Original Articles

Expression of α5β1 Integrin and Fibronectin during Early Pregnancy in Pigs

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Abstract. The implantation in pig is superficial and non-invasive, involving phases of apposition, adhesion and attachment of conceptuses to endometrial surface epithelium. The role of integrins and ECM proteins is suggested. In the study, the expression of α5β1 integrin and FN on conceptus trophectoderm and endometrium during implantation and early pregnancy was investigated. The immunohistochemical localization of α5β1 integrin and FN was performed on formalin-fixed, paraffin-embedded tissue sections using the ABC method. The results indicate that both conceptus and uterus expressed α5β1 integrin and FN during early porcine pregnancy. The most intensive staining for α5β1 integrin and FN was found in conceptus trophectoderm and endometrial surface epithelium in all investigated periods. During placentation the immunohistochemical staining for both α5β1 integrin and FN was increased in trophectoderm and all endometrial structures. Since placenta in pigs is non-invasive, it can be suggested that both α5β1 integrin and FN participate in molecular events leading to successful implantation and placentation in species with true epitheliochorial placenta.

Placenta in pigs is diffuse, epitheliochorial, accomplished with interdigitation of the microvilli of trophectoderm and surface uterine epithelium and without penetration of uterine epithelium and endometrial stroma. The contact between conceptus and endometrium depends on precise cell-cell and cell-extracellular matrix interaction events, mediated by cell surface receptors and their ligands.

Integrins are transmembrane glycoproteins, composed of at least 18 α and 9 β non-covalently linked subunits (Burghardt et al., 1997). They possess the ability to bind different extracellular matrix (ECM) proteins to mediate adhesion, migration and differentiation as well as to transduce signals into the cells (Albelda and Buck, 1990; Hynes, 1992). Since the integrins are receptors for matrix proteins, a hypothesis exists for the participation of bifunctional ligands through which interaction between trophectoderm and uterine epithelium is accomplished (Bowen and Hunt, 2000). Fibronectin (FN) is one of the ubiquitous homodimeric ECM proteins that undergoes alternative splicing to provide a variety of binding sites for different integrins. Up to 20 different isoforms of FN are known to be formed by alternative splicing of the primary RNA, which play a role in cell adhesion and migration, implantation, embryogenesis and oncogenic transformation (Vahery and Mosher, 1978; Hynes and Yamada, 1982; Mosher, 1984; Zetter and Brightman, 1990; Kornblihtt et al., 1996).

Integrins are regulated spatially and temporally within the uterus throughout the reproductive cycle and early pregnancy. Recent studies showed that integrins and different ECM proteins have been involved in implantation events in human (Lessey et al., 1994; Aplin et al., 1997), mice (Sutherland et al., 1993), baboon (Fazleabas et al., 1997), pigs (Bowen et al., 1996; Burghardt et al., 1997), sheep (Johnson et al., 2001), goat (Garcia et al., 2004) and cattle (MacLaren and Wildeman, 1995; Johnson et al., 1999).

To date, there are no studies concerning the expression and possible involvement of integrin heterodimers and their ligands during pregnancy in pigs. Thus, the aim of the study is to investigate the expression of α5β1 integrin and FN during early pregnancy in pigs.

Material and Methods
Animals

Crossbreed gilts (Large White x Landrace) were artificially inseminated 12 and 18 h after oestrus detection (the day of oestrus was designated as day 0) (Camberow Meat Production Farm). Animals were slaugh-

tered (3 per group) on days 15, 20, 30 and 35 of pregnancy. The uterine horns were removed and immediately dissected along the antimesometrial region. The conceptuses and chorion-endometrium were collected and fixed in 10% buffered formalin.

Tissue preparation for histology and immunohistochemical localization

Tissue samples, including foetal and maternal placenta, were processed after fixing in 10% buffered formalin for 12–24 h. The tissues were washed in running tap water and dehydrated by sequential washing as follows: 1 x 1 h in 60%, 70% and 80%, 2 x 2 h in 95% and 3 x 1 h in 100% ethanol. The alcohol was removed using cedar oil until the samples become translucent, after that they were rinsed in xylene and embedded in paraffin. The primary antibodies used were rabbit anti-human FN (Sigma-Aldrich Inc., St. Louis, MO) and goat anti-FN receptor (integrin α5β1; Chemicon International Inc., Temecula, CA). The ABC method was performed according to the established procedure. Tissue sections were blocked with 2.5% horse serum for 30 min at 37 °C in humidity chamber. Primary antibodies were added in appropriate dilutions and sections were incubated overnight at 4 °C in humidity chamber. Sections were incubated consecutively with biotinylated antibodies and streptavidin-HRP (Vectastain Universal Quick Kit; Vector Laboratories Inc., Burlingame, CA) for 1 h at 37 °C in humidity chamber. The antibody binding was visualized for 10 min using 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich Inc.) as chromogen. The sections were counterstained with haematoxylin, washed, dehydrated and cover-slipped in canada balsam. For controls, the primary antibody was replaced with isotype-matched control antibody. All sections were stained immunohistochemically under the same conditions. The staining intensity was evaluated by a semi-quantitative scoring system as follows: absent (-); weak (+), moderate (++), strong (+++) or very strong (++++). For each section, multiple fields were viewed and recorded. The average score was taken from slides of 3 animals on each day of pregnancy and the photomicrograph taken from 1 of 3 slides of each day of

pregnancy is presented here. Photomicrographs of representative fields of immunohistochemistry were evaluated using an Olympus BX 40 microscope fitted with an Olympus C5050Z digital camera (Olympus Optical Co, Ltd). Digital images were captured using Adobe Photoshop v.4.0 (Adobe Systems Inc.) and photomicrographs were electronically printed.

Results
Expression of FN

On day 15 of pregnancy FN exhibited strong apical and basal staining in conceptus trophectoderm and extra-embryonic endoderm (Fig. 1A; Table 1). The same pattern of expression was observed in uterine surface epithelium (Fig. 1A). Diffuse immunohistochemical reaction for FN in glandular epithelium and through endometrial stroma was found (Fig. 1B). Quantitative and qualitative changes in staining for FN were observed after attachment of the conceptus to the uterine surface epithelium. Gradually increased apical staining was observed in conceptus trophectoderm between days 20 and 35 of pregnancy. In the same periods, strong diffuse cytoplasm reaction, which is more intensive on the apical and basal parts of uterine surface epithelial cells, was found (Fig. 1C and D). In endometrium, immunoreactive FN demonstrates diffuse stromal reaction on day 20 (Fig. 1C), but with more compact fibrils on day 30, especially near to the surface epithelium (Fig. 1D). An increase of staining intensity in apical and basal poles of glandular epithelium was also found (not shown). The staining for FN was weaker at the sites of attachment in comparison with that in the bases of the trophoblastic folds (Fig. 1D and E). In the endometrial stromal and endothelial cells, diffuse cytoplasm staining for FN was also observed. In all studied periods the expression of FN was stronger in trophectoderm in comparison with endometrial surface epithelium, and with a more intensive reaction in the area of embryo-uterine contact.

Expression of α5β1 integrin

On days 15 and 20 of pregnancy there was strong immunohistochemical staining for α5β1 integrin in

Table 1. Relative assessment of the intensity of immunohistochemical staining for α5β1 integrin and fibronectin in the porcine conceptus and uterine tissues

Tissue [a]	Staining intensity for α5β1 integrin on:				Staining intensity for fibronectin on:			
	day 15	day 20	day 30	day 35	day 15	day 20	day 30	day 35
en	+++	+++	——	——	+++	+++	——	——
tr	+++	+++	++++	++++	+++	+++	++++	++++
se	++	++	+++	+++	+++	+++	+++	+++
ge	+	+ [b]	++ [b]	+++ [b]	++	++ [b]	++ [b]	++
end	++	+	++	++	+	++	+++	+++

[a]en - extra-embryonic endoderm; tr – trophectoderm; se – surface epithelium of uterine endometrium; ge – glandular epithelium of uterine endometrium; end – endometrial stroma; [b]photographs not shown
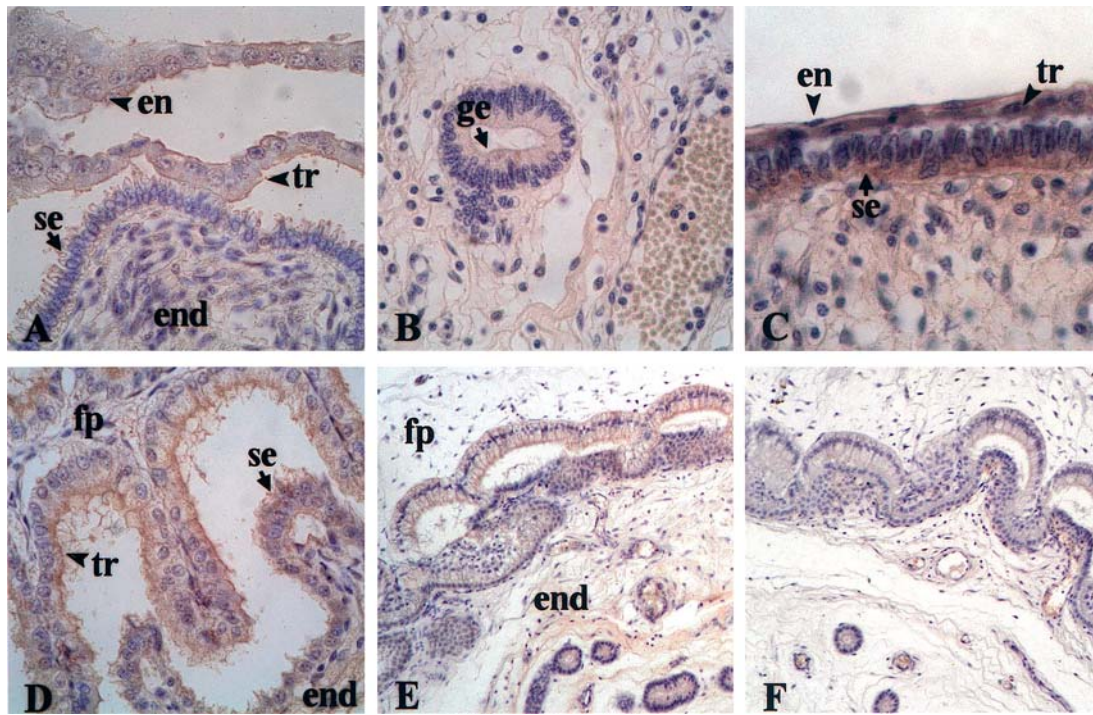
Fig. 1. Immunohistochemical localization of fibronectin in porcine conceptuses and endometrium during attachment/implantation and placentation. Conceptuses and endometrium were obtained on day 15 (A and B), day 20 (C), day 30 (D) and day 35 (E and F) of pregnancy and were fixed in 10% buffered formalin for immunohistochemical characterization. Tissue sections were stained with rabbit anti-human fibronectin (A, B, C, D and E) or isotype-matched antibody (F). Magnification: A, B and D, x 300; C, x 400; E and F, x 100.

porcine trophectoderm and extra-embryonic endoderm (Fig. 2A and C), which was more intensive in supranuclear cytoplasm. Strong staining in endometrial surface epithelium and moderate staining in glandular epithelium with a diffuse cytoplasm pattern of expression were observed (Fig. 2A and B). Weak staining was found on some stromal and endothelial cells (Fig. 2B).

As pregnancy progressed, an increase in the staining intensity for α5β1 integrin was observed. On days 30 and 35, strong apical and basal staining in trophectoderm and uterine surface epithelium was found, with more intensive staining in contact points between the two epithelial layers (Fig. 2D and E).

Discussion

The investigations of integrin subunits in human were designed to search for markers of endometrial receptiveness, clarification of their role in implantation, infertility and pathology of pregnancy. These markers are characterized with spatial and temporal expression during the period of blastocyst adhesion to endometrial epithelium, so-called "implantation window" (Lessey et al., 1992; Klentzeris et al., 1993; Lessey and Castelbaum, 1995). Integrins are the main group of adhesion molecules with a proven role in the processes of blastocyst adhesion and invasion of endometrial stroma and endometrial capillaries. Many integrins are receptors for proteins of ECM, which are expressed by trophoblast and endometrial epithelial cells and serve as bifunctional molecules between the two epithelia. In the cases with diffuse epitheliochorial placenta (pig placenta), implantation ends with the blastocyst adhesion and attachment to the endometrial epithelium without a subsequent penetration of basal membrane and invasion of endometrial stroma. This type of placenta is an excellent model for the investigation of early interactions between the foetus and endometrial epithelium, and the role of apically expressed adhesion molecules and the components of extracellular matrix during implantation and early pregnancy.

In studies on the expression of integrin subunits and components of ECM during attachment/implantation in pig pregnancy, Bowen et al. (1996, 1997) found that trophectoderm and uterine surface epithelium express α4, α5, αv, β1 and β3 integrin subunits. In combination with β1 and β3 integrin subunits they can form integrin heterodimers α4β1, α5β1, αvβ1 and αvβ3, which are receptors for ECM proteins (FN, vitronectin, osteopontin and others). The maximal degree of expression was observed during the period of elongation and maternal recognition.

In our study we found that α5β1 integrin and FN are expressed in both porcine conceptus and uterine endometrial tissues during early pregnancy, with increased staining during placenta formation.

During attachment of the blastocyst to the endometrial surface epithelium there is an intensive reaction for FN in both conceptus trophectoderm and uterine sur-
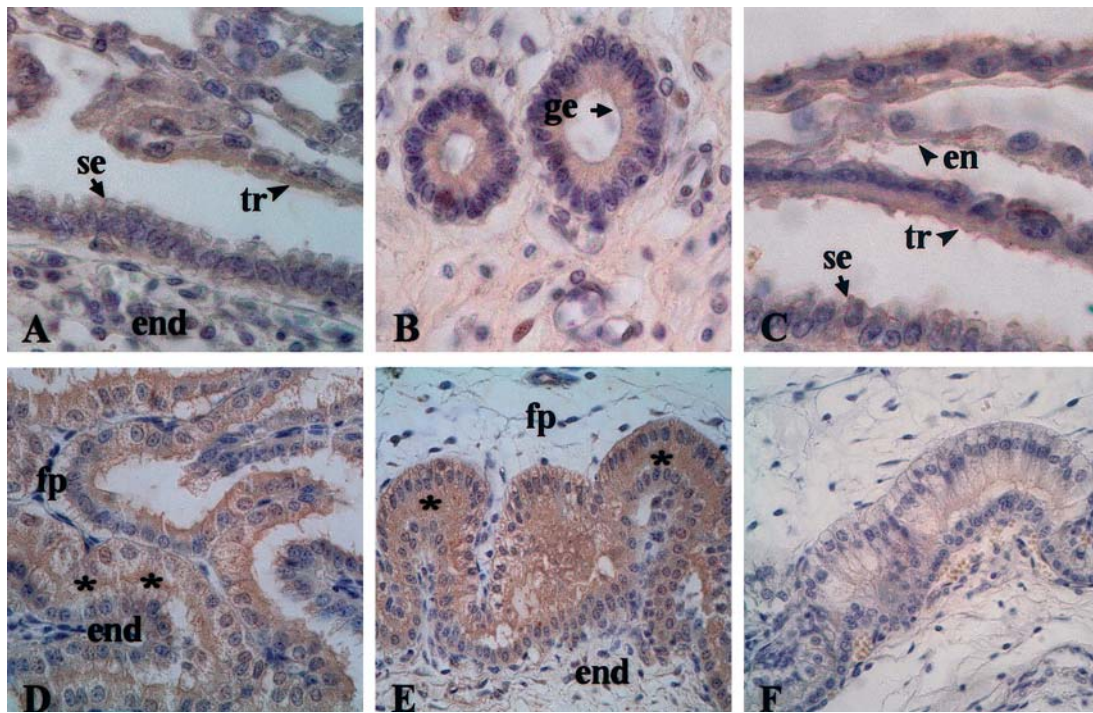
Fig. 2. Immunohistochemical localization of α5β1 integrin in porcine conceptuses and endometrium during attachment/implantation and placentation. Conceptuses and endometrium were obtained on day 15 (A and B), day 20 (C), day 30 (D) and day 35 (E and F) of pregnancy and were fixed in 10% buffered formalin for immunohistochemical characterization. Tissue sections were stained with goat anti-fibronectin receptor (integrin α5β1) (A, B, C, D and E) or isotype-matched antibody (F). The stars indicate the sites of embryo-uterine contacts. Magnification: A, B and C, x 400; D, x 300; E and F, x 200.

face epithelium. These data are in accordance with the results of Tuo and Bazer (1996), who proved the expression of oncofoetal FN in trophectoderm and uterine surface epithelium throughout porcine pregnancy. On the other hand, Bowen et al. (1996) have not found FN expression on the uterine surface epithelium. One possible explanation for the differences in this expression could be the specificity of the used antibodies, possibly recognizing different variable epitopes in the FN molecule.

Studies on the structure of the FN molecule showed that it consists of two semi-identical polypeptide chains (Mosher, 1984), each of which contains the RGD sequence (the recognition site of α5β1 integrin), so that each of them can bind α5β1 integrin. This tripeptide sequence was also found in other matrix proteins (vitronectin and osteopontin) and recognized by other integrin heterodimers, which are expressed at attachment sites during porcine pregnancy (Bowen et al., 1996).

Our data show that α5β1 is expressed on both superficial and glandular epithelia during all studied periods. The expression of α5β1 increases with advancing pregnancy and may be steroid-dependent. As reported by Bowen et al. (1996), P_4-treated gilts demonstrated high intensity of expression of α5 and β1 integrin subunits. Therefore, when the conceptus secretes oestrogen during maternal recognition, the expression of α5β1 integrin will not be altered because progesterone will stimulate the uterine epithelium to continue to express the α5β1 integrin heterodimer.

Lessey et al. (1996) reported that the α5 integrin subunit is not expressed on endometrial surface epithelium in human endometrium during the menstrual cycle. Thus, it can be supposed that the expression of α5β1 integrin is closely linked with the mechanisms of implantation in both species.

It was proved that α5β1 contributes to FN network formation, which can be triggered upon binding the RGD sequence into the FN molecule and is efficient in stimulating the polymerization process (Wennerberg et al., 1996; Johansson et al., 1997). Three different mechanisms exist for blastocyst-endometrial interactions: (1) integrin expressed on the trophectoderm binds to ligands on the uterine surface epithelium; (2) integrin expressed on the uterine epithelium binds to ligands on the trophectoderm; or (3) integrins are expressed on both epithelia and bind to ECM proteins found in the maternal-foetal interface. Since both porcine trophectoderm and uterine surface epithelium express α5β1 integrin and FN, it can be assumed that both epithelia have capacity to bind FN, as well as accumulate and assemble FN molecules on the maternal-foetal interface. Perhaps it leads to a blocking or restriction of blastocyst invasion (Damsky et al., 1994).

In conclusion, it can be suggested that α5β1 integrin and FN participate in molecular events leading to successful implantation/placentation in species with epitheliochorial placenta.

Future studies will be necessary to identify other apically expressed integrin heterodimers and ECM proteins at the maternal-foetal interface during porcine pregnancy.

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