RESEARCH

Placental vascularization in middle and late gestation in the pig

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Abstract

The aims of this study were to determine the changes in the capillary area density in relation to fetal development, to determine immunoexpression of angiogenic factors and to compare their mRNA expression throughout pig gestation. Samples were collected from the maternal-chorioallantoic interface at days 40, 77, 85 and 114 of pregnancy for immunohistochemistry analysis and the measurement of mRNA expression of *VEGFA, ANGPT1, ANGPT2, FGF2* and its receptors *KDR, TEK, FGFR1*, *FGFR2* respectively. Morphometric measurement of blood vessels was performed. We found a significant increase in capillary area density throughout gestation ($P < 0.05$). On the maternal side, at day 77, we observed a significant increase in the number of vessels from small vascular areas ($P < 0.05$) and the vascular area was significantly higher on day 85 ($P < 0.05$). On the fetal side, the number of vessels and the vascular area increased between days 40 and 77 ($P < 0.05$) and between days 77 and 114 ($P < 0.05$), respectively. Immunohistochemical findings revealed intense VEGFA staining and a trend for increased expression towards the end of gestation ($P < 0.05$). We also demonstrated a high *VEGFA*, *FGF2, FGFR1, ANGPT1* and *ANGPT2* mRNA expression at day 77 ($P < 0.05$). In conclusion, our findings suggest that an active angiogenesis would be present even until late-middle gestation at day 77 of pregnancy with the predominance of angiogenic stimulation by VEGFA/KDR, FGF2/FGFR1 and a balance between ANGPT1 and ANGPT2/TEK.

Lay summary

Critical moments occur at different stages of placental formation in pigs, where the expression of angiogenic factors, that is, molecules that stimulate the formation of blood vessels must be adequate to promote their development. This exchange is necessary to cover the increasing nutritional demands of fetuses in continuous development. Determining the changes in the area of capillary density in relation to fetal development and the expression of angiogenic factors throughout pregnancy in pigs could contribute to understanding the causes of fetal loss. Placental samples were obtained at gestational days 40, 77, 85 and 114 (n = 7, 10, 7 and 5, respectively). We found that the capillary area density increases accompanying fetal growth with advancing gestation and an increase in capillary area density in late-middle gestation, around day 77, is due to the expansion in the number of small blood vessels on the maternal side. The present findings suggest that an intense angiogenesis would be present even until late-middle gestation at day 77 of pregnancy, with the predominance of angiogenic stimulation by specific molecules that promote this process.

Key Words: ▸ angiogenesis ▸ placenta ▸ pig ▸ VEGFA ▸ FGF2 ▸ ANGPT1/2

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Introduction

In the last few years, along with the appearance of hyperprolific pigs, a higher number of piglets/gilt/years are weaned (Guimarães *et al.* 2017). The increase in litter size with the decrease in birth weight is a manifestation of the limitations in the growth of the embryo, which is related to uterine capacity. This is associated with a higher number of embryonic, fetal and postpartum mortality, resulting in a substantial economic loss (Kridli *et al.* 2016).

The supply of nutrients through the placenta determines the weight of the piglet at birth, and it is influenced by the size of the placenta and the blood flow (Van Rens *et al.* 2005). Considerable changes in the position of the placental capillaries have been described throughout the gestation in pigs (Friess *et al.* 1980, 1981). Therefore, blood vessel formation plays a pivotal role in placenta formation, and thus the successful establishment of pregnancy, ensuring the proper development of the fetus (Kaczynski *et al.* 2020).

The placenta grows very rapidly during early gestation in gilts until middle gestation at day 40. The placental vascularization, as indicated by the number and size of placental blood vessels, also increases markedly during this period. The endometrium is remodelled and requires a large amount of nutrients, while fetal growth is slow (Wright *et al.* 2016, Wu *et al.* 2017, Zhang *et al.* 2020).

In late-middle gestation, around day 70 of gestation, endometrial remodelling is almost complete, and the fetal placenta unit grows steadily, requiring abundant nutritional supplements to meet the maternal–fetal exchange (Zhang *et al.* 2020). Studies have shown a greater vascular development in the middle third of gestation, while after day 80 of gestation the vascular network tends to stabilize, and only the diameter of the vessels increases (Guimarães *et al.* 2017).

Until the end of pregnancy, at day 114 approximately, the size of the placenta stabilizes, and the fetuses grow exponentially (Merkis *et al.* 2005, Guimarães *et al.* 2017, Cristofolini *et al.* 2018). Previous work from our laboratory demonstrated that in advanced gestations, the new blood vessels formation and the reduction in the haemotrophic diffusion distance are involved in the maternal/fetal haemotrophic exchange efficiency (Cristofolini *et al.* 2018).

Other authors have reported associations between fetal size and placental vasculature and the expression of some genes, which are influenced by gestational day (Stenhouse *et al.* 2019). In addition, Seo *et al.* (2020) revealed that the increases in the maternal placenta vascularization and blood flow are associated with higher placental fold length.

It is widely accepted that the formation of blood vessels occurs through the angiogenesis process, in which numerous angiogenic factors act by binding to specific receptors. The main factor involved in the regulation of vascular development is the vascular endothelial growth factor (VEGF) (Vonnahme *et al.* 2001, Vonnahme & Ford 2004, García Fernández *et al.* 2015, Sanchis *et al.* 2015, Guimarães *et al.* 2017). During advance gestation in the pig, we have observed that angiogenic molecules, which are non-VEGF family members, induce angiogenesis (Sanchis *et al.* 2015). Although many authors studied this event, little is known about the role of other angiogenic factors involved in placental vascular development in pigs, as well as, angiopoietins (ANGPT1 and ANGPT2) and basic fibroblast growth factor (FGF2) (Javerzat *et al.* 2002, Lobov *et al.* 2002, Welter *et al.* 2004, Presta *et al.* 2005, Seval *et al.* 2008, Edwards *et al.* 2011, Tomao *et al.* 2014, Kappou *et al.* 2015, Fiorimanti *et al.* 2018).

We hypothesize that the expression of VEGFA, angiopoietins and FGF2 in critical moments of placentation in pigs promotes the angiogenesis of small vessels. This exchange is necessary to cover the increasing physiological demands of fetuses in continuous development.

Thus, the aims of our study were (i) to determine the changes in capillary area density in relation to fetal development; (ii) to determine immunoexpression of angiogenic factors and compare their mRNA expression throughout pig gestation.

Materials and Methods

All procedures were performed with approval from the National University of Río Cuarto Ethical Committee of Research in animals (CoEdI), Res. 186/2016.

Animals and tissue collection

Reproductive tracts of healthy crossbred gilts (Yorkshire x Landrace) from slaughterhouses from Río Cuarto, Argentina (33.11°S; 64.3°O) were used. Placental samples were obtained at gestational days (GD) 40, 77, 85 and a term 114 (n = 7, 10, 7 and 5, respectively) and were collected at the maternal-chorioallantoic interface from the middle fetuses of each horn immediately after slaughter, washed with saline solution of Hank's containing sodium penicillin G, streptomycin sulphate and fungizone (Gibco) and maintained at 4°C until processing within 30 min. The uterine horns were opened with an incision on the anti-mesometrial edge. Fetuses were removed and gestational

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day was determined according to the crown-rump length of the litter, using the formula: gestational days (GD) = cephalocaudal length (CL) cm × 3 + 21, where 3 and 21 are constant. These results were checked with the table's Marrable (Marrable 1971). Samples were gathered from the central zone of maternal-chorioallantoic interface and snap-frozen in liquid nitrogen and then stored at −80°C until they were processed for mRNA extraction (Stenhouse *et al.* 2019). The placental tissues were processed with the conventional histological technique for immunohistochemistry (Fiorimanti *et al.* 2018).

Conventional histological technique

Portions of approximately 6 mm^3 of placental tissue were fixed by immersion in 10% (v/v) buffered saline formaldehyde, pH 7.2–7.4 at 4°C, dehydrated with alcohol and embedded in paraffin. Then, they were cut in 4-µm histological sections with a microtome (Micron, Germany) and mounted on slides. The sections were used for staining with haematoxylin and immunohistochemistry.

Immunohistochemistry

Tissue sections were deparaffined with xylol and then hydrated in alcohol of decreasing concentrations (100, 90, 80 and 70%) and distilled water. For antigenic retrieval (CD31, FGFs and angiopoietins), the slides were treated in a microwave oven in 10 mM citrate buffer, pH 6.0, for 15 min, and left to cool for 20 min. After two washes in PBS, endogenous peroxidase activity was blocked by incubating slides with 0.3% H_2O_2 in PBS for 20 min, followed by two PBS washes. The slides were blocked for 1 h at room temperature with 5% horse serum to block non-specific binding. The sections were incubated overnight at 4°C with rabbit polyclonal antibodies (anti-CD31 ab28364, Abcam), (anti-VEGF (A-20):sc-152, anti-FGFR1 (Flg (C-15): sc-121), anti-FGFR2 (Bek (C-17): sc-122), anti-ANGPT1 (Ang-1 (H-123): sc-9044), anti-TEK (Tie-2 (H-176): sc-9026), mouse MAB (anti-FLK-1 (A-3):sc6251) and goat polyclonal antibodies (anti-FGF-2 (FGF-2 (C-18): sc-1360 and anti-ANGPT2 (Ang-2 (C-19): sc-7015), Santa Cruz Biotechnology Inc., all at working dilution 1/100. The concentrations of the antibodies used are detailed in Table 1. Then, the tissues were rinsed twice in PBS and incubated for 30 min with biotinylated secondary antibodies pool ready to use (Polivalent Biotinylated Link Hi Def Amplifier (rabbit and mouse) Cell Marque, USA) and 30 min with Hi Def Detection™ HRP Polymer Detector (951D-20 HiDefDetection™ HRP Polimer System Cell Marque, USA). In case of FGF2 and Ang-2, tissues were incubated for 30 min with biotinylated secondary antibodies pool (Santa Cruz Biotechnology Inc.; ImmunoCruz™ goat ABC Staining System, sc-2023) and then 30 min with the complex Avidine-HRP AB enzyme reagent (Santa Cruz Biotechnology). After two PBS washes, the antibody reaction was visualized using 3,3´-diaminobenzidine chromogen solution (DAB Cell Marque, USA) for 10 min. The sections were counterstained with Mayer´s hematoxylin (Dako), washed, dehydrated and cover-slipped in Entellan (Merck). Slides were treated with isotype control antibody, instead of primary antibody, as a negative control. Photomicrographs were evaluated using an Axiophot microscope (Carl Zeiss) fitted with high-resolution digital camera Powershot G6 7.1 megapixels (Canon Inc., Tokyo, Japan). Digital images were captured using Axiovision 4.6.3 software (Fiorimanti *et al.* 2018).

High score analysis

Immunohistochemical staining intensity for angiogenic factors were ranked with the designation between 0 (absent), 1 (weak), 2 (moderate) and 3 (intense). For each slide, five randomly selected areas were evaluated using a light microscope (20× magnification).

For each tissue, an high score (HSCORE) value was derived by summing the percentages of placental tissue that stained at each intensity ranking and multiplying this value by the weighted intensity of the staining (Selam *et al.* 2001, Fiorimanti *et al.* 2018), using the formula, HSCORE = $\sum$ Pi (i+1), where i represents the intensity scores and Pi is the corresponding percentage of the intensity. The values were determined by two investigators at different times, and the average score was used.

Morphometric measurements of blood vessels

Morphometric measurement analysis was performed from hematoxylin staining and CD31 immunohistochemical

Table 1 Antibody concentrations used for immunohistochemical analysis.

Antibody	**Concentration** (µg/mL)
VEGFA	2
FLK-1	2
FGF2	2
FGFR1	2
FGFR2	2
ANGPT1	2
ANGPT2	2
TEK	2
CD31	0.2

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Table 2 Gene specific primers used for real-time PCR.

Genes	Primers (5´–3´) Forward	Reverse	Product size (bp)	Accession number
VEGFA	GCTCTCTTGGGTGCATTGGA	GCAGCCTGGGACCACTTG	69	NM_214084
KDR	GGAGCAACACACAGCGAACA	GAGATGGTGGCCAATGTGAAT	72	XM_003128987.6
FGF2	CTTCTATTCTCTGCTTCATCCACACT	GGGTAAGCCACAACAGGATCA	79	XM_021100546
FGFR1IIIc	GATAACACCAAACCAAACCGTATG	CGCATGCAATTTCTTTTCCA	78	XM_005671767.3
FGFR2IIIb	CCGGCAGTGCACAAGCT	CTGGACTCGGCAGAAACTGTT	68	NM_001099924.2
ANGPT1	CTTCCTCGCTGCCATTCTG	CCCACCGTTTTCTGGACTTC	67	NM_213959.1
ANGPT2	ACTGCGCAGAAGCATTCAAA	CTCCTCAGTGGAGTTAGGAAAGGT	77	NM_213808.1
TEK	TGTGCTACAGGCTGGAAAGGT	AAGCTTACAGTCTGGCCCATAATAA	75	XM_021062647.1
ACTB	CCAACCGTGAGAAGATGACC	CCAGAGGCGTACAGGGACAG	97	XM_003124280.5

staining (Supplementary Fig. 1, see section on supplementary materials given at the end of this article). The images captured (1.3) were analyzed using AxioVision 4.6.3 software (Carl Zeiss). Morphometric analyses were performed to measure the number of blood vessels, the capillary area (CA) and the capillary area density (CAD) on the maternal and fetal side separately. The capillary area density was calculated with the formula CAD= Σ CA/TA; where CA is capillary area and TA is total area of placental tissue (Reynolds *et al.* 2005).

Quantitative reverse transcriptase PCR

mRNA was extracted from the maternal-chorioallantoic interface of placental tissues using Trizol reagent according the manufacturer's protocol (Thermo Scientific). Isolated RNA concentration was determined with Nanodrop, and RNA was stored at 80°C until cDNA conversion. An aliquot of the extracted RNA (1 µg of total RNA) was converted to cDNA using M-MLV Reverse Transcriptase (Promega) following the methodology recommended by the manufacturer. The synthesized cDNA was stored at −20°C until quantitative reverse transcriptase PCR was performed. The genes selected for mRNA measurements were *VEGFA*, kinase insert domain receptor (*KDR*), *FGF2*, fibroblast growth factor receptor 1 (*FGFR1IIIc*), fibroblast growth factor receptor 2 (*FGFR2IIIb*), angiopoietin 1 (*ANGPT1*) , angiopoietin 2 (*ANGPT2*), tyrosine kinase, endothelial (*TEK*) and β-actin (*ACTB*) as housekeeping gene. Relative expressions of selected genes were determined using primers (Invitrogen) and SYBR Green PCR Master Mix (Applied Biosystems). Primers were designed with Primer Express software (Applied Biosystems) from the corresponding *Sus scrofa* mRNA. All gene names, abbreviations and accessories numbers are listed in Table 2. All primer pairs had efficiencies greater than 95%. All samples were run in triplicate for each gene and for β-actin. There were no differences in *ACTB* expression among the GD. No-RT controls were performed by omitting RT, and no template controls were performed by addition of nuclease-free water. Relative mRNA expression of each gene was calculated by determining change in threshold cycle (DCt) between the mean Ct for each gene and the mean Ct for β-actin mRNA from the same sample.

Statistical analysis

Data were analyzed with InfoStat Version 2019e software (Di Rienzo *et al.* 2019). An ANOVA test and *a posteriori* LSD-Fisher test were performed in every case. When a parametric ANOVA test could not be accomplished, even with transformations of the variable, a nonparametric ANOVA by ranks (Kruskal–Wallis test) was used.

Pearson's correlations were performed within gestational day to determine the association between the CL and the weight of the fetuses.

Data are expressed as mean ± s.e.m., and means were considered to be statistically different at $P < 0.05$.

Results

A positive correlation between the CL and the weight of the fetuses was observed at days 40, 77 and 85 of gestation ($P < 0.05$). Fetal weight and CL had a significant correlation at the beginning of the middle of the gestation and in late-middle gestation, at days 40 and 77, respectively ($P < 0.05$) (Table 3). Interestingly, a strong positive correlation was obtained at day 85 of gestation between fetal weight and CL ($P < 0.05$). We had no fetal data at day 114, so the correlation analysis could not be determined, as placentas from this day were collected after natural farrowing of sows and fetal data were not considered.

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Table 3 Pearson correlation (R^2) values and respective probability values (*P*) between the fetal weight and the fetal cephalocaudal length at different stages of gestation, days 40, 77 and 85.

Variable		R^2	*P*-value
40 days			
Fetuses, *n*	10		
FW (g)	21.71 ± 1.29		
FCL (cm)	6.9 ± 0.39		
FW × FCL		0.82	<0.05
77 days			
Fetuses, *n*	10		
FW (g)	347.40 ± 50.60		
FCL (cm)	18.70 ± 0.89		
FW * FCL		0.80	<0.05
85 days			
Fetuses, *n*	7		
FW (g)	445.71 ± 93.16		
FCL (cm)	21.21 ± 1.78		
FW × FCL		0.91	<0.05

FW, fetal weight; FCL, fetal cephalocaudal length.

Morphometric analysis

We demonstrate a significant increase in capillary area density throughout gestation, both on the maternal and fetal side by morphometric analysis (Fig. 1).

At day 77 we observed a significant increase in the number of vessels on the maternal side, although these were predominantly from small vascular areas ($P < 0.05$). The vascular area was significantly higher on day 85; however, the number of vessels was not increased ($P < 0.05$).

On the fetal side, the number of vessels had a significant increase between days 40 and 77 ($P < 0.05$), then it remained constant at days 85 and 114. Interestingly, the vascular area was larger between days 77 and 114 ($P < 0.05$).

Immunolocalization and expression of mRNA for VEGFA and receptor KDR

We observed positive staining of VEGFA in cells of the endometrial and chorionic epithelium, in the endothelium of maternal blood vessels, in the endothelium of fetal blood vessels and in the uterine glandular epithelium throughout the gestation (Fig. 2).

We observed an increasing trend of VEGFA HSCORE towards the end of gestation. There was intense staining and a significant increase in VEGFA HSCORE values at day 114 of gestation and its receptor KDR at day 77 ($P < 0.05$) (Fig. 3A and E).

Accordingly, the relative mRNA expression of *VEGFA* was higher at day 77 compared to days 40, 85 and 114 of gestation. However, KDR expression was decreased at 40 and 77 days compared to days 85 and 114 of gestation (Fig. 4A and E).

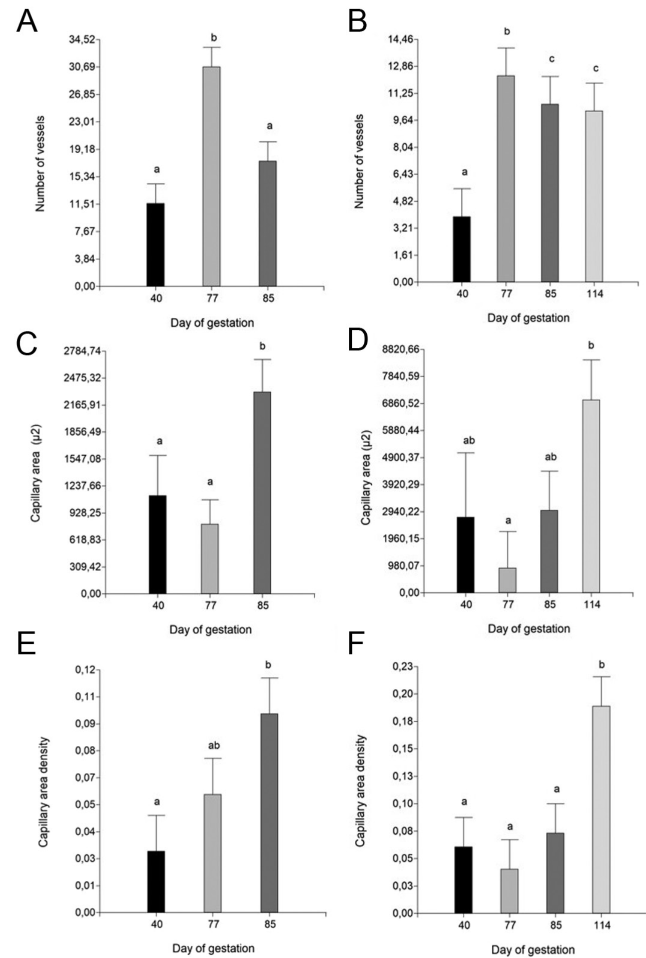

Figure 1 Blood vessel quantification in maternal (A) and fetal side (B). Measurement of the mean capillary area in maternal (C) and fetal (D) side. Measurement of the mean capillary area density in maternal (E) and fetal (F) side. Data are expressed as mean ±S.E.M. ($P \leq 0.05$).

Immunolocalization and expression of mRNA for FGF2 and receptors FGFR1 and FGFR2

Immunoexpression of FGF2 HSCORE and receptors FGFR1 HSCORE and FGFR2 HSCORE were detected throughout the gestation. FGF2 staining was detected in uterine luminal epithelium and in the uterine glandular epithelium. Moderate immunoexpression was found in chorionic cells. Endothelial cells from fetal and maternal blood vessels were negative to FGF2, while the smooth muscle cells from medium and extern layers from arterioles expressed weak immunoexpression (Fig. 5). FGF2 HSCORE increased with advancing gestation, with a statistically significant

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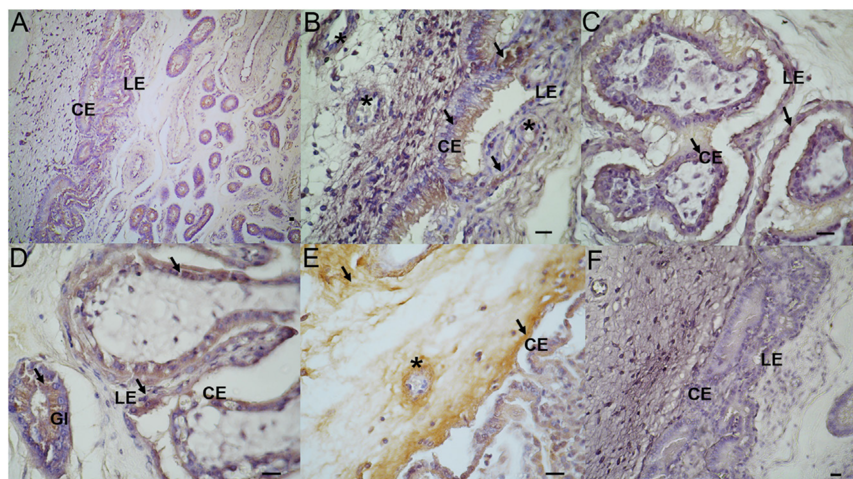

Figure 2 Representative images of immunohistochemical analysis of VEGFA in pig placental tissues (A) and (B) 40 days of gestation, 100× and 400×, respectively, (C) 77 days of gestation, (D) 85 days of gestation (E) 114 days of gestation and (F) negative control. Asterisks indicate positive immunostaining in blood vessels. Arrows indicate positive staining in chorionic epithelium (CE), uterine luminal epithelium (LE) and uterine glands (Gl). Scale bar: 20 µm.

increase observed at days 77 and 85 compared to day 45. In addition, a significant increase of FGF2 expression was observed at day 114 (Fig. 3D).

FGFR1 and FGFR2 HSCORE values showed a moderate fluctuation throughout gestation (see Supplementary Figs 2 and 3). The higher FGFR1 HSCORE was observed at day 77, while the lowest FGFR2 HSCORE was detected at day 114 ($P < 0.05$) (Fig. 3G and H).

These results coincide with the expression of *FGF2* mRNA and *FGFR1 mRNA*. We detected a significant increase at day 77 ($P < 0.05$). Increased *FGFR2* expression was observed at days 40 and 85 of gestation (Fig. 4D, G and H).

Immunolocalization and expression of mRNA for ANGPT1, ANGPT2 and receptor TEK

ANGPT1 and ANGPT2 were detected in the uterine luminal epithelium, in the endothelium of maternal and fetal blood vessels, and in the cytoplasmic apical part of chorionic cells by immunohistochemistry (Figs 6 and 7).

The highest ANGPT1 HSCORE values and *ANGPT1* mRNA were detected at day 77 of gestation ($P < 0.05$) (Figs 3B and 4B).

There was a significant increase in the ANGPT2 HSCORE values at days 77 and 85 of gestation ($P < 0.05$) (Fig. 3C). Increased *ANGPT2* expression was observed at days 77 and 114 of gestation (Fig. 4C).

The TEK immunoreactivity was observed as a moderate staining in endothelial cells of maternal and fetal blood vessels and in chorionic cells throughout the gestation (see Suplementary Fig. 4). There was a significant decrease in TEK HSCORE at day 77 and 85, in agreement with the expression of *TEK* mRNA ($P < 0.05$) (Figs 3F and 4F).

Discussion

Several studies have shown that the development of a placental vascular network is key for placental exchange and fetal growth during pregnancy in mammals. Morphological changes in the placenta and increased

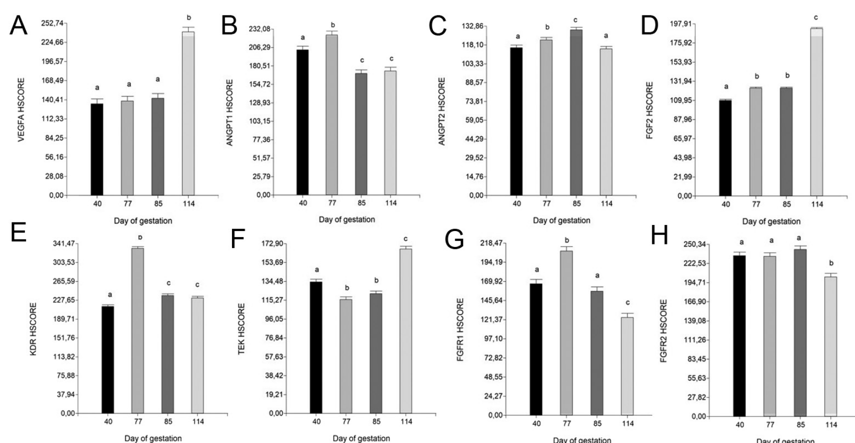

Figure 3 HSCORE values of angiogenic factors and their receptors (A) VEGFA, (B) ANGPT1, (C) ANGPT2, (D) FGF2, (E) KDR, (F) TEK, (G) FGFR1 and (H) FGFR2. Means followed by different letters differ by ANOVA with test a posteriori LSD Fisher ($P < 0.05$).

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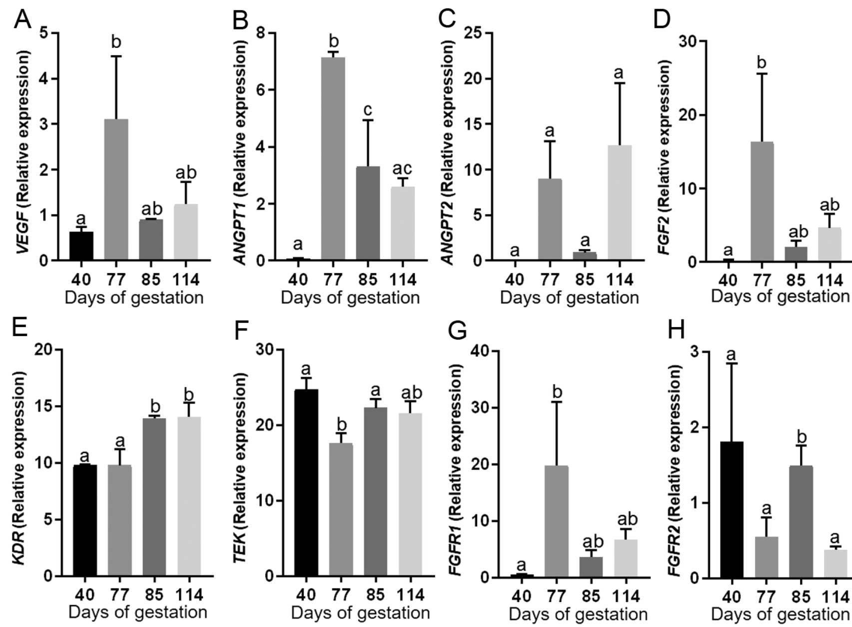

Figure 4 mRNA expression of vascular endothelial growth factor (*VEGFA*) and *KDR* receptor, angiopoietins 1 and -2 (*ANGPT1* and *ANGPT2*) and TEK receptor, basic fibroblast growth factor (*FGF2*) and its receptors (*FGFR1* and *FGFR2*) in maternal-chorioallantoic interface at days 40, 77, 85 and 114 of gestation. Different letters indicate significant differences according to ANOVA and test a posteriori LSD Fisher ($P < 0.05$).

fluid volume are important for early fetal survival (Wright *et al.* 2016). In pigs, for conceptuses to survive to term, coordinated placental development and pregnancy-specific adaptations for growth and development are crucial (Kridli *et al.* 2016). Placental expression of angiogenic factors responsible for regulating these processes is temporally variable throughout gestation, and changes in these factors regulate stages of placental angiogenesis during pregnancy (Umapathy *et al.* 2020).

The present study found that the capillary area density increases accompanying fetal growth with advancing gestation. These results can be explained, in part, by the increase in the number of small vessels at late-middle gestation in the maternal side and by the increase in diameter and, consequently, in the vascular area, towards the end of gestation. These results are consistent with previous findings from our laboratory showing that the vascular area depends on the gestational period in pigs

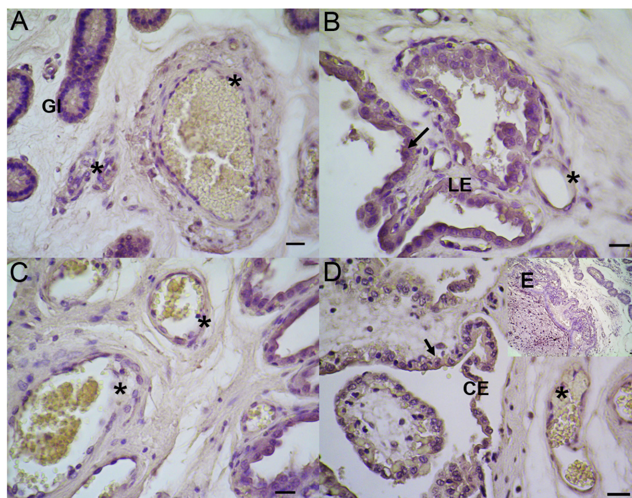

Figure 5 Representative images of immunohistochemical analysis of FGF2 in pig placental tissues (A) 40 days of gestation, (B) 77 days of gestation, (C) 85 days of gestation (D) 114 days of gestation and (E) negative control. Asterisks indicate positive immunostaining in blood vessels. Arrows indicate positive staining in chorionic epithelium (CE), uterine luminal epithelium (LE). Gl: uterine glands. Scale bar: 20 µm.

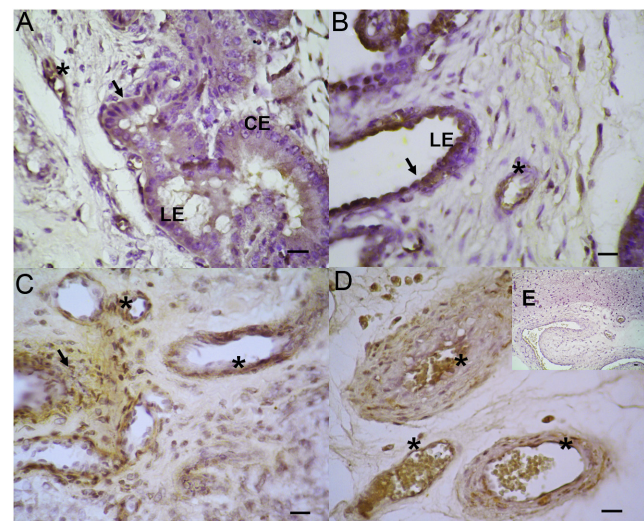

Figure 6 Representative images of immunohistochemical analysis of ANGPT1 in pig placental tissues (A) 40 days of gestation, (B) 77 days of gestation, (C) 85 days of gestation and (D) 114 days of gestation and (E) negative control. Asterisks indicate positive immunostaining in blood vessels. Arrows indicate positive staining in chorionic epithelium (CE), uterine luminal epithelium (LE). Scale bar: 20 µm.

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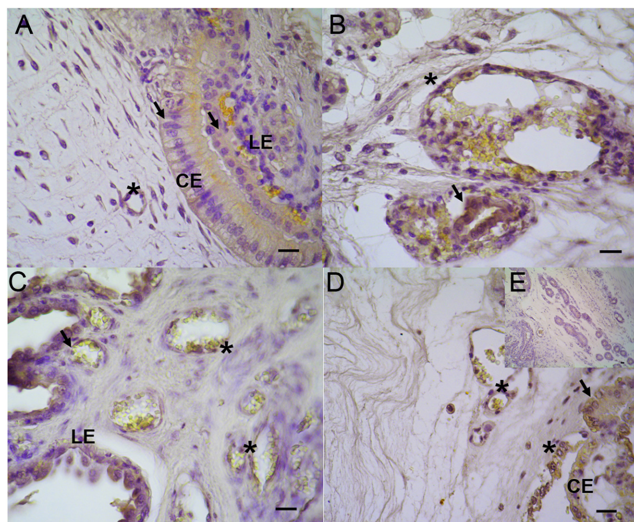

Figure 7 Representative images of immunohistochemical analysis of ANGPT2 in pig placental tissues (A) 40 days of gestation, (B) 77 days of gestation, (C) 85 days of gestation and (D) 114 days of gestation and (E) negative control. Asterisks indicate positive immunostaining in blood vessels. Arrows indicate positive staining in chorionic epithelium (CE), uterine luminal epithelium (LE). Scale bar: 20 µm.

(Cristofolini *et al.* 2018). Indeed, Vonnahme *et al.* (2001) have detected a slight decrease at day 44 in the number of vessels per unit area before increasing progressively from day 44 to 112 of gestation. Recently, increases in vascularization of the maternal placenta and the blood flow were associated with higher placental fold length (Seo *et al.* 2020). Other studies suggested that most uterine vascular development occurs before the middle third of pregnancy (Guimarâes *et al.* 2017). During early gestation, the greatest increase in placenta weight occurs between 37 and 42 days (Wright *et al.* 2016). In fact, when the trophoblast-endometrial bilayer fully develops at day 40, increased vascularization occurs in the chorion (Zhang *et al.* 2020), whereas the unoccupied areas of the uterus develop folds with changes in endometrial cell size and morphology (Wright *et al.* 2016).

In this study, we observed that the increase in capillary area density at day 77 is due to the expansion in the number of small blood vessels, predominantly on the maternal side. Our data agree with those reported by Vonnahme and Ford (2004) who determined a higher density of the capillary area from day 70 to 90 of gestation. However, the increase in the number of vessels per unit area was only observed on the fetal side. This appears to be mainly a consequence of more nutrient requirements due to the increased fetal size. Indeed, this is consistent with our data by the strong association between CL and fetal weight found from days 77 and 85 of gestation due to increased fetal growth.

In addition, we observed that the vascular areas augmented markedly at day 114 of gestation on the fetal side. Probably, these findings are due to a second phase of angiogenesis in mid-gestation until term to increase the availability of nutrients to the exponentially growing fetus (Vonnahme *et al.* 2001, Stenhouse *et al.* 2019). Consistent with the findings of Guimarâes *et al.* (2017), we observed that at day 85 there is a greater vascular area, but the number of vessels is unchanged, which could be attributed to a higher diameter of the vessels.

Interestingly, our immunohistochemical findings revealed intense VEGFA staining and a trend towards increased expression of this factor by the end of gestation, coinciding partly, with the expression of *VEGFA* mRNA. Similar results were reported by several authors (Vonnahme *et al.* 2001, Vonnahme & Ford 2004, Guimarâes *et al.* 2017) who found an increase in placental expression of the VEGF with advancement gestation in pigs. In addition, in Iberian pigs, *VEGF* mRNA and *VEGFR2* mRNA expression was determined by García Fernández *et al.* (2015) who observed an upregulated *VEGF* mRNA and *VEGFR2* mRNA expression in the uterus, during early pregnancy.

Our results also showed the higher *VEGFA* mRNA expression at day 77, probably related to the increase in the capillary area density given the higher number of blood vessels. Furthermore, in this study, we found a high KDR immunoexpression at day 77 of gestation (Supplementary Fig. 5). Possibly, these findings were due to the activation of the VEGFA/KDR pathway to stimulate endothelial cell migration and proliferation in late-middle gestation. KDR signaling is therefore considered essential for the differentiation of endothelial precursor cells into vascular endothelial cells and their proliferation (García Fernandez *et al.* 2015). However, Sanchis *et al.* (2015) have denoted the absence of VEGF in placental blood vessels, suggesting that angiogenesis in pigs during advanced gestation could be due to other angiogenic factors.

A synergic activity between VEGFA and FGF2 was demonstrated, with different effects on vessel size and function (Presta *et al.* 2005, Edwards *et al.* 2011, Tomao *et al.* 2014). In the present study, we have detected FGF2 in uterine luminal epithelium, glands and stroma cells during early gestation, similar to the results obtained by Welter *et al.* (2004) but in the pre-implantation period, at day 12. We also found FGF2 in the trophoblastic epithelium and the chorion throughout gestation. In addition, we demonstrated here a high *FGF2* mRNA and *FGFR1* mRNA expression at day 77 of gestation, whereas FGF2 HSCORE values were increased throughout pregnancy. It has been reported that the activation

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of FGFR1 triggers proliferation, migration, protease production and tubular morphogenesis, whereas FGFR2 only stimulates cell motility (Javerzat *et al.* 2002). Other authors have observed that mRNA *FGF2* levels and its receptors were elevated at the sites of fetal arrest at days 20 and 50 of gestation, suggesting a compensatory survival mechanism when other angiogenic factors are decreasing at the sites of fetal arrest (Edwards *et al.* 2011). Considering these data, high *FGF2* mRNA and *FGFR1* mRNA expression levels at day 77 of gestation, at the normal placental interface could be attributed to synergistic activity of the expression of the FGF2 and VEGFA proteins to induce angiogenesis.

On the other hand, in this study, ANGPT1 was mainly expressed in endothelial cells, which is consistent with its role in promoting vascular maturation and stabilization (Seval *et al.* 2008, Kappou *et al.* 2015). We also demonstrated a high expression of *ANGPT1* and *ANGPT2* mRNA expression at day 77 of gestation. The expression of ANGPT2 could be attributed to activating endothelial cells in response to the angiogenic stimulus of VEGFA, whereas the *ANGPT1* would allow stabilizing the newly formed blood vessels (Kappou *et al.* 2015). Furthermore, the upregulation of ANGPT2 mRNA at day 114 could promote a rapid increase in capillary diameter at the end of gestation. This result agrees with those reported by Lobov *et al.* (2002) which demonstrated that *in vivo*, in the presence of endogenous VEGFA, ANGPT2 promotes an increase in capillary diameter, remodeling of the basal lamina, proliferation and migration of endothelial cells, and stimulates sprouting of new blood vessels.

In summary, our previous findings indicate that after the first wave of angiogenesis, the expression of VEGFA/KDR may have a greater role in placental vascular development by paracrine signaling on day 40 of gestation (article in press). The present findings suggest that the second wave of angiogenesis would be present even until late-middle gestation at day 77 of pregnancy, with the predominance of angiogenic stimulation by VEGFA/KDR, FGF2/FGFR1 and a balance between ANGPT1 and ANGPT2/TEK.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/RAF-21-0092.

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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Author contribution statement

M R F conceived the study and wrote the paper. A L C performed experiments and analyzed data. M J M E performed experiments and analyzed data. M B R interpreted and revised the data. C G B interpreted the data, revised the article critically for important intellectual content. C I M contributed to the conception and design of the study, analyzed and interpreted the data and revised the article critically. All co-authors have read, approved and concur with the submitted manuscript. The authors have ensured the integrity of the work.

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