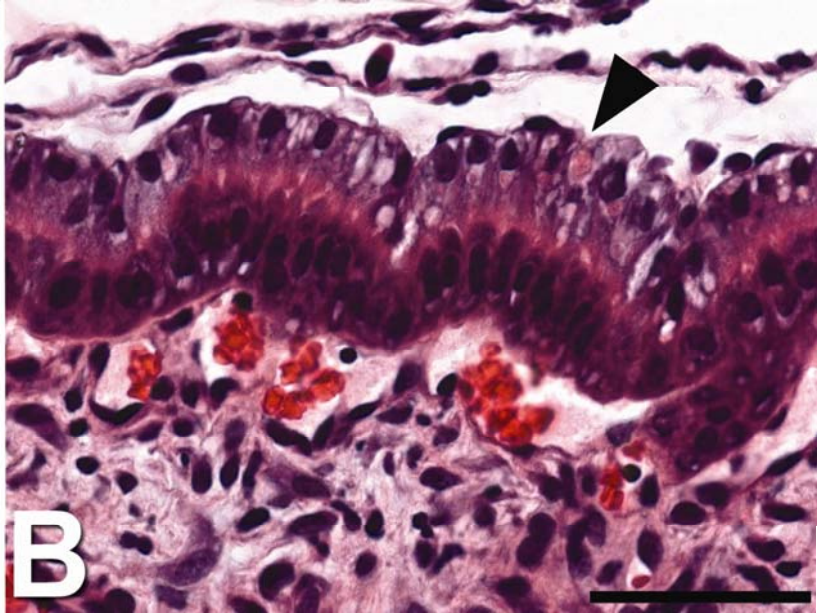
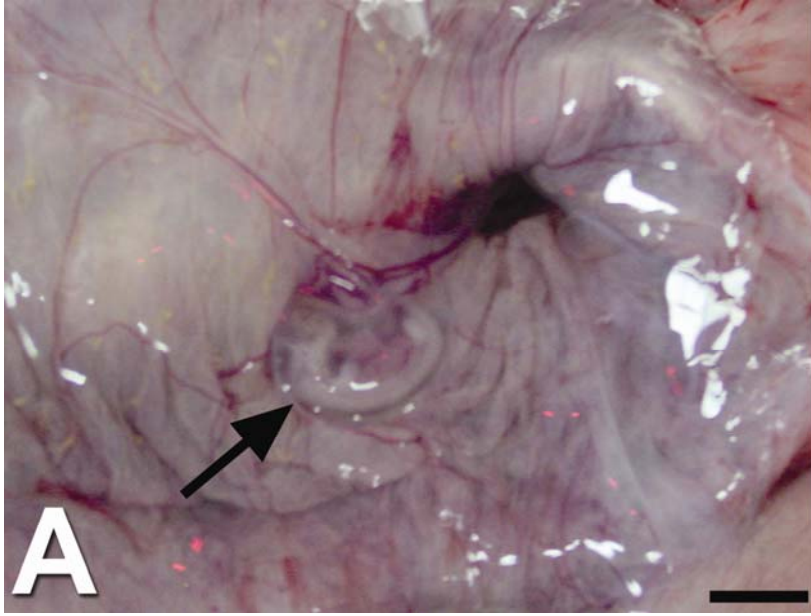
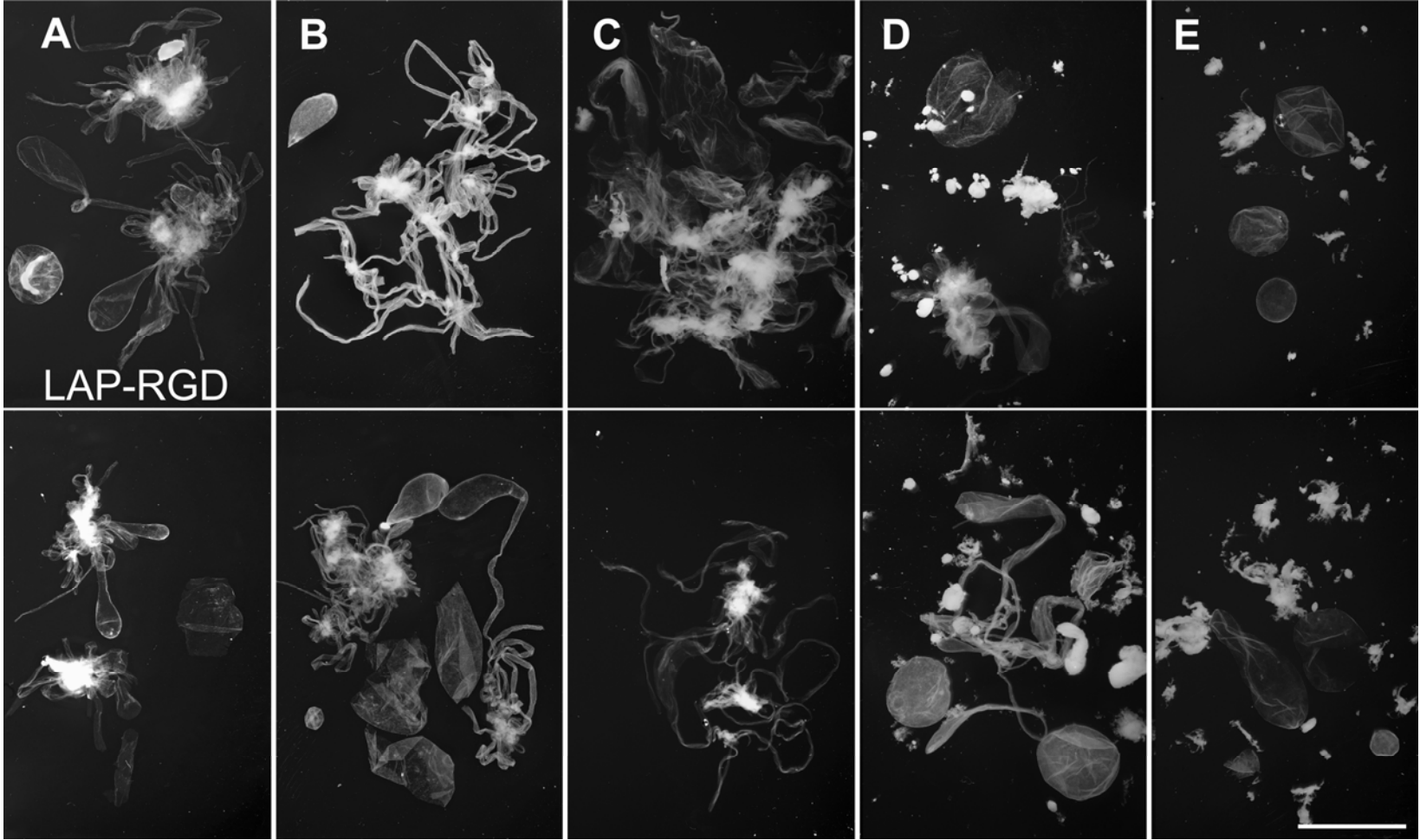


Figure 4.5. Morphology of Day 13 Conceptuses Flushed from Uterine Horns of LAP-RGD, LAP-RGE, and PSA Infused Pregnant Gilts. Conceptuses were flushed from right and left horns of LAP-RGD treated pregnant gilts (n = 5; top two rows; right horn, top row; left horn, second row). Litters A-E show spherical and filamentous conceptuses. Note litter E exhibited no elongation. Filamentous conceptuses were seen in LAP-RGE (third and fourth rows) and PSA (fifth and sixth rows) treatment groups. Bar = 1 cm.



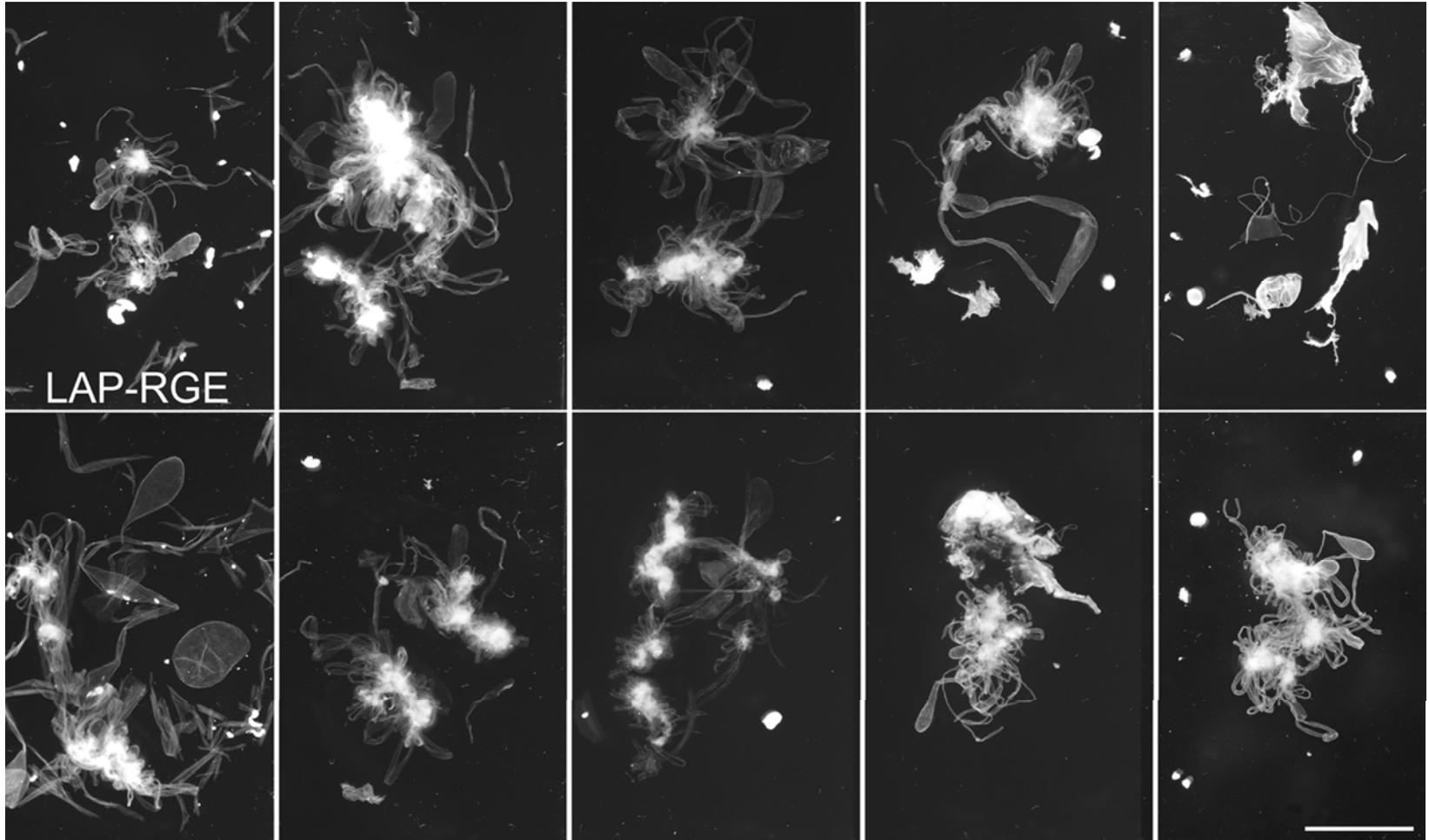


Figure 4.5. Continued

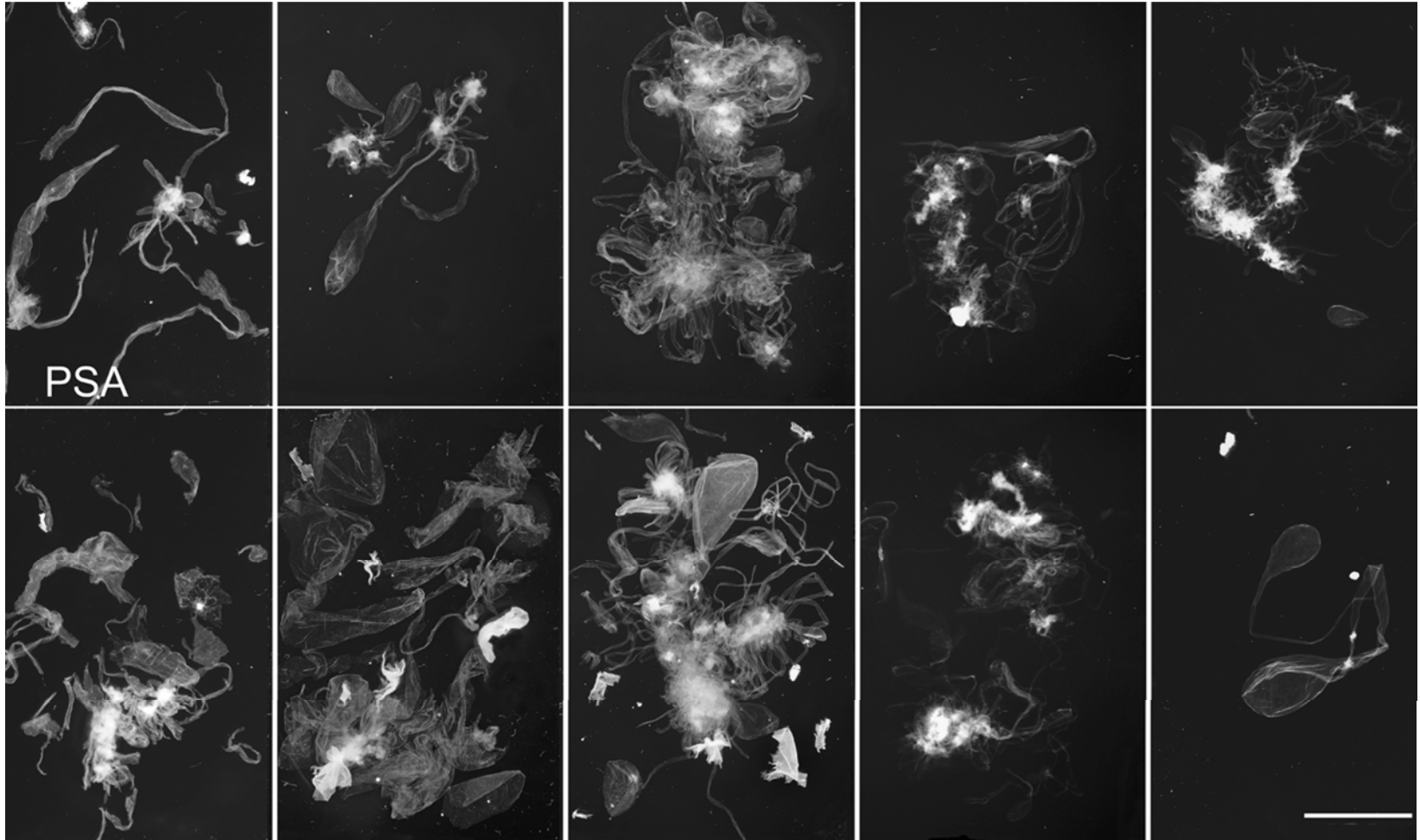


Figure 4.5. Continued

filamentous conceptuses were present, and 5 spherical conceptuses had sizes 0.15 cm, 0.5 cm, 0.9 cm, 1.0 cm, and 1.0 cm. In litter C, conceptuses appeared to elongate; however, some filamentous conceptuses appeared to have lost their normal structure and presented as sheets of trophoblast. In litter D, spherical and filamentous conceptuses with bulbous ends were present, and three spherical conceptuses had sizes 0.6 cm, 0.75 cm, and 0.8 cm. In litter E, all conceptuses failed to elongate; conceptuses were characterized as 5 spherical and 1 tubular and ranged in sizes 0.3 cm, 0.4 cm, 0.5 cm, 0.7 cm, and 0.9 cm; and 1.5 cm.

In LAP-RGE and PSA infused gilts, overall morphology of conceptuses in both treatment group were grossly normal (**Figure 4.5**). Nearly all conceptuses from all gilts in LAP-RGE and PSA treatment groups were filamentous, and few filamentous conceptuses contained bulbous ends. Only 1 spherical conceptus was reported for each LAP-RGE and PSA infused litters, and their sizes were 0.8 cm and 1.0 cm, respectively.

p-SMAD 2/3 Immunohistochemistry (Study 2)

SMAD proteins 2 and 3 are phosphorylated in response to TGFB signaling through TGFBRs and are subsequently translocated to nuclei. Therefore, immunohistochemical analysis of nuclear p-SMAD2/3 was performed on day 13 conceptus and endometrial tissues of each treatment group (LAP-RGD, LAP-RGE, or PSA) as an assessment of TGFB activity within uterine and conceptus tissues. In all treatment groups, p-SMAD2/3 was detected in nuclei of the conceptus trophectoderm, endometrial LE, endometrial GE, fibroblast cells, and endothelial cells (**Figure 4.6**).

Immunostaining for p-SMAD2/3 in the LAP-RGE treatment group appeared less intense in endometrial LE compared to immunostaining in LE of LAP-RGD and PSA treatment groups and was graded a lower score in blind-ended evaluations (**Table 4.1**).

Intrauterine Infusions of Recombinant LAP-RGE Affects Placental and Fetal Development (Study 2)

Conceptus development was impaired by day 13 of pregnancy when LAP-RGD was infused into the uterus; however, conceptuses from LAP-RGE treatment groups achieved elongation, and no disruption of pregnancy was apparent. Therefore, additional studies were employed to determine if infusing LAP-RGE resulted in long-term effects on conceptus attachment and/or fetal and placental development. Pregnant gilts were infused with LAP-RGE or PSA control beginning on day 9 of pregnancy and hysterectomized on day 24. These infusions, which spanned from day 9 to approximately day 15.5 of pregnancy, were designed to determine effects on implantation and conceptus development that would result from LAP-induced inhibition of TGFB activity that was not accompanied by concurrent alteration of integrin activity from the native RGD site present in LAP.

H&E sections of placental attachment sites in LAP-RGE and PSA groups indicated that infusions of LAP-RGE resulted in no detectable histopathological abnormalities in attachment (**Figure 4.7**). Fetal weight (g), fetal length (cm), allantois length (cm), allantoic fluid volume (mL), and number of fetuses for LAP-RGE and PSA

Figure 4.6. Immunohistochemical Localization of p-SMAD2/3 protein in paraffin-embedded uterine cross-sections from day 13 PSA (first column), LAP-RGD (second column), and LAP-RGE (third column) infused pregnant pig endometrium and conceptuses. The *brown color* indicates positive immunostaining for p-SMAD2/3. All sections were counterstained with Shandon Eosin-y aqueous. Sections stained with nonimmune rabbit IgG (fourth column) serve as negative controls. LE, luminal epithelium; GE, glandular epithelium; Tr, trophoctoderm. Width of fields are 230 μ m.

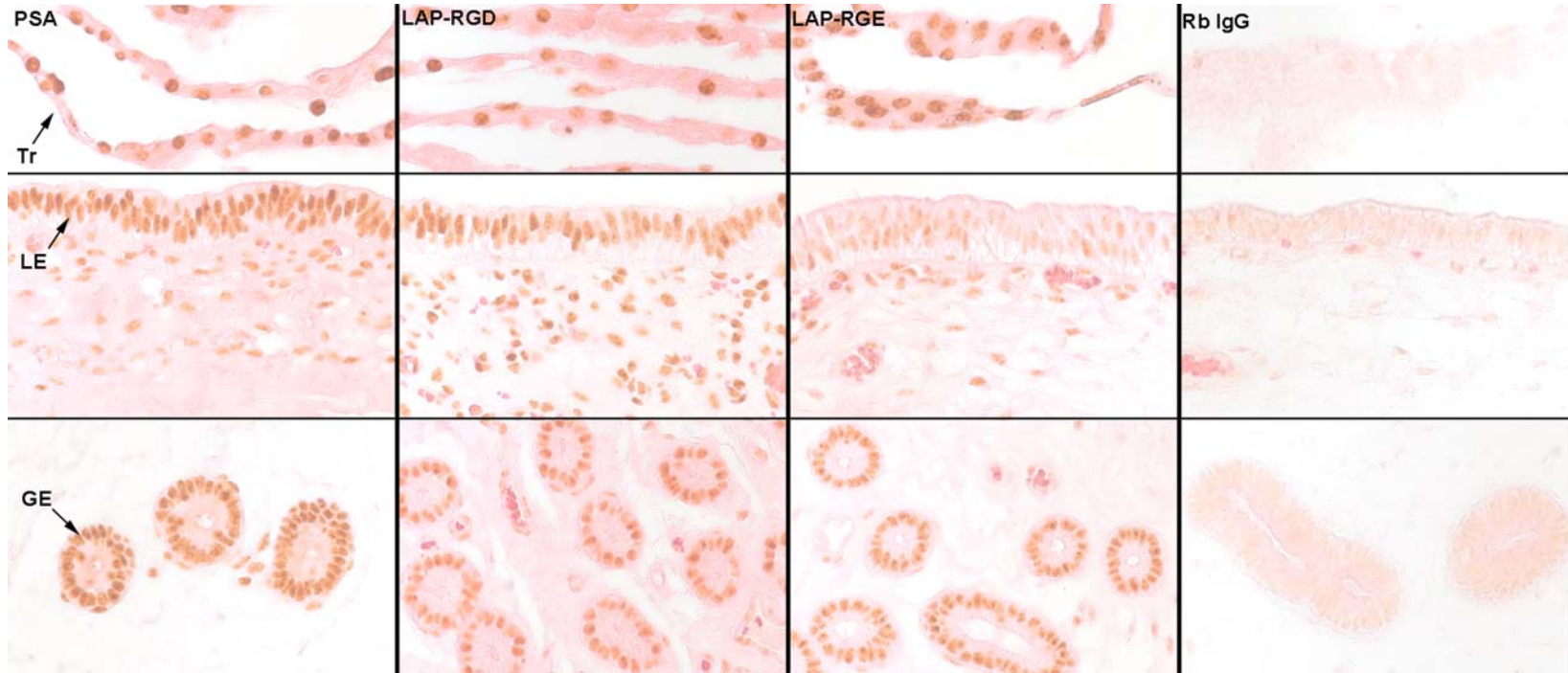


Table 4.1. p-SMAD 2/3 Porcine Endometrial and Conceptus Immunostaining for LAP-RGD, LAP-RGE, or PSA Infused Gilts (Day 13 of Pregnancy). p-SMAD 2/3 immunostaining was evaluated visually within cell population, (-) no immunostaining, (+) weak, (++) moderate, or (+++) strong.

IHC p-SMAD2/3 Detection: Comparison
Across Treatment Groups

	LE	GE	Tr
PSA	+++	+++	+++
LAP-RGD	+++	++	+++
LAP-RGE	++	+++	+++

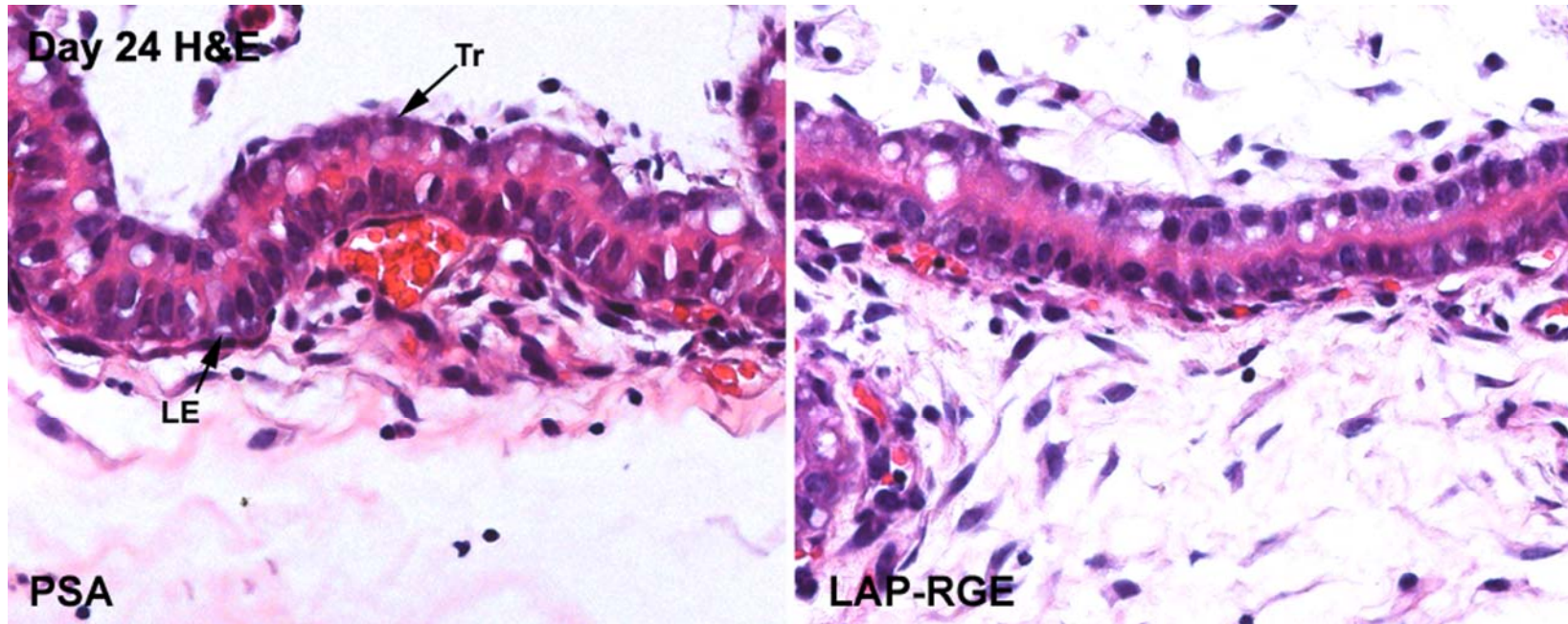


Figure 4.7. Conceptus Attachment Site From PSA and LAP-RGE Infused Gilts, Day 24 Gestation. PSA and LAP-RGE exhibited normal conceptus attachment and placentation appears to be proceeding in a normal manner. LE, luminal epithelium; Tr, trophoblast. H&E stain.

treatment groups were recorded at ovariohysterectomy on day 24 of pregnancy. The least square means and standard errors for LAP-RGE treatment group were 0.47 ± 0.04 g fetal weight, 1.55 ± 0.08 cm fetal length, 11.85 ± 0.97 cm allantois length, 54.37 ± 5.38 mL allantoic fluid volume, and 8.60 ± 0.71 number of fetuses per litter. Means and standard deviations for PSA control group were 0.29 ± 0.04 g fetal weight, 1.36 ± 0.08 cm fetal length, 8.17 ± 1.03 cm allantois length, 24.16 ± 4.89 mL allantoic fluid volume, and 8.20 ± 0.71 number of fetuses (**Table 4.2**). Compared to PSA infusions, LAP-RGE infusions resulted in larger fetal mass ($P < 0.05$), allantois length ($P < 0.05$), and allantoic fluid volume ($P < 0.01$; **Figure 4.8**).

Discussion

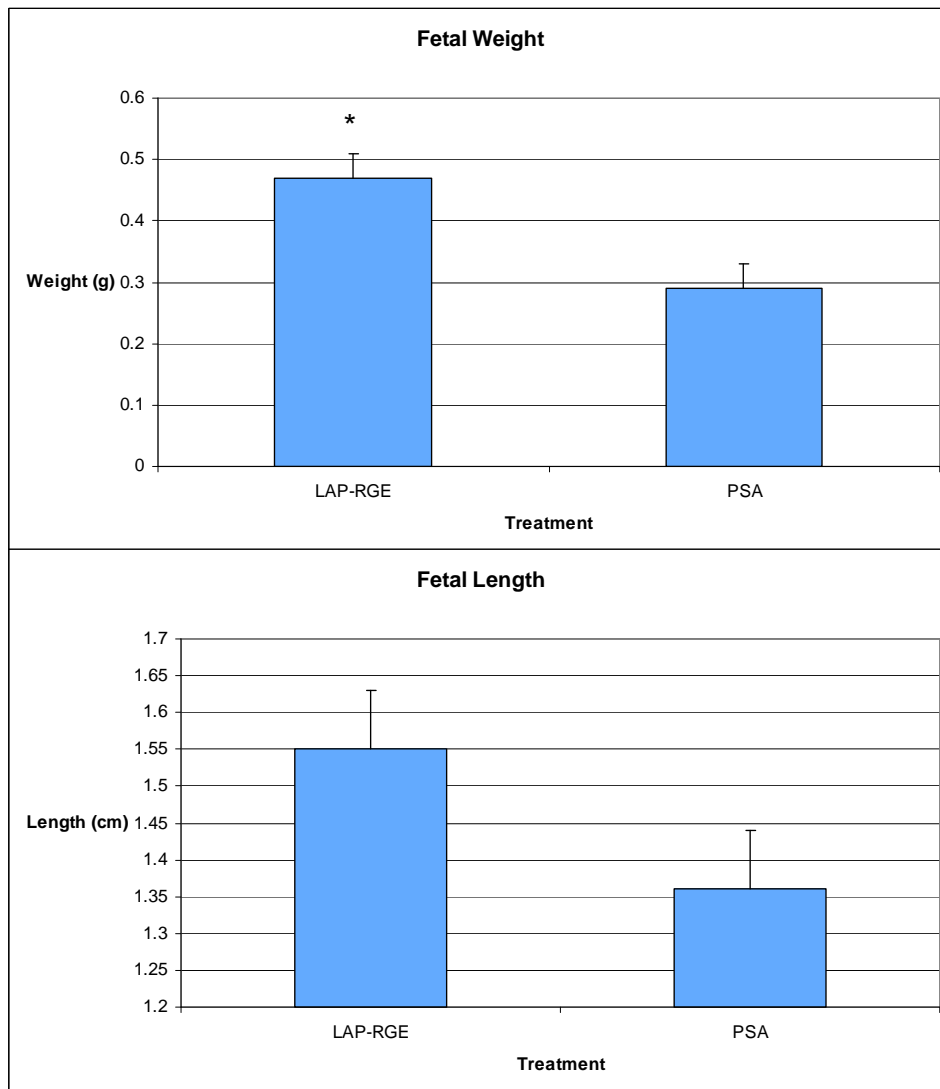
Results of *study 1* indicate that substances can be infused in a continuous, controlled manner to the intrauterine lumen of pregnant gilts and, further, that such infusion is compatible with maintenance of pregnancy. The surgical techniques described in this report were successful in allowing continual infusions into the uterine lumen over the entire time periods defined. Compared to alternative methods that use exteriorized catheters (Harney & Bazer 1990, Sample *et al.* 2000, Sample *et al.* 2004, Jana *et al.* 2007), the use of surgically implanted, commercially available osmotic pumps permits minimal manipulations and disruptions to the animal during the period of treatment, continual infusion of substances rather than multiple bolus treatments, and decreased risk of infection from environmental sources. The process of infusion did not result in any gross or histopathologically significant alterations of the endometrium and

Table 4.2. Mean \pm SEM for Fetal Weight, Fetal Length, Allantois Length, Allantoic Fluid Volume, and Number of Fetuses. Tests of significance were performed using the appropriate error terms according to the expectation of the means squares for error, and $P < 0.05$ was considered statistically significant. NS, not significant ($P > 0.05$).

Treatment	Fetal Weight (g)	Fetal Length (cm)	Allantois Length (cm)	Allantoic fluid Volume (ml)	Number of Fetuses
LAP-RGE	0.47 \pm 0.04	1.55 \pm 0.08	11.85 \pm 0.97	54.37 \pm 5.38	8.6 \pm 0.71
PSA	0.29 \pm 0.04	1.36 \pm 0.08	8.17 \pm 1.03	24.16 \pm 4.89	8.2 \pm 0.71
	P<.005	NS	P<.05	P<.01	NS

Figure 4.8. Fetal Weight, Fetal Length, Allantois Length, Allantoic Fluid Volume, and Number of Fetuses from Day 24 LAP-RGE and PSA Infused Gilts in *Study 2*.

Mean \pm SEM for fetal weight, fetal length, allantois length, allantoic fluid volume, and number of fetuses are represented in bar graphs. Fetal weights ($P < 0.05$), allantois length ($P < 0.05$), and allantoic fluid volume ($P < 0.01$) were larger in LAP-RGE treatment group compared to PSA treatment group.



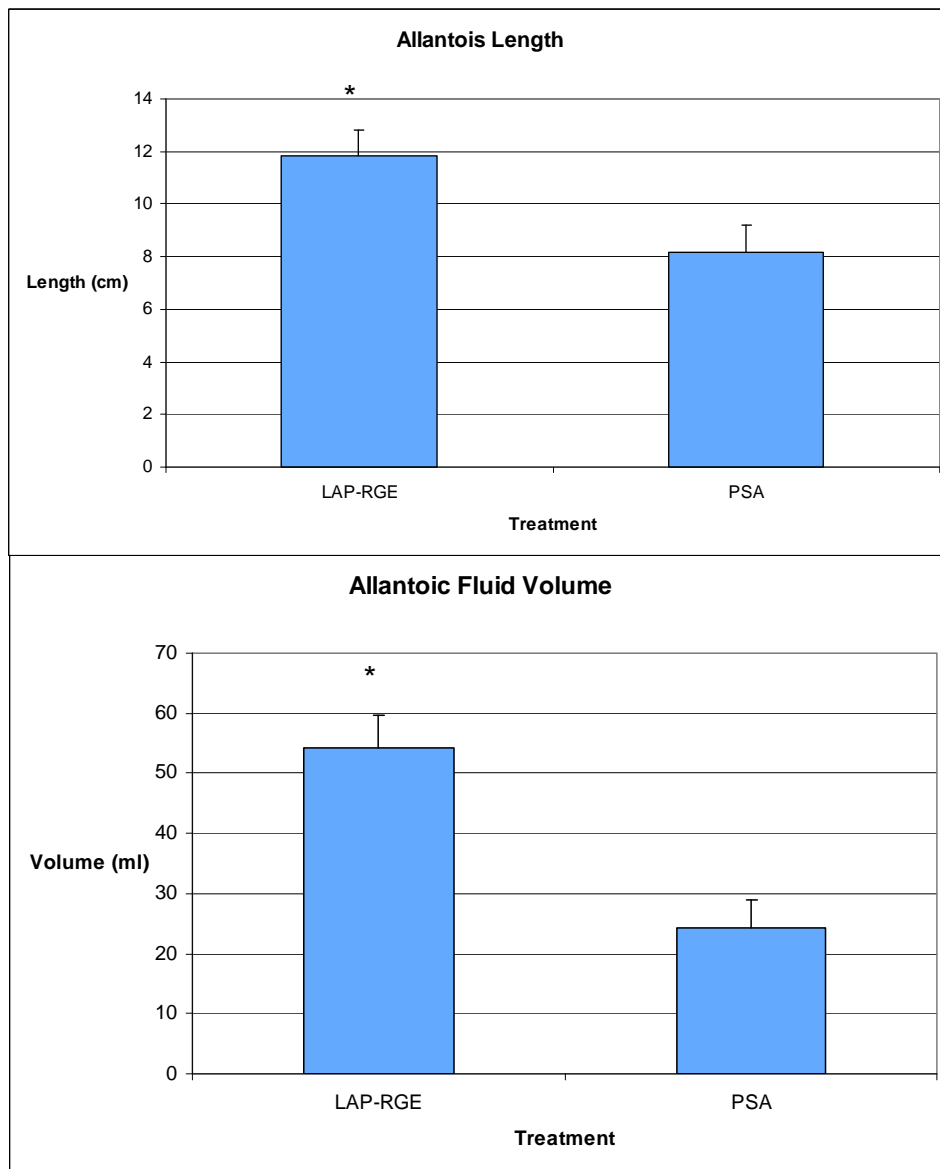


Figure 4.8. Continued

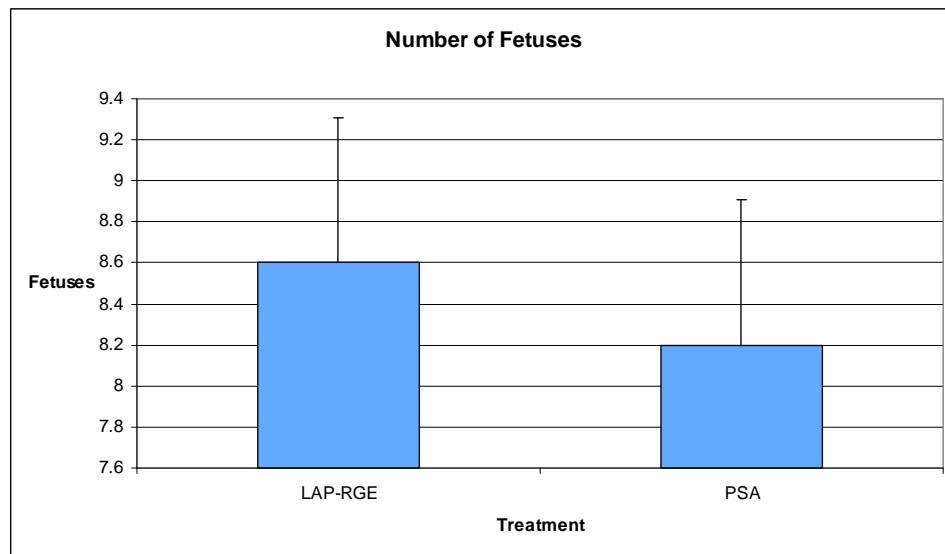


Figure 4.8. Continued

conceptus tissues or conceptus attachment sites at day 24 of pregnancy. Mild submucosal edema, dilated lymphatics, mild margination of neutrophils, and submucosal eosinophils were observed in the endometrium from all uteri; all changes were consistent with procedural effects of surgery resulting from clamping, suturing, and/or handling and are not considered to be pathologically significant. Thus, it appears that the infusion technique reported in *study 1* would be broadly applicable to a wide range of studies focused on endometrial function during pregnancy. Finally, results of this study clearly demonstrate that uterine infusion is compatible with maintenance of pregnancy through the peri-implantation period.

Results of *study 2* demonstrate that intrauterine infusions of LAP-RGD, but not LAP-RGE or PSA, beginning on day 9 of pregnancy in gilts resulted in a reduction of conceptus elongation. Further, intrauterine infusions of LAP-RGE through the peri-implantation period grossly resulted in normal implantation and placental and fetal development. Further assessment of fetal and placental parameters indicated that infusing with LAP-RGE results in larger fetal weight, allantois length, and allantoic fluid volume compared to infusing with PSA.

Alterations in Integrin-Ligand Interactions Reduces Conceptus Elongation

Conceptus loss can reach 18% during pre-implantation, and during this time, conceptus extraembryonic tissues elongate from a 5 mm sphere to a 150 mm filamentous form at a rate of 30-40 mm/hr between days 10 and 12 of pregnancy (Geisert *et al.*

1982b, Bennett & Leymaster 1989). The elongated conceptus provides increased surface area for exchange of nutrients between the conceptus and uterus and is essential for successful implantation. Investigating which factors mediate conceptus elongation is complicated by the failure of conceptuses to elongate *in vitro* (Stroband & Van der Lende 1990).

We are the first to demonstrate *in vivo* inhibition of conceptus elongation via intrauterine infusions during early pregnancy in pigs. Infusions of LAP-RGD inhibited conceptus elongation, whereas similar infusions of LAP-RGE had no visible effect on conceptus elongation. LAP-RGD, but not LAP-RGE, supports binding to integrins, and intrauterine infusions of LAP-RGE did not affect conceptus elongation, implicating integrin-mediated effects in inhibiting conceptus elongation. In studies involving mice, intrauterine injections of RGD containing peptides and neutralizing monoclonal antibodies against integrin subunits resulted in the reduction of implantation (Illera *et al.* 2000). In addition, phenotypes in mice with ITGA4, ITGA5, ITGAV, and ITGB1 null mutations resulted in failure of chorioallantois fusion, placental defects, and embryonic deaths (Dickson *et al.* 1995, Fassler & Meyer 1995, Stephens *et al.* 1995, Hynes & Bader 1997, Bader *et al.* 1998, Chung *et al.* 2000). In recent studies, intracellular signaling pathways involving TGF β and integrins were identified during conceptus elongation suggesting mechanisms for growth factor signaling and cytoskeletal rearrangement/cell motility during conceptus elongation (Blomberg *et al.* 2008). These studies, along with our current study, provide evidence that integrins are critical during the peri-implantation period.

Integrins via receptor-ligand induced formation of multi-protein intracellular focal adhesions can serve to provide links to the actin cytoskeleton and activate multiple downstream signaling pathways resulting in reorganization of the cytoskeleton and regulating cellular behaviors such as cell proliferation, differentiation, and migration (Gilcrease 2007). A potential mechanism for LAP-RGD inhibition of conceptus elongation is that infused LAP-RGD binds one or more integrins inappropriately, thus preventing other integrin-ligand interactions or, alternatively, inappropriately stimulates ITGAV-containing heterodimers, therefore, causing interruptions in linking the actin cytoskeleton and intracellular signaling cascades that may critical for conceptus elongation.

In the LAP-RGD treatment group, some conceptuses achieved elongation. Osmotic pumps installed on day 9 of pregnancy delivered infusate at a rate of 10 $\mu\text{l/hr}$. A slow continuous infusion of protein is optimal for maintaining physiological concentrations; however, the infusate may not reach the entire length of the uterine horn in time to completely disrupt receptor-ligand interactions. As a result, the conceptuses that achieved morphological elongation may not have been exposed to exogenous LAP-RGD within a critical period which, based on morphology of conceptuses, appears to encompass 10 of pregnancy.

TGFB is Functional in LAP-RGD and LAP-RGE Treatment Groups

Infusing LAP-RGD and LAP-RGE is expected to disrupt the balance of active

TGFB at the conceptus-maternal interface. Detection of p-SMAD2/3 in day 13 conceptus and endometrial tissues of LAP-RGD, LAP-RGE, and PSA treatment groups provides evidence that TGFB signals through TGFBRs and activates downstream SMAD signaling in conceptus and endometrial tissues during LAP intrauterine infusions. LAP-RGD and LAP-RGE infusions may initially recombine with active TGFB, rendering it latent; however, several mechanisms involving integrins and proteases can activate TGFB.

Integrins have been implicated in activation of TGFB *in vivo* (Sato & Rifkin 1989, Shull *et al.* 1992, Taipale *et al.* 1992, Huang *et al.* 1996, Bader *et al.* 1998, Zhu *et al.* 2002). Integrins bind to latent TGFB via the RGD sequences in LAP and activate TGFB via traction forces or with the aid of proteases (Munger *et al.* 1998, Munger *et al.* 1999, Annes *et al.* 2002, Mu *et al.* 2002, Asano *et al.* 2005a, Asano *et al.* 2005b, Wipff & Hinz 2008). ITGAV- containing heterodimers are present in trophoctoderm and uterine LE during porcine implantation (Bowen *et al.* 1996, Burghardt *et al.* 2002), and in addition to integrins, proteases such as plasmin and matrix metalloproteinases (MMPs) have been identified during porcine pregnancy. Proteases involved in TGFB activation include, but not limited to, plasmin and MMPs, which activate TGFB mostly by proteolytic cleavage of LAP (Lyons *et al.* 1990, Yu & Stamenkovic 2000, Jenkins 2008). MMP2 is present during porcine pregnancy with increasing detectable amounts between days 10 to 14 of pregnancy (Foxcroft *et al.* 2000, Kayser *et al.* 2006). Plasminogen, plasmin's proprotein, was also found within the uterine lumen, and its activator urokinase plasminogen activator (uPA) is expressed in porcine blastocysts,

suggesting potential for temporal production of plasmin (Fazleabas *et al.* 1983). In LAP-RGD treated gilts, TGFB scavenged by LAP-RGD can presumably be activated via integrins and proteases such as MMP2 and plasmin. However, in LAP-RGE treated gilts, because LAP-RGE does not support binding to integrins, it is reasonable to assume that TGFB scavenged by LAP-RGE may be activated only by proteases and is not available for integrin-mediated activation.

LAP-RGE Affects Placental Size

Porcine implantation is non invasive; therefore, there is a need for a large surface area between placental and endometrial tissue for exchange of nutrients to support the growing fetus. Placental efficiency is measured by fetal weight-to-placental weight ratio, and large placental sizes are implicated as a limiting factor to litter size (Wilson *et al.* 1999, Wilson & Ford 2001). Novel experiments by Wilson and Ford, have demonstrated that administration of estradiol-17B around the time of conceptus elongation results in increased placental size at term. Increases in estradiol-17B may affect endometrial secretions of growth factors, which may ultimately affect placental size (Wilson & Ford 2000). In support of this, our studies have demonstrated that infusing exogenous LAP-RGE during the time of conceptus elongation results in increased fetal weight, allantois length, and allantoic fluid volume; however, litter size and fetal length were not affected. In our model, infusing LAP-RGE appeared to result in a decrease in SMAD signaling in the uterine LE. The apparent decrease in SMAD signaling in epithelial cells may affect critical TGFB-regulated processes such as cell

growth arrest, proliferation, apoptosis, migration, and differentiation during porcine pregnancy. TGF β participates in regulating cellular growth, apoptosis, and differentiation in many cell types including epithelial, endothelial, and mesenchymal cells (Rahimi & Leof 2007). TGF β can induce cell cycle arrest by up-regulating cell cycle inhibitors such cyclin-dependent kinase (CDK) inhibitors via SMAD signaling pathways. In addition, TGF β signaling via SMADs can also up-regulate pro-apoptotic proteins, down-regulate pro-survival proteins, and induce epithelial-mesenchymal transition (Rahimi & Leof 2007). We hypothesize that alterations in TGF β induced SMAD signaling regulating cellular behavior such as growth, proliferation, migration, and apoptosis during the porcine peri-implantation period result in larger placental and fetal parameters, particularly allantois lengths and fetal weight as seen in day 24 LAP-RGE infused gilts.

In summary, we have developed a successful technique for immobilizing osmotic pumps in the porcine reproductive tract and delivering substances into the luminal cavity of the uterus. This methodology should prove useful for studies of uterine physiology and pathophysiology during early conceptus development that are relevant to pigs and other mammals. By use of these novel techniques, we demonstrate that infusing LAP-RGD beginning on day 9 of porcine pregnancy reduces conceptus elongation suggesting that appropriate integrin-ligand interactions are necessary for successful conceptus elongation during porcine pregnancy. In addition, infusing LAP-RGE beginning on day 9 of porcine pregnancy results in larger fetal weight, allantoic length, and allantoic fluid volume compared to infusing with PSA. The phenotype seen at day 24 of pregnancy

may be a result of altering TGF β induced SMAD signaling in epithelial cells during the critical time of conceptus elongation and implantation ultimately affecting fetal weight, allantoic length, and allantoic volume. Although the precise mechanisms are not known at this time, current results implicate TGF β and integrins as contributing factors in the regulation of conceptus elongation and placental and fetal size.

CHAPTER V
INVESTIGATIONS INTO MECHANISMS UNDERLYING
LAP-RGD INDUCED INHIBITION OF CONCEPTUS ELONGATION

Introduction

Porcine implantation is superficial and non-invasive, and prior to their attachment to the uterine luminal epithelia (LE), conceptuses undergo elongation. Porcine conceptus elongation occurs between days 11 and 12 of pregnancy, and conceptuses elongate from spherical to filamentous forms. Spherical day 10 porcine blastocysts (2-6 mm) undergo morphological transition to larger spheres (10 to 15 mm) to tubular (15 mm by 50 mm) and then to filamentous (1 mm by 100-200 mm) forms by day 12 of pregnancy (Geisert *et al.* 1982b). The elongated conceptus provides increased surface area for exchange of nutrients between the conceptus and uterus and is essential for successful implantation (Stroband & Van der Lende 1990).

Molecules such as transforming growth factor betas (TGFBs) and integrins are among the various signaling molecules that have been reported to be present in uterine and placental tissues prior to and during implantation in pigs (Bowen *et al.* 1996, Gupta *et al.* 1998a, Burghardt *et al.* 2002). TGFB is a homodimer containing non-covalent associations with its prepropeptide homodimer, latency associated peptide (LAP) (Lawrence *et al.* 1984, Gentry *et al.* 1988). Like many ECM proteins, LAP1 and 3 monomers, which correspond to their respective TGFB1 and 3 isoforms, contain an arginine-glycine-aspartate (RGD) site. Previous studies have demonstrated that LAP

binds to integrin heterodimers via this RGD sequence (Munger *et al.* 1998, Mu *et al.* 2002, Ludbrook *et al.* 2003). Interactions between LAP and integrins, along with TGFB and TGFB receptors (TGFBRs) and the signals they transmit, may be important in supporting events of conceptus elongation and attachment. Mechanisms supporting conceptus elongation are largely unknown; however, our studies indicate that infusion of exogenous LAP with a native RGD sequence into the uterine lumen of pregnant gilts, beginning day 9 of pregnancy, reduced conceptus elongation. Conceptus elongation was not affected in gilts similarly infused with exogenous LAP which contained a mutated sequence (RGE). *In vitro*, LAP decreases TGFB activity by recombining with active TGFBS, rendering them latent, and thus inhibiting TGFB from binding to TGFBRs; therefore, infusing exogenous LAP into the uterine lumen of pregnant gilts was expected to alter receptor-ligand interactions of TGFB and TGFBRs. Further, infusions of LAP-RGD, but not LAP-RGE, are also expected to alter normal integrin signaling.

In an attempt to investigate mechanisms of action that resulted in reduction of conceptus elongation in LAP-RGD infused gilts, a wide range of experiments was employed in this study. Based on our results, we hypothesize that infused LAP-RGD bound to integrin ligands via the RGD sequence, preventing other ligands from binding to integrins, and therefore, impeding integrin signaling critical to mediating initiation of conceptus elongation and successful attachment. It is important to note that, at the time some of the experiments were initiated, we had conducted only LAP-RGD infusions and not the LAP-RGE infusions, so the possibility that the observed inhibition of conceptus

elongation could be attributed to altered TGFB levels was still a viable alternative hypothesis.

Materials and Methods

Animals

Experimental procedures were approved by Texas A&M University's Animal Care and Use Committee (AUP # 2007-154). Crossbred gilts were checked daily for estrus, and bred approximately 12 and 24 hours after their second detected estrus. Anesthesia was induced in gilts, on day 9 of pregnancy, by intramuscular injection of a tiletamine hydrochloride and zolazepam hydrochloride (6.6mg/kg) and was maintained with isoflurane (2-4% in oxygen). Sterile ALZET[®] osmotic pumps (Durect Corp., Cupertino, CA), previously prepared as per manufacturer's instructions, were installed into each uterine horn. The polyethylene catheter (1.2 mm outside diameter) was inserted into the transected end of the uterine tube and threaded toward the uterine horn until 1-2 cm of the catheter resided in the uterine horn.

Sterile pumps contained either exogenous recombinant LAP-RGD, LAP-RGE, or PSA control (9.6 micrograms LAP-RGD or LAP-RGE per horn per day delivered in PSA vehicle for a total protein concentration of 1 mg/ml; n=5 gilts/treatment group) beginning on day 9 of pregnancy. Pumps delivered either LAP-RGD, LAP-RGE, or PSA into each uterine horn at a rate of 10 ul/hour.

Ovariohysterectomies were performed on day 13 pregnant gilts. Immediately after removal, reproductive tissues were assessed visually to assure placement and

patency of catheters and document the conditions of the ovaries and any gross abnormalities of the reproductive tract. Conceptuses were flushed from uterine horns with 10 mM Tris. Uterine horns were opened along the anti-mesometrial border, and uterine tissues were preserved by embedding in Tissue-Tek Optimal Cutting Temperature Compound (OCT, Miles, Inc, Onenta, NY) and freezing in liquid nitrogen. Remaining uterine tissues were preserved in phosphate buffered 4% paraformaldehyde (PAF) and embedded in paraffin.

Immunofluorescence

Antibodies used for immunofluorescence staining included: goat anti-LAP (#AB-246-NA) and chicken anti-TGFB (#AF-101-NA) from R&D Systems (Minneapolis, MN); rabbit anti-ITGAV (#AB1930), ITGB1 (#AB1952), ITGB3 (#AB1932) and ITGB5 (#AB1926) integrin subunits from Chemicon (Temecula, CA); and rabbit anti-ITGB6 (beta 6, #SC15329) and ITGB8 (beta 8, #SC25714) integrin subunits from Santa Cruz (Santa Cruz, CA). Secondary antibodies used for immunofluorescence staining included FITC-conjugated rabbit anti-chicken, Texas Red-conjugated mouse anti-goat from Pierce Biotechnology Inc. (Rockford, IL) and FITC-conjugated donkey anti-rabbit from Southern Biotech (Birmingham, Alabama). Anti-LAP detected dimeric and monomeric LAP from TGFB isoform 1 in western blotting. In addition, anti-LAP was specific for latent TGFB1 immunostaining (Barcellos-Hoff et al. 1994; Ehrhardt et al. 1997). Anti-TGFB recognizes active TGFB1 not associated with LAP (Ehrhardt et al. 1997).

Frozen uterine and conceptus tissues were sectioned (8 μ M), mounted on Superfrost Plus glass slides (Anapath, Cheyenne, WY), and immunofluorescence co-localization of LAP and TGFB were performed as described by Ewan et al., (2002) with minor modifications. Frozen sections were fixed with 2% PAF, washed with 0.1 M glycine in .02 M phosphate buffered saline (PBS), blocked with 0.5% casein in PBS, and incubated overnight at 4°C with TGFB and LAP primary antibodies at concentrations of 1.25 μ g/ml and 15 μ g/ml, respectively. Controls included separate sections incubated with TGFB and irrelevant goat IgG; LAP and irrelevant chicken IgG; and with chicken IgG and goat IgG. Sections were incubated with appropriate FITC- or Texas Red-conjugated secondary antibodies for 1 h at room temperature at a concentration of 3.75 μ g/ml and 2.5 μ g/ml, respectively. Slides were overlaid with Prolong antifade mounting reagent containing the nuclear counterstain DAPI (Invitrogen, Molecular Probes, Eugene, OR) and then cover slipped.

For integrin single labeled immunofluorescence, sections were fixed and washed as described above then blocked in 10% normal goat and normal donkey sera. Sections were incubated overnight at 4°C with each integrin subunit ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, or ITGB8 primary antibody (5 μ g/ml). Controls included separate sections incubated with irrelevant rabbit IgG. Sections were incubated with FITC-conjugated secondary antibodies and cover slipped as described above.

Photomicrography

Representative immunofluorescence images were collected and analyzed using an

Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) interfaced with an Axioplan HR digital camera and Axiovision 4.6 software. Because of Axiovision's multidimensional acquisition with individual fluorophore channels, each fluorophore was captured as separate channels, and both single and double labeled sections were visualized. Images were converted to TIFF file format, and Adobe Photoshop CS2 (version 9.0, Adobe Systems Inc., San Jose, CA) was used to generate photographic composite images.

In Situ Hybridization

Expression of *TGFBI*, plasminogen activator (*PA*), matrix metalloproteinases 2 and 9 (*MMP2*, 9), and fibronectin (*FN*) was localized in paraffin-embedded porcine uterine tissue by in situ hybridization followed by the Wilcox protocol (Wilcox 1993). Paraffin embedded tissues were sectioned (5 μ M) and mounted on Superfrost Plus glass slides. Sections were deparaffinized and fixed in 4% PAF. Hybridizations were performed with 5×10^5 cpm [α -³⁵S-UTP] labeled antisense *TGFBI*, *PA*, *MMP2*, *MMP9*, and *FN* cRNAs at 55° C overnight. Non specific hybridization was determined using 5×10^5 cpm [α -³⁵S-UTP] labeled sense estrogen receptor (ER) cRNAs. Sections were then washed and treated with 20 ug/ml RNase A for 30 min. Sections were washed and dehydrated with graded ETOH and allowed to dry for 1 to 4 hr at 37°C. The signal was visualized by exposing the sections to Kodak autoradiographic emulsion NTB-2 at 4°C for 6 to 8 weeks. Sections were counterstained with hematoxylin.

Cell Culture

Mink lung epithelial cells (MLEC) transfected with an expression construct containing a truncated PAI-1 promoter fused to a firefly luciferase reporter gene was kindly provided by Dr. D. B. Rifkin. MLEC were cultured as previously reported (Abe et. al. 1994). Briefly, cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Gemini Bio-Products, West Sacramento, CA), 200ug/ml of Geneticin (G-418 sulfate, Invitrogen Corporation, Carlsbad, CA), L-glutamine (2 mM, Thermo Fisher Scientific Inc., Waltham, MA), and penicillin G/ streptomycin sulfate (100 units/ml and 100 ug/ml, respectively, Thermo Fisher Scientific Inc., Waltham, MA). Cells were kept at 37°C in a 5% CO₂ humidified environment.

TGFB Bioassay

Total TGFB (active and latent TGFB isoforms 1, 2, and 3) in flushes from day 13 infused pregnant gilts was determined using MLEC transfected with the PAI-1 promoter luciferase construct and similar methods as previously described (Abe et. al., 1994). MLEC grown to confluence in 25 cm² culture flasks were passaged using 0.0625% trypsin in 0.02% of Moscana/EDTA. Cells were plated at a concentration of 2.0 X10⁵ cells per ml on 96-well culture dishes with DMEM supplemented with 10% FBS, L-glutamine (2 mM), and penicillin G/streptomycin sulfate (100 units/ml and 100 ug/ml, respectively), and were allowed to attach for 3 hours at 37°C in a 5% CO₂ humidified environment. After removing culture media, a TGFB standard using TGFB1 (R&D Systems) and DMEM supplemented with 0.1% BSA, L-glutamine(2 mM), and penicillin

G/streptomycin sulfate (100 units/ml and 100 ug/ml, respectively) was serial diluted (500 pg, 250 pg, 125 pg, 62.5 pg/ml, 31.25 pg/ml, 15.625 pg/ml, 7.8125 pg/ml, 3.90625 pg/ml, 1.953125 pg/ml, 0.976563 pg/ml) and each dilution was added to three separate wells. Flushes from day 13 pregnant gilts were also acid activated with Tris and 1N HCl (pH 2.2-3.5) for 1 hour on ice to cause cleavage of LAP from TGFB, thereby, activating TGFB. 1N NaOH and Tris were then added at a dilution of 1:10 to neutralize the acid. Samples were sterile filtered with 0.2um syringe filter (Pall Life Sciences, East Hill, NJ), and serial dilutions of samples were made (1:100, 1:200, and 1:400) with DMEM supplemented with 1.66% bovine serum albumin (BSA), L-glutamine (2 mM), and penicillin G/streptomycin sulfate (100 units/ml and 100 ug/ml, respectively). Diluted samples and standards were then added to wells (3 wells/sample) and incubated for 12-16 hours at 37°C in a 5% CO₂ humidified environment. Sample and standard media were removed from MLEC, briefly washed with 1XPBS, and lysed with Promega Cell Culture Lysis Reagent (Promega, Madison, WI) for 20 minutes at 4°C on an orbital shaker. Lysates were transferred to opaque 96 well plates and Luciferase Assay Reagent (Promega, Madison, WI) was added to each well. Plates were read on a luminometer according to manufacturer's instructions, Luciferase Assay System (Promega Corp).

Statistical Analysis

Data from TGFB bioassay was subjected to ANOVA using general linear models procedures of SPSS (SPSS, Inc., Chicago, IL). ANOVA was used to determine statistical

significance between treatment groups (LAP-RGD, LAP-RGE, or PSA) of total TGFB amounts within the uterine lumens of infused pregnant gilts.

Results

TGFB and LAP Colocalization

Immunofluorescence analyses were used to co-localize LAP and TGFB in day 13 uterine tissue of PSA and LAP-RGD infused gilts, with the expectation that these analyses would aid our understanding of the fate of infused LAP-RGD and its affect on the net balance of active and latent TGFB in the uterus.

In PSA infused gilts, LAP (Texas Red) was detected on the surfaces of endometrial luminal epithelium (LE), endometrial glandular epithelium (GE), and with the endometrial stroma. TGFB (FITC) was low but detectable on the apical surface of LE and within the stroma (data not shown).

In LAP-RGD infused gilts, LAP was also detected on apical surfaces of GE and within the endometrial stroma. LAP immunostaining was variable between gilts, but in most cases, immunostaining appeared more intense in endometrial tissues compared to that in PSA infused gilts. TGFB immunostaining was low but detectable on the apical surfaces of LE and within the stroma (data not shown).

The intensity of LAP immunostaining was significantly higher compared to TGFB immunostaining in uterine tissue of PSA and LAP-RGD infused gilts; the net result was that co-localization of LAP and TGFB (appearing yellow in color) was low and, in most cases, undetectable (data not shown).

Integrin Immunofluorescence

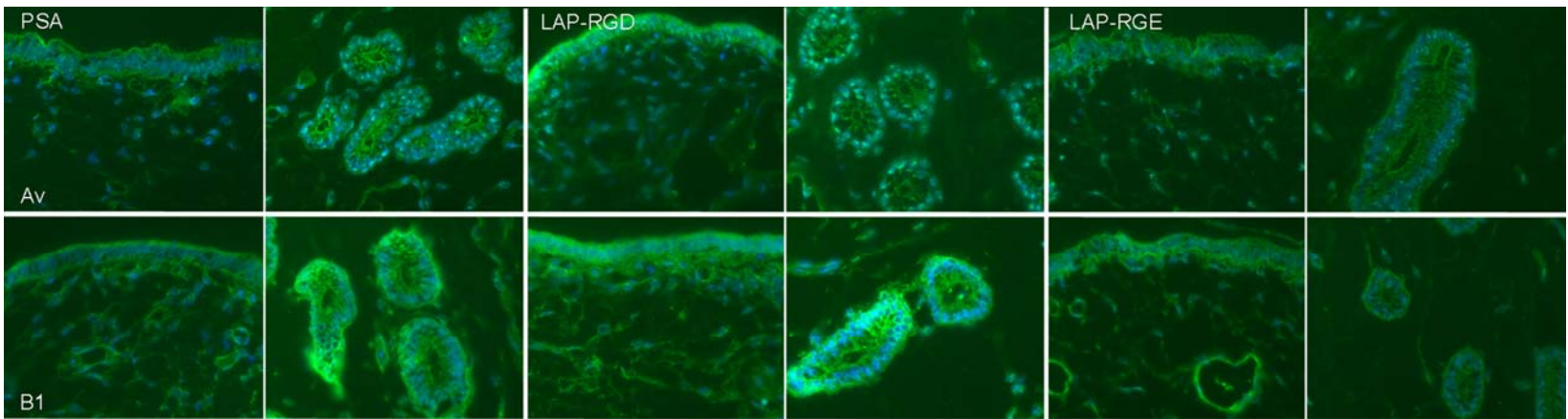
Because active TGFB can alter integrin expression on cell surfaces, and LAP-RGD can bind to integrins and, potentially, alter their distribution on cell surfaces, immunofluorescence analyses were used to localize integrin subunits ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, or ITGB8 (FITC) on day 13 uterine tissue of PSA, LAP-RGD, and LAP-RGE infused gilts. In all three treatment groups immunostaining intensities for ITGB6 and ITGB8 were low and, in some cases, undetectable (**Figure 5.1**). No appreciable differences were noted for ITGB6 and ITGB8 in uterine tissue among treatment groups.

Immunostaining for ITGAV and ITGB5 was detectable and similar in intensity in LE and GE. ITGB3 was also detectable but intensity was low in the LE and GE (**Figure 5.1**). No appreciable differences were noted for ITGAV, ITGB3, and ITGB5 in uterine tissue among treatment groups. Immunostaining for ITGB1 was detectable in the LE and GE and appeared more intense in the LAP-RGD treatment groups as compared to that in control PSA and LAP-RGE treatment groups.

TGFB Bioassay

Flushes from day 13 infused LAP-RGD, LAP-RGE, and PSA pregnant gilts were subjected to TGFB bioassay to determine active and total TGFB (active and latent TGFB isoforms 1, 2, and 3) using MLEC transfected with the TGFB-responsive PAI-1 promoter luciferase construct. Uterine flushes of all treatment groups (LAP-RGD, LAP-RGE, and

Figure 5.1. Integrin Subunit Immunofluorescence in Day 13 Porcine Endometrium of PSA, LAPRGD, and LAPRGE Treated Gilts. Immunofluorescence of integrin subunits (ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, ITGB8) in frozen cross-sections of day 13 pregnant pig endometrium of PSA, LAPRGD, and LAPRGE infused gilts. Integrin subunit immunoreactivity was detected using FITC-conjugated anti-rabbit IgG (green fluorescence). Compare the antibody staining with staining using rabbit and goat IgG (bottom row). LE, luminal epithelium; GE, glandular epithelium. Width of fields are 230 μm .



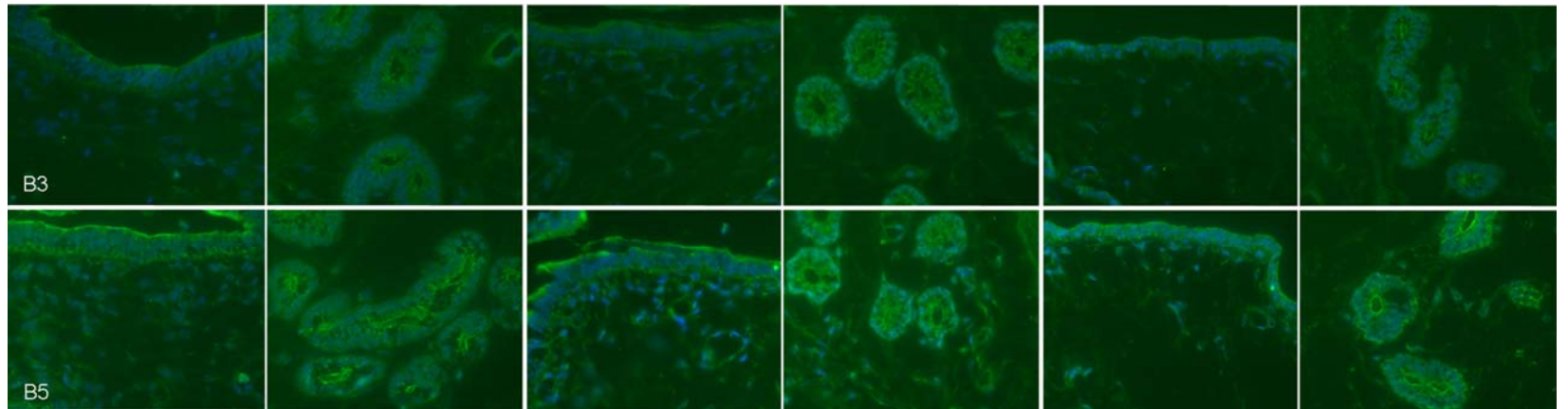


Figure 5.1. Continued

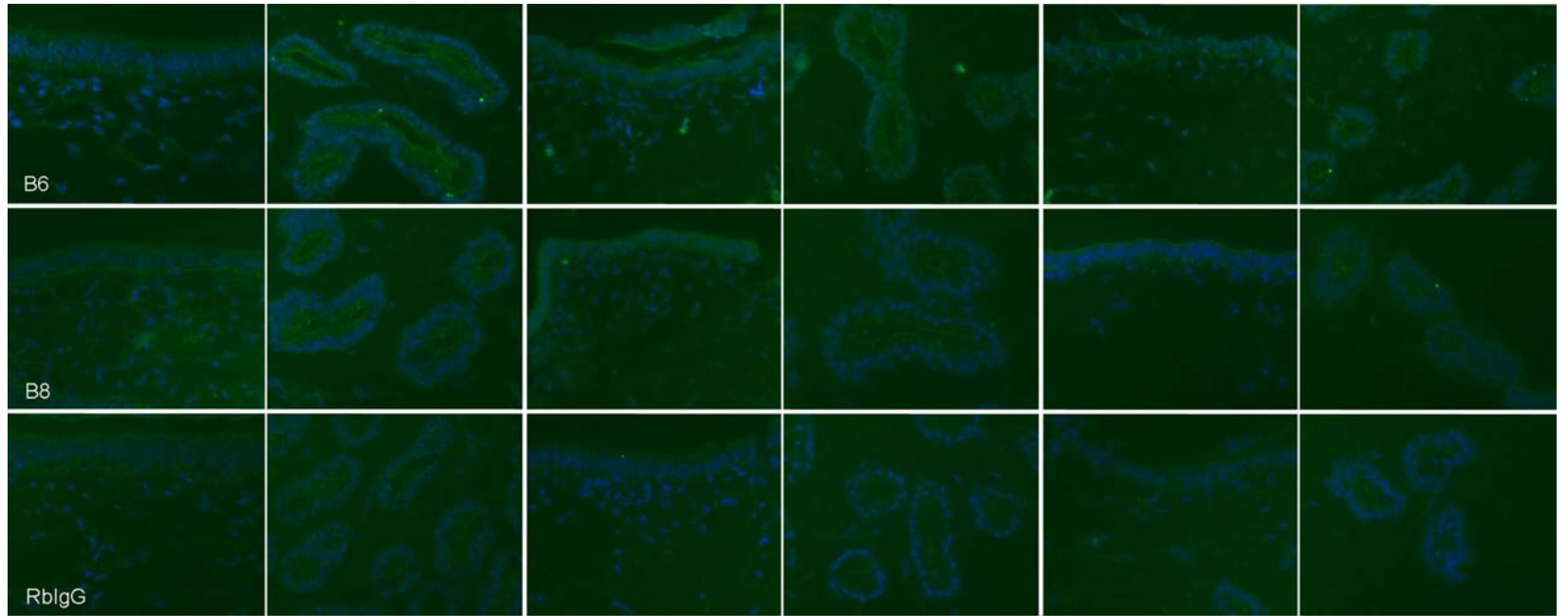


Figure 5.1. Continued

PSA) were treated with HCl to activate any latent TGFB present in the flushes; total amounts detected represent the summation of TGFB that was present in active and latent forms at the time of collection. Uterine flushes were also assayed, using varying dilutions, without acid-activation, in order to determine amounts of active TGFB present at the time of collection. Amounts of active TGFB were below detectable limits. The means and standard deviations for total TGFB in LAP-RGD, LAP-RGE, and PSA treatment groups were 0.77 ± 0.71 ug, 1.65 ± 1.10 ug, 1.26 ± 0.55 ug per horn, respectively (**Figure 5.2**). Means between LAP-RGD, LAP-RGE, and PSA were subjected to ANOVA, and differences were not statistically significant ($P > 0.05$, **Table 5.1**).

In Situ Hybridization

TGFB1, *PA*, *MMP2*, *MMP9*, and *FN* were localized via in situ hybridization in day 13 uterine tissue from LAP-RGD, LAP-RGE, and PSA infused gilts. Expression of *TGFB1* was low but detectable in LE and GE. Expression of *PA* was detected in LE and GE, and low levels were detected within the endometrial stroma, particularly within the stratum compactum stroma. Expression of *MMP2* was localized in the LE and GE with lower levels detected within the stratum compactum stroma. Expression of *MMP9* was lower compared to *MMP2* but was localized to the LE and GE (data not shown). In all transcripts tested in uterine tissues of all treatment groups, *FN* exhibited the highest expression. Expression of *FN* was intense and expressed in the outer longitudinal smooth muscle layer and, to lesser extent, within the inner circular smooth muscle layer within

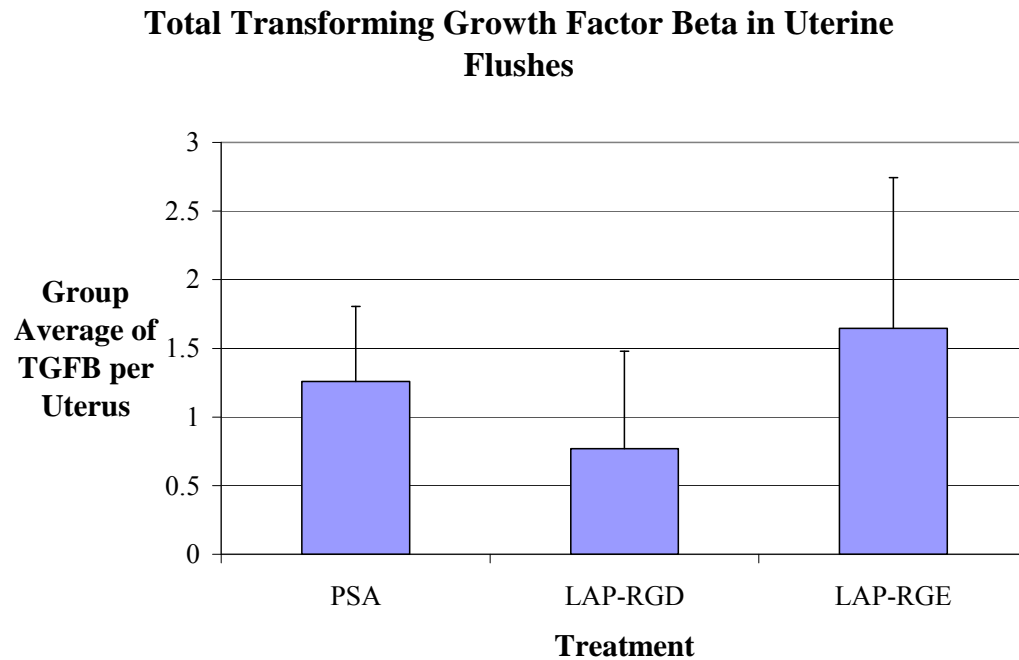


Figure 5.2. Total TGFβ (Active and Latent TGFβ isoforms 1, 2, and 3) Detected in Day 13 Luminal Flushes of LAP-RGD, LAP-RGE and PSA Infused Gilts. Mean ± standard deviations are represented in bar graphs. No differences were detected in total TGFβ amounts.

Table 5.1. Total TGFB Activity. Mean \pm standard deviations for total TGFB in day 13 luminal flushes of LAP-RGD, LAP-RGE, and PSA treated gilts. Tests of significance were performed using the appropriate error terms according to the expectation of the means squares for error, and $P < 0.05$ was considered statistically significant. No differences were detected in total TFGB amounts.

Treatment	TGFB Activity (ug)
PSA	1.2592 \pm 0.5455
LAP-RGD	0.7699 \pm 0.3172
LAP-RGE	1.6457 \pm 0.4910

TGFB Activity Mean \pm SEM ($P < 0.05$)

the myometrium. *FN* was also abundantly expressed within the walls of blood vessels found in the endometrial stroma. Lower levels of *FN* were detected within the extracellular matrix and GE of the stratum compactum stroma and LE (data not shown).

Expression of *TGFBI*, *PA*, *MMP2*, *MMP9*, and *FN* did not appear to be different between LAP-RGD, LAP-RGE, or PSA treatment groups (data not shown).

Discussion

In an attempt to understand the underlying mechanisms resulting in reduction of conceptus elongation as seen in LAP-RGD infused gilts, multiple experiments were employed. Results of the present study indicate that there were no detected differences between LAP-RGD, LAP-RGE, or PSA infused gilts in: 1) amounts of total TGFB within the uterine lumen; 2) integrin expression patterns on endometrial apical surfaces and; 3) TGFB regulated genes such as *PA*, *MMP2*, *MMP1*, *FN*, and *TGFBI* in uterine tissues.

TGFB

Amounts of active TGFB were below detectable limits. However, in the presence of sera; TGFB activity may be masked. Indeed, previous studies, have reported that factors in sera may cause interference with bioassays (Garrigue-Antar *et al.* 1995). Further alpha 2 macroglobulin which is found in serum is known to modify TGFB activity and therefore cause interference. When latent TGFB was acid activated, active TGFB could therefore be detected. Possibly, the addition of acid may neutralize the

effect of alpha 2 macroglobulin while activating latent TGFB; therefore, interference is minimal when detecting total TGFB.

Integrins

Integrins are implicated in mediating conceptus attachment to the uterine LE during porcine pregnancy; however, mechanisms mediating conceptus elongation remain unknown. Reorganization and remodeling of the cells of the extra-embryonic tissues and hyperplasia have both been implicated in playing a role in conceptus elongation (Geisert *et al.* 1982a, Blomberg *et al.* 2008). Pathways involving integrin and TGFB signaling have been identified in elongating conceptuses (Blomberg *et al.* 2008). Based on results of our previous studies, we suggest that reduction in conceptus elongation exhibited in LAP-RGD treatment gilts was a result of inappropriately stimulating integrins or preventing other integrin-ligand interactions. Integrins bind to ECM proteins and upon stimulation of integrins, they will aggregate to enhance receptor activation and intracellular signals. Aggregation of integrins and differences in apical integrin expression were not detected in day 13 porcine endometrium in LAP-RGD, LAP-RGE, or PSA treatment groups. These results do not provide any evidence that integrins inappropriately aggregated in response to infused LAP-RGD. LAP-RGD binding to integrins may prevent other ECMs from interacting with the occupied integrins. Preventing other integrin-ligand interactions may interrupt intracellular signaling events, which may be responsible for up regulation of genes or establishing associations with the actin cytoskeleton. Proper associations with the actin cytoskeleton and up regulation of

particular genes may be critical for reorganization and hyperplasia, which may be critical in mediating conceptus elongation.

Expression of TGFB Regulated Genes during Porcine Pre-Implantation

In previous studies, differences were detected in intracellular SMAD signaling between LAP-RGD, LAP-RGE, and PSA treatment groups. TGFB can signal via SMADs to regulate gene expression of multiple genes including its own gene, *PA*, *MMP2*, *MMP9*, and *FN*. No difference was detected between treatment groups for these TGFB regulated genes; however, spatial and temporal distributions in these genes were noted.

Expression of TGFB regulated genes *PA*, *MMP2*, *MMP1*, and *FN* have been previously identified in the porcine uterus during pregnancy. PA converts plasminogen to plasmin, and plasmin has been implicated in regulating conceptus invasion in species such as mice, which undergo conceptus invasion and decidualization (Salamonsen 1999). Plasmin supports conceptus invasion by its actions on degrading the ECM. In addition, plasmin proteolytically activates latent TGFB (Lyons *et al.* 1990). PA is produced by porcine blastocysts during days 10 to 16 in culture; however, PA could not be detected within the uterine lumen after day 12 of pregnancy (Mullins *et al.* 1980, Fazleabas *et al.* 1983). In situ hybridization results indicate that *PA* is produced by day 13 endometrial epithelium and stratum compactum stroma, suggesting that PA is secreted within the uterine lumen. Plasminogen activator inhibitor (PAI) has been detected in porcine

uterine tissues suggesting that PA is inhibited within the porcine uterine lumen as a mechanism to prevent ECM degradation and conceptus invasion.

Other proteases identified during porcine pregnancy include MMP2 and MMP9. In previous reports, MMP2 transcripts were detected in blastocysts and uterine tissues from days 11 to 16 of pregnancy, while MMP9 transcripts were identified in both embryo and uteri between days 15 and 16 and, in some cases, undetectable (Menino *et al.* 1997, Foxcroft *et al.* 2000). Results of our studies indicate that *MMP9* was indeed present in day 13 porcine uterine tissues particularly in the uterine LE, and abundant expression of *MMP2* was present in the LE and GE.

Multiple ECM proteins have been identified during porcine pregnancy, including FN, which was identified during porcine pregnancy on the apical surfaces of the uterine LE and trophoctoderm (Rashev *et al.* 2005). FN is a ubiquitous homodimeric ECM protein that has roles in cellular adhesion, migration, and implantation, and a fetal form – oncofetal FN – has been characterized as trophoblastic glue (Jaeger *et al.* 2001). While our results support the finding that *FN* is found within the porcine uterine LE, FN is most abundantly expressed within the myometrium, walls of blood vessels found in the endometrial stroma, and to a lesser extent within the extracellular matrix and GE of the stratum compactum stroma.

FN is found within the ECM in many tissues and abundantly found in injured tissues because it plays a role in wound healing. FN associates with fibrillar adhesions consisting of integrin $\alpha 5\beta 1$ and tensin, and FN can stretch which accommodates for certain cellular activities such as migration and protrusion. FN is also associated with

providing a stable matrix and fibroblast differentiation into myofibroblasts, which is critical during these events. FN is often seen at the edges of wound healing and within cardiac valve matrices where migration and cellular contraction is evident (Fayet *et al.* 2007). It is likely that FN found within the porcine uterus, particularly within the myometrium and walls of blood vessels, is associated with cell-matrix adhesions supporting events of cellular stretch in these structures during pregnancy.

In summary, mechanisms supporting conceptus elongation remain unknown, and no evidence was obtained to attribute reduction of conceptus elongation as seen in LAP-RGD treatment groups to LAP-RGD inappropriately stimulating focal adhesion formation. Likewise, we could not associate the observed gross morphological changes in expression of *TGFB1*, *PA*, *MMP2*, *MMP9*, and *FN* in endometrium, as assessed by in situ hybridization. Further analysis of TGF β - and integrin- regulated factors may provide insight in determining mechanisms responsible for inhibiting conceptus elongation. Although the precise nature of the LAP-integrins interactions in the infused gilts have not been defined, it is possible that the resultant alterations in downstream integrin signaling regulating cellular behaviors such as proliferation and cytoskeletal reorganization will result in phenotypic changes such as inhibiting conceptus elongation.

CHAPTER VI

CONCLUSIONS

Introduction

There is a high incidence of conceptus and embryonic mortality during the porcine peri-implantation period. The complex networking of autocrine, paracrine, and juxtacrine communication at the conceptus-maternal interface is poorly understood; however, secretions from both conceptuses and uterus and their interactions are important for conceptus attachment and survival. Among the many growth factors, adhesion molecules, steroid hormones, and cytokines found within histotroph or expressed on the apical surfaces of the uterine LE and trophectoderm, TGFB and ITGA4, ITGA5, ITGAV, ITGB1, ITGB3, and ITGB5 subunits have been detected in both porcine conceptus and uterine tissues during the peri-implantation period (Gupta *et al.* 1998a, Bowen & Hunt 2000, Burghardt *et al.* 2002). The work in this dissertation addressed the roles TGFB and integrins have in conceptus-uterine interactions during the porcine peri-implantation period.

There were two main objectives in this study. The first objective identified interactions between latent TGFB (LAP) and integrins which may result in conceptus adhesion and TGFB activation. Studies were conducted to identify temporal and spatial distributions of LAP, TGFB, and integrin subunits (ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, and ITGB8), identify LAP as a functional ligand for integrin receptors, and provide evidence for TGFB activity in conceptus and uterine tissues during

preimplantation and at the conceptus-maternal interface in pigs. We used immunofluorescence to colocalize LAP and TGFB along with LAP and relevant integrin subunits in conceptus and endometrium during the porcine peri-implantation period. To investigate LAP's capacity to serve as a functional ligand at the conceptus-maternal interface, we use cell surface labeling, affinity chromatography, and immunoprecipitation. Finally, to identify evidence of functional TGFB, we used immunohistochemistry to detect a TGFB downstream signal, p-SMAD2/3, in both conceptus and uterine tissues and at the conceptus-maternal interface.

The second objective examined the effects of LAP and TGFB on conceptus development, conceptus attachment, and/or placental and fetal development by infusing day 9 pregnant gilts with LAP-RGD, LAP-RGE, or PSA control. Morphology of day 13 conceptuses was assessed grossly in LAP-RGD, LAP-RGE, and PSA infused gilts. In addition, integrity of implantation sites, along with assessment of placental and fetal parameters, were examined in day 24 fetuses of LAP-RGE or PSA infused gilts. In an attempt to determine the mechanism(s) underlying the inhibition of conceptus elongation in LAP-RGD infused gilts, we performed various experiments including immunofluorescence for LAP, TGFB, and integrin subunits (ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, and ITGB8); immunohistochemistry on p-SMAD2/3; and in situ hybridization on TGFB regulated genes (*PA*, *MMP2*, *MMP9*, *FN*, and *TGFB*). Our aim was to identify differences between LAP-RGD and LAP-RGE treatment groups leading to possible mechanisms responsible for mediating conceptus elongation.

LAP and TGFB Are Present at the Conceptus-Maternal Interface

In Chapter III, we identified the spatial and temporal distribution of LAP and TGFB in porcine conceptus and endometrium during the peri-implantation period. Specificity of the antibodies was critical for this study, and anti-TGFB was specific for active TGFB1 (TGFB) not associated with LAP while anti-LAP (LAP) was specific for dimeric and monomeric LAP1 and latent TGFB1 in the forms of the SLC and LLC (Barcellos-Hoff *et al.* 1994, Ehrhardt *et al.* 1997). Colocalization of LAP and TGFB during the peri-implantation period in pigs (days 10, 12, 16, 20, and 24 of pregnancy) provided a means by which to visualize temporal and spatial activation of TGFB, sequestration of latent TGFB, and distribution of LAP that may serve as an integrin ligand.

Results of this study demonstrated LAP and TGFB at the conceptus-maternal interface. The detection of LAP in conceptus and endometrial tissues, along with earlier data (Gupta *et al.* 1996, Gupta *et al.* 1998a), support the finding that both the conceptus and uterus are potential sources of secreted TGFB. Further, abundant LAP immunostaining detected on the apical surfaces of GE on days 16 and 20 of pregnancy suggests that the GE secrete latent TGFB as a component of histotroph. Results of this study also provide evidence that activation of TGFB occurs at the apical surfaces of uterine LE, trophoctoderm, and at the conceptus-maternal interface, potentially resulting in TGFB and TGFBR functional interactions. Activation of TGFB at these apical surfaces may be occurring, in part, by proteases and integrins.

Large aggregates of LAP at the conceptus-maternal interface by day 24 of pregnancy provide evidence that LAP is a potential adhesion molecule promoting attachment of the conceptus to the uterine LE via binding to integrin receptors. In addition, aggregation of LAP suggests sequestration of latent TGFB as a reservoir for later in pregnancy; however, detection of low levels of immunostaining for TGFB during the late stages of porcine implantation (day 24 of pregnancy) also indicate that there is ongoing activation of TGFB, and suggests a need for active TGFB during this time.

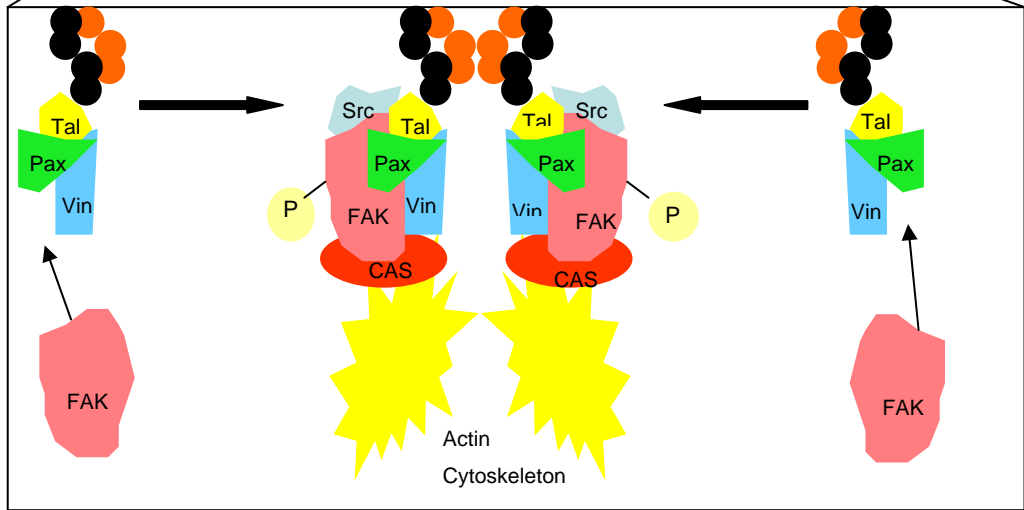
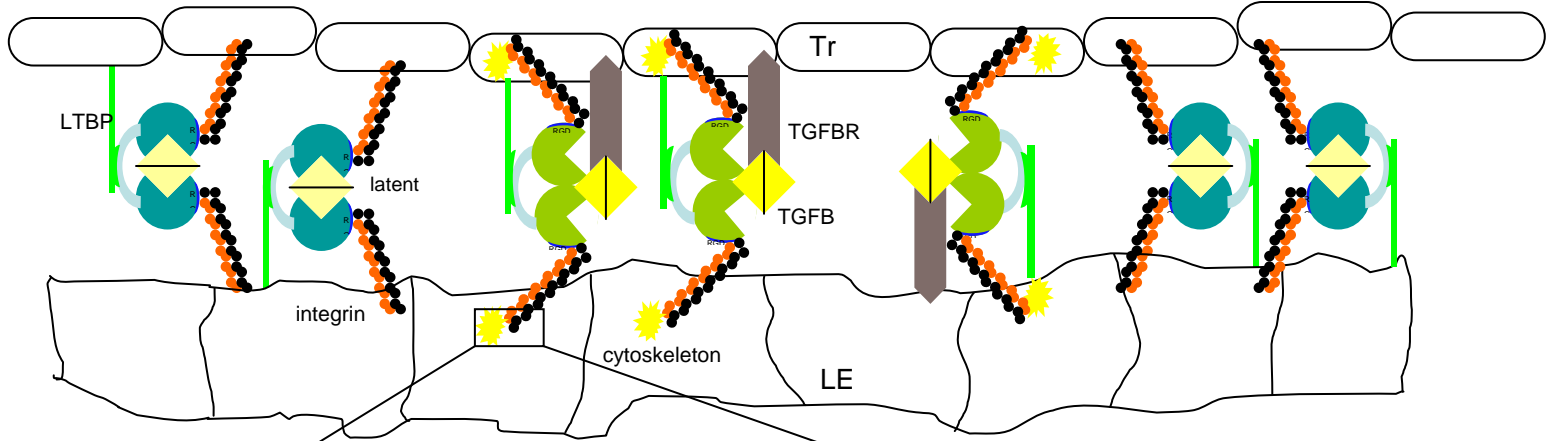
LAP and Integrin Interactions Potentially Support Conceptus Attachment

Dual label immunofluorescence of LAP with each integrin subunit (ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, and ITGB8), described in Chapter III, was essential in placing LAP in close proximity to integrin receptors to support the hypothesis that LAP is a potential integrin ligand during the porcine peri-implantation period. Immunostaining for ITGAV, ITGB1, ITGB3, and ITGB5 were detected in endometrial and conceptus tissues from days 10 through 24 of pregnancy, became more apical in distribution as pregnancy progressed, and aggregated at the conceptus-maternal interface. ITGB1 and ITGB5 formed large distinct aggregates, particularly in the LE, at the conceptus-maternal interface by day 20 of pregnancy and were maintained through day 24. Intermittent aggregates for ITGB3 were detected at the conceptus-maternal interface by day 24. Aggregates for ITGB1, ITGB3, and ITGB5 were absent at the apical surface of LE at non-attachment sites, strongly suggesting that aggregates form in response to ligand binding that serve to attach trophectoderm to LE for stable attachment. Further, LAP did

not precisely co-localize with ITGB1, ITGB3, and ITGB5 but appeared to be distributed in the matrix that resides between the trophoctoderm and LE, which perfectly places this protein to bind ITGB1, ITGB3, and ITGB5 integrins and serve as an adhesion molecule. Indeed, ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, and ITGB8 expressed on the apical surfaces of a porcine trophoctoderm cell line bound to LAP immobilized on an affinity column, further supporting the hypothesis that LAP binds to integrins at the porcine conceptus-maternal interface to support conceptus attachment.

Results from this study were the first to report ITGB1 and ITGB5 aggregation at the conceptus-maternal interface in any species; however, aggregation of integrins and focal adhesion assembly at the conceptus-maternal interface in sheep and pigs was recently reported (Burghardt *et al.* 2009, Erikson *et al.* 2009). Upon binding of ECM proteins, integrins aggregate to enhance signal transduction and form cytoplasmic focal adhesions that associate with the actin cytoskeleton (Wozniak *et al.* 2004), and our detection of ITGB1 and ITGB5 aggregates provides evidence of induced focal adhesions at the conceptus-maternal interface in pigs. Formation of focal adhesions stabilizes cell attachments to the ECM by connecting ECM proteins to the actin cytoskeleton via actin filaments (Bershadsky *et al.* 2006a). Focal adhesions also serve as signaling centers from which numerous intracellular pathways can regulate cell growth, proliferation, survival, gene expression, development, tissue repair, migration and invasion. Our results imply that integrins bind to ECM proteins such as LAP to induce integrin aggregation and focal adhesion assembly that stabilizes conceptus attachment (**Figure 6.1**) and potentially induces intracellular pathways to regulate cellular behavior such as cell growth,

Figure 6.1. Proposed Model for Actions of TGFB and Integrins in Conceptus and Uterine Tissues at the Conceptus-Maternal Interface. Integrin heterodimers on porcine trophoderm (Tr) and uterine LE bind to LAP via its RGD sequence, induce integrin aggregation, and induce focal adhesion assembly to support conceptus attachment to the LE during porcine implantation.



proliferation, and migration which may be critical to support conceptus development and attachment.

It is noteworthy that aggregates of ITGB3 were not detected until the later stage of porcine implantation, while aggregates of ITGB1 and ITGB5 appeared by mid implantation. Changes in the integrin repertoire on LE, have been detected during implantation in many species and, particularly with $\alpha\beta3$, are associated with stabilizing stronger conceptus adhesions (Johnson *et al.* 2001, Burghardt *et al.* 2002, Lessey 2002, Armant 2005). During porcine implantation, ITGB1 and ITGB5, presumably as the heterodimers $\alpha\beta1$ and $\alpha\beta5$, may play important roles for stabilizing initial attachments of the conceptus to the LE. As implantation progresses, up regulation of ITGB3, presumably as $\alpha\beta3$, along with $\alpha\beta1$ and $\alpha\beta5$, may be necessary to support stronger placental attachments of the growing embryo.

TGFB Activation at the Porcine Conceptus-Maternal Interface

Abundant immunostaining for active TGFB was detected along the apical surfaces of filamentous conceptuses by day 12 of pregnancy and at the conceptus-maternal interface beginning by day 16 of pregnancy, suggesting that activation occurs along these surfaces. Activation of TGFB can occur via proteases and integrins in the presence and absence of proteases (Jenkins 2008, Wipff & Hinz 2008).

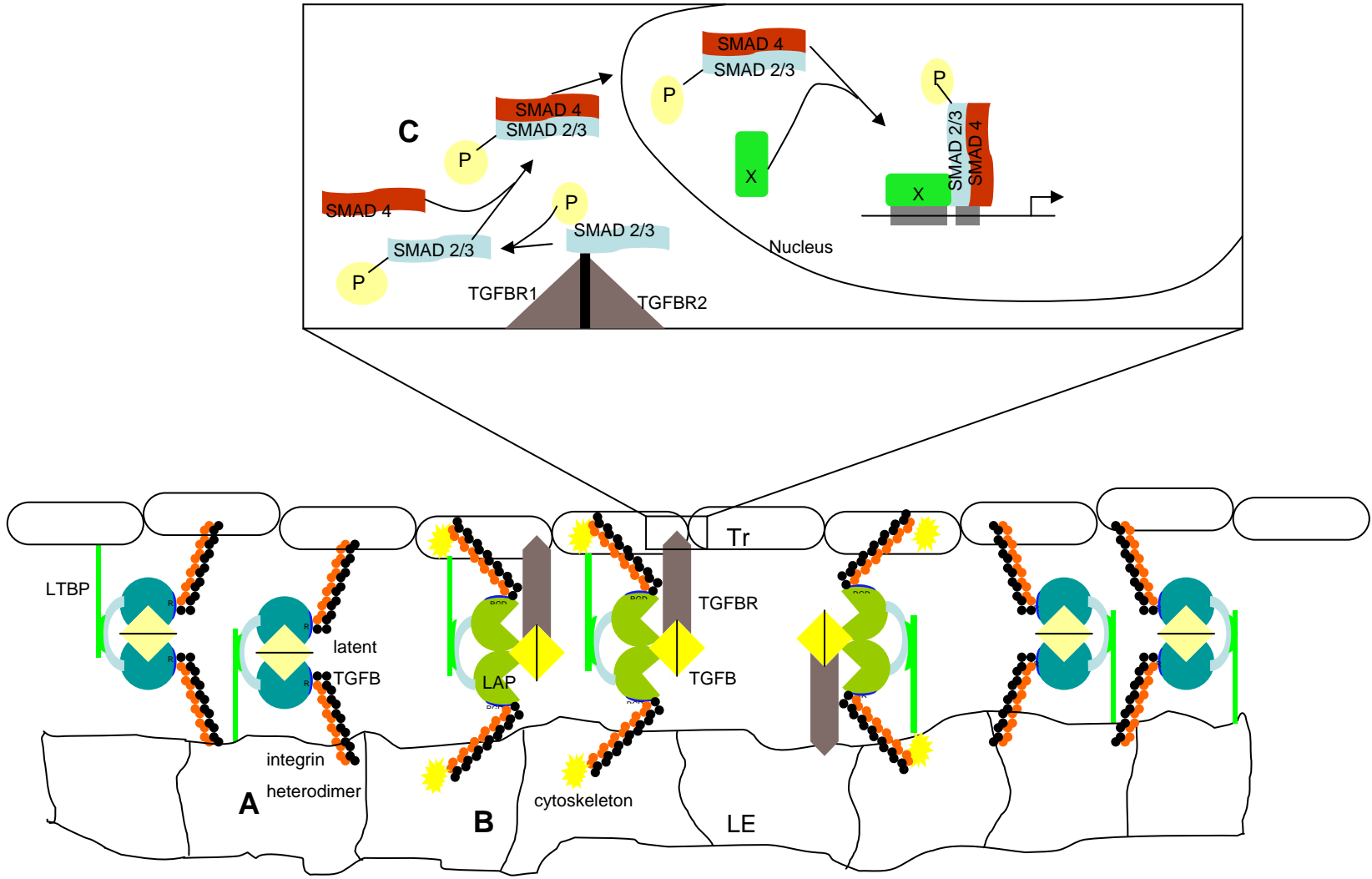
LAP and ITGAV, ITGB1, ITGB3, ITGB5, and low levels of ITGB6 and ITGB8 were detected along the uterine LE. Notably, $\alpha\beta3$, $\alpha\beta5$, $\alpha\beta6$, and possibly $\alpha\beta1$, have been determined to activate latent TGFB by mechanical forces such as cellular

contraction and external stretching (Jenkins *et al.* 2006, Wipff *et al.* 2007). During porcine implantation, the conceptus migrates along the uterine LE, and the migrating conceptus may be a source of external force applied to the LAP-integrin adhesion complex. Results from Chapter III suggest that the apically bound LAP-integrin adhesion complex, in addition to supporting conceptus attachments, can also activate TGFB by inducing conformational changes in the latent complex. The conformational change generated by conceptus movement along the uterine LE may cause external stretch on the LLC and therefore liberate TGFB (**Figure 6.2**).

Intracellular forces such as cellular contraction generated by cross-bridging of ACTA2 and myosin may also play a role in activating TGFB during porcine implantation. The subepithelial stroma of pigs undergoes remodeling during pregnancy that is associated with the up regulation of α -smooth muscle actin (ACTA2) within myofibroblast-like stromal cells (Johnson *et al.* 2003). It is possible that in the porcine uterus, contraction of the actin filaments associated with integrins generates a force on the LAP- integrin complex, causing a conformational change in the LLC, and liberating TGFB from its latent complex. As reported here, latent TGFB localized within the ECM of the stroma during porcine implantation may be subjected to activation by integrins transmitting intracellular mechanical forces.

Proteases involved in TGFB activation include, but not limited to, plasmin and MMP's, which function by proteolytic cleavage of LAP (Jenkins 2008). Independent of traction forces, integrins also aid in proteolytic cleavage of LAP from TGFB during TGFB activation. For example, $\alpha\beta 8$ forms a cell surface complex with the

Figure 6.2. Proposed Model for Actions of TGFB and Integrins in Conceptus and Uterine Tissues at the Conceptus-Maternal Interface. **A**, Integrin heterodimers on porcine trophectoderm (Tr) and uterine LE bind to LAP via its RGD sequence serving to support conceptus attachment to the LE during porcine implantation. **B**, LAP bound to integrins $\alpha\beta3$, $\alpha\beta5$, $\alpha\beta6$, and possibly $\alpha\beta1$ may serve to activate TGFB. **C**, TGFB signals via TGFBRs to activate intracellular signaling pathways, most commonly involving SMADs to control transcription of many genes.



transmembrane matrix metalloproteinase MMP14 (MT1-MMP). Integrin $\alpha\beta 8$ binds LAP that sequesters latent TGFB at the luminal surface and presents the latent TGFB to MT1-MMP where it cleaves LAP and releases active TGFB (Mu *et al.* 2002). MT1-MMP has been identified in the synepitheliochorial placenta in sheep and goats (Uekita *et al.* 2004), but to our knowledge MTI-MMP has not yet been investigated in pig implantation. Integrin $\alpha\beta 3$ is also hypothesized to participate in proteolytic activation of TGFB since $\alpha\beta 3$ may serve as a docking station for MMP2 and MMP9 along with latent TGFB. Similar to MT1-MMP, MMP2 and MMP9 activates TGFB by cleaving LAP and releasing TGFB (Wipff & Hinz 2008). MMP2 was detected in porcine blastocysts and peri-implantation uterine tissues (Menino *et al.* 1997, Foxcroft *et al.* 2000). Not surprisingly, tissue inhibitors of matrix metalloproteinases (TIMP1, 2, and 3) have been localized in the trophoctoderm and endometrial stroma, and TIMP2 was detected in uterine flushes during porcine pre-implantation and early implantation (Menino *et al.* 1997, Kayser *et al.* 2006). While inhibiting the proteolytic actions of MMPs via TIMPs may be important in inhibiting conceptus invasion during porcine epitheliochorial implantation, MMP2 may temporally activate TGFB at the apical surfaces of LE and GE, where expression of TIMPS1, 2, and 3 were not detected (Menino *et al.* 1997).

We propose therefore that ITGAV-containing heterodimers participate in TGFB activation during porcine implantation via mechanisms including, but not limited to, traction forces. Forces generated by the conceptus may be a mechanism for activating TGFB at sites of conceptus contact, and forces generated from within the cell may be a mechanism for activating TGFB within the endometrial stroma. In addition, proteases

such as MMP-2 alone or in combination with integrins may also aid in activating TGFB at non-attachment sites or within glandular lumens. Active TGFB can therefore, bind and activate TGFBRs at both conceptus attachment and non-attachment sites (**Figure 6.2**).

LAP-RGD Reduces Conceptus Elongation

Mechanisms supporting conceptus elongation are largely unknown, but altering the uterine environment during the peri-implantation period may alter conceptus development and survival (Wilson *et al.* 2001). In attempt to uncover mechanisms supporting conceptus elongation and attachment, experimental intrauterine infusions with implantable osmotic pumps were utilized to deliver LAP-RGD, LAP-RGE, or PSA directly into the uterine horns of early pregnant gilts, described in Chapter IV. Such experimental infusions have not been used to investigate conceptus development and attachment during the peri-implantation period in pregnant swine. We successfully established a delivery system with the use of mini osmotic pumps that remained contained within the abdominal cavity, enabled controlled delivery of substances to the uterine lumen of pregnant gilts, and maintained pregnancy throughout the implantation period. This system allowed for exogenous LAP, at physiological concentrations, to be directly infused into the uterus of pregnant gilts with interiorized catheters and minimal post surgery manipulation or restraints. Inappropriate LAP-RGD infused into the uterine lumen of pregnant gilts beginning at day 9 of pregnancy was expected to disrupt the balance of active TGFB and alter normal integrin signaling, whereas LAP-RGE infusions were expected to similarly affect the TGFB activity, but not alter integrin interactions.

Day 13 conceptuses collected from pregnant gilts infused with LAP-RGD exhibited a dramatic reduction in elongation; whereas, the overall morphology of conceptuses in both LAP-RGE and PSA treatment group was grossly normal. In attempt to understand the underlying mechanisms resulting in reduction of conceptus elongation as seen in LAP-RGD infused gilts, multiple experiments including immunofluorescence, immunohistochemistry, and in situ hybridization were employed. Detection of p-SMAD2/3 in day 13 conceptus and endometrial tissues, described in Chapter IV, provides evidence that activity of TGFB was not inhibited in LAP-RGD infused gilts. Further, results from Chapter V provide no evidence of differences in 1) amounts of total TGFB within the uterine lumen; 2) integrin expression patterns on endometrial apical surfaces and; 3) expression of TGFB regulated genes such as *PA*, *MMP2*, *MMP1*, *FN*, and *TGFB1* in uterine tissues that could be linked to the inhibition of conceptus elongation. Further analysis of TGFB- and integrin- regulated factors via microarray analyses and detection of phosphorylated intracellular signaling proteins may provide insight in determining mechanisms responsible for inhibiting conceptus elongation.

Based on the findings that LAP-RGD treatment groups exhibited a reduction in conceptus elongation, whereas, LAP-RGE did not affect elongation, we hypothesize that the RGD sequence in LAP-RGD treatment groups mediated the inhibition of conceptus elongation, and the observed phenotype. It is possible that LAP-RGD bound to integrins prevented other ligands such as FN, SPP1, or VN from binding to integrins, thus resulting in inappropriate integrin intracellular signaling or prevention of signaling. In addition, the presence of spherical conceptuses on day 13 of porcine pregnancy, a phenotype

normally detected prior to day 11, provides evidence that altering or interfering with downstream integrin and/or TGFB signaling within a critical window encompassing day 10 of pregnancy inhibits critical, initiating signals mediating conceptus elongation.

LAP-RGE Increases Placental Size

Because conceptus elongation in LAP-RGD infused gilts was severely inhibited and, in many cases, conceptuses appeared fragmented or as trophoblastic sheets, it is unlikely that the pregnancy would have continued to day 24 of pregnancy. In many gilts that received LAP-RGD from days 9 to 13, degenerating cells and necrotic tissue found in luminal flushes suggested that pregnancy resorption was occurring. In *study 1* in Chapter IV, LAP-RGE infused gilts exhibited normal conceptus elongation; therefore, a second study was designed to evaluate effects of LAP-RGE or PSA infusions on placental and fetal parameters such as fetal weight, number, and crown-rump length and allantoic length and allantoic fluid volume. Infusions began on day 9 of pregnancy, continued for 6.5 days, and ovariohysterectomies were performed on day 24 of pregnancy. Infusing LAP-RGE resulted in larger fetal weight ($P < 0.05$), allantoic length ($P < 0.05$), and allantoic fluid volume ($P < 0.01$) compared to the PSA treatment group.

Results from Chapter IV provide evidence that infusing LAP-RGE results in a decrease in SMAD2/3 signaling in the uterine LE by day 13 of pregnancy, suggesting that the phenotype seen at day 24 of pregnancy may be a result of altering TGFB induced SMAD2/3 signaling in epithelial cells during the critical time of conceptus elongation. Further, the apparent decrease in p-SMAD2/3 in epithelial cells, potentially caused by

reduced TGFB activity, may alter cellular growth arrest, proliferation, and migration in epithelial cells, which may ultimately affect porcine placental and fetal parameters including fetal weights, allantoic length, and allantoic fluid volume.

Functional TGFB at the Conceptus-Maternal Interface and Potential TGFBR and Integrin Cross-Talk

TGFB signals via SMAD and less characterized SMAD-independent pathways and functions to elicit different cellular behaviors such as growth, proliferation, apoptosis, and differentiation. In multiple species, TGFB has been indirectly linked to regulating trophoblast attachment and invasion by enhancing production of ECM proteins such as oFN (Feinberg *et al.* 1994), inducing expression of integrins (Zambruno *et al.* 1995, Kagami *et al.* 1996, Lai *et al.* 2000, Pechkovsky *et al.* 2008), and reducing production of proteases such as MMPs and plasmin (Graham & Lala 1991, Graham 1997, Kallapur *et al.* 1999, Karmakar & Das 2002). TGFBR downstream p-SMAD2/3 signaling identified in porcine conceptus and uterine tissues, described in Chapter III, may function to regulate transcription of genes encoding for adhesion molecules, such as oFN and SPP1; protease inhibitors, such as PAI and TIMPs; and integrins during the peri-implantation period in pigs. Therefore, TGFB acting via intracellular SMAD signaling may be functioning in regulating processes such as adhesion and invasion in porcine pregnancy.

There is evidence to support that integrins cluster with TGFBRs, and cross-talk between growth factor receptors and integrins have been implicated in growth,

proliferation, migration, and angiogenesis. At the porcine conceptus-maternal interface, integrins clustering with TGFBRs may not only be important during TGF β activation and subsequent TGF β and TGFBR interactions, but may be critical for intracellular signaling cross-talk. Cooperation between integrins and growth factors can activate signaling cascades such as Ras-ERK and MAPK c-Jun NH₂-terminal kinase (JNK) that can regulate cell cycle progression and growth (Eliceiri 2001). TGFBR and integrin cytoplasmic domains, such as ITGB1 and ITGB3, may be linked via ILK, PINCH-1, and NCK2 resulting in synergy between TGF β and integrin intracellular signaling regulating growth, proliferation, and migration in epithelial cells at the porcine conceptus-maternal interface.

Integrin-mediated cell adhesion to the correct ECM appears to be important for optimal growth factor stimulation, and it appears that integrins preferentially associate with specific growth factor receptors. For example, in the presence of certain integrin ligands, $\alpha\beta$ 5 requires growth factor stimulation for integrin-mediated cell migration, while $\alpha\beta$ 3 exhibits growth factor-independent migration. Indeed, cell migration occurs in the presence of EGF or insulin when $\alpha\beta$ 5-vitronectin interactions support cell attachments. ITGB3 and ITGB5 have been identified at the porcine conceptus-maternal interface, and while $\alpha\beta$ 3 and $\alpha\beta$ 5 may support adhesion of the conceptus to the uterine LE, they may also mediate other cellular events such as migration in the presence of particular adhesion molecules and growth factors, potentially including LAP and TGF β , respectively. Further, at the porcine conceptus-maternal interface TGF β activity via SMAD-dependent and/or SMAD-independent pathways may regulate these events by

inducing expression of *ITGB3* and *ITGB5* and subsequent apical expression of $\alpha\beta3$ and $\alpha\beta5$ and regulating components of the ECM, particularly FN and VN. Mechanisms controlling TGFB and integrin activity, as described here, may aid in the underlying causes of the phenotypic changes detected in conceptus, placental, and fetal development, as described in Chapter IV.

Results in Chapter III provide evidence of focal adhesion assembly to support conceptus attachment. TGFB may aid in initiating focal adhesion assembly during porcine implantation by activating FAK and $\alpha\beta3$ via SMADs and MAPK, respectively. TGFB may also regulate size of focal adhesions by increasing intracellular stress by mechanisms such as increasing ECM composition and activating ACTA2, Rho, and Rho kinase that increase myosin contraction. In addition, TGFB may regulate the differentiation and remodeling of the subepithelial stroma in pigs during pregnancy by up regulating ACTA2 that is associated with myofibroblast-like stromal cells.

Final Conclusions

The series of studies conducted have revealed that interactions among active TGFB, latent TGFB (LAP), and integrins are complex, and appear to play important roles during the peri-implantation period in pigs. Prior to this project, TGFB was detected during porcine pregnancy; however, evidence for functional TGFB and the potential actions of TGFB during the porcine peri-implantation period were largely unknown. Overall, this project provided evidence for 1) TGFB activity in porcine conceptus and uterine tissues; 2) receptor-ligand interactions of LAP and integrins; 3)

integrin aggregation and potential focal adhesion formation at the conceptus-maternal interface; and 4) TGF β - and/or integrin-associated mechanisms during pre-implantation regulate porcine conceptus elongation and placental and fetal size. Collectively, results of this project suggest that TGF β and integrins are extensively involved in autocrine, paracrine, and juxtacrine communication at the porcine conceptus-maternal interface, particularly that regulating conceptus development, adhesion, and placental and fetal development. Future studies are warranted and should include those focused on determining the exact mechanisms of TGF β and integrin intracellular signaling that regulate events of implantation and placentation. Better understanding of these processes provides insight into improving the reproductive success in many mammals including humans.

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