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Porcine placental immunoexpression of vascular endothelial growth factor, placenta growth factor, Flt-1 and Flk-1

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Abstract

Porcine embryo mortalities cause economic losses. Development of the placental vascular bed is required for successful gestation and postnatal survival. We studied the temporal and spatial distributions of vascular endothelial growth factor (VEGF), placenta growth factor (PlGF) and their receptors, Flt-1 and Flk-1. We used crossbred swine placental tissues from 30, 60, 80, 90 and 114 (term) days of gestation. Both VEGF and PlGF were present during gestation. At early pregnancy and at term, VEGF probably acts through Flt-1; during intermediate periods, its function is mediated by Flk-1. By day 90, factors other than members of VEGF family appear to be involved.

Key words: angiogenesis, Flk-1, Flt-1, PlGF, pigs, placenta, vascularization, VEGF

The placenta is a highly vascularized organ (Wooding and Burton 2008, Burton et al. 2009) that transports substances between fetal and maternal circulations (Greenwood et al. 2000, Mayhew 2002, Barrio et al. 2003). In every mammalian placenta, extensive fetal and maternal capillary networks develop parallel to their interface to maximize the efficiency of hemotrophic exchange (Friess et al. 1980, Abd-Elnaeim et al. 2006) by minimizing the diffusion distance for gases and solutes (Cristofolini et al. 2012b, Sanchis et al. 2012b).

Adaptation, expansion and remodeling of the adult vascular system occur during placentation (Charnock-Jones et al. 2004, Merkis et al. 2006). We established earlier the importance of extracellular matrix and apoptosis for the formation, development and support of the placental vascular system (Sanchis et al. 2011, 2012a, 2013) as well as in placental remodeling of gilts (Cristofolini 2010, Cristofolini et al. 2012a,c, Merkis et al. 2010).

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Several angiogenic and non-angiogenic factors are involved in placental vascular development. Better characterized angiogenic factors include vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) (Vonnahme et al. 2001, Tjwa et al. 2003, Santi et al. 2011); these factors act by binding to specific receptors (Ahmed et al. 2000, Zachary and Gliki 2001, Roskoski 2008). Both VEGE and PlGF participate in the formation of the fetal-maternal vasculature of several mammalian species (Ahmed et al. 2000, Vonnahme et al. 2001, Wulff et al. 2002, Zygmunt et al. 2003, Vonnahme and Ford 2004, Valdés and Corthorn 2011). VEGF acts by binding to two specific tyrosine-kinase receptors, Flt-1 and Flk-1 or KDR (Park et al. 1994, Ferrara et al. 2003, Kaczmarek et al. 2009, Moliva et al. 2011).

PlGF is a dimeric glycoprotein that shares significant homology with VEGF (Tjwa et al. 2003) and exhibits potent mitogenic activity in endothelial cells in vitro (Luttun et al. 2002) and angiogenic effects in vivo (Luttun et al. 2002, Odorisio et al. 2002). PlGF is involved in angiogenic activity; it acts especially on non-endothelial cells by binding to Flt-1 (Tjwa et al. 2003, Roskoski, 2008). PlGF is expressed abundantly in the placenta (Hauser et al. 1993). Experiments with knock-out mice helped

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clarify the importance of VEGF receptors (Demir et al. 2004). On one hand, Flk-1 was important for specification and early differentiation from hemangioblastic precursors to feto-placental capillaries. On the other hand, Flt-1 was found to participate in the rearrangement of early endothelial cells to form endothelial tubes. In the placenta, both receptors are expressed in both endothelial and non-endothelial cells (Ahmed et al. 2000). VEGF and its receptors have been found in the endometrium, decidua and trophoblast of human (Demir et al. 2004) and non-human primates (Wulff et al. 2002), in the synepitheliochorial placenta of ewes (Cheung et al. 1995) and in the epitheliochorial placenta of mares (Allen et al. 2007) and gilts (Charnock-Jones et al. 2001, Vonnahme and Ford 2004).

We investigated the temporal and spatial localization of the angiogenic factors, VEGF and PlGF, and their receptors, Flt-1 and Flk-1, in porcine placenta during gestation.

Material and methods

Animals and tissue collection

Our protocol was approved by the National University of Río Cuarto Ethical Committee of Research. We used reproductive tracts from crossbred healthy swine from slaughterhouses in Río Cuarto City, Argentina. The reproductive tract was obtained immediately after slaughter, washed with a saline Hanks´ solution (SSH) prepared as instructed by the manufacturer with the addition of 10,000 U/ml penicillin, 10 mg/ml streptomycin and 2.5 μg/ml fungizone (Gibco, Grand Island, NY) and maintained at 4° C until processing. The uterine horns were opened longitudinally with an incision on the anti-mesometrial edge. Embryos or fetuses were removed and gestational age was determined by crown-rump length (Marrable 1971). Twenty-five placentas were selected for study at 30, 60, 80 and 90 days of gestation, and at maturity (114 days), n = 5 for each group.

Tissue samples were taken from five placentas at each gestational age; one placenta was chosen randomly from each animal. Samples were taken from mesometrial, endometrial and fetal placental tissues and used for immunohistochemistry.

We investigated both maternal and fetal tissues from different placental regions and structures: endometrium, fetal mesenchyme, uterine and chorionic epithelium of placental villi, trophoblastic spaces, endothelium, adventitia and vascular wall of placental vessels, myometrium, areolar region where uterine glands open, uterine glands and histiotroph.

Conventional histological technique

Approximately 6 mm^3 portions of placenta were fixed in 10% (v/v) buffered saline formaldehyde, pH 7.2 − 7.4, at 4° C, dehydrated through a series of alcohols and embedded in paraffin. Then, they were cut at 4 μm using a microtome (Microm, Walldorf, Germany) and mounted on slides.

Immunohistochemistry

Paraffin embedded sections were deparaffinized with alcohols, and washed with distilled water and PBS. Washed sections were placed in 3% H_2O_2 for 20 min to block endogenous peroxidase activity, followed by three washes with PBS. After blocking with 5% normal horse serum to reduce nonspecific binding, sections were incubated for 1 h with primary antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) as described earlier (Sanchis et al. 2011). Antibodies included rabbit polyclonal anti-human VEGF (sc-152) diluted 1:100, goat polyclonal rabbit anti-mouse PlGF (sc-1882) diluted 1:100, mouse monoclonal anti-human Flt-1 diluted 1:200 (sc-57139), and mouse monoclonal anti-mouse Flk-1 (sc-6251) diluted 1:200. Tissues were rinsed twice in PBS, then incubated for 20 min with biotinylated anti-rabbit, anti-mouse, anti-goat antibodies (LSAB® + Systems HRP; Dako Cytomation, Carpintería, CA) and 20 min with streptavidin conjugated to horseradish peroxidase (LSAB® + Systems HRP, Dako Cytomation). After two washes in PBS, the antibody reaction was visualized using 3, 3´-diaminobenzidine chromogen solution (Liquid DAB+ Substrate Chromogen System; Dako Cytomation) for 10 min. Sections were counterstained with Mayer´s hematoxylin, washed, dehydrated and coverslipped in Entellan (Merck, Darmstadt, Germany).

For controls, sections were treated with the appropriate mouse, rat or rabbit IgG, depending on the primary antibody used, and diluted to the same final concentration as the primary antibody. All sections were stained under the same conditions at room temperature.

Photomicrographs were evaluated using an Axiophot microscope (Carl Zeiss, Thornwood, NY) fitted with a high resolution digital camera Powershot G6 7.1 megapixels (Canon Inc., Tokyo, Japan). Digital images were captured using Axiovision 4.6.3 software.

High score (HSCORE) analysis

Immunohistochemical staining intensity was ranked as: (−) negative; (+) weak; (++) moderate

and (+++) strong. For each tissue, an HSCORE value was calculated by summing the percentages of cells that stained at each intensity multiplied by the weighted intensity of the staining (Selam et al. 2011), e.g.,

$$\text{HSCORE} = \sum P_i\,(i+1)$$

where i is the intensity score and P_i is the corresponding percentage of cells. Five randomly selected areas per slide were evaluated by light microscopy at 100 x; the values were determined by two investigators at different times and the average score was used.

Statistical analysis

HSCORE data were analyzed by one-way ANOVA with post hoc comparison of means by Tukey's multiple comparison statistics. When a parametric ANOVA test could not be performed, a nonparametric ANOVA by ranks (Kruskal-Wallis test) was used. Statistical significance was defined as $p \leq 0.05$. Data were analyzed using InfoStat Version 2009 software (Di Rienzo et al. 2009).

Results

Distribution of VEGF

VEGF was present in nearly every placental tissue analyzed and was expressed in the endothelium and adventitia of placental vessels, stroma, fetal mesenchyme and myometrium during most periods analyzed (Fig. 1a–f). VEGF stained weakly in the trophoblastic cavities of placenta at day 80 and had an apical location in chorionic cells (Fig. 1c). During later gestation, uterine and trophoblastic epithelia

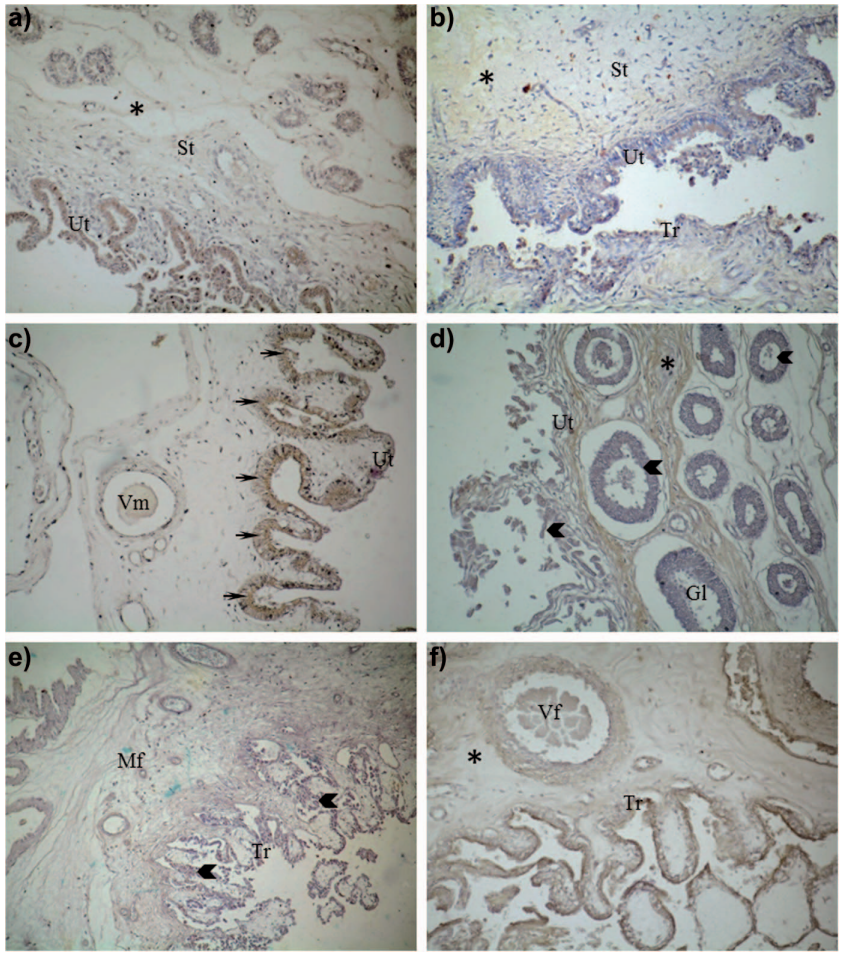

Fig. 1. VEGF immunohistochemistry in porcine placental tissues. a) Placental interface at day 30. ×200. b) Placental interface at day 60. ×200. c) Placental interface at day 80. ×200. d) Maternal placenta at day 90. ×200. e) Fetal placenta at day 90. ×100. f) Fetal placenta at term. ×200). Arrows indicate trophoblastic cells positive for VEGF in uterine cavities at day 80. Arrowheads indicate epithelial cells negative for VEGF. Asterisks indicate maternal and fetal connective tissues positive for VEGF.

that form the placental interface, and uterine glands did not express VEGF (Fig. 1d, e).

Statistical analyses of VEGF HSCORE using the Kruskal Wallis test showed its relation to gestational period ($p = 0.001$). A posteriori test revealed that the highest values for VEGF HSCORE occurred at days 30 and 60 ($p < 0.05$) (Fig. 5).

Distribution of PlGF

No PlGF immunoreactivity was detected in uterine and chorionic epithelial tissues or endothelium (Fig. 2a–f). By contrast, abundant expression was found in the endometrium and fetal mesenchyme throughout pregnancy (Fig. 2a–f). In every case, the immunoexpression was less or negative near the fetal/maternal interface. Fetal and maternal vessels showed evidence of PlGF in the smooth muscle cells of vessels from 30 to 80 days of pregnancy (data not shown).

The ANOVA test indicated statistically different PlGF immunostaining scores among different periods of gestation ($p = 0.0001$). The Tukey a posteriori test showed the highest values of PlGF HSCORE at days 60 and 114 ($p < 0.05$) (Fig. 5).

Distribution of Flt-1

Abundant expression of Flt-1 was found in placental villi at day 30 and weak intensity was found at term (Fig. 3a, f). At intermediate periods of gestation, no staining was observed in placental villi, connective tissue or vessels (Fig. 3b,e). Uterine glands were positive only in the epithelium and histiotroph of early gestation (data not shown).

The nonparametric Kruskal Wallis test showed statistical differences among the periods analyzed ($p = 0.0001$). The highest Flt-1 HSCOREs were found during early and late gestation ($p < 0.05$) (Fig. 5).

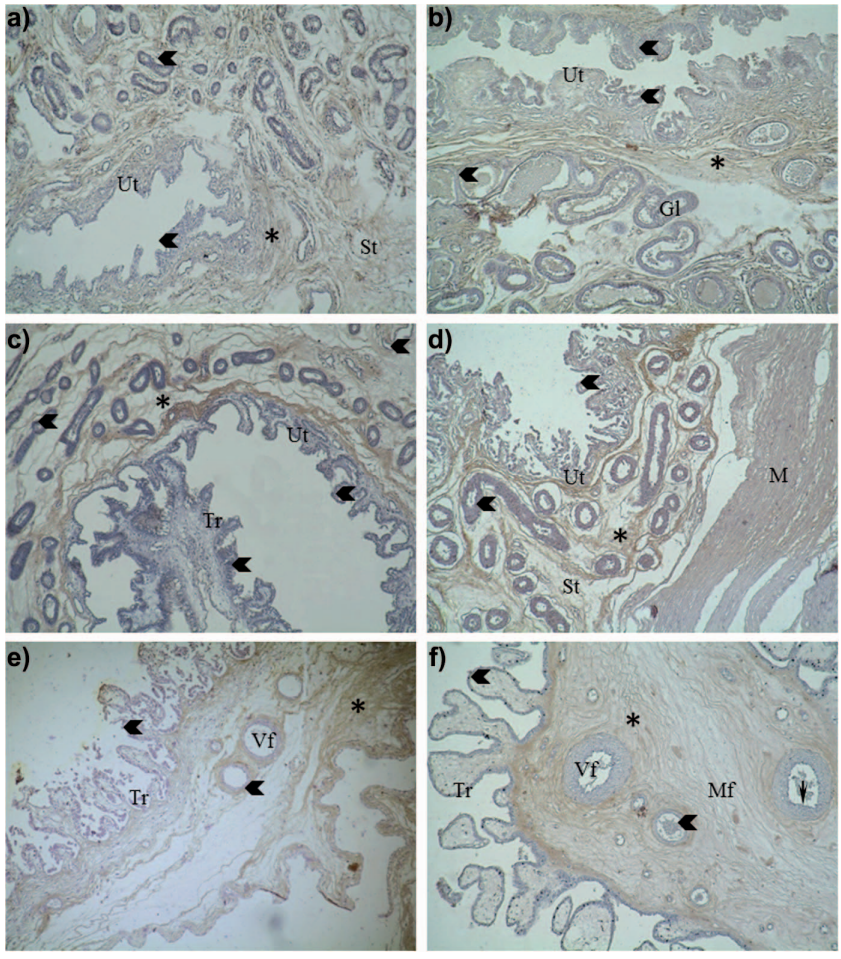

Fig. 2. PlGF expression in porcine placental tissues. a) Placental interface at day 30. ×100. b) Placental interface at day 60. ×100. c) Placental interface at day 80. ×100. d) Maternal placenta at day 90. ×100. e) Fetal placenta at day 90. ×100. f) Fetal placenta at term. ×100. Arrowheads indicate epithelial and endothelial cells negative for PlGF. Asterisks indicate maternal and fetal connective tissues positive for PlGF.

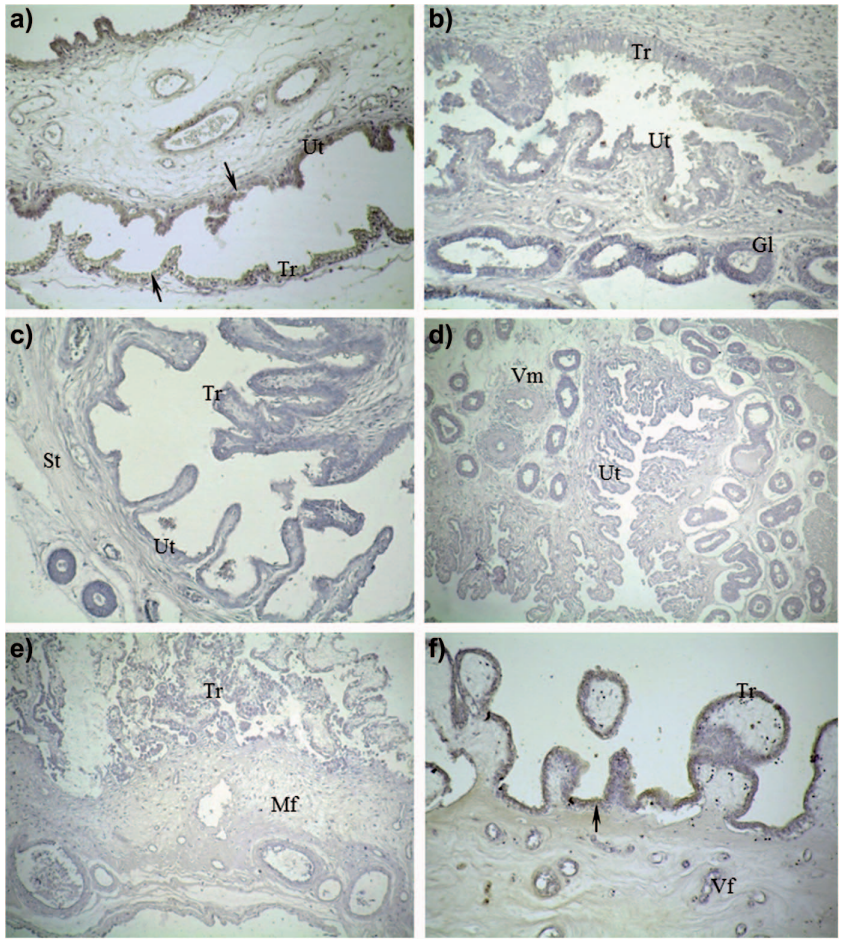

Fig. 3. Immunohistochemical expression of Flt-1 in placental tissues. a) Placental interface at day 30. ×200. b) Placental interface at day 60. ×200. c) Placental interface at day 80. ×200. d) Maternal placenta at day 90. ×100. e) Fetal placenta at day 90. ×100. f) Fetal placenta at term. ×100. Arrows indicate uterine and trophoblastic cells that show VEGF staining.

Distribution of Flk-1

Uterine epithelium showed weak Flk-1 immunoexpression only at day 30, while trophoectoderm showed staining from days 30 to 80. Placental vessels were negative for Flk-1 (Fig. 4).

Statistical analyses revealed the relation of Flk-1 expression to day of gestation ($p = 0.0005$). The a posteriori test showed the lowest Flk-1 HSCORE at day 90 of gestation and the highest at day 30 ($p < 0.05$) (Fig. 5).

Discussion

The placenta contributes to fetal growth through the transfer of nutrients and oxygen to the fetus, by regulating the diffusion of fetal metabolites to maternal blood and by acting as an endocrine organ (Barrio et al. 2003).

VEGF, the primary stimulator of angiogenesis, was detected throughout gestation in uterine and chorionic epithelia. The presence of VEGF in the placental villi during most periods analyzed is similar to that found in the mare (Allen et al. 2007), which presents a noninvasive chorioallantoic placentation as does the gilt. While in the mare VEGF was expressed in the uterine and trophoblastic epithelia during pregnancy, in gilts VEGF was absent in at term gestation (Wooding and Burton 2008). Our findings are consistent with earlier reports by Vonnahme et al. (2001) and Charnock-Jones et al. (2001), who detected VEGF in the placenta of white composition gilts (Landrace, Large White and/or Yorkshire) and Large White pure gilts. We also found VEGF in smooth muscle cells of myometrium, endometrial and fetal mesenchymal connective tissue cells, and glandular epithelial cells as has been reported by Charnock-Jones et al. (2001). The VEGF HSCORE,

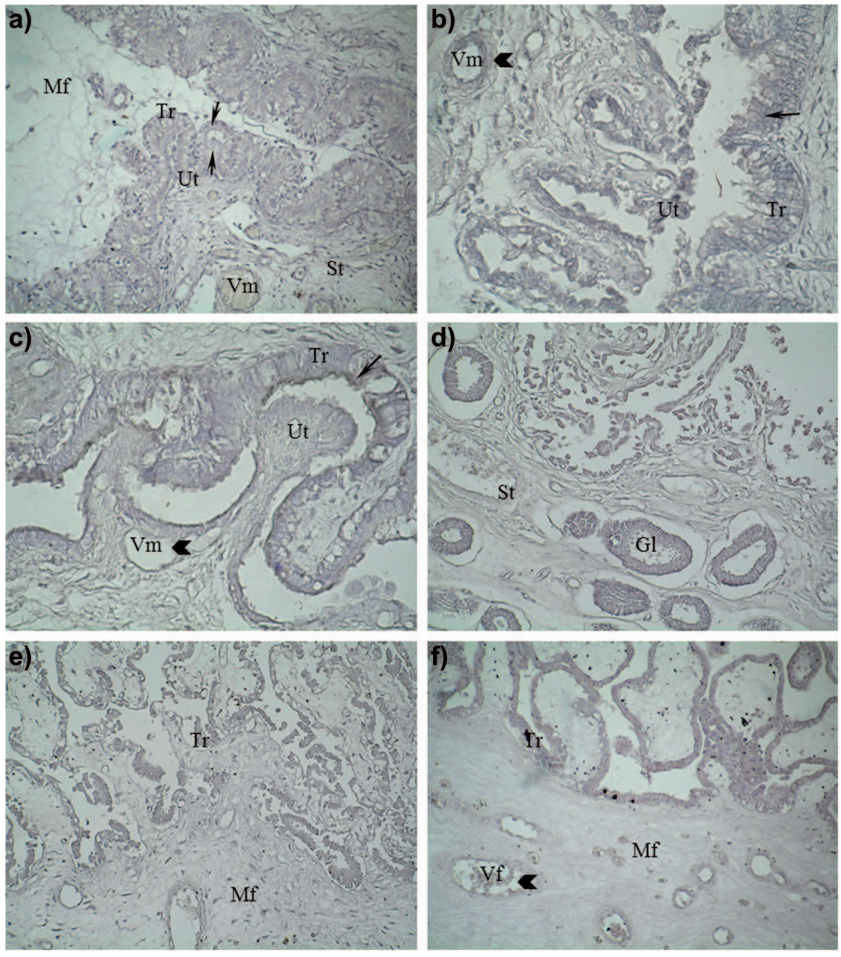

Fig. 4. Immunohistochemistry of Flk-1. a) Placental interface at day 30. ×200. b) Placental interface at day 60. ×400. c) Placental interface at day 80. ×400. d) Maternal placenta at day 90. ×200. e) Fetal placenta at day 90. ×200. f) Fetal placenta at term. ×400. Arrows indicate weak staining for Flk-1 in uterine and trophoblastic cells. Arrowheads indicate negative staining of maternal and fetal vessels with Flk-1.

however, decreased after day 60 of pregnancy. This decrease was accompanied by decreased Flt-1 and Flk-1 levels during mid-gestation; these observations are consistent with observations of decreased vascular area after day 80 (Sanchis 2013).

Reports in the literature together with the absence of VEGF in placental blood vessels suggest that angiogenesis in crossbred swine during advanced gestation could be due to other angiogenic factors. Because PlGF activity is mediated solely by Flt -1 (Tjwa et al. 2003, Roskoski 2008, Valdés and Corthorn 2011), which is absent during gestation of 80 and 90 days, other members of the VEGF family may be responsible for the angiogenic process during this period.

VEGF was detected only in the trophoblastic cavities of the placental villi at 80 and 90 days of pregnancy. Friess et al. (1980) showed morphological and histological evidence that transport of less diffusible material takes place in this zone, where the chorionic and uterine epithelia are columnar. Moreover, the immunostaining coincided with the areolar region, which is rich in blood vessels that are close to the basal membrane.

Charnock-Jones et al. (2001) postulated that VEGF produced by the epithelial cells of the uteroplacental interface could act in paracrine manner on endothelial cells of the capillaries, which would favor their development and proximity to the epithelial surface to increase the exchange capacity of the placenta.

Immunostaining of VEGF in placental blood vessels during most of the periods we analyzed indicates that VEGF binds to endothelial cells by specific receptors (Vonnahme and Ford 2004). Several investigators have emphasized the importance of the VEGF membrane receptors, Flt-1 and Flk-1, in VEGF-mediated angiogenesis (Ferrara

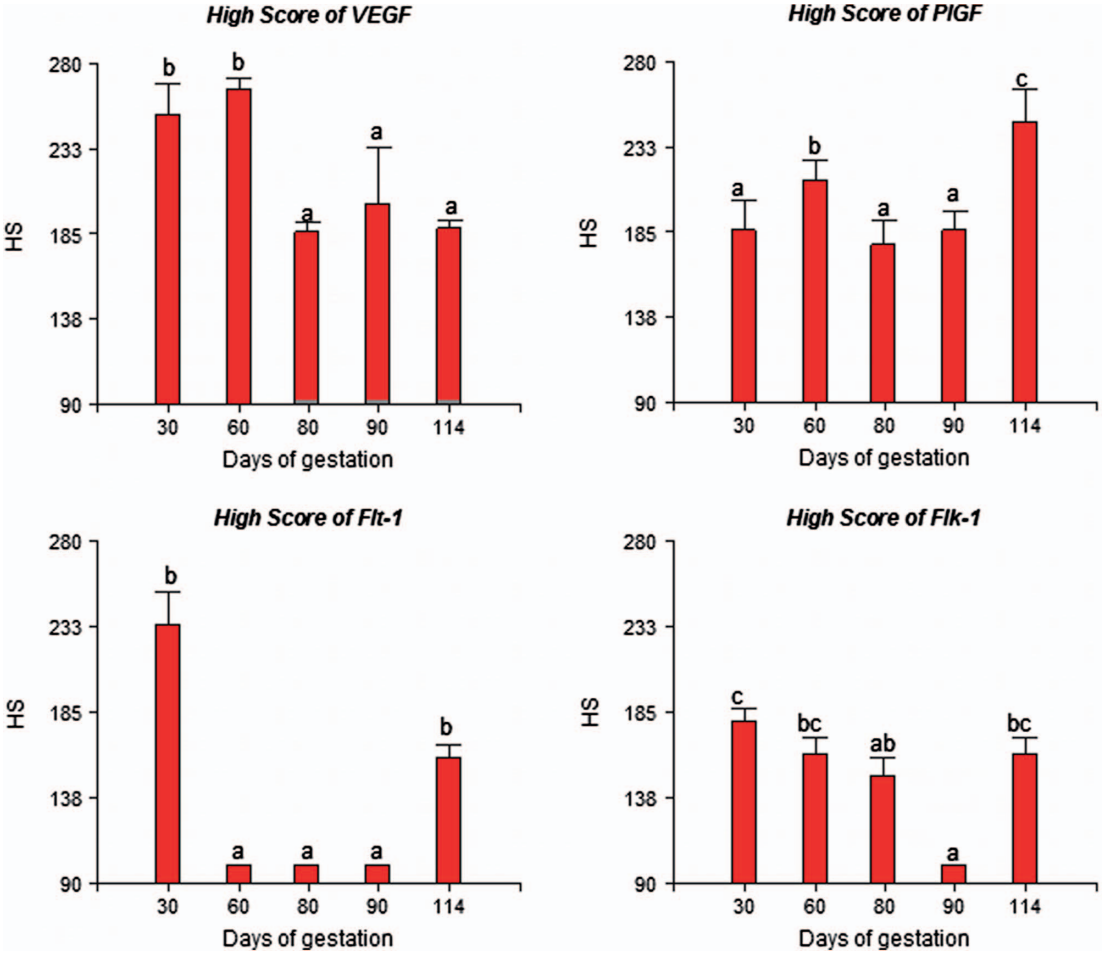

Fig. 5. Immunostaining intensities of angiogenic molecules and receptors. Bars with different letters are significantly different ($p < 0.05$).

et al. 2003, Demir et al. 2004, Kaczmarek et al. 2009). We observed that at early gestation and at term, VEGF acted through the Flt-1 receptor. During these periods, both receptors Flk-1 and Flt-1 were present; however, Flt-1 showed greater binding affinity to VEGF than Flk-1 (15 pM and 750 pM, respectively) (Roskoski 2008). VEGF action is mediated preferentially by Flk-1 (Ferrara et al. 2003); however, our results are consistent with reports by Kaczmarek et al. (2009) and Moliva et al. (2011), who suggested a substantial role for Flt-1 during early gestation in pigs. Failures in angiogenesis would cause defective placentation, one of the main causes of embryonic losses by the end of the first trimester in human gestation (Goldman-Wohl et al. 2000) and during early gestation in pigs (Bosch et al. 2001, Van der Lende and Van Rens 2003). The expression of Flt-1 by trophoblastic cells of term placentas observed here is consistent with observations reported by Ahmed et al. (2000) for humans.

It has been reported that other growth factors are potent angiostimulating agents for vasculogenesis and angiogenesis during placental development, including PlGF (Zygmunt et al. 2003). In a previous report, both forms of PlGF, $PlGF_{152}$ y and $PlGF_{131}$, showed high affinity for binding to Flt-1 (250 ± 35 pM and 186 ± 40 pM, respectively) (Park et al. 1994). Placental growth factor immunostaining was detected in every period analyzed, which coincided with VEGF expression. Nevertheless, because VEGF is more competitive than PlGF for binding to the Flt-1 receptor, we hypothesize that during early and late gestation, vascularization is stimulated mainly by VEGF.

During intermediate periods of pregnancy, e.g., 60 days, the absence of Flt-1 immunoexpression precludes the action of PlGF in placental angiogenesis. The presence of VEGF in porcine placentas during this period is consistent with previous descriptions (Sanchis et al. 2012a). Our findings indicate that VEGF acts exclusively by the Flk-1 receptor in the

epithelia that form the maternal-fetal interface, in the connective tissue and in the myometrium. It has been reported that cell signaling that affects the functionality of VEGF is mediated mainly by Flk-1, or at least includes Flk-1 (Zachary and Gliki 2001).

At day 80, the situation was similar to that observed at day 60; however, at day 90 no receptor was present in placental tissues. This finding, together with the absence of VEGF in most placental structures, indicates that during advanced periods of porcine gestation, angiogenesis is due to angiogenic molecules other than members of VEGF family.

Gestation demands placental development and remodeling (Cristofolini 2010, Cristofolini et al. 2012a,c, Merkis et al. 2010). Failures in this process are related to embryonic losses at the end of the first trimester in human gestation (Goldman-Wohl et al. 2000). Because the pork industry is affected by frequent fetal loss (Bosch et al. 2001, Van der Lende and Van Rens 2003), knowledge about vascularization and angiogenesis in pregnant gilts could suggest future strategies for improving productivity of this species.

We investigated the expression profiles of the angiogenic factors, VEGF and PlGF, and their receptors, Flt-1 and Flk-1. We demonstrated the localization of both VEGF and PlGF during several gestational periods and localized Flt-1 and Flk-1 receptors at days 30 and 114 of pregnancy.

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