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Immunohistochemical distribution of early pregnancy factor in ovary, oviduct and placenta of pregnant gilts

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Abstract

Early pregnancy factor (EPF) is an immunosuppressant that promotes maternal immune system tolerance of the allogenic fetus. Little is known about localization of this factor in different tissues and nothing has been reported about localization in swine reproductive and placental tissues. We determined the concentration of EPF in serum of gilts and porcine placenta conditioned medium (PPCM). We also analyzed the expression of EPF in different reproductive tissues of pregnant gilts at 10, 30, 60 and 90 days of pregnancy. EPF concentration in serum and PPCM was determined by western blot and densitometry. EPF expression in reproductive tissue was assessed by immunohistochemistry. The highest concentration of EPF was observed at 30 days in serum and PPCM; the concentration was higher in PPCM than in serum at the stages we evaluated. All reproductive tissues from the gestational stages analyzed showed specific labeling of EPF, but this labeling did not appear in non-pregnant gilts. At 30 days pregnancy, the EPF expression in the ovary was predominantly in follicular lutein cells, probably owing to its function as a luteotrophic factor. In the oviduct, EPF was expressed in unciliated secretory epithelial cells and in the cilia of ciliated cells. In the placenta, EPF was expressed in the fetal portion (mesoderm chorioallantois and epithelium of endoderm). EPF acts as an autocrine and paracrine growth factor for the trophoblast during the peri-implantation period.

Key words: early pregnancy factor, immunolocalization, ovary, oviduct, placenta, swine

The maternal immune system must tolerate an allogenic fetus. Early pregnancy factor (EPF) is one early indicator of immunosuppressive activity (Shahani et al. 1994, Cheng et al. 2000). EPF purified from human platelets is identical to chaperonin 10 (cpn-10) or heat shock protein 10 (HSP-10) (Cavanagh and Morton 1994, Quinn et al. 1994, Cavanagh 1996).

EPF was first detected in the serum of mice during early pregnancy as an immunosuppressive factor by using the rosette inhibition test (Morton et al. 1974). EPF is composed of two fractions, EPF-A and EPF-B. EPF-A is formed in the oviduct during estrus and pregnancy. By contrast, EPF-B is associated only with pregnancy and it is produced by the ovary (pre-implantation) and embryo (peri- and post-implantation) as the result of a combination of signals from the pituitary and the zygote (Morton et al. 1983, 1992b).

EPF can be detected in serum at 6 h after mating in mice (Morton et al. 1976) and sheep (Clarke et al. 1980), between 24 and 48 h in cattle (Ito et al. 1998) and within 48 h after insemination in humans (Morton et al. 1992b). Bemis et al. (2012) determined that day 8 equine embryos cultured for 48 h produces mRNA for EPF and may be involved in embryonic signaling in the mare. In pigs, EPF

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exhibits biphasic production that consists of a first peak between 24 and 48 h after mating after which levels decline until about 21 days after mating, then a second increase occurs toward the end of pregnancy and continues until the end of pregnancy (Morton et al. 1983, Merkis 2000).

EPF has been described as both a growth factor and an immunosuppressive agent. EPF activity has been demonstrated in tumor cell cultures (Quinn et al. 1990), which suggests a role as an autocrine or paracrine growth factor (Morton et al. 1992a,b). EPF has been found in the circulation of patients with ovarian cancer (Summers et al. 1998, Akyol et al. 2006). In addition, EPF has immunosuppressive properties that are mediated by at least two lymphokines, called EPF-induced suppressor factor 1 and EPF-induced suppressor factor 2. EPF is found in platelets (Rolfe et al. 1988, 1995, Cavanagh and Morton 1994), which suggests that it plays a role in inflammation and wound healing.

The protective and suppressive effects of EPF have been investigated in Lewis rats with experimental autoimmune encephalomyelitis (EAE), which suppresses acute and chronic EAE and increases the ability of interferon beta to suppress clinical signs of the disease (Athanasas-Platsis et al. 2003, Harness et al. 2003, Zhang et al. 2003). Also, EPF has been shown to enhance survival of the oligodendrocyte lineage O4 + cells (McCombe 2008).

Merkis (2000) reported that the serum fractions of 72 h and 7 day pregnant pigs showed EPF activity. The N-terminal amino acid of pig EPF was determined (Lys-Thr-Tyr-Lys-Ser-Glu-Ile-Ala-His-Arg-Asp-Phe-Lys-Leu-Asp-Gly-Gln-Gln-Leu-Tyr) and had a molecular weight of 29 kDa. The N-terminal amino acid sequence was 98% homologous to bovine serum albumin (Di Trapani et al. 1991).

We produced polyclonal antibodies to synthetic peptides that corresponded to different parts of the amino acid sequence of EPF to produce a more robust array of reagents (Grosso et al. 2008).

There are few reports concerning localization of EPF in reproductive tissues. In an earlier study, we established the location of EPF in rat placenta. EPF was present in giant and decidual cells, blood vessels and trophoblastic lacuna cells (Grosso el al. 2012). Cruz et al. (2001) conducted an immunohistochemical analysis of ovaries from pregnant and non-pregnant marsupials (*Sminthopsis macroura*). In a study of rat tissues, using electron microscopy and polyclonal antibodies, Sadacharan et al. (2001) found EPF in extramitochondrial compartments. EPF localization studies of human colon carcinoma tissue showed diffuse punctate cytoplasmic staining (Somodevilla-Torres et al. 2000). Immunolocalization of EPF has been reported in early equine embryos and reproductive tissues, i.e., ovary, oviduct and uterine tissue (Hatzel et al. 2012). There is no information, however, about EPF localization in the reproductive and placental tissues of swine. Therefore, we investigated the concentration of EPF in the serum of gilts and porcine placenta conditioned medium (PPCM) and to analyze the expression in different reproductive tissues of pregnant gilts throughout pregnancy.

Material and methods

Animals

Our research protocol was approved by Ethics Committee of the Universidad Nacional de Río Cuarto. Careful use of animals conformed to the specifications of the Canadian Council Animal Care Guide (1993). Blood was collected from 39 gilts at 10 (n = 6), 30 (n = 8), 60 (n = 8), 90 (n = 7) days of pregnancy and from nonpregnant animals (n = 10) from Río Cuarto, Córdoba (Salesian Agro-technical School Ambrosio Olmos, Córdoba, Argentina).

Tissue sampling

Blood samples were collected from the cranial vena cava and serum was obtained. Briefly, freshly collected blood was allowed to clot in a sterile glass tube for 30 min at room temperature, then centrifuged at approximately 2000 x g for 10 min to separate the clot. The serum then was aliquoted and stored at -20° C until they were analyzed.

Whole reproductive tracts were collected at slaughter (Rio Cuarto and Penny Lane) from 24 gilts. Tissues were taken from non-pregnant gilts (n = 5) and pregnant gilts on days 10 (preimplantation stage, n = 4), 30 (n = 6), 60 (n = 5) and 90 (n = 4). Fetal placental tissues were taken from three animals at 114 days of pregnancy (term). Tissue samples were washed with normal saline solution and transported immediately to the laboratory at 4° C where they were processed within 6 h after slaughter. Once in the laboratory, the tracts were washed with Hanks' buffered salt solution (HBSS Sigma C5502, St. Louis, MO) that contained 2% antibiotic-antimycotic solution, (10,000 units/ml penicillin, 10,000 µg/ml streptomycin and 25 µg/ml of fungizone, 15240062; Gibco Life Technologies, Grand Island, NY). These tissues were used to perform PPCM and immunohistochemistry as described below.

Gestational age determination

The location of the embryos or fetuses was detected by palpation. The uterine horns were opened longitudinally with an incision on the anti-mesometrial edge to observe the implantation sites and to gather samples of mesometrial, endometrial and fetal placental tissues. The gestational age of the placentas was determined according to the crown-rump length of the embryos and/or fetuses obtained from each pregnant gilt (Marrable 1971). Pregnancy during the pre-implantation stage was confirmed by visualization of endometrial autofluorescence under UV light, a characteristic of early pregnancy in pigs (Keys et al. 1989, Schweigert et al. 1999).

PPCM

Placentas from 30, 60 and 90 days of pregnancy were weighed and 5 g samples of the maternal-fetal interface were placed on sterile Petri dishes and homogenized in a tissue grinder. Tissue homogenates were cultured in a flask after diluting 1:20 with McCoy's 5A medium (M4892; Sigma) supplemented with 5% fetal calf serum (Natocor, Villa Carlos Paz, Córdoba, Argentina), 25 mM HEPES (H4034; Sigma), 1% antibiotic-antimycotic (10,000 units/ml penicillin, 10,000 µg/ml streptomycin and 25 µg/ml of fungizone, 15240062; Gibco Life Technologies, Grand Island, NY).

Cultures were incubated for 120 h at 37° C in a 5% CO_2 atmosphere (Incubator Labline Instruments, Tripunithura, India). The culture then was homogenized by stirring, filtered through sterile gauze and ultracentrifuged at 22,000 x g for 30 min (Heraeus Biofuge Stratos™, Thermo Fisher Scientific Inc., Waltham, MA), sterilized by filtration through a 0.22 µm sterile syringe filter, aliquoted and stored at -20° C until analyzed (Koncurat et al. 1998).

EPF determination

Rabbit polyclonal anti-porcine EPF antibodies (IgG) developed in our laboratory (Grosso et al. 2008) were used to determine the presence of EPF in serum and PPCM by western blot and in reproductive tissues by immunohistochemistry.

EPF concentration in serum and PPCM

Western immunoblotting was used to assess the presence of EPF. Proteins from serum (10 µg) or PPCM (15 µg) were applied to lanes of a 12% SDS-PAGE gel; 10 µg of synthetic EPF was applied as standard (Grosso et al. 2008). The proteins were separated electrophoretically according to Laemmli (1970) and analyzed by western immunoblot by probing with rabbit polyclonal anti-porcine EPF (1:100 in 10% skim milk in TBS-T) overnight at 4° C as the primary antibody, and peroxidase-conjugated anti-rabbit immunoglobulin as the secondary antibody (A6154; Sigma) 1:1000 in 10% skim milk in TBS-T for 90 min at room temperature. The bound immune complexes were visualized using 3.3′-diaminobenzidine (DAB) (D4418; Fast Tablets™, Sigma) and quantified using densitometry.

Densitometry

Membranes containing labeled EPF bands were digitized and analyzed using image processing software ("ImageJ" http://rsbweb.nih.gov/ij/). The differences between the relative intensities of the EPF bands and the background are net intensities of the color. An EPF standard (10 µg) was used as a positive control. The concentration was defined as the ratio of net intensity of the bands for serum or PPCM versus the intensity of EPF band of the standard.

Immunohistochemistry (IHC)

The reproductive tissues from pregnant gilts, 10 each at pre-implantation stage and at 30, 60 and 114 days of gestation, were processed for IHC using the avidin–biotin complex (Vectastain ABC Elite Kit 6200; Vector Laboratories, Inc., Burlingame, CA) (Hsu et al. 1981). Briefly, samples from ovary, oviduct and uterus or placenta tissue were fixed in buffered formalin, pH 7.4, dehydrated through graded alcohols to xylene, paraffin embedded at 56–58° C for at least 3 h (1172601; Cicarelli Santa Fe, Argentina) and 3–4 µm sections were cut (Jung ultramicrotome Leica RM 206; Wetzlar, Germany) and placed on Vectabond® slides (Vector Laboratories). Microwave antigen retrieval with antigen unmasking solution (H-3300; Vector Laboratories) was applied to the deparaffinized sections to detect EPF according to the manufacturer's instructions.

A standard avidin-biotin immunoperoxidase technique (BA-1000; Vectastain® ABC kit for rabbit IgG; Vector Laboratories) was used. Endogenous peroxidase activity was blocked by hydrogen peroxidase in methanol. Nonspecific protein binding was diminished using goat serum. The sections were incubated overnight with the primary antibodies: rabbit polyclonal anti-porcine EPF IgG

(Grosso et al. 2008) or pre-immune serum to controls. In the final step, DAB (Sigma) was added as chromogen and all sections were counterstained with Mayer's hematoxylin. One reference section was run in every IHC batch as an inter-assay control. All slides were coded. Photographs were taken using a Canon PowerShot G6, 7.1 megapixel, (Canon Inc., Ōta, Tokyo, Japan) through an Axiostar Plus Carl Zeiss microscope (Nikon Inc., Tokyo, Japan).

Semiquantitative evaluation of IHC labeling was conducted using a light microscope and slides were examined using a double grading system based on the intensity and distribution of stained cells by three scientists blinded to the identity of the sample. Staining intensity was graded as 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining. The values were expressed as median. Sections of oviducts, ovaries and placentas were evaluated. We evaluated surface epithelium and subepithelial connective tissue (lamina propria) of the isthmus and ampulla of the oviduct and the uterus, maternal and fetal portions of placentas and lutein cells, and stroma from the corpora lutea.

Statistical analysis

All statistical analyses were performed using the InfoStat software (Di Rienzo et al. 2008). EPF concentrations were expressed as means ± SD. Differences were considered significant at $p \leq 0.05$. A nonparametric test (Kruskal Wallis) was used to detect differences in EPF concentrations in serum or PPCM during gestation.

Results

EPF concentration in serum and PPCM

Western blot analysis showed a 29 kDa band in serum and PPCM of pregnant gilts at all stages of pregnancy, but not in nonpregnant animals (Fig. 1). The EPF concentrations in both serum and PPCM reached their highest levels at 30 days of pregnancy. In serum, EPF was present at 30 days of gestation at a level of 1.390 ± 0.080 ug/ul; this was significantly higher than that of nonpregnant (0.017 ± 0.003 ug/ul) animals. EPF also was present at 10 (0.631 ± 0.050 ug/ul) and 60 (0.568 ± 0.080 ug/ul) days of pregnancy. In PPCM at 30 days of gestation, EPF was present at a level of 3.090 ± 0.150 ug/ul; this value was significantly higher than that found at 90 days of gestation (0.180 ± 0.010 ug/ul) (Fig. 2). Also, we found significantly higher EPF levels in PPCM than in serum at all gestation days studied (Fig. 2).

EPF in reproductive tissues

All reproductive tissues of pregnant animals at all gestational stages analyzed showed specific EPF labeling, but no labeling was observed in tissues from nonpregnant gilts.

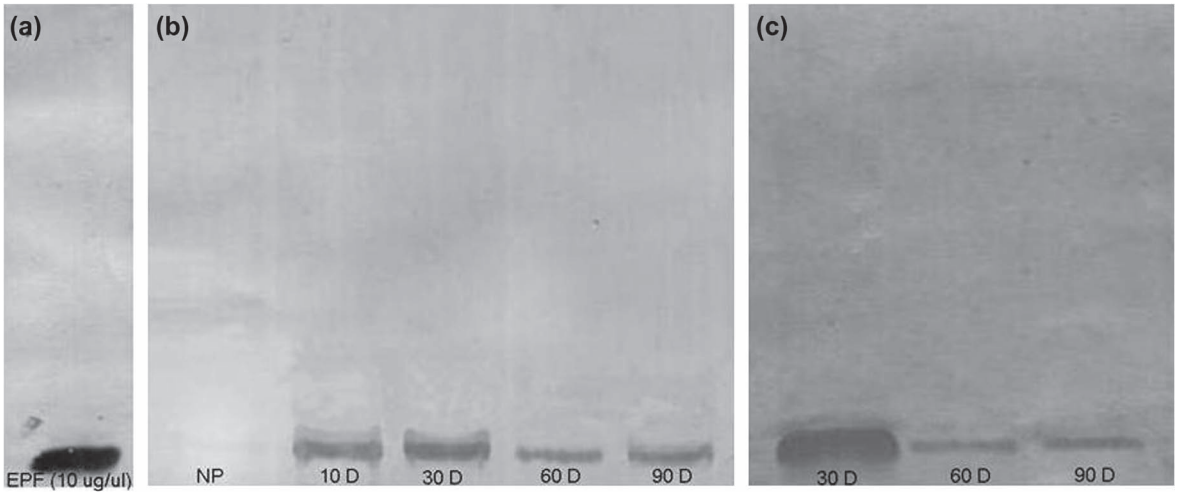

Fig. 1. (a) Representative western blot of EPF standard (10 μg). (b) Representative western blot of gilts serum tested for EPF. NP, nonpregnant; 10 D, 10 days of pregnancy; 30 D, 30 days of pregnancy; 60 D, 60 days of pregnancy; 90 D, 90 days of pregnancy. Rabbit anti-porcine EPF IgG (1/100) was used. Results are from four independent experiments. (c) Representative western blot of PPCM tested for EPF. 30 D, 30 days of pregnancy; 60 D, 60 days of pregnancy; 90 D, days of pregnancy. Rabbit anti-porcine EPF IgG (1/100) was used. Results are from four independent experiments.

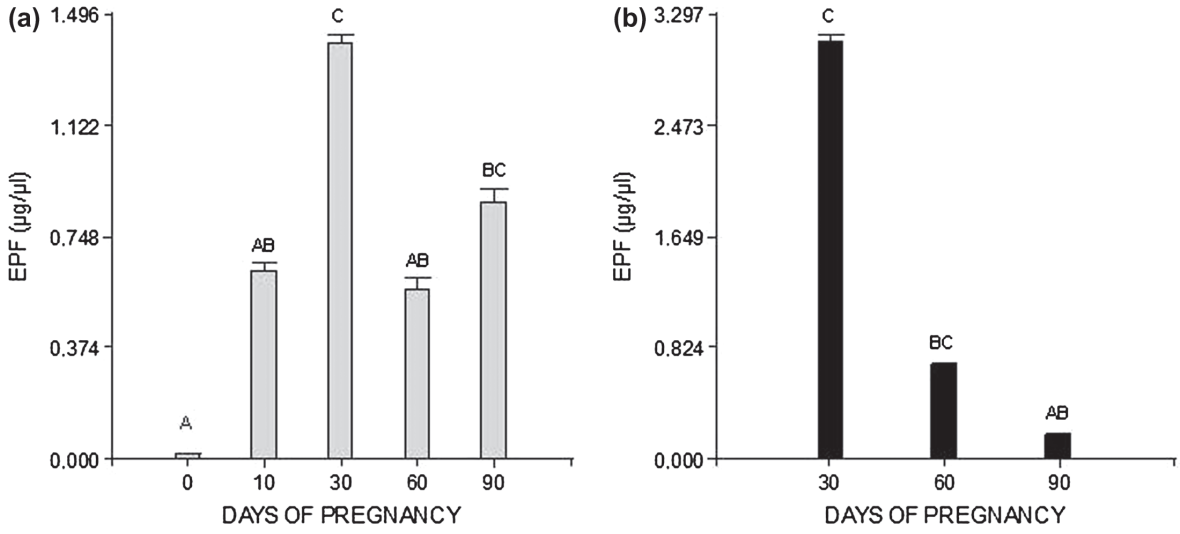

Fig. 2. Concentration evaluated by densitometry of EPF in serum (a) and PPCM (b) at different days of pregnancy. Values are means ± SD. Different letters indicate significant differences ($p < 0.05$).

Ten days of pregnancy (pre-implantation stage)

In the ovary, EPF was expressed mainly in the stroma. In the oviduct ampulla, EPF was located on the apical membrane of epithelial cells and in the secretion of these cells. In the isthmus, EPF was located in the connective tissue (lamina propria) and the endometrial glands (Fig. 3). In the uterus, the labeling was detected in the endometrium, mainly in connective tissue of the submucosa of the chorion and in the apical membrane of glandular epithelial cells (Table 1).

Thirty days of pregnancy

In the ovary, EPF expression was mainly in the corpus luteum and on the luteal follicular cells, but not in luteal theca cells (Fig. 4). In the oviduct, EPF distribution varied depending on the region of oviduct studied. The ampulla proximal to the ovary exhibited significant expression of EPF in the mucosal and submucosal folds, and in the unciliated secretory epithelial cells. In the ciliated epithelial cells, EPF expression was limited to the cilia. Some sectors showed EPF expression predominantly at

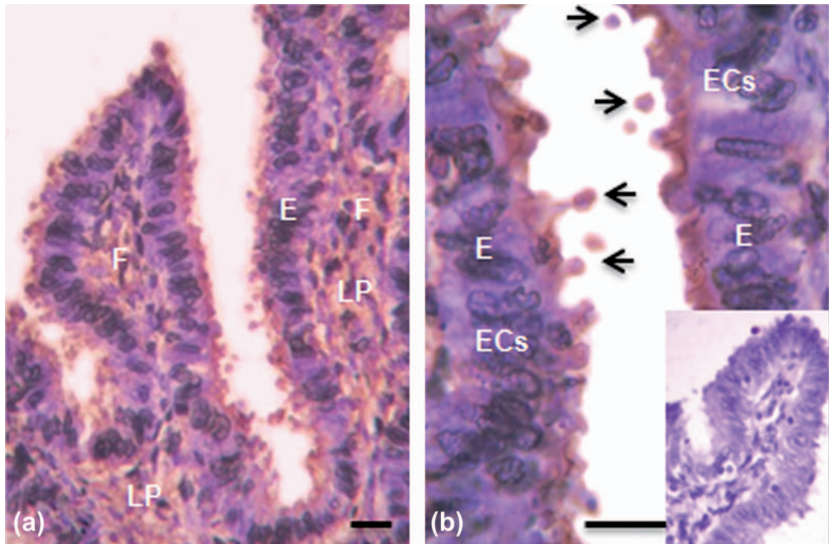

Fig. 3. Representative photomicrographs of immunolocalization of EPF in gilt oviduct ampulla at 10 days of pregnancy (pre-implantation). (a) (b) Scale bar = 20 µm. Inset shows negative control (× 100). ECs, secretory epithelial cells (unciliated); E, epithelium; F, mucosal folder. Arrows indicate secretion epithelial cells.

Table 1. Expressions and distributions of EPF in gilt reproductive tissues at different gestational stages

Tissues	Non pregnant	10 days of pregnancy	30 days of pregnancy	60 days of pregnancy	114 days of pregnancy
endometrium	0	2	NA	NA	NA
fetal placenta	NA	NA	2	1	1
maternal placenta	NA	NA	0	1	NA
oviduct	0	3	3	3	NA
ovary	0	2	2	2	NA

0, no staining; 1, weak; 2, moderate; 3, strong; NA, sample not available.
Numbers are median values.

unciliated secretory cells ("peg" cells), interspersed with smaller areas of ciliated epithelial cells positive to EPF. The ampulla distal to ovary showed ciliated and unciliated secretory cells distributed homogeneously and alternately. Few secretory cells were present in the isthmus and these expressed EPF in the cytoplasm. In ciliated cells of the isthmus, EPF labeling was observed in the apical membranes and cytoplasm (Fig. 5, Table 2). EPF appeared in the fetal placenta, mainly in the connective tissue of the chorioallantois and epithelium of the allantois wall. Staining appeared as intracytoplasmic dots in the apical membrane of allantoic cells (Fig. 6).

Sixty days of pregnancy

In the ovary, EPF was expressed in luteal follicular cells, but not in luteal theca cells. The epithelium of the oviduct showed a predominance of ciliated cells that expressed EPF in the apical membrane and in the cytoplasm. Ciliated epithelial cells were interspersed with epithelial secretory cells that contained specific EPF staining of secretory vesicles (Fig. 7). The fetal placenta showed specific EPF in the epithelium of allantoic epithelium, the chorionic epithelium of the villi and low intensity staining appeared in the chorionic connective tissue. The maternal placenta at 60 days of pregnancy showed EPF staining in the connective tissue of the endometrium.

One hundred fourteen days of pregnancy (term)

No EPF expression was detected in ovary, oviduct or maternal portion of the placenta at this stage of pregnancy. EPF was expressed in the chorionic epithelial tissue and connective tissue from the wall of the allantois in the fetal portion of the placenta (Table 1).

Discussion

The interplay of signals between mother and fetus plays an important role in maternal immunological tolerance for fetuses, half of whose genes are foreign. We investigated the presence of EPF, which is not species-specific and appears only during pregnancy, within 6–24 h of fertilization. EPF has both immunomodulatory and growth factor properties (Morton et al. 1983, Rolfe et al. 1988, 1995, Quinn et al. 1990).

We detected EPF in serum and PPCM of pregnant gilts during pregnancy, which is consistent with the report by Morton et al. (1983) that in the

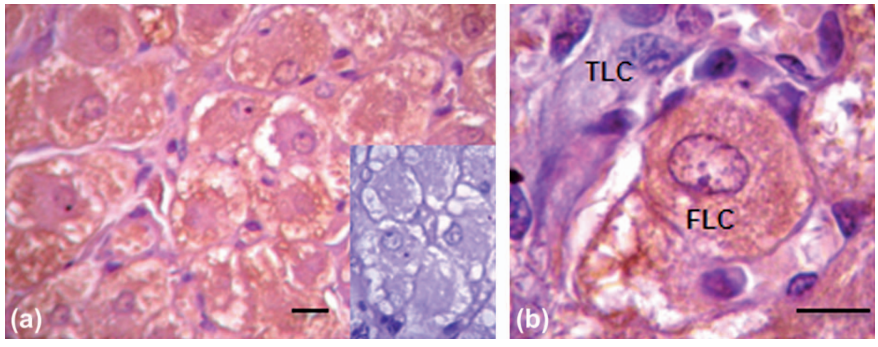

Fig. 4. Representative photomicrographs of immunolocalization of EPF in gilt corpus luteum at 30 days of pregnancy. (a) (b) Scale bar = 20 μm. Inset shows negative control (× 100). TCL, luteal theca cells; FLC, luteal follicle cells.

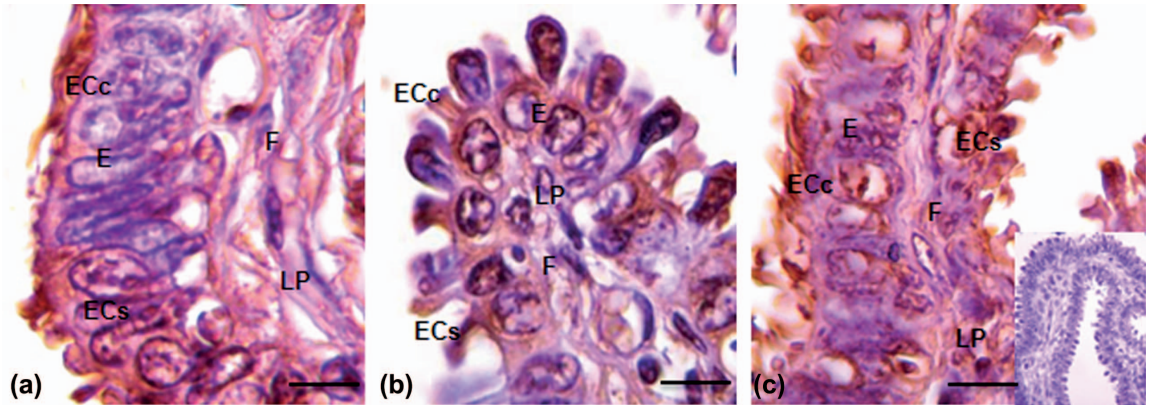

Fig. 5. Representative photomicrographs of immunolocalization of EPF in gilt oviduct at 30 days of pregnancy. Scale bar = 20 μm. (a) Ampulla of oviduct proximal to the ovary. (b) Ampulla of oviduct distal to the ovary. (c) Isthmus of oviduct. Inset shows negative control (×50). F, mucosal fold; E, epithelium; LP, lamina propria; ECs, secretory epithelial cells (unciliated); ECc, ciliated epithelial cells.

pig, EPF is present virtually until the end of pregnancy. We found the highest concentration of EPF in both serum and PPCM at day 30 of pregnancy, which is related to the peri-implantation stage in pigs. EPF could be one of the immunomodulators involved in developing the immune tolerance that is required for implantation.

The literature contains no reports concerning the expression of EPF in porcine reproductive tissues throughout pregnancy. We found that the highest concentration of EPF in sera and placentas was at 30 days of pregnancy; in sera, there was a second minor peak within 90 days of pregnancy. The highest concentration of EPF at day 30 of pregnancy could be related to the immunomodulatory function related to implantation. The second EPF peak in serum occurred at the end of pregnancy and likely was related to the role of EPF as a growth factor during a period of exponential growth (Morton et al. 1992).

We did not detect EPF in tissues from nonpregnant gilts. EPF is a pregnancy marker that normally is detected during pregnancy. It has been reported that the oviducts of animals in estrus can produce low levels of EPF-B, which could lead to a false positive interpretation in the rosette inhibition test (Cavanagh et al. 1982). Our results are similar to those observed in marsupials (Cruz et al. 2001) in which EPF was detected by immunohistochemistry in ovaries from pregnant, but not nonpregnant animals.

EPF was detected in tissues of pregnant gilts during the pre-implantation stage; the EPF expression was significant in ovarian stroma. This finding could be related to ovum factor (OF), which is secreted by the ovum when sperm penetration occurs. OF signals fertilization to the ovary, which releases EPF-B (Cavanagh et al. 1982). In culture, the ovaries of pregnant mice produce EPF for 24–48 h (Morton et al. 1980). EPF expression in the oviduct was intense, mainly in secretory vesicles in the apical part of epithelial secretory cells, which suggests that at the pre-implantation stage, EPF originates from the oviduct.

We propose that EPF expressed in tissue during the pre-implantation stage is produced by the ovary and oviduct. We believe that EPF released into the lumen of the oviduct creates a beneficial

Table 2. Expressions and distributions of EPF in epithelial cells of different parts of the oviduct of gilts at 30 days of gestation

Part of the oviduct	EPF	Secretory cells (peg)	Ciliated cells
ampulla proximal to ovary	distribution	in groups, interspersed	
	expression	cytoplasm 3	cilia 3
ampulla distal to ovary	distribution	individually, interspersed	
	expression	cytoplasm 3	cytoplasm and cilia 3
isthmus	distribution	predominantly in ciliated cells	
	expression	cytoplasm 1	cytoplasm and cilia 3

0, no staining; 1, weak; 2, moderate; 3, strong.

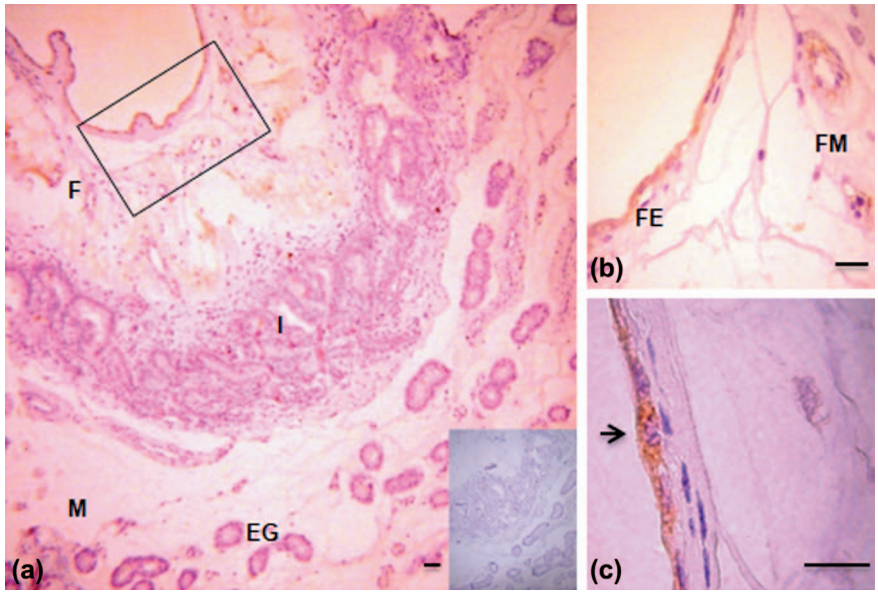

Fig. 6. Representative photomicrographs immunolocalization of EPF in gilt placenta at 30 days of pregnancy. Scale bar = 20 μm scale. (a) Maternal fetal interface. Selected areas are shown at higher magnification in (b) × 400 and (c) × 1000. (b) Fetal placenta. Immunoreactivity is present in epithelium and connective tissue. (c) Arrows indicate intracytoplasmic immunoreactivity at epithelium of endoderm. Inset shows negative control (× 50). F, fetal placenta; M, maternal placenta; I, Maternal-fetal interface; EG, endometrial glands; FE, fetal epithelium; FM, fetal connective tissue.

environment for fertilization, which ensures the survival of embryos. EPF expression in connective tissue of submucosa of the chorion is due to the EPF produced in the oviduct, which reaches the endometrium by transport through the blood and the lumen of the oviduct. In this location, EPF could act as an immunomodulator and play a role in endometrial growth in preparation for receiving embryos.

At 30 days of pregnancy, EPF appeared in fetal placental tissues (endoderm, allantois wall and connective tissue, and fetal chorion), but not in maternal placental tissues. At this stage of pregnancy, when implantation is well established, we found EPF in the fetal placenta, which corresponds to EPF produced by the embryo after implantation (Morton et al. 1983 1980). Unlike our observations, day 8 horse embryos exhibited cytoplasmic EPF staining throughout the ectodermal cells that form the trophoblast, except in the nuclei. Also, day 25 horse embryos demonstrated intense localization

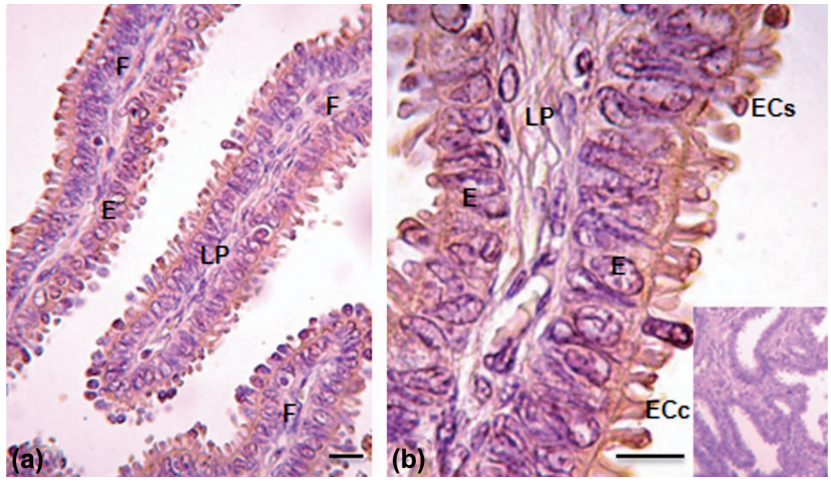

Fig. 7. Representative photomicrographs of immunolocalization of EPF in gilt oviduct at 60 days of pregnancy. (a) (b) Scale bar = 20 μm scale. Inset shows negative control (× 50). F, mucosal fold; E, epithelium; LP, lamina propria; ECs, unciliated secretory epithelial cells; ECc, ciliated epithelial cells.

along the apical boarder of the ectodermal cells that form the trophoblast layer of the developing chorion (Hatzel et al. 2012). We found that at 60 days of pregnancy, EPF was expressed equally in fetal and maternal placental tissues. At 114 days of pregnancy, EPF expression also was evident in the epithelium and connective tissue of the chorioallantois. Owing to the type of epitheliochorial placentation, it is not possible to recover the maternal placenta, because is not expelled at birth. At 60 and 114 days of pregnancy, EPF would originate from the oviduct or the embryo and probably would play a role as an autocrine growth factor for the embryos during the last third of pregnancy when their exponential growth occurs.

In the ovary at 30 and 60 days of pregnancy, EPF appeared in luteal follicular cells, but not in luteal theca cells. Luteal follicle cells remained after ovulation and contained lipid, which is characteristic of cells that secrete steroid hormones such as estradiol and progesterone. Other investigators have described EPF staining in the ovarian granulosa and luteal cells at 8 and 25 days of pregnancy (Hatzel et al. 2012). The expression of EPF that we observed in the ovary at 30 and 60 days of pregnancy is at variance with the report by Morton et al. (1983). EPF, in the ovary, probably acts as luteotrophic factor, but originates in the oviduct or embryo. The significant expression of EPF in the unciliated secretory epithelial cells (plug cells) and in the cilia of epithelial cells of the oviduct suggests a correspondence with EPF produced by oviduct plug cells throughout pregnancy and moved by the cilia to the uterus to act as a growth factor for embryos. In addition, EPF could reach the endometrium by an endocrine pathway.

The anti-EPF antibodies used for immunohistochemistry are specific against EPF obtained from swine serum at 7 days of gestation, so the antibodies would be directed against EPF produced by maternal tissues (ovary and oviduct) prior to implantation (Grosso et al. 2008). These antibodies recognize and bind specifically to the EPF produced by the ovary and oviduct (pre-implantation) and to that produced by the embryo (post-implantation). This suggests that EPF-A and EPF-B share epitopes or are different isoforms of the same protein whose sites of production may differ (Bose and Lacson 1995, Bose 1997).

EPF acts as an autocrine or paracrine growth factor for regenerating normal liver cells (Quinn et al. 1994). The fact that EPF was expressed in the fetal trophoblast suggests that it acts as growth factor for trophoblastic cells during the post-implantation period. Our findings consistent with those of Nahhas and Bernea (1990) who reported that trophoblast cells from human embryos from 7 to 9 weeks of pregnancy secreted significant amounts of EPF. EPF activity was not detected in the culture medium of pre-implantation embryos (36 h) and these investigators concluded that pre-implantation EPF is of maternal origin, whereas post-implantation EPF is of fetal origin.

We conclude that EPF is present throughout pregnancy in swine; it is required for normal embryonic development and attainment of full term pregnancy. It remains to confirm the synthesis *de novo* of EPF from oviduct epithelial cells by measuring mRNA in this tissue.

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