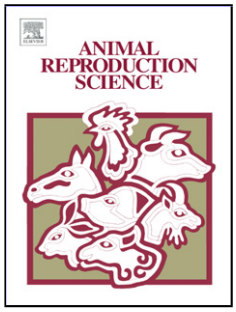


Title: Immunohistochemical determination of Ang-1, Ang-2 and Tie-2 in placentas of sows at 30, 60 and 114 days of gestation and validation through a bioinformatic approach

Authors: Fiorimanti Mariana Rita, Rabaglino María Belén, Cristofolini Andrea Lorena, Merkis Cecilia Inés

PII: S0378-4320(18)30198-2
DOI: https://doi.org/10.1016/j.anireprosci.2018.06.001
Reference: ANIREP 5871

To appear in: *Animal Reproduction Science*

Received date: 24-2-2018
Revised date: 21-5-2018
Accepted date: 1-6-2018

Please cite this article as: Mariana Rita F, Belén RM, Lorena CA, Inés MC, Immunohistochemical determination of Ang-1, Ang-2 and Tie-2 in placentas of sows at 30, 60 and 114 days of gestation and validation through a bioinformatic approach, *Animal Reproduction Science* (2018), https://doi.org/10.1016/j.anireprosci.2018.06.001

Immunohistochemical determination of Ang-1, Ang-2 and Tie-2 in placentas of sows at 30, 60 and 114 days of gestation and validation through a bioinformatic approach

Fiorimanti Mariana Rita*[a,c], Rabaglino María Belén[b,c], Cristofolini Andrea Lorena[a,c], Merkis Cecilia Inés[a]

[a]*Area of Electron Microscopy, Department of Animal Pathology. School of Agronomy and Veterinary, National University of Río Cuarto, Argentina*

[b]*Department of Animal Reproduction. School of Agronomy and Veterinary. National University of Río Cuarto, Argentina*

[c]*National Scientific and Technical Research Council (CONICET), Argentina*

**Corresponding author at: Fiorimanti Mariana Rita, Area of Electron Microscopy, Department of Animal Pathology, School of Agronomy and Veterinary, National University of Río Cuarto. Ruta Nac. 36, Km. 601 – C.P. X5804BYA, Río Cuarto, Córdoba, Argentina. Tel: +54 0358 4676165; Fax: +54 0358 4680280*

E-mail addresses: mfiorimanti@ayv.unrc.edu.ar

ABSTRACT

Angiopoietins (Ang-1, Ang-2) participate in vascular development and placental growth, both bind to Tie-2. This study aimed to determine the localization of angiopoietins in placental development of sows by immunohistochemistry and to validate the gene expression during gestation through a bioinformatic approach. Samples were collected from fifteen maternal-fetal interface from approximately 30 (n = 5), 60 (n = 5) and 114 (n = 5) days of gestation for immunohistochemistry. A bioinformatic approach was performed by re-analysis of public datasets to determine the increase or decrease of genes involved in angiogenesis during pregnancy. There was no significant statistical difference of Ang-1 during gestation, although there was a tendency to increase from mid- to term-gestation (P = 0.7680). A notable decrease of Ang-2 was observed from early- to term-pregnancy ($P \leq$ 0.05), consistent with the gene expression determined through bioinformatics. Furthermore, there were greater abundances of Tie-2 at both early and at term periods, but lesser abundances at mid-gestation ($P \leq 0.05$). The bioinformatics approach indicated that genes related to biological processes such as angiogenesis (i.e., development and morphogenesis of blood vessels) were expressed to a greater extent in early gestation as compared with later in gestation. The Ang-1 gene expression related to cell maturation, response to hypoxia and apoptosis, however, increased as gestation period advanced. In conclusion, angiopoietins may have an important role in the vascular development thus ensuring adequate placental growth in sows. The presence of angiopoietins in the trophoblast suggests a specific role for these pro-angiogenic factors in the tissue formation at the maternal-fetal interface.

Keywords:

Angiogenesis; Tie-2; Gestation; Immunohistochemistry; Bioinformatics; Swine

1. Introduction

Physiological angiogenesis in the placenta of sows is critical for peri-attachment development at the maternal-fetal interface, and an essential developmental stage for efficient placental function (Kridli et al., 2016). Thus, angiogenesis is tightly controlled by proangiogenic and antiangiogenic factors at the maternal-fetal interface (Krawczynski et al., 2015). During pregnancy, these pro-angiogenic factors, such as angiopoietin-1 and -2 (Ang-1 and Ang-2), are mainly produced by the placenta and have an important role in endothelial cell survival and vessel remodeling (Eklund and Olsen, 2006; Fagiani and Christofori, 2013; Kappou et al., 2015). Ang-1 and Ang-2, also known as ANGPT-1 and ANGPT-2 (Augustin et al., 2009), are ligands for Tie-2 (TEK), one the tyrosine kinase receptors of vascular endothelial cells, with similar affinity for both ligands (Gale et al., 2002; Thurston, 2003; Fiedler and Augustin, 2006; Thomas and Augustin, 2009; Linares et al., 2014; Wang and Lash, 2017). The Tie-2 gene expression, however, does not only occur in the vascular endothelium, but also in the placental tissue of humans (Tseng et al., 2006; Seval et al., 2008).

The angiopoietins together with vascular endothelial growth factors (VEGFs) and the relevant receptors, form the two signaling pathways that are almost exclusively endothelial cell (EC) specific (Eklund and Saharinen, 2013). The *in vivo* biologic effects of the angiopoietins also depend on concentrations of VEGFA in tissues having receptors of the angiopoietins (Wang and Lash, 2017). The angiogenic phase might be initiated by an increase in Ang-2 and VEGF, while the microvessel maturation phase might be initiated by a relative increase in Ang-1 and a decrease in VEGF (Lobov et al., 2002; Wakui et al., 2006; Yuan et al., 2007; Thurston and Daly, 2012; Biel et al., 2014). Sanchis et al., (2015) investigated the temporal and spatial localization of VEGFA in the sow placenta and this factor was detected in the endothelium, stroma, fetal mesenchyme, trophoblast and myometrium during gestation. In other studies, the binding site of Ang-1 and Ang-2 and the Tie-2 receptor for these angiopoietic factors have been detected in the human (Zhang et al., 2001; Dunk et al., 2000; Geva et al., 2002; Tseng et al., 2006; Seval et al., 2008; Schiessl et al., 2009) and baboon placenta (Babischkin et al., 2007). It was reported that changes in the specific gene expression patterns of Ang-1, Ang-2 and Tie-2 are the leading cause of alterations in abnormal placental development. The implication of these angiopoietins in vascular bed formation and placental development in sows, however, is still unknown. In addition, it is important to note that these biological processes are closely regulated by the differential expression of specific genes during placental development.

There has been investigations of differential gene expression resulting in production of microRNAs in the trophoblastic elongation and adhesion to the sow endometrium from days 10 to 11 post-fertilization (Ross et al., 2009; Krawczynski et al., 2015), and at the onset of placentation around days 20 to 26 and days 50 to 90 of gestation (Su et al., 2010; Wessels et al., 2013; Su et al., 2014). The study of placental gene

expression has become an important aspect on days 12 to 14 post-fertilization (Østrup et al., 2010; Gu et al., 2014), and subsequent development until days 75 and 90 of gestation (Zhou et al., 2009). Through use of bioinformatics, gene expression datasets from different studies that are available in public databases can be combined and re-analyzed as one dataset to identify times when gene expression is changed during the sow gestation period.

The hypothesis in the present study is that abundance of the angiopoietin synthesis in the sow placenta is modified from early to late gestation and that these modifications correspond with changes in gene expression during these periods. The aims of the present study, therefore, were to determine the localization of angiopoietins (Ang-1, Ang-2) and Tie-2 during placental development in sows by utilization of immunohistochemistry and to validate gene expression during gestation through a bioinformatics approach.

2. Material and methods

2.1. Animals and tissue collection

All procedures were approved by the National University of Río Cuarto Ethical Committee of Research in animals (CoEdI), Res. 186/2016. Reproductive tracts of healthy crossbred gilts (Yorkshire x Landrace) from different slaughterhouses from Río Cuarto, Argentina (33.11º S; 64.3º O) were used.

Tissues (n = 15) at the maternal-fetal interface were obtained from the reproductive tracts immediately after slaughter or after parturition, washed with Hank's saline solution (SSH) containing sodic penicillin G, streptomycine sulphate and fungizone (Gibco, Grand Island, NY USA) and maintained at 4 °C until processing within 30 min (Sanchis et al., 2015).

The lumen of the uterine horns was accessed longitudinally by making an incision on the anti-mesometrial edge. Fetuses were removed and gestational age was determined according to the crown-rump length of the litter using the methods previously reported by Marrable (1971).

The periods evaluated in the present study were: early (approximately day 30 of gestation; *n* = 5 placentas), mid (approximately days 60 of gestation; *n* = 5 placentas), and term gestation (approximately days 114; *n* = 5 placentas). Samples were collected from maternal-fetal interface. The tissues were processed using the conventional histological technique for immunohistochemistry.

2.2. 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. Tissues were subsequently dissected into 4 µm histological sections with a microtome (Micron, Germany) and mounted on slides. Paraffin embedded sections were used for immunohistochemistry.

2.3. Immunohistochemistry

Tissue sections were deparaffinized in xylol and then hydrated in alcohol of decreasing concentrations (100%, 90%, 80% and 70%) and distilled water. Antigenic retrieval was performed in which 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 three washes in phosphate buffered saline (PBS), endogenous peroxidase activity was quenched by use of

3% hydrogen peroxide in PBS for 20 min and subsequently there were three washes with PBS.

After incubating for 1 h at room temperature with 5% horse serum (Ang-1 and Tie-2) or goat serum (Ang-2) to inhibit non-specific binding, sections were incubated over night at 4 °C with primary antibodies: rabbit polyclonal anti-human Ang-1 antibody at a working dilution of 1/50 (Santa Cruz Biotechnology, Inc., USA; Ang-1 (H-123): sc-9044), goat polyclonal anti-human Ang-2 antibody, working dilution 1/50 (Santa Cruz Biotechnology, Inc., USA; Ang-2 (C-19): sc-7015), and rabbit polyclonal anti-human Tie-2 antibody, working dilution 1/50 (Santa Cruz Biotechnology, Inc., USA; Tie-2 (H-176): sc-9026).

The tissues used to assess Ang-1 and Tie-2 were rinsed twice in PBS and then incubated for 20 min with Polivalent Biotinylated Link HiDefAmplifier (rabbit and mouse) and subsequently for 20 min with HiDefDetection™ HRP Polymer Detector (HiDefDetection™ HRP Polimer System, Cell Marque, USA). The tissues used to assess Ang-2 were rinsed twice in PBS and then incubated for 30 min with biotinylated secondary antibodies pool (Santa Cruz Biotechnology Inc., USA; ImmunoCruz™ goat ABC Staining System, sc-2023) and subsequently for 30 min with the complex Avidine-HRP AB enzyme reagent (Santa Cruz Biotechnology).

After two washes in PBS, the antibody reaction was visualized using 3, 3´-diaminobenzidine chromogen solution (DAB Cell Marque, USA) for 10 min. For all tissues, sections were counterstained with Mayer´s haematoxylin (Dako, USA), washed, dehydrated and cover-slipped in Entellan (Merk, Darmstadt, Germany). Negative slides

were treated identically except an isotype control antibody was used instead of primary antibody.

All sections were stained using the same procedures 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.

2.4. HSCORE analysis

Immunohistochemical staining intensity for Ang-1, Ang-2 and Tie-2 was ranked with the designation 0 (absent), 1 (weak), 2 (moderate), and 3 (intense). For each slide, five randomly selected areas were evaluated using a light microscope (40x magnification).

For each tissue, an 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; Sanchis et al., 2015), 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.

2.5. Statistical analysis

Data from immunohistochemistry technique were analyzed with InfoStat Version 2009 software (Di Rienzo et al., 2016). Dependency of Ang-1 and Ang-2 and receptor Tie-

2 with gestational periods was evaluated. When significant differences were detected ($P \leq 0.05$), the means were compared using the *post hoc* LSD-Fisher test for all tissue assessments.

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. For *post hoc* comparisons, the Dunn's test was applied. Statistical significance was defined as $P \leq 0.05$.

2.6. Bioinformatic approach

Microarray dataset searches were performed in a public functional genomics data repository: Gene Expression Omnibus (GEO) from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/geo/), which supports MIAME-compliant data submissions.

To search for data, the keywords entered were (porcine AND endometrium OR placenta) AND "Susscrofa"[porgn:__txid9823]. The following datasets were considered for this study: GSE18641 (Østrup et al., 2010); GSE46332 (Gu et al., 2014) and GSE11853 (Zhou et al., 2009). Samples from these datasets obtained from nonpregnant sows (n = 4) and pregnant sows at 12 days (n = 4), 75 days (n = 4) or 90 days of gestation (n = 4) were selected for further analyses. All these samples were hybridized to the Affymetrix Porcine Genome Array. Animals were all from occidental breeds.

The gcRMA package (Wu et al., 2017) for the R software environment (http://www.r-project.org) was used to import the raw data into R, perform background correction, and normalize the data. Batch effects (different studies) were removed using the ComBat

function of the SVA package, adjusting the data for gestational age (quantitative variable; Johnson et al., 2007).

The result after using these procedures for data analysis was development of a table indicating the expression of several genes from the non-pregnant endometrium to the 90 days of gestation for sows. The genes of interest were manually selected to determine the change in expression throughout gestation.

Also, there was a desire to explore the biological processes that are activated or inhibited from the non-pregnant endometrium to 90 days of gestation. Thus, The Bayesian Estimation of Temporal Regulation (BETR) (Aryee et al., 2009) algorithm was used to identify the differentially expressed genes (DEG) throughout gestation at a False Discovery Rate (FDR) of <0.05. The non-pregnant endometrium was considered as the baseline measurement and was compared to subsequent gestational periods. This method, which is applied with the BETR package, provides the probabilities of differential expression for each gene in the data set. Genes with a probability greater than 99.99% were considered as DEG.

The list of DEG increasing (or decreasing) in expression toward the end of gestation was input into the DAVID software (Huang da et al., 2009) to determine the significant biological processes ($P \leq 0.05$) that were stimulated (or inhibited) at the different gestational ages when tissues were collected in the present study.

3. Results

3.1. Immunohistochemical determination for angiopoietins-1, 2 and Tie-2

Ang-1 was detected in the endothelium of maternal and fetal blood vessels, uterine epithelium, chorion and cytoplasmic apical part of trophoblast cells, as evidenced by weak

staining (+) by 30 days of pregnancy. The staining for the glandular epithelium was negative (-) for Ang-1. In addition, some weakly stained sites were detected in the endometrium, mainly distributed in the perivascular region of the blood vessels (Figure 1 A). Spatial location of Ang-1 was similar throughout the three gestational periods when tissues were collected, although a greater intensity of staining was observed towards mid- to term-gestation (Figure 1 B and 1 C). According to the HSCORE analysis, there was no difference in abundance of Ang-1 HSCORE values between early-, mid- and term-gestation, although there was a tendency ($P = 0.077$) for increases in HSCORE values in the tissues collected at mid- and late-gestation (Figure 4 A).

The Ang-2 was immuno-detected in the basal portion of uterine epithelial cells as a result of weak cytoplasmic staining by day 30 of gestation. A moderate perivascular staining pattern was observed around maternal and fetal blood vessels (Figure 2 A). By day 60 of pregnancy, alternated zones of weak and moderate Ang-2 staining were detected in the endometrium. During this period of gestation, the uterine glands stained positive for Ang-2. In trophoblast cells, there was weak to moderate amounts of staining in the apical portion of the cytoplasm of most cells (Figure 2 B). By day 114 of gestation, moderate immunoreactivity was observed, mainly in the trophoblast cells and in some parts of the chorion (Figure 2 C). There was an increase in the Ang-2 HSCORE values by day 30 of gestation when compared to days 60 and 114 ($P \leq 0.05$) as pregnancy progressed (Figure 4 B).

The Tie-2 immunoreactivity was detected as a result of moderate cytoplasmic staining in endothelial cells of maternal and fetal blood vessels at all three gestational periods when tissues were collected (Figure 3 A, 3 B and 3 C). Weak to moderate positive staining for Tie-2 was detected in trophoblast cells and the chorion by days 60 and 114 of

pregnancy (Figure 3 B and 3 C). The Tie-2 immuno-staining decreased by day 60 and subsequently increased again by term gestation ($P \leq 0.05$; Figure 4 C).

3.2. Bioinformatic analysis of gene expression in placental tissues of 12, 75 and 90 days of gestation

Compared to the non-pregnant endometrium, the decreasing gene expression toward the end of gestation in the tissues that were collected was related to biological processes such as angiogenesis, development and morphogenesis of blood vessels, among others (Table 1). There are actions of Ang-1 (ANGPT1) and Ang-2 (ANGPT2) in these biological processes.

As depicted in Figure 5, there was a greater ANGPT2 gene expression in both the non-pregnant and pregnant endometrium by day 12 of gestation. The relative ANGPT2 gene expression decreased by days 75 and 90 of gestation and these findings are consistent with the immuno-histochemical results where there was greater staining for ANGPT2 during early gestation than at term of gestation (Figure 4, B). As previously described, genes involved in vascular development, migration and cell proliferation processes were inhibited, which might explain why there was a decrease in Ang-2. The Ang-1 gene was also expressed on days 12, 75 and 90 of gestation and results indicated there was an overexpression (Figure 5) of this gene with a similar trend for gene expression as detected using immunohistochemistry from mid- to term-gestation (Figure 4, A).

In addition, an overexpression of the VEGFA gene was detected by days 75 and 90 of gestation (Figure 5). These genes are involved in the biological processes which are significantly enriched by DEG expression increases around day 90 of gestation. These biological processes are, among others, regulation of apoptosis, inhibition of cell death,

response to oxygen concentrations, and response to hypoxia, proliferation of epithelial cells and smooth muscle cells, cell maturation and regulation of endothelial cell migration in blood vessels (Table 2).

4. Discussion

The results of the present study determined the localization and expression of angiopoietin-1, -2 and Tie-2 from days 30 to 114 of gestation in the tissues of the maternal-fetal porcine interface and the gene expression from early to late gestation of sows. Results from both the immuno-histochemical and bioinformatics approaches indicate that Ang-1 tended to increase while Ang-2 decreased in late gestation.

The Ang-1 is expressed, mainly in perivascular cells that cover mature blood vessels (Gale et al., 2002) and the present results are consistent with the localization in placental tissues of sows, and support the previous findings that there is an increase in expression of the Ang-1 gene in perivascular cells from mid- to term-gestation to maintain quiescent blood vessels. In the human placenta, expression of the Ang-1 is also restricted to the perivascular stroma at term gestation (Dunk et al., 2000). Consistent with these previous findings, in the present study the Ang-1 and receptor Tie-2 were expressed in the perivascular stroma surrounding the larger blood vessels.

Expression of the Ang-1 and its receptor was detected in the tissues collected at the various stages of gestation but the gene expression was predominant during mid- and term-gestation. These findings suggest that Ang-1 might function through paracrine mechanisms by binding to Tie-2 in the blood vessels. There have been several studies indicating the location Ang-1 in blood vessels is consistent with the role of Ang-1 in maturation and

maintenance of vessels after actions of VEGF, to complement the late stages of angiogenesis (Thurston, 2003; Eklund and Olsen, 2006; Yuan et al., 2007; Thurston and Daly, 2012).

In addition, Ang-1 inhibits the effects of VEGF by enhancing adhesion among endothelial cells and decreasing the cell permeability (Eklund and Saharinen, 2013) so as to maintain the endothelial barrier and the quiescent vasculature (Fiedler and Augustin, 2006; Fagiani and Christofori, 2013; Biel et al., 2014; Linares et al., 2014). While the results of HSCORE Ang-1 values did not indicate that there was any increase in Ang-1, there was a trend for increasing Ang-1 gene expression by days 75 and 90 of gestation. During mid to term gestation, the biological processes related to Ang-1 (determined through the bioinformatic approach) were cell maturation and regulation of endothelial cell migration in blood vessels. Zhou et al., (2009) reported that vascular development and permeability was associated with different gene expression patterns for VEGF. These changes varied depending on the breed of pigs by days 75 and 90 of pregnancy.

Abundance of Ang-2 is normally basal in quiescent mature vessels regardless of physiological condition (Thurston and Daly, 2012) and is mainly secreted by endothelial cells at locations of active vascular remodeling and is thought to function in an autocrine manner (Fagiani and Christofori, 2013). These previous findings are consistent with the results from the present study where Ang-2 immuno-expression decreased by mid- to term-gestation, when quiescent mature vessels would be predominant.

The greater HSCORE values for Ang-2 compared to Ang-1 in early gestation could be related to actions of Ang-2 in the destabilization of endothelial cells for vascular remodeling during this gestational period that is characterized by rapid development and growth of both the uterus and embryos.

During maternal recognition of pregnancy and embryo implantation, there are significant differences in gene expression that is related to miRNA in developing porcine conceptuses and trophoblasts (Krawczynski et al., 2015). These miRNAs are involved in several processes, including angiogenesis, cell proliferation, cell migration, cell adhesion and cytoskeleton organization (Wessels et al., 2013; Su et al., 2014).

In the present study, the bioinformatic analysis indicated that biological processes in early gestation were related to angiogenesis, blood vessel development, regulation of cell proliferation and migration, and processes of anti-apoptosis. During mid- and term-gestation, Ang-2 immunostaining and relative gene expression decreased. The Ang-2 is an antagonist of Ang-1-mediated activation of Tie-2. Studies by Gale (2002) with Ang-2 deficient mice, however, suggest that Ang-2 may have an agonist role depending on the tissue environment and experimental conditions.

In other studies, Ang-2 was a potent inhibitor of Ang-1 induced-phosphorylation in some regards, although if Tie-2 is expressed in non-endothelial cells, Ang-2 may function as an agonist, depending on its concentration, source (autocrine or paracrine) and duration of action (Thurston, 2003). Lobov et al. (2002) reported that Ang-2 stimulated pupillary membrane capillary angiogenesis *in vivo*, as a function of endogenous VEGF presence. Sanchis et al., (2015) detected VEGFA throughout gestation in the trophoblast and uterine cells of sows but VEGFA was not detected in endothelial cells. The action of Ang-2 combined with VEGFA, however, could have a positive effect on the placenta of sows by days 30 and 60 of gestation, as it would increase the diameter of the blood vessels, remodeling the basal lamina and aid in proliferation of endothelial cells.

These angiogenesis processes decreased by mid-gestation (days 75 and 90) while homeostatic processes, response to oxygen concentrations and hypoxia, regulation of cell

death, apoptosis, reproductive development and cell maturation increased as ascertained by conducting the bioinformatic analysis in the present study. Additionally, Tie-2 was detected in vascular and mesenchymal placental tissues in the present study. This finding may reflect the capacity of endothelial cells in the early development of the placenta to respond to angiogenic stimuli through Ang-1 binding to Tie-2. For Tie-2, there were greater HSCORE values in the early- and term-gestation, and lesser HSCORE values at mid-gestation. The fluctuation of Tie-2 HSCORE values may be related to both ligands, with its signaling being regulated by the Ang-1/Ang-2 interactions and, thus, the Tie-2 capacity to respond to other angiogenic signals, stimulating or inhibiting the angiogenic process. The Tie-2 paracrine activation leads indirectly to recruitment of supporting perivascular cells, probably through the action of Ang-1 released by endothelial cells (Kappou et al., 2015; Wang and Lash, 2017). In other reports (Dunk et al., 2000; Seval et al., 2008), there has been confirmation that angiopoietin and Tie-2 genes were co-expressed in trophoblasts suggesting an autocrine function for angiopoietins in the human placenta.

To our knowledge, however, this study is the first where there has been immuno-histochemical localization of angiopoietins in the placenta of sows during pregnancy. Findings in the present study also indicate the location of angiopoietins-1, -2 and Tie-2 in the placental trophoblast tissues throughout gestation.

5. Conclusions

In the present study, the localization of Ang-1, Ang-2 and Tie-2 in the placenta of sows was ascertained on days 30, 60 and 114 of gestation, and there was validation of the expression of these genes during this gestational period through a bioinformatic approach. The angiopoietins may have an important role in the vascular development, thus having

important functions for adequate placental growth. The presence of Tie-2 in the trophoblast indicates there is a specific role of the angiopoietins for the structural tissue formation in the tissues of the maternal-fetal interface, independent of the functions of Tie-2 in the vascular endothelium. Future research is needed to elucidate the role of angiopoietins in the placenta of sows and the interaction with other angiogenic factors such as VEGF.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgments

The authors are grateful to the SECyT (Secretaría de Ciencia y Técnica, Universidad Nacional de Río Cuarto) and ANPCYT (FONCYT-PICT), which supported this study through grants. Mariana Fiorimanti thanks CONICET for fellowship support.

References

Aryee, M., Gutierrez-Pabello, J., Kramnik, I., Maiti, T., Quackenbush, J., 2009. An improved empirical bayes approach to estimating differential gene expression in microarray time-course data: BETR (Bayesian Estimation of Temporal Regulation). BMC Bioinformatics 10, 409-420.

Augustin, H., Koh, G., Thurston, G., Alitalo, K., 2009. Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. Nat. Rev. Mol. Cell. Biol. 10, 165–177.

Babischkin, J., Suresch, D., Pepe, G., Albrecht, E., 2007. Differential expression of placental villous angiopoietin-1 and -2 during early, mid and late baboon pregnancy. Placenta 28, 212–218.

Biel, N., Lee, J., Sorg, B., Siemann, D., 2014. Limitations of the dorsal skinfold window chamber model in evaluating anti-angiogenic therapy during early phase of angiogenesis. Vasc. Cell. 6, 17-37.

Di Rienzo, J., Casanoves, A., Balzarini, M., Gonzalez, L., Tablada, M., Robledo, C., 2016. InfoStat Group, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentina. http://www.infostat.com.ar.

Dunk, C., Shams, M., Nijjar, S., Rhaman, M., Qiu, Y., Bussolati, B., Ahmed A., 2000. Angiopoietin-1 and angiopoietin-2 activate trophoblast Tie-2 to promote growth and migration during placental development. Am. J. Pathol. 156, 2185–2199.

Eklund, L., Olsen, B., 2006. Tie receptors and their angiopoietin ligands are context-dependent regulators of vascular remodeling. Exp. Cell. Res. 312, 630–641.

Eklund, L., Saharinen, P., 2013. Angiopoietin signaling in the vasculature. Exp. Cell. Res. 319, 1271–1280.

Fagiani, E., Christofori G., 2013. Angiopoietins in angiogenesis. Cancer Lett. 328, 18–26.

Fiedler, U., Augustin, H., 2006. Angiopoietins: a link between angiogenesis and inflammation. Trends Immunol. 27, 552–558.

Gale, N., Thurston, G., Hackett, S., Renard, R., Wang, Q., McClain, J., Martin, C., Witte, C., Witte, M., Jackson, D., 2002. Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by angiopoietin-1. Dev. Cell. 3, 411-423.

Geva, E., Ginzinger, D., Zaloudek, C., Moore D., Byrne, A., Jaffe, R., 2002. Human placental vascular development: vasculogenic and angiogenic (branching and nonbranching) transformation is regulated by vascular endothelial growth factor-A, angiopoietin-1, and angiopoietin-2. J. Clin. Endocrinol. Metab. 87, 4213–4224.

Gu, T., Zhu, M., Schroyen, M., Qu, L., Nettleton, D., Kuhar, D., Lunney, J., Ross, J., Zhao, S., Tuggle, C., 2014. Endometrial gene expression profiling in pregnant Meishan and Yorkshire pigs on day 12 of gestation. BMC Genomics 15, 156-185.

Huang da, W., Sherman, B., Lempicki, R., 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–57.

Johnson, W., Li, C., Rabinovic, A., 2007. Adjusting batch effects in microarray expression data using empirical Bayes methods. Biostatistics 8, 118–127.

Kappou, D., Sifakis, S., Konstantinidou, A., Papantoniou, N., Spandidos, D., 2015. Role of the angiopoietin/Tie system in pregnancy (Review). Exp. Ther. Med. 9, 1091–1096.

Krawczynski, K., Najmula, J., Bauersachs, S., Kaczmarek, M., 2015. MicroRNAome of porcine conceptuses and trophoblasts: expression profile of micrornas and their potential to regulate genes crucial for establishment of pregnancy. Biol. Reprod. 92, 21-34.

Kridli, R., Khalaj, K., Bidarimath, M., Tayade C., 2016. Placentation, maternal-fetal interface, and conceptus loss in swine. Theriogenology 85, 135–144.

Linares, P., Chaparro, M., Gisbert, J., 2014. Angiopoietins in inflammation and their implication in the development of inflammatory bowel disease. A review. J. Crohns Colitis 8, 183–190.

Lobov, I., Brooks, P., Lang, R., 2002. Angiopoietin-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival in vivo. Proc. Natl. Acad. Sci. U S A. 99, 11205–11210.

Marrable, A., 1971. The Embryonic Pig: a Chronological Account. London: Exeter, Pitman Medical.

Østrup, E., Bauersachs, S., Blum, H., Wolf, E., Hyttel, P., 2010. Differential endometrial gene expression in pregnant and nonpregnant sows. Biol. Reprod. 83, 277–285.

Ross, J., Ashworth, M., Stein, D., Couture, O., Tuggle, C., Geisert, R., 2009. Identification of differential gene expression during porcine conceptus rapid trophoblastic elongation and attachment to uterine luminal epithelium. Physiol. Genomics 36, 140–148.

Sanchis, E., Cristofolini, A., Merkis, C., 2015. Porcine placental immunoexpression of vascular endothelial growth factor, placenta growth factor, Flt-1 and Flk-1. Biotech Histochem. 90, 486–494.

Schiessl, B., Innes, B., Bulmer, J., Otun, H., Chadwick, T., Robson, S., Lash, G., 2009. Localization of angiogenic growth factors and their receptors in the human placental bed throughout normal human pregnancy. Placenta 30, 79–87.

Selam, B., Kayisli, U., Mulayim, N., Arici, A., 2001. Regulation of Fas ligand expression by estradiol and progesterone in human endometrium. Biol. Reprod. 65, 979–985.

Seval, Y., Sati, L., Celik-Ozenci, C., Taskin, O., Demir, R., 2008. The distribution of angiopoietin-1, angiopoietin-2 and their receptors tie-1 and tie-2 in the very early human placenta. Placenta 29, 809–815.

Su, L., Liu, R., Cheng, W., Zhu, M., Li, X., Zhao, S., Yu, M., 2014. Expression patterns of microRNAs in porcine endometrium and their potential roles in embryo implantation and placentation. PLoS One. 9, e87867.

Su, L., Zhao, S., Zhu, M., Yu, M., 2010. Differential expression of microRNAs in porcine placentas on days 30 and 90 of gestation. Reprod. Fertil Dev. 8, 1175–1182.

Thomas, M., Augustin H., 2009. The role of the Angiopoietins in vascular morphogenesis. Angiogenesis 12, 125–137.

Thurston, G., 2003. Role of Angiopoietins and Tie receptor tyrosine kinases in angiogenesis and lymphangiogenesis. Cell Tissue Res. 314, 61–68.

Thurston, G., Daly, C., 2012. The complex role of angiopoietin-2 in the angiopoietin-tie signaling pathway. Cold Spring Harb. Perspect. Med. 2, a006550.

Tseng, J., Hsu, S., Ho, E., Hsieh, Y., Wen, M., Chou, M., 2006. Differential expression of angiopoietin-1, angiopoietin-2, and Tie receptors in placentas from pregnancies complicated by placenta accreta. Am J. Obstet. Gynecol. 194, 564–571.

Wakui, S., Yokoo, K., Muto, T., Suzuki, Y., Takahashi, H., Furusato, M., Hano H., Endou H., Kanai, Y., 2006. Localization of Ang-1, -2, Tie-2, and VEGF expression at endothelial-pericyte interdigitation in rat angiogenesis. Lab. Invest. 86, 1172–1184.

Wang, Q., Lash, G., 2017. Angiopoietin 2 in placentation and tumor biology: The yin and yang of vascular biology. Placenta 56, 73-78.

Wessels, J., Edwards, A., Khalaj, K., Kridli, R., Bidarimath, M., Tayade, C., 2013. The microRNAome of pregnancy: deciphering miRNA networks at the maternal-fetal interface. PLoS One 8, e72264.

Wu, J., Irizarry, R., 2017. Gentry WCFJMJ, gcrma: Background Adjustment Using Sequence Information.

Yuan, H., Venkatesha, S., Chan, B., Deutsch, U., Mammoto, T., Sukhatme, V., Woolf A., Karumanchi, S., 2007. Activation of the orphan endothelial receptor Tie1 modifies Tie2-mediated intracellular signaling and cell survival. FASEB J. 21, 3171–3183.

Zhang, E., Smith, S., Baker, P., Charnock-Jones, D., 2001. The regulation and localization of angiopoietin-1, -2, and their receptor Tie2 in normal and pathologic human placentae. Mol. Med. 7, 624–635.

Zhou, Q., Fang, M., Huang, T., Li, C., Yu, M., Zhao, S., 2009. Detection of differentially expressed genes between Erhualian and Large White placentas on day 75 and 90 of gestation. BMC Genomics 10, 337-349.

Figure captions

Fig.1. Immuno-histochemical localization of Ang-1 in placental tissues at 30 (A), 60 (B), and 114 (C) days of gestation, and the negative control at 30 days of gestation, no staining is observed (D); Arrowheads indicate positive immuno-staining in the trophoblast (Tr) and the uterine epithelium (Ut); Asterisks indicate positive immuno-staining in the endothelial cells of the maternal or fetal vessels; Mv = maternal vessels; Fv = fetal vessels; Gl = uterine glands; Ut= uterine epithelium; Tr = trophoblast; Scale bar: 20 µm

Fig.2. Immuno-histochemical localization of Ang-2 in placental tissues at 30 (A), 60 (B), and 114 (C) days of gestation and the negative control at 60 days of gestation, no staining is observed; Arrowheads indicate positive immuno-ostaining in the trophoblast (Tr) and the uterine epithelium (Ut); Asterisks indicate positive immuno-staining in the endothelial cells of the maternal or fetal vessels; Mv = maternal vessels; Fv = fetal vessels; Gl = uterine glands; Ut = uterine epithelium; Tr = trophoblast; Scale bar: 20 µm

Fig.3. Immuno-histochemical localization of Tie-2 in placental tissue at 30 (A), 60 (B), and 114 (C) days of gestation, and the negative control at 114 days of gestation, no staining is observed; Arrowheads indicate positive immuno-staining in the trophoblast (Tr) and the uterine epithelium (Ut); Asterisks indicate positive immunostaining in the endothelial cells of the maternal or fetal vessels; Mv = maternal vessels; Fv = fetal vessels; Gl = uterine glands; Ut = uterine epithelium; Tr = trophoblast; Scale bar: 20 µm

Fig.4. Distribution of HSCORE values for Ang-1 (A), Ang-2 (B) and Tie-2 (C) immuno-staining intensity in placental tissues of sows at different stages of gestation; Data represented as mean ± SEM; Bars with different letters differ ($P \leq 0.05$)

Fig.5. Expression of selected genes in endometrium or placenta of sows at different gestational ages, as determined through a bioinformatics approach; NP: Non-pregnant endometrium; 12, 75 and 90 days: placenta at 12, 75 and 90 days of gestation, respectively; Data represent the means and SEM, with differences ($P \leq 0.05$) indicated by an asterisk

Fig 1

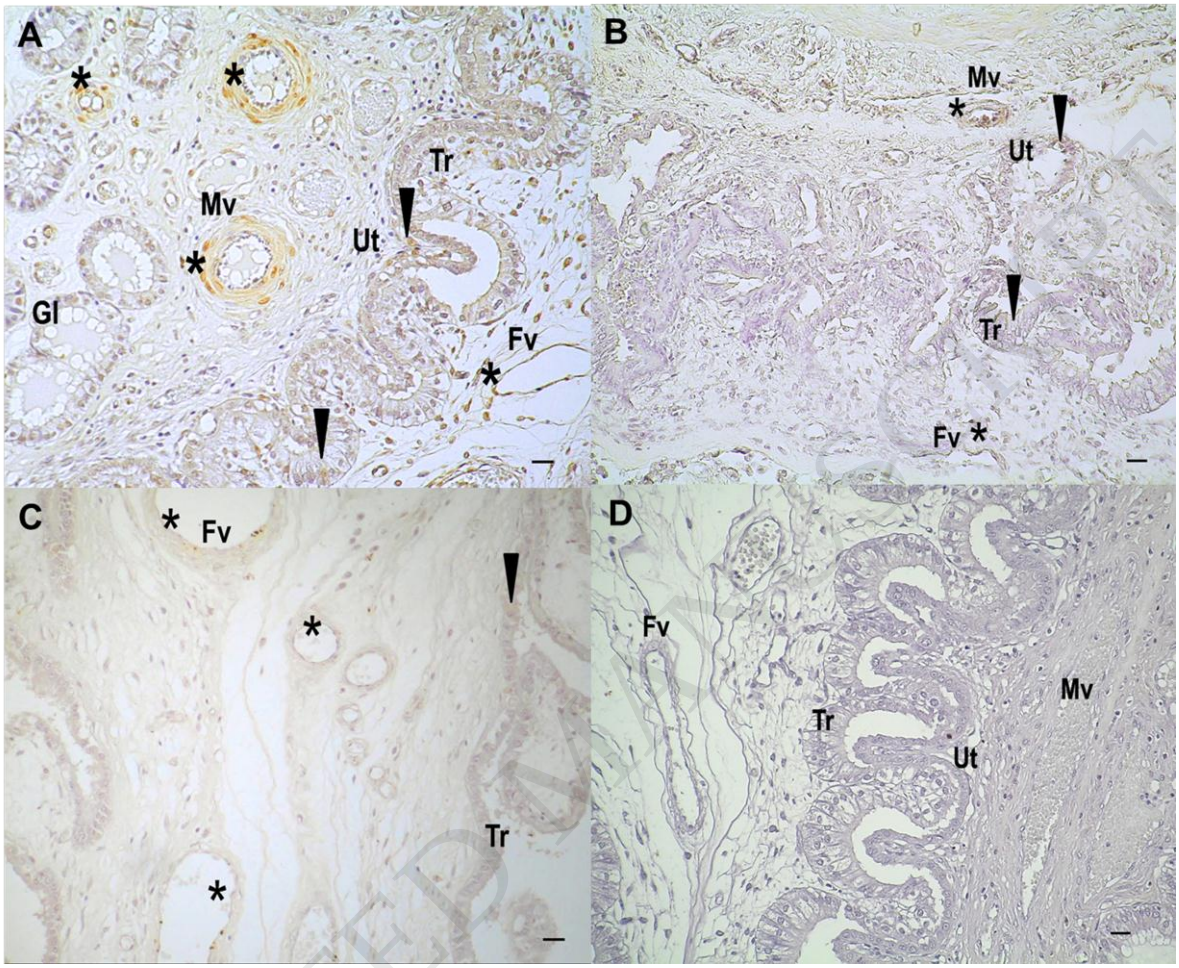


Fig 2

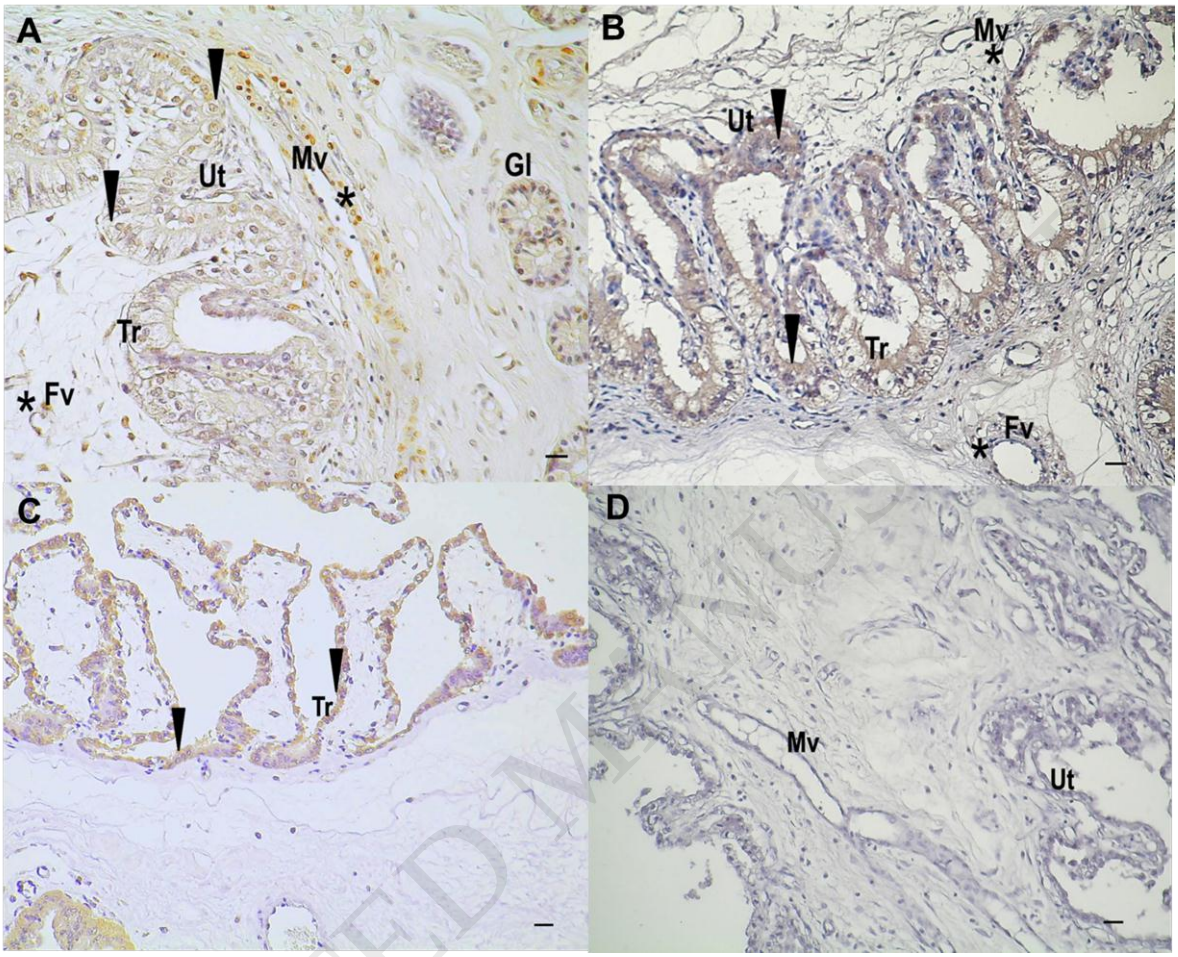


Fig 3

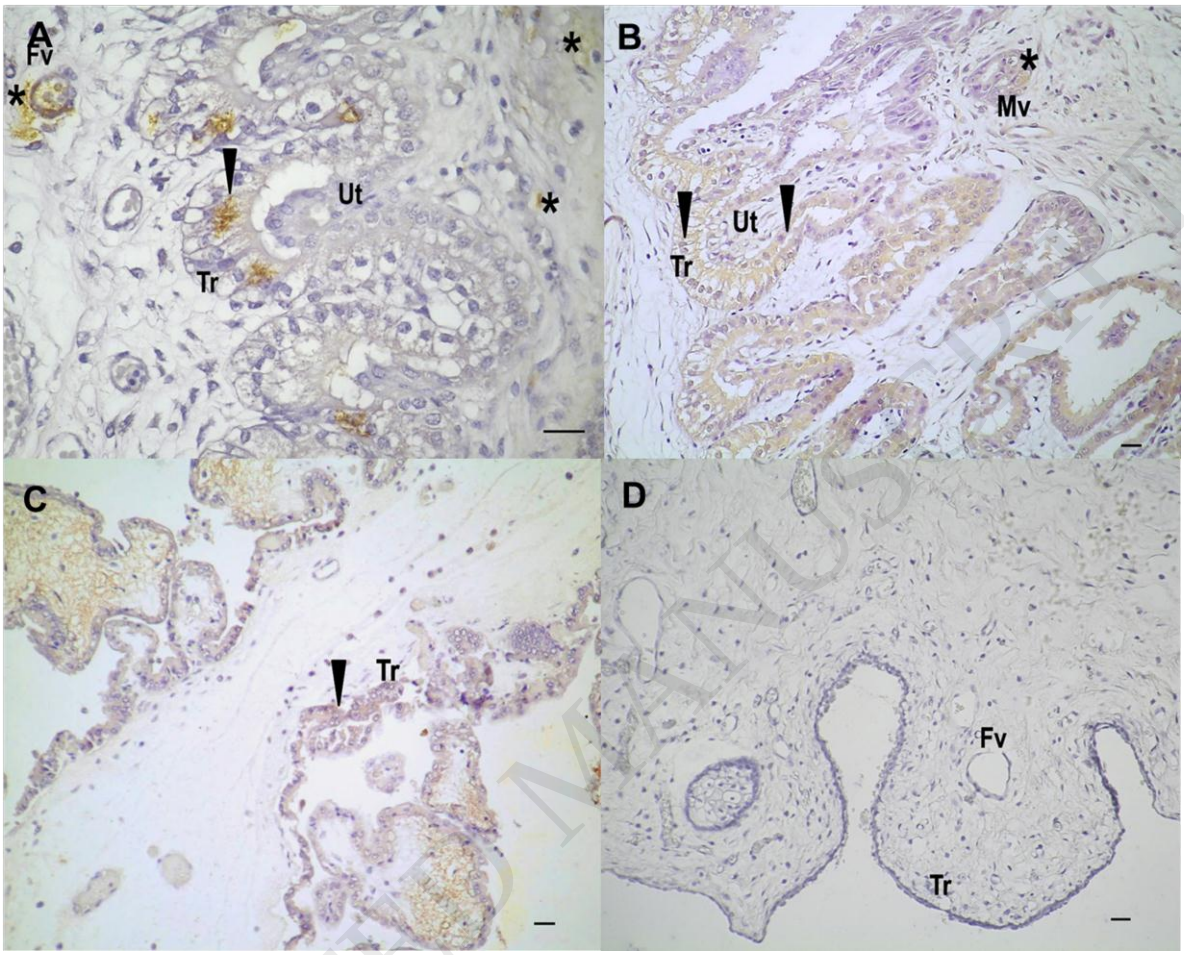


Fig 4

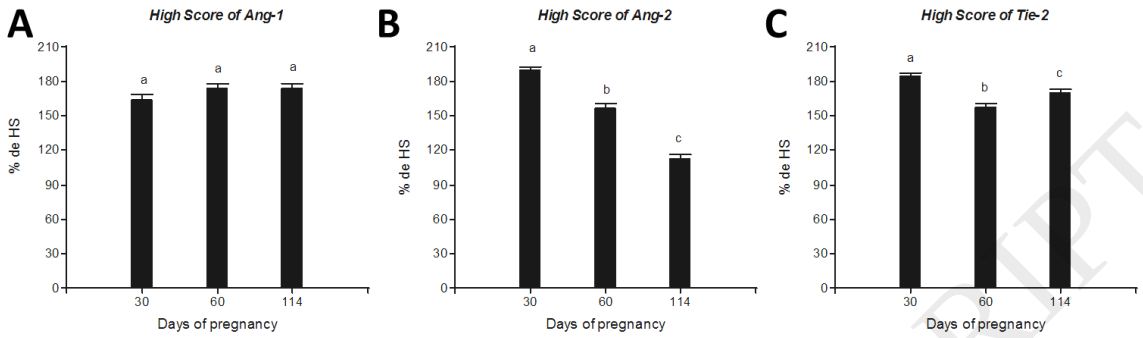
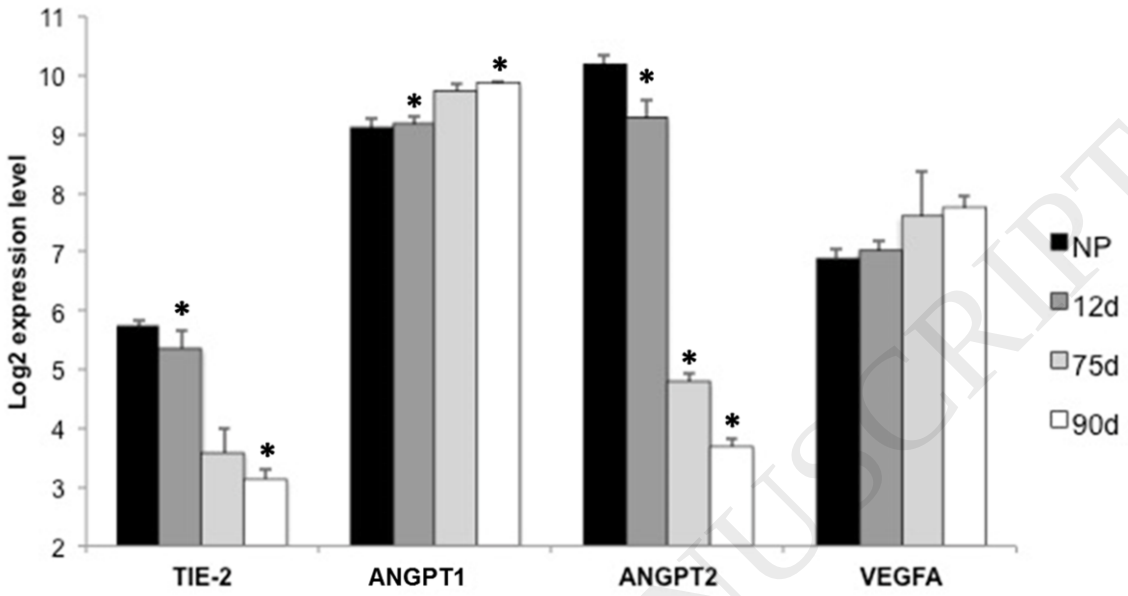


Fig 5



Tables

Table 1

Statistically over-represented biological processes with the genes decreasing in expression toward the end of gestation

Biological processes	**Genes expression**
Angiogenesis	EPAS1, LEPR, EDN1, JAG1, MYH9, CTNNB1, EDNRA, MAP3K7, CDH13, HIF1A, NUS1, NOTCH4, CASP8, ANGPT1, THBS1, RBPJ, ANGPT2, CYR61
Vasculature development	ZFAND5, LMO2, LEPR, COL3A1, EDN1, JAG1, GJA4, CDH5, CTNNB1, MAP3K7, EDNRA, ITGAV, CASP8, HEY2, QKI, ANGPT1, THBS1, ANGPT2, CYR61, PLAT, SELP, EPAS1, TGFBR1, EFNB2, MYH9, CDH13, HIF1A, NUS1, NOTCH4, COL1A2, TGFBR3, RBPJ
Blood vessel development	LMO2, LEPR, EDN1, COL3A1, JAG1, GJA4, CDH5, CTNNB1, EDNRA, MAP3K7, ITGAV, CASP8, HEY2, QKI, ANGPT1, THBS1, ANGPT2, CYR61, PLAT, SELP, EPAS1, TGFBR1, MYH9, CDH13, HIF1A, NUS1, NOTCH4, COL1A2, TGFBR3, RBPJ
Regulation of cell proliferation	NAMPT, EDN1, NAP1L1, GJA1, JAG1, PRDX3, GLI1, CTNNB1, EDNRA, CUL2, GPC3, CCL3L1, KIFAP3, HEY2,

	CALCRL, IL13RA1, CCNA2, CTBP2, ADAM10, IL6R, CD164, MECOM, PPP1CB, HES1, PPM1D, HIF1A, BTG2, CD80, NCK1, SCIN, ADAMTS1, SMARCA2, CAV2, CNBP, PTH1R, TIPIN, ITGB1, CDH5, ARNT, IL12RB2, VCAM1, MORC3, TEK, LAMB1, THBS1, TRAF5, DNAJA3, TXNIP, BECN1, KLF10, TGFBR1, SMAD4, SMAD1, GUCY2C, CDH13, CDKN1B, NOTCH4, FABP4, TGFBR3, JAK2, NR5A2, F2R, BMPR1A, HTR2A, IL2
Cell migration	ZFAND5, CAV2, CCK, FUT8, ITGB1, VCAM1, SCARB1, TOP2B, THBS1, FN1, PLAT, SELP, ROCK1, TGFBR1, NR4A2, NRD1, SCYL3, IL6R, MYH9, CDH13, HIF1A, CD34, NCK1, LYST, SIX1, LRP6, TGFBR3, VCAN
Blood vessel morphogenesis	PLAT, SELP, EPAS1, LEPR, TGFBR1, EDN1, JAG1, MYH9, CTNNB1, EDNRA, MAP3K7, CDH13, HIF1A, NUS1, CASP8, NOTCH4, HEY2, QKI, ANGPT1, RBPJ, THBS1, ANGPT2, CYR61

Genes such as ANGPT1 and ANGPT2 decrease in expression toward the end of gestation be participating in the pathways of angiogenesis, vasculature development, blood vessel development, regulation of cell proliferation, cell migration and blood vessel morphogenesis

Table 2

Statistically over-represented biological processes with the genes increasing in expression toward the end of gestation

Biological processes	**Genes expression**
Regulation of apoptosis	PTGS2, STAT5A, MMP9, FOXO3, CIAPIN1, SART1, TGFB1, MYD88, PROP1, APOE, NGFRAP1, NQO1, NUDT2, ZC3HC1, RARG, CD3E, AARS, ACTN1, ACTN2, FADD, HBXIP, NME6, NME2, MSX1, NME3, CD40LG, IFNB1, NME1, F3, VEGFA, CSTB, UBA52, NMNAT1, CLU, BCL2L1, GCLM, PEA15, ALB, INS, RAC1, DIABLO, FAIM, ARHGDIA, GRIN1, MUL1, CIDEA, SMAD3, TRADD, ATP7A, CDKN1A, SFRP1, VCP, IKBKG, BMP7, DAP3, FASTK, PRDX5, PRDX2, NFKB1, AKT1, BAG3, PAX7, DHCR24, IHH, KNG1, CLN3, PPP2R1A, IRAK1, ARHGEF2, NDUFA13, INHA, CDK5, INHBA, ADRB2, CAPN10, IGF2R, IL12A, SERPINB2, SEMA4D, CLN8, CAMK1D, DPF2, CSF2, PRKCZ, MGMT, PML, ASNS, SFN, MIF, SH3GLB1, MAP3K1, DAD1, POU3F3, NEFL, ERCC1, IL4, FOXL2, IL2RA, YWHAB, IGF2, BAD, PLG, NCSTN, PRLR, EEF1E1, SMPD1, ID3, TIAF1, SMPD2
Response to oxygen concentrations	PPARA, TF, TXN2, CLDN3, IL18, ALDOC, PML, BCL2L1, TRH, MMP2, TGFB1, LONP1, ECE1, SERPINA1, NOS2, DPP4, EPO, FLT1, CYP1A1, SMAD3, CAPN2, CDKN1A,

	PYGM, VEGFA, ALDH2, NPPC, ENG
Inhibition of cell death	STAT5A, PRDX5, NFKB1, PRDX2, CIAPIN1, AKT1, PROP1, MYD88, APOE, PAX7, BAG3, DHCR24, IHH, IRAK1, CLN3, ZC3HC1, AARS, HBXIP, NME6, NME2, MSX1, NME1, CD40LG, F3, VEGFA, SERPINB2, SEMA4D, CLN8, UBA52, CSF2, PRKCZ, CLU, ASNS, BCL2L1, GCLM, MIF, PEA15, INS, SH3GLB1, ALB, DAD1, POU3F3, FAIM, NEFL, ARHGDIA, ERCC1, IL4, GRIN1, SMAD3, CIDEA, IGF2, CDKN1A, PRLR, SFRP1, TIAF1
Response to hypoxia	PPARA, TF, FLT1, CYP1A1, CLDN3, TXN2, ALDOC, IL18, PML, SMAD3, BCL2L1, TRH, CAPN2, MMP2, TGFB1, LONP1, ECE1, PYGM, VEGFA, NPPC, SERPINA1, NOS2, ENG, DPP4, EPO
Regulation of epithelial cell proliferation	SMAD3, TGFB1, KDR, NME2, CDKN2B, CCND2, NME1, GRN, TSC2, VEGFA, TGFA, TINF2, PRL, IHH
Regulation of smooth muscle cell proliferation	FLT1, PTGS2, VEGFA, IL12A, NPPC, COMT, AGER, IGFBP5, AGPAT1
Cell maturation	CEBPA, SOX10, PLP1, PPARG, PICK1, FOXO3, KDR, VEGFA, KCNE1, GDF11, TRIP13, EPO, IHH
Regulation of blood vessel endothelial cell migration	HDAC5, APOE, VEGFA, PLG, TGFB1

Other genes increase in expression toward the end of gestation be participating in the pathways of regulation of apoptosis, negative regulation of cell death, response to oxygen concentrations, and response to hypoxia, proliferation of epithelial cells and smooth muscle cells, cell maturation and regulation of endothelial cell migration of blood vessels