Transforming growth factor beta (TGFB) signaling is activated during porcine implantation: proposed role for latency-associated peptide interactions with integrins at the conceptus–maternal interface

Dana A Massuto¹, Eric C Kneese¹, Gregory A Johnson¹, Robert C Burghardt¹, R Neil Hooper², Nancy H Ing³ and Laurie A Jaeger¹

Departments of ¹Veterinary Integrative Biosciences, ²Veterinary Large Animal Clinical Sciences and ³Animal Science, Texas A&M University, College Station, Texas 77843-4458, USA

Correspondence should be addressed to L A Jaeger who is now at Department of Basic Medical Sciences, School of Veterinary Medicine, Purdue University, West Lafayette, Indiana 47907, USA; Email: ljaeger@purdue.edu

Abstract

The process of implantation is mediated by a complex network of signaling and adhesive factors. In the pig, latent and active transforming growth factor beta (TGFB), TGFB receptors (TGFB_R), and integrins (ITGs) are present during the peri-implantation period. TGFB signals via TGFB_R and activates downstream effector SMAD proteins 2 and 3 (p-SMAD2/3). Latency-associated peptide (LAP), part of the latent TGFB complex, is known to bind to ITG heterodimers and activate TGFB. We hypothesize that active TGFBs and TGFB_Rs along with LAP and ITGs functionally interact at the conceptus–maternal interface to mediate events essential for conceptus development and attachment in pigs. Uteri and conceptuses from days 10, 12, 16, 20, and 24 pregnant gilts were immunostained for TGFB, LAP, and ITG subunits (ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, and ITGB8). Activation of TGFB_Rs was evaluated by the presence of phosphorylated downstream effector SMAD2/3. Binding of LAP to ITGs was also evaluated using porcine trophectoderm cells. Abundant active TGFB was detected at the apical surfaces of epithelia at the conceptus–maternal interface, and p-SMAD2/3 was detected at both conceptus attachment and nonattachment sites during implantation. Separate aggregates of LAP, ITGB1, ITGB5, and later ITGB3 were detected at the porcine conceptus–maternal interface, and binding of LAP to ITGs on apical surfaces was demonstrated. Results suggest that functional LAP–ITG adhesion complexes support conceptus attachment and promote TGFB activation leading to TGFB interaction with TGFB_R supporting events of porcine implantation.

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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 have epitheliochorial implantation and their conceptuses undergo an exaggerated morphological transformation in which they elongate from 5 to 150 mm between days 10 and 12 of pregnancy (Geisert *et al.* 1982). 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 trophectoderm (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). Signaling molecules such as transforming growth factor betas (TGFBs) and integrins (ITGs) 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, 1998, Burghardt *et al.* 2002).

The TGFB isoforms (TGFB1, 2, and 3) signal through TGFB receptor types I and II (TGFB_R1 and TGFB_R2). TGFBs bind TGFB_R2 and recruit TGFB_R1 to the receptor–ligand complex, ultimately causing serine and threonine transphosphorylation of TGFB_R1 by TGFB_R2 (Wrana *et al.* 1994, Chen & Weinberg 1995). TGFB_R1 then phosphorylates the downstream effector SMAD (homologues of mothers against decapentaplegic in *Drosophila* and sma-2, -3, and -4 in *Caenorhabditis 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 regulates transcriptional activity of target genes (Nakao *et al.* 1997).

The cell secretes latent TGFB as a homodimer containing a noncovalent 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 extracellular matrix (ECM) proteins such as fibronectin and fibrillin-1 via transglutaminase (Nunes *et al.* 1997, Isogai *et al.* 2003). Conformational changes in the latent complex or dissociation of TGFBs from LAP activate TGFB and allow it to become available for receptor interactions. This activation occurs through multiple extracellular mechanisms, which may involve proteases (Jenkins 2008), thrombospondin-1 (Murphy-Ullrich & Poczatek 2000), and ITGs (Munger *et al.* 1999, Annes *et al.* 2002, 2003, Mu *et al.* 2002, Sheppard 2005). Like many ECM proteins, LAP1 and 3 monomers, corresponding to respective TGFB1 and 3 isoforms, contain an arginine-glycine-aspartate (RGD) site. Previous studies have demonstrated that LAP binds to ITG heterodimers via this RGD sequence (Munger *et al.* 1998, 1999, Mu *et al.* 2002, Ludbrook *et al.* 2003).

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

TGFBs, TGFBs, and 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, 1998, Burghardt *et al.* 2002). We hypothesize that active TGFBs and TGFBs, along with LAP and ITGs, functionally interact at the conceptus-maternal interface to mediate events essential for conceptus development and attachment in

pigs. Although the presence of TGFB 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 ITG 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 TGFB and ITGs during porcine implantation. Therefore, the present studies were designed to 1) colocalize and identify the temporal and spatial distributions of TGFB, LAP, and ITGs in porcine attachment sites; 2) demonstrate that LAP directly binds to ITGs on the surface of porcine conceptuses; and 3) demonstrate the activation of TGFBs through the presence of phosphorylated downstream effector SMAD2/3 in porcine conceptus and endometrium.

Results

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

Distribution of LAP and TGFB

On days 10–16 of pregnancy, LAP (Texas Red) and TGFB (FITC) were detected primarily on the surfaces of conceptus trophoctoderm, endometrial LE, and endometrial glandular epithelium (GE; data not shown). Trophoctoderm, LE, and GE immunostaining gradually increased from days 10 to 16 and became more apical in distribution (Fig. 1). By day 16, LAP and TGFB were also prominently expressed at the basal surface of endometrial epithelia. Significant colocalization 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 pregnancy (Fig. 1). By day 20 of pregnancy, LAP immunostaining at the conceptus-maternal interface decreased compared with day 16; however, TGFB immunostaining was predominant between the matrix of LE and trophoctoderm. Further, intense immunostaining for LAP was observed at apical surfaces of GE, and this colocalized with lower levels of TGFB immunostaining (data not shown). Uterine sections devoid of conceptus tissues (nonattachment sites) from days 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

Figure 1 Distribution of TGFB and LAP during porcine implantation. Immunofluorescence colocalization (yellow color) of TGFB (green color) with LAP (red color) in pregnant pig endometrium and conceptuses. Compare the antibody staining with staining using chicken and goat IgG (Fig. 2; bottom row). Immunostaining for LAP and TGFB colocalized at the apical surface of trophoblast and LE at maximal levels by day 16 (arrows). LE, luminal epithelium; Tr, trophoblast. Width of fields is 230 μ m.

prominent at the apical surfaces of LE and trophoblast at the conceptus–maternal interface (Fig. 2). In contrast, few small aggregates of TGFB were detected by day 24 of pregnancy. 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 (data not shown). Low-intensity immunostaining for TGFB was detected on the apical surfaces of GE, and low levels of LAP and TGFB colocalization were evident. The intensity of LAP and TGFB immunostaining was markedly lower on day 24 at nonattachment sites compared with

attachment sites. At nonattachment sites on day 24, aggregates of LAP were no longer evident along the luminal surfaces (Fig. 2).

Detection of p-SMAD2/3

SMAD proteins 2 and 3 are phosphorylated in response to TGFB interacting with TGFBRs. Therefore, immunohistochemical analysis of p-SMAD2/3 was performed on conceptus and uterine tissues to evaluate functional interactions between TGFBRs and TGFBRs during porcine implantation. On all days of the peri-implantation period

Figure 2 Distribution of TGFB and LAP during porcine implantation. Immunofluorescence colocalization (yellow color) of TGFB (green color) with LAP (red color) in pig attachment and nonattachment sites. Compare the antibody staining with staining using chicken and goat IgG (bottom row). Large aggregates of LAP were prominent at the apical surfaces of LE and trophoctoderm at the conceptus–maternal interface. LE, luminal epithelium; Tr, trophoctoderm. Width of fields is 230 μ m.

(10 to 24; only on days 16, 20, and 24 are shown) p-SMAD2/3 was detected in nearly all the nuclei of endometrial LE, GE (data not shown), fibroblasts, and endothelia in both attachment and nonattachment sites (Fig. 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 with that in the uterine epithelium (Fig. 3).

Detection of ITG subunits and LAP

Immunofluorescence analyses were used to colocalize ITG subunits ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, or ITGB8 (FITC) with LAP (Texas Red) on days 10 and 12 pre-implantation uterine tissues and on days 16, 20, and 24 conceptus attachment and nonattachment sites. On all days observed, immunostaining intensities for ITGB6 and ITGB8 were low and, in some cases, it was undetectable (data not shown). On days 10 to 16, ITGB6 immunostaining was slightly higher in GE compared with LE; whereas, ITGB8 was slightly higher in LE as compared with GE. Both ITGB6 and ITGB8 were undetectable by day 20 of pregnancy.

Immunostaining for ITGAV was detectable on all endometrial and conceptus tissues from days 10 to 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 (Fig. 4). Immunostaining for ITGB3 was detectable, but low in all endometrial and conceptus tissues from days 10 to 20. Interestingly, by day 24, ITGB3 became more apical in

distribution in GE, but formed intermittent aggregates at the conceptus–maternal interface (Fig. 5).

Immunostaining for ITGB1 and ITGB5 was 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 ITGs. 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 (Figs 6 and 7). Note the absence of ITG aggregation at the apical surface of LE for ITGAV, ITGB1, ITGB3, and ITGB5 immunostaining at day 24 nonattachment sites (Figs 4–7).

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 24 of pregnancy. Notably, LAP appeared to be distributed in the matrix that resides between the trophoctoderm and LE, and did not precisely colocalize with aggregates of ITGB1, ITGB3, and ITGB5 (Figs 5–7).

LAP binds to ITGs on pTr2 cells

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 nonreducing 7.5% SDS-PAGE gel. This resulted in biotinylated protein bands of $\sim$ 150 and 100 kDa. The molecular weight of 150 kDa corresponded with the known molecular weights of the nonreduced ITGAV subunit. The molecular weight of 100 kDa was within the range of multiple beta ITG subunits including ITGB1, ITGB3, ITGB5, ITGB6, and ITGB8 (Fig. 8A).

Figure 3 Detection of p-SMAD2/3 implies functional TGFB during implantation. Immunohistochemical localization of p-SMAD2/3 protein in days 16 and 20 conceptus attachment sites, and day 24 conceptus attachment and nonattachment sites. The brown color indicates positive immunostaining for p-SMAD2/3. On all days, p-SMAD2/3 was detected in nearly all the nuclei of endometrial LE and trophoblast. LE, luminal epithelium; GE, glandular epithelium; Tr, trophoblast. Width of fields is 230 μ m.

Immunoprecipitations performed on these eluates using antibodies directed against ITG subunits ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, and ITGB8 successfully confirmed binding of each of these ITG 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 (Fig. 8B). ITGAV immunoprecipitates also yielded a broad band at $\sim$ 100 kDa corresponding to the beta subunit of the heterodimer. ITGB1 has a reduced molecular weight of 130 kDa, and the ITGB1 immunoprecipitates yielded two bands in the range of 125–130 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 110–130 kDa. ITGB6 and ITGB8 immunoprecipitates yielded bands in the range of 95–110 kDa (Fig. 8B).

Discussion

Results of the present study in pigs provide evidence that during the porcine peri-implantation period: 1) latent and active TGFB are present in conceptus and endometrium; 2) TGFB is functional in both conceptus

and endometrium; and 3) LAP is a ligand for ITGAV-containing heterodimers in conceptus trophoblast and uterine LE. Our detection of LAP in conceptus and endometrial tissues, along with earlier data (Gupta *et al.* 1996, 1998), supports the finding that both the conceptus and uterus are sources of secreted TGFB. Further, detection of active TGFB predominantly at the apical surfaces of filamentous conceptuses and at the conceptus–maternal interface suggests that activation of latent TGFB appears to occur primarily at sites of conceptus attachments. Further, detection of p-SMAD2/3 at both conceptus attachment and nonattachment sites during porcine implantation suggests that TGFB binds and activates TGFBRs at both attachment and nonattachment sites. Presumably, levels of p-SMADs 2/3 detected in these studies may also be affected by the levels of certain phosphatases, although those were not determined in the current study.

Large aggregates of LAP, lower levels of TGFB, and abundant p-SMAD2/3 at the conceptus–maternal interface on day 24 of pregnancy provide evidence for the maintenance of functionally active TGFB along with latent TGFB reservoirs during the later stages of implantation. In addition, the large aggregates of LAP

Figure 4 ITGAV and LAP colocalization during porcine pregnancy. Immunofluorescence colocalization of ITGAV (FITC; column 1) with LAP (Texas Red; column 2) and their colocalization (yellow; column 3) in day 12 endometrium, days 16 and 20 conceptus attachment sites, and day 24 conceptus attachment and nonattachment sites. ITGAV was detectable on all endometrial and conceptus tissues from days 10 to 24. Compare the antibody staining with staining using rabbit and goat IgG (bottom row). LE, luminal epithelium; GE, glandular epithelium. Width of fields is 230 μ m.

in the matrix between trophoblast and LE perfectly place this protein to bind ITGB1, ITGB3, and ITGB5 ITGs functioning as an adhesion molecule promoting attachment of the conceptus to the uterine LE. Indeed, affinity chromatography results demonstrate that LAP binds to the ITG subunits ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, and ITGB8 on the surfaces of porcine trophoblast cultured cells. These results, along with the detection of ITG aggregation at the conceptus–maternal interface, provide mechanistic and functional evidence for the hypothesis that LAP binding to ITGAV-containing

ITG heterodimers serves as an adhesion complex that promotes and stabilizes conceptus attachment to the uterine LE during porcine implantation.

Aggregation of ITGs at the conceptus–maternal interface in sheep and pigs was recently reported (Burghardt *et al.* 2009, Erikson *et al.* 2009). However, this is the first report of ITGB1 and ITGB5 aggregation at the conceptus–maternal interface in any species. Upon binding of ECM proteins, ITGs aggregate to enhance signal transduction and form cytoplasmic focal adhesions that associate with the actin cytoskeleton (Wozniak *et al.* 2004). Formation

Figure 5 ITGB3 and LAP colocalization during porcine pregnancy. Immunofluorescence colocalization of ITGB3 (FITC; column 1) with LAP (Texas Red; column 2) and their colocalization (yellow; column 3) in day 12 endometrium, days 16 and 20 conceptus attachment sites, and day 24 conceptus attachment and nonattachment sites. ITGB3 was low on all endometrial and conceptus tissues from days 10 to 20, but by day 24, formed aggregates at the conceptus–maternal interface. LE, luminal epithelium; GE, glandular epithelium. Width of fields is 230 μ m.

of focal adhesions can serve as signaling centers but also stabilizes cell attachments to the ECM by connecting ECM proteins to the actin cytoskeleton via actin filaments (Bershadsky *et al.* 2006). Immunostaining at porcine conceptus attachment sites identified intense and intermittent immunoreactive aggregates of ITG subunits ITGB1 and ITGB5 (beginning day 20) and ITGB3 (beginning day 24). These aggregates likely represent a response to ligand binding and assembly of focal adhesions. Indeed, the absence of ITG aggregation at the apical surface of LE strongly suggests that aggregates form in response to trophoblast attaching to LE. These aggregates imply that ITGs bind to ECM proteins, such as LAP, to assemble focal adhesions that stabilize conceptus attachment during porcine implantation. Changes in the ITG repertoire on LE, particularly with α v β 3, have been detected during implantation in other species and are associated with stabilizing stronger conceptus adhesions (Johnson *et al.* 2001, Burghardt *et al.* 2002, 2009, Lessey

2002, Armant 2005). During porcine implantation, α v β 1 and α v β 5 may play important roles for stabilizing initial attachments of the conceptus to the LE, and as implantation progresses, α v β 1, α v β 5, and α v β 3 may be necessary to support stronger placental attachments.

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 matrix metalloproteases (MMPs) can activate TGFB; however, ITGs also activate TGFB *in vivo* (Sato & Rifkin 1989, Lyons *et al.* 1990, Shull *et al.* 1992, Huang *et al.* 1996, Yu & Stamenkovic 2000, 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 ITG binding of LAP plays a significant role in activating TGFB *in vivo* (Yang *et al.* 2007). TGFB activation via ITGs can result from conformational changes in the latent complex.

Figure 6 ITGB1 and LAP colocalization during porcine pregnancy. Immunofluorescence colocalization of ITGB1 (FITC; column 1) with LAP (Texas Red; column 2) and their colocalization (yellow; column 3) in day 12 endometrium, days 16 and 20 conceptus attachment sites, and day 24 conceptus attachment and nonattachment sites. ITGB1 became more apical in distribution and formed aggregates at the conceptus–maternal interface by day 20. LE, luminal epithelium; GE, glandular epithelium. Width of fields is 230 μ m.

Activation of TGF β via α v β 3, α v β 5, α v β 6, and an unidentified ITGB1 heterodimer occurs if TGF β is part of the LLC (Annes *et al.* 2004, Wipff & Hinz 2008). LAP associated with the LLC binds ITGs that are linked to the actin cytoskeleton; and since ITGs can sense and respond to intracellular forces generated through the actin cytoskeleton and extracellular forces such as ECM rigidity, it is hypothesized that ITGs can activate TGF β via traction forces (Keski-Oja *et al.* 2004, Larson *et al.* 2006). Another mechanism of TGF β activation is plausible. LAP and ITG subunits ITGAV, ITGB1, ITGB3, ITGB5, and low levels of ITGB6 and ITGB8 were detected along the uterine LE, and ITGs α v β 3, α v β 5, α v β 6, and possibly α v β 1 have been determined to activate latent TGF β 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 TGF β of the LLC tethered to an ECM that resists traction forces exerted on the LLC, b) LAP bound to

ITG receptors, and c) presence of the actin cytoskeleton to generate force or provide resistance to extracellular forces (Wipff & Hinz 2008). During porcine implantation, the conceptus migrates along the uterine LE where the migrating conceptus may be a source of external force applied to the LAP–ITG adhesion complex. We hypothesize that the apically bound LAP–ITG adhesion complex, in addition to supporting conceptus attachments, can also activate TGF β 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 TGF β (Fig. 9).

Intracellular forces may also play a role in activating TGF β 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).

Figure 7 ITGB5 and LAP colocalization during porcine pregnancy. Immunofluorescence colocalization of ITGB1 (FITC; column 1) with LAP (Texas Red; column 2) and their colocalization (yellow; column 3) in day 12 endometrium, days 16 and 20 conceptus attachment sites, and day 24 conceptus attachment and nonattachment sites. ITGB5 became more apical in distribution and formed aggregates at the conceptus–maternal interface by day 20. LE, luminal epithelium; GE, glandular epithelium. Width of fields is 230 μ m.

Increased ECM and incorporation of ACTA2 into stress fibers significantly augment the contractile activity of fibroblastic cells and are hallmarks of connective tissue remodeling (Hinze *et al.* 2001), which is expected to take place in a mechanically stressed environment such as the expanding uterine wall during pregnancy. Cellular contraction is mediated by the cross bridging of 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 ITGs generates a force on the LAP–ITG complex, causing a conformational change in the LLC, and liberating TGFB from its latent complex.

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. Active TGFB can therefore bind and activate TGFBRs at both conceptus attachment and nonattachment sites.

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 (Feinberg *et al.* 1994), inducing expression of ITGs (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

Figure 8 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 nonreducing 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. Immuno-precipitants were separated by 7.5% SDS-PAGE under reducing conditions, transferred to nitrocellulose, and probed for biotin using ABC.

intracellular signaling within conceptus and uterine tissues, possibly regulating transcription of genes involved in, but not limited to, conceptus development and attachment during porcine implantation.

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 ~12 and 24 h 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 i.m. injection of tiletamine hydrochloride and zolazepam hydrochloride (6.6 mg/kg, Telazol, Fort Dodge Animal Health, Fort Dodge, IA, USA), and was maintained with isoflurane (2–3% in oxygen). After removal of the uterus, days 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 (greater than ten tissues per animal; OCT, Miles, Inc., Onenta, NY, USA) and freezing in liquid nitrogen. Remaining uterine and conceptus tissues were preserved in phosphate-buffered 4% paraformaldehyde (PAF) and embedded in paraffin (a minimum of ten tissues per animal). 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 nonattachment sites absent of conceptus tissues were dissected from the uterine horns. All attachment and nonattachment uterine sections from each animal were preserved using both methods stated above.

Figure 9 Working model for force-generated activation of TGF β at the conceptus–maternal interface. (A) Integrin heterodimers on porcine trophoblast (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 $\alpha\text{v}\beta 3$, $\alpha\text{v}\beta 5$, $\alpha\text{v}\beta 6$, and possibly $\alpha\text{v}\beta 1$ may serve to activate TGF β . 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 TGF β . TGF β is liberated from the latent complex and is available to bind to TGFBR on porcine Tr and uterine LE.

Three to four tissues from each attachment and nonattachment site were embedded in OCT or paraffin. For immunofluorescence and immunohistochemical staining, three areas from each of these tissues from all animals were examined.

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, USA); rabbit anti-ITGAV (#AB1930), ITGB1 (#AB1952), ITGB3 (#AB1932), and ITGB5 (#AB1926) ITG subunits from Chemicon (Temecula, CA, USA); and rabbit anti-ITGB6 (beta 6, #SC15329) and ITGB8 (beta 8, #SC25714) ITG subunits from Santa Cruz (Santa Cruz, CA, USA). Secondary antibodies used for immunofluorescence staining included FITC-conjugated rabbit anti-chicken, Texas Red-conjugated mouse anti-goat from Pierce Biotechnology Inc. (Rockford, IL, USA) and FITC-conjugated donkey anti-rabbit from Southern Biotech (Birmingham, AL, USA).

Discriminating between latent and active TGFB depends on specific antibodies, fixation methods, and tissue preparation (Barcellos-Hoff *et al.* 1994, 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 μ M), mounted on Superfrost Plus glass slides (Anapath, Cheyenne, WY, USA), and immunofluorescent colocalization 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 0.02 M PBS, blocked with 0.5% casein in PBS, and incubated overnight at 4 °C with anti-TGFB and anti-LAP primary antibodies at concentrations of 1.25 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 concentrations of 3.75 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, USA) and then cover slipped.

ITGs 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 μ g/ml) along with each ITG subunit ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, or ITGB8 primary antibody (5 μ g/ml). Controls included separate sections incubated with each anti-ITG 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 cover slipped 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 μ M) 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 μ g/ml) or control Rabbit IgG (0.4 μ g/ml) in 2% 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, USA) for 30 min at room temperature, and then rinsed again with PBS and 0.5 M Tris-HCl. Immunoreactions were detected with peroxidase solution (diaminobenzidine/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, USA), rinsed with distilled water, dehydrated with the reverse series of graded alcohol, and covered slipped with Clarion Mounting Medium (Biomedica Corp, Foster City, CA, USA).

Photomicrography

Representative immunofluorescence and immunohistochemistry images were collected and analyzed using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY, USA) 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 into TIFF file format, and Adobe Photoshop CS2 (version 9.0, Adobe Systems Inc.) 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). Cell culture and biotinylation were performed in three separate experiments; and lysates were subjected to chromatography and subsequent immunoprecipitations. Cells were rinsed with Ca²⁺-Mg²⁺-free PBS following with a short incubation of 5 mM EDTA for 60 s 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*-hydroxysulfosuccinimido-biotin, EZ-Link Sulfo-NHS-Biotin (Pierce Biotechnology, Inc.)

for 1 h in the dark at room temperature. The reaction was stopped with 0.1 M glycine in PBS. Cells were then lysed with 50 mM *n*-octyl- β -D-glucopyranoside (OSGP) lysis buffer (1 mM each Ca^{2+} / Mg^{2+} / Mn^{2+} and 3 mM phenylmethylsulphonyl fluoride), and the monolayer of cells was scraped and passed through a 25-gauge needle. Lysates were centrifuged (16 000 g) for 20 min at 4 °C, and the supernatant was retained for subsequent affinity chromatography and immunoprecipitation.

Affinity chromatography and immunoprecipitation

Recombinant simian LAP, produced by Sf9 insect cells (Invitrogen) infected with a recombinant baculovirus (generously provided by Dr J S Munger, New York University School of Medicine, New York, NY, USA) and purified as previously described (Munger *et al.* 1998), was dialyzed using Slide-A-Lyzer dialysis cassette (Pierce Biotechnology, Inc.) as per the manufacturer's instructions. LAP (4.3 mg) was coupled to cyanogen bromide-activated sepharose (Sigma #c9142) as per the manufacturer's instructions. A small sample of LAP coupled with sepharose beads was subjected to 12% reducing and nonreducing SDS-PAGE to verify that sepharose beads were coupled with nondenatured 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% OSGP, 1.5 mM Mg^{2+} , and 1.5 mM Mn^{2+} ; and with TBS containing 1% OSGP, 1.5 mM Mg^{2+} , and 1.5 mM Mn^{2+} . Eight fractions (0.5 ml/fraction) were eluted with 1% OSGP in TBS and 10 mM EDTA. Eluate fractions were run on a 7.5% nonreducing SDS-PAGE, blotted to nitrocellulose, blocked with 5% BSA in TBS containing 0.03% Tween 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, USA).

Eluate fractions containing biotinylated proteins were concentrated using Centrprep Centrifugal Filter Device (Millipore Corporation, Billerica, MA, USA). Eluate fractions were immunoprecipitated with antibodies to ITG subunits (ITGAV, ITGB1, ITGB3, ITGB5, ITGB6, or ITGB8) or normal rabbit serum control at a concentration of 2.5 $\mu\text{g}/\text{ml}$ and A-protein G agarose conjugated beads (Santa Cruz Biotechnology) as previously described (Jaeger *et al.* 2005). Briefly, eluates were precleared with normal rabbit serum and A-protein G agarose, and incubated with anti-ITG subunits or control IgG overnight with end-over-end rotation at 4 °C. A-protein G agarose beads were washed with RIPA buffer (0.02 M 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.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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