Six placenta permeability-related genes: molecular characterization and expression analysis in pigs

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The nutrient transportation ability of placenta depends on placental size, vascular density and permeability. Regulation of angiogenesis in the placenta is critical for successful gestation. Placenta vascularity exhibits disparity in different gestation stages and different pig breeds. To investigate the expression of genes related to permeability in the porcine placenta of different gestation stages and breeds, molecular cloning and gene expression analysis of six porcine genes, vascular endothelial growth factor (VEGF), VEGF-R1, VEGF-R2, endothelial nitric oxide synthase (eNOS), vascular endothelial cadherin (CDH5) and β-arrestin2 (Arrb2), were performed in this study. The results demonstrated that from gestation day 33 to day 90, Landrace exhibited significant increase ($P < 0.05$) in placental VEGF and Arrb2 mRNA expression. Moreover, expression levels of VEGF, VEGF-R1, VEGF-R2 and eNOS mRNA were higher ($P < 0.01$) in the placenta of Erhualian than those in Landrace on day 90 of gestation. In contrast, CDH5 placental mRNA expression level exhibited significant decrease ($P < 0.05$) from day 33 to day 90 gestation in Landrace. Erhualian placental CDH5 and Arrb2 expression levels were significantly lower ($P < 0.01$) than those in Landrace conceptuses on day 90 of gestation. Our study offered new data on the expression of genes in VEGF signal transduction pathway in porcine placenta.

Keywords: pig, placenta, expression, permeability

Introduction

The pig placenta is responsible for nutrient transport from the sow to the fetus during pregnancy. In the placenta, maternal blood must be brought into direct contact with the villous trophoblast of the fetal placenta, where sow and fetal nutrient and substrate supply occur within folds of the two epithelial cell layers (Vallet and Freking, 2007). Therefore, the capability of placenta nutrient transport depends on the density and permeability of blood vessels on the placental surface besides placenta size. The placental surface area of Meishan was similar between day 70 and day 110 of gestation, but their vascular density significantly increased during this interval (Biensen et al., 1998). Whereas the placental surface area of Yorkshire placentaes was markedly larger on day 110 than on day 90, placental vascular density was similar during this interval. A large number of potentially important growth factors may directly or indirectly regulate angiogenesis at the maternal–fetal interface. Angiogenesis can occur by sprouting, intussusception and recruitment of endothelial progenitor cells (Bates and Harper, 2002). There is substantial evidence that permeability increases are associated with sprouting. Placental vascularization as well as its permeability reflects a complex interaction of many regulatory factors.

Vascular endothelial growth factor (VEGF) is a potent angiogenic, vasoactive and permeability-increasing molecule (Michel, 1984; Taylor and Granger, 1984; Bates et al., 1999). VEGF-binding sites were identified in vascular endothelial cells corresponding to VEGF-R1 (Flt-1) (Shibuya et al., 1990) and VEGF-R2 (Flk-1/KDR) (Matthews et al., 1991). VEGF up-regulation and VEGF-R activation promote numerous downstream targets of receptor tyrosine kinases of signal transduction pathways such as endothelial nitric oxide synthase (eNOS) and vascular endothelial cadherin (CDH5), which lead to vascular endothelia cells proliferation and vascular permeability (Wang et al., 2004).

Nitric oxide (NO) is the endothelium-derived relaxing factor, which regulates blood flow and vascular permeability. Endothelial NO synthase is the primary physiological source of NO in the vascular system. VEGF increases eNOS expression via activation of the VEGF-R2 receptor tyrosine...
kinase and a downstream protein kinase C signaling pathway (Shen et al., 1999). NO and prostacyclin were produced by the interaction of VEGF with its VEGF-R2 receptor as mediators of VEGF/WPF-induced vascular permeability (Murohara et al., 1998).

Down-regulation of vascular endothelial cadherin in endothelial tumors may have important consequences for tumor growth and bleeding complications (Zanetta et al., 2005). CDH5 is located strictly at endothelial junctions and appears to be a major adhesive component of cell-to-cell contacts (Huber et al., 1996). Activation of VEGF-R results in the dissolution of endothelial cell-specific adhesion through the β-arrestin2-dependent endocytosis of CDH5, thereby promoting cell migration and vascular permeability (Gavard and Gutkind, 2006). β-arrestins are mainly associated with receptor desensitization and endocytosis by means of binding to the phosphorylated receptors or interacting with proteins of the endocytic machinery (Goodman et al., 1996; Kohout and Lefkowitz, 2003). β-arrestin2 (Arrb2) is involved in G-protein-coupled receptors (GPCRs) signal transduction pathway, and GPCRs is the best example of phospho-serine-threonine-targeted endocytosis.

Pig placental VEGF mRNA level is positively correlated with placental and adjacent endometrial vascularity (Vonahnme et al., 2001); moreover, placental growth and vascular development have been shown to be markedly different in the prolific Chinese Meishan pig v. the less-prolific Yorkshire breed (Biensen et al., 1998). In this study, we cloned and characterized the partial mRNA sequences of porcine VEGF, VEGF-R1, VEGF-R2, eNOS, CDH5 and Arrb2 from porcine placenta, and further investigated their tissue expression profile. Real-time quantitative polymerase chain reaction (RT-Q-PCR) was used to measure the relative expression levels of the six genes in placentas on days 33, 60 and 90 of gestation in Landrace pigs. We also compared placental mRNA expression of the six genes on day 90 of gestation in the most prolific Chinese Erhualian pig v. the Landrace breed.

**Material and methods**

**Animals and tissue collection**

Landrace and Chinese indigenous Erhualian pigs were used in this study. Landrace (n = 9) and Erhualian (n = 3) sows were mated with boars of the same breed, respectively. Landrace pig placentas were collected on day 33 (n = 3), day 65 (n = 3) and day 90 (n = 3) of gestation. Erhualian pig placentas were collected on day 90 (n = 3) of gestation. The placentas were snap frozen in liquid nitrogen for future RNA isolation. The heart, liver, spleen, lung, kidney, skeletal muscle and placenta from Landrace fetus on day 90 of gestation were also harvested.

**RNA preparation**

Total RNAs were isolated from approximately 200 mg of frozen tissue by using Trizol reagent (Gibco-BRL, Rockville, USA) according to the manufacturer’s protocol, and treated with DNaseI by a TURBO DNA-free kit (Ambion, Austin, TX, USA). RNA integrity was checked using denaturing gel electrophoresis and the concentration was estimated with a Beckman DU® 640 spectrophotometer. Two micrograms of total RNA was reverse-transcribed into cDNA with 300 U M-MLV reverse transcriptase (Promega Corporation, USA) using random and oligo-dT primers as described previously (Feng et al., 2007).

**Cloning of porcine VEGF, VEGF-R1, VEGF-R2, eNOS, CDH5 and Arrb2 genes**

The cDNA of the six genes were cloned by RT-PCR. cDNA fragments were amplified from the single-stranded cDNAs prepared from Landrace placenta on day 90 of gestation. Sixteen primer pairs were designed based on the sequences of cDNA or EST of these porcine genes (Table 1). The polymerase chain reaction amplification was performed using 1 μl cDNA template, 2 μl 10 × PCR buffer (containing 100 mM Tris–HCl (pH 8.0), 500 mM KCl, 10 mM of MgCl2 and 0.1% glutin), 2.0 μl 10 mM dNTPsMix, 0.5 μl forward and reverse primers (10 pmol/ml), 0.2 μl AmpliTaq DNA polymerase (5 U/ml) and 14.3 μl double-distilled water. The PCR profile was 3 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at annealing temperature, 30 s at 72°C and a final extension of 5 min at 72°C. The PCR products were separated on 2% agarose gels, visualized by ethidium bromide staining and analyzed using an Alpha Innotech (San Leandro, CA, USA) imaging system. The amplified fragments were sequenced to verify the correct amplification.

**mRNA expression of porcine VEGF, VEGF-R1, VEGF-R2, eNOS, CDH5 and Arrb2 genes in different tissues**

Total RNAs isolated from heart, liver, spleen, lung, kidney, skeletal muscle and placenta of Landrace fetus on day 90 gestation were reverse-transcribed into cDNA. The tissue expression patterns of these genes were investigated by RT-PCR. Primers used for expression pattern analysis were designed across introns (Table 2). Ribosomal protein L32 (RPL32) gene was used as an internal control.

**SYBR Green RT-PCR analysis of VEGF, VEGF-R1, VEGF-R2, eNOS, CDH5 and Arrb2 gene expression patterns**

Expression differences of the six genes in different developmental stages of placenta in Landrace pigs, and on day 90 gestation between two breeds were detected using SYBR Green I-based quantitative real-time polymerase chain reaction (Q-RT-PCR). RPL32 gene was used as a control gene. Each RT-PCR reaction (in 20 μl) contained 1 × RT-PCR SYBR Green master buffer (Toyobo, Osaka, Japan), 0.25 μM primers (Table 2) and 1 μl template cDNA. The cycling conditions consisted of an initial cycle of 3 min at 95°C followed by 35 cycles of cycling consisting of 15 s at 94°C, 20 s at 61°C, 20 s at 72°C and fluorescence acquisition at 82°C for 1 s. The specific PCR products were confirmed by melting curve analysis. cDNAs from three fetus placenta samples in each stage were used to detect the expression changes of the target gene, and all PCRs were...
performed in triplicate and gene expression levels were quantified relative to the expression of RPL32 using Gene Expression Macro software (Bio-Rad, Richmond, CA, USA) employing an optimized comparative Ct (ΔΔCt) value method. Expression levels were considered undetectable when the Ct value of the targeted gene exceeded 35 in the sample tissues. The t-test was conducted to identify genes differing in expression; \( P < 0.05 \) was considered significant.

**Results**

Molecular cloning and sequence analysis of porcine VEGF, VEGF-R1, VEGF-R2, endothelial nitric oxide synthase (\( \text{eNOS} \)), vascular endothelial cadherin (\( \text{CDH5} \)) and \( \beta \)-arrestin2 (\( \text{Arrb2} \)) genes in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequences (5'–3')</th>
<th>( T_m ) value (°C)</th>
<th>Product size (bp)</th>
<th>Contig length (bp)</th>
<th>Sequence information</th>
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<td>530</td>
<td>1785</td>
<td>Including CDS 570 bp, spanned 7 exons</td>
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<td></td>
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<tr>
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<td><strong>eNOS</strong></td>
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</tr>
<tr>
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<td>1102</td>
<td>3434</td>
<td>Including CDS 2349 bp, 11 exons</td>
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|        | Reverse       | CTAGCTAGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
mRNA expression of six genes in different tissues

Porcine VEGF, VEGF-R1, VEGF-R2, eNOS, CDH5 and Arrb2 genes showed expression in all seven tissues: heart, liver, spleen, lung, kidney, skeletal muscle and placenta (Figure 2).

Temporal expression of VEGF, VEGF-R1, VEGF-R2, eNOS, CDH5 and Arrb2 gene during placenta development

Q-RT-PCR results are shown in Figures 3 and 4. The results showed several different patterns for the six genes. VEGF and Arrb2 gene expressions were increased ($P < 0.05$) from 33-day and 65-day to 90-day gestation in Landrace pigs, whereas VEGF-R1 did not show different expression in the same stages. Placental eNOS mRNA expression exhibited significant increase ($P < 0.05$) from 33- to 65-day gestation in Landrace placenta, which remained the same through 90-day gestation. VEGF-R2 and CDH5 expressions have no expression difference between 33- and 65-day gestation placentas; however, their expression was significantly

Table 2 Primers used for semi-quantitative reverse transcription (RT)-polymerase chain reaction (PCR) and quantitative (Q)-RT-PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward Primer Sequences (5′−3′)</th>
<th>Reverse Primer Sequences (5′−3′)</th>
<th>Product size (bp)</th>
<th>$T_m$ value (°C)</th>
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<tr>
<td>VEGF</td>
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<td>Reverse GCACACAGGACGGCCTGAA</td>
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<td>60</td>
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<tr>
<td>VEGF-R1</td>
<td>Forward TCTCTAGTGGAGACCATGAAA</td>
<td>Reverse CATTCTCCAAGTTCA</td>
<td>162</td>
<td>60</td>
</tr>
<tr>
<td>VEGF-R2</td>
<td>Forward CGGGCTTATCCCTGCTCA</td>
<td>Reverse CTGCCCTCTCAGTTCTCA</td>
<td>127</td>
<td>60</td>
</tr>
<tr>
<td>eNOS</td>
<td>Forward AAGGGAGTGAAGGCCGCAAA</td>
<td>Reverse ATCCCCGTCCAAAGGT</td>
<td>189</td>
<td>60</td>
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<tr>
<td>Arrb2</td>
<td>Forward GGTGTGGGACCCTCTGACTA</td>
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<td>136</td>
<td>60</td>
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<tr>
<td>CDH5</td>
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<td>Reverse TCCCGTTCGCCAGATGTA</td>
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<td>Forward GGGCATGAAACCATGAGAAGT</td>
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<td>60</td>
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VEGF = vascular endothelial growth factor; eNOS = endothelial nitric oxide synthase; Arrb2 = β-arrestin2; CDH5 = vascular endothelial cadherin; RPL32 = ribosomal protein L32.
decreased ($P < 0.05$) from 65 day to 90 day. Comparison of expression levels in placenta on day 90 of gestation was also analyzed between Landrace and Erhualian breeds. The results showed that $VEGF$, $VEGF-R1$, $VEGF-R2$ and $eNOS$ genes had significantly higher ($P < 0.01$) expression levels in Erhualian than those in Landrace. However, $CDH5$ and $Arrb2$ showed contrary tendency, their expression levels were significantly lower ($P < 0.01$) in Erhualian than in Landrace.

**Discussion**

In this study, we obtained the porcine $VEGF$, $VEGF-R1$, $VEGF-R2$, $eNOS$, $CDH5$ and $Arrb2$ partial cDNA sequences. The porcine sequences of these genes showed high similarity with their counterparts in human and mouse, indicating the conserved function of these genes in the pig. We assume that the proteins are produced in close correlation to the total RNA levels we measured. Expression analysis showed that these six genes were expressed in various porcine tissues (heart, liver, spleen, lung, kidney, skeletal muscle and placenta). The high level of $VEGF$ expression in different tissues showed that this gene plays important roles in maintaining normal function of these organs. The $CDH5$ gene showed a lower level of expression in placenta compared to other tissues. $CDH5$ is a major adhesive component of cell-to-cell contacts (Huber et al., 1996); its low-level expression may help to promote placenta permeability.

Our study demonstrated that during porcine placenta development, the expression of $VEGF$ was up-regulated, which is generally in agreement with the previous research results (Vonnahme and Ford, 2004). $VEGF$s are crucial regulators of vascular development (Olsson et al., 2006) and also directly stimulate increased vascular permeability to water and large molecular weight proteins and vasodilatation (Bates and Harper, 2002). Thus, up-regulation in placental $VEGF$ mRNA expression will promote placenta growth and placenta permeability. According to a previous study, $VEGF$ increases permeability acutely by activating
VEGF-R2, then phospholipase C was stimulated, which results in diacylglycerol (DAG) production (Wu et al., 1996). DAG directly stimulates calcium influx, to increase intracellular calcium, which stimulates eNOS to produce NO (Hatakeyama et al., 2006). Unexpectedly, significantly lower VEGF-R2 and eNOS expressions were detected in Landrace placenta of 90-day gestation comparing to the two earlier stages, which showed a tendency in contrary to VEGF. Biensen et al. (1998) reported that less-prolific Yorkshire pig placenta size increased markedly compared to prolific Meishan pig throughout gestation. Here, we found significantly increased expression of VEGF but decreased expression of VEGF-R2 and eNOS, which may weaken the function per unit placenta (placenta efficiency) during Landrace pig gestation, and may be one reason of the enlarged placenta in late gestation stages in Landrace.

In the study of Youngs et al. (1993), the Meishan pig can farrow three to five more live pigs per litter than Western breeds (Ford and Youngs, 1993; Youngs et al., 1993). Wilson found that the Meishan placenta exhibits a marked and progressive increase in the density of blood vessels on the placental surface (Wilson et al., 1998). Our study showed significantly high levels of VEGF, VEGF-R1, VEGF-R2 and eNOS expression in 90-day gestation Erhualian placenta compared to that in Landrace, indicating that VEGF, VEGF-R1 and VEGF-R2 may promote vascular growth and eNOS increases vascular permeability, thereby strengthening placenta function/efficiency in the Erhualian pig breed, which is a breed considered to have higher litter size than Meishan pig in China.

Gavard and Gutkind (2006) proposed that the effects of VEGF on endothelial-cell permeability may result from the transient disruption of the CDH5 cell–cell adhesion, and Arrb2 may aid CDH5 endocytosis. Therefore, down-regulation of CDH5 and up-regulation of Arrb2 in Landrace placenta found in our study will increase placenta vascular permeability in late gestation stages, thereby promoting placenta function. Significant low expression of CDH5 detected in Erhualian than in Landrace placenta on 90-day gestation indicated high endothelial-cell permeability in Erhualian placenta. The low expression of both CDH5 and Arrb2 in Erhualian pig might suggest that Arrb2 is a cofactor of CDH5 endocytosis and help in promoting placenta permeability.

Conclusion

In summary, the results of our study showed that high expression of VEGF and its receptors and low expression of CDH5 and Arrb2 may be related high placental efficiency in the Erhualian pig, and may explain in one aspect why Erhualian pig is more prolific than the Landrace breed. The differential expression of VEGF, VEGF-R1, VEGF-R2, eNOS, CDH5 and Arrb2 genes during placenta development and between different breeds indicates these genes have important relationship with reproduction traits in the pig. Understanding the regulation of vascular growth and permeability in porcine placenta will provide much-needed insight into placentation and the mechanism of litter size difference between pig breeds. This result also contributes new data of the VEGF signal transduction pathway in porcine placenta development.

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References


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