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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 placentae 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

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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*/VPF-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 (Vonnahme *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 $MgCl_2$ 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

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Table 1 *Primers used for cloning of vascular endothelial growth factor (*VEGF*),* VEGF-R1, VEGF-R2*, endothelial nitric oxide synthase (*eNOS*), vascular endothelial cadherin (*CDH5*) and β-arrestin2 (*Arrb2*) genes in this study*

Gene	Primer	Sequences (5′–3′)	T_m value (°C)	Product size (bp)	Contig length (bp)	Sequence information
VEGF	Forward	CCTTGCTGCTCTACCTCCAC	58	530	1785	Including CDS 570 bp, spanned 7 exons
	Reverse	CCTCGGCTTGTCACATCTG				
	Forward	GGGTGAGGCGGCTGTGTG	60	225		
	Reverse	AGCAAGGCAAGGCTCCAATG				
	Forward	CAACGACGAAGGTCTGGAGT	57	1783		
	Reverse	GAGGGCAGAGTTGAGTGTTAG				
VEGF-R1	Forward	CCCTGGTTGTGGCGGACT	61	794	1765	Exon 5–17
	Reverse	CCCACTTGCTGGCATCGTAA				
	Forward	TGCGAACGGCTGCCTTAC	60	1005		
	Reverse	GGTCTTTGTGCCAACAGTCCA				
VEGF-R2	Forward	GGAAGACTCAGGTATCGTGCTAA	63	892	2031	Exon 12–22
	Reverse	GAGATGCTCCAAGGTCAGAAAG				
	Forward	CAGATGAACTGCCCTTGGATG	60	1732		
	Reverse	TGGCGGTGGAAAGAACAAC				
eNOS	Forward	GGGGAGCATCACCTACGACA	61	1770	3588	25 exons, including termination codon
	Reverse	GCACGAGCAAAGGCACAGA				
	Forward	AAGCGAGTGAAGGCGACAA	61	2270		
	Reverse	ACCCAAGGGAGGGAAACAG				
CDH5	Forward	CAGACAGCCAGACAGACGGA	59	1102	3434	Including CDS 2349 bp, 11 exons
	Reverse	CTCAGGTAGCGGAGGTCGA				
	Forward	TGAAGCCCCTGGACTATGAAC	60	711		
	Reverse	TGAGTATGGGAAGGTGGTGGA				
	Forward	CAGCCAACATCACAGTCAAGC	59	975		
	Reverse	CCCAGCCCAGACAGAACAC				
	Forward	GGACACCACCAGCTACGACG	60	1417		
	Reverse	TGAGTGAAGCCATTTCCCAGA				
Arrb2	Forward	CGGGGACCAGGGTCTTCA	61	488	1571	Exon 2–14, including termination codon
	Reverse	CAGCCGCACAGAGTTCCTTT				
	Forward	GATGGCGTGGTGCTTGTG	59	1039		
	Reverse	CGTCGTCCGTGGCATAGTT				
	Forward	AGGTGTCGCCCAGTTCCA	59	800		
	Reverse	GGTGTCGGGGTGAGGTTG				

CDS = coding sequence.

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, eNOS, CDH5 and Arrb2 gene

A 1875 bp (GenBank: EU714324) porcine *VEGF* fragment was identified from the sequencing of PCR products, which contains an open-reading frame of 576 nucleotides (nt). We also cloned the partial mRNA sequences of porcine *VEGF-R1*, *VEGF-R2*, *eNOS*, *CDH5* and *Arrb2* (Table 1), and submitted to the NCBI database (GenBank accession nos.: EU714325, EU714326, EU714327, EU714329 and EU714330). Basic local alignment search tool analysis revealed 88–100% sequence homology with the published porcine ESTs or human cDNA sequences for these genes. A GenBank database search using BLAST revealed that the predicted porcine *VEGF*, *VEGF-R1*, *VEGF-R2*, *eNOS*, *CDH5* and *Arrb2* gene coding sequence (CDS) has high similarity with other mammalian counterparts (Table 1). We compared the amino acid sequences of *VEGF*, *VEGF-R1*, *VEGF-R2*, *eNOS*, *CDH5* and *Arrb2* with those of these genes from other species retrieved from the EMBL and GenBank databases. Multiple sequence alignments were generated using Clustal W. Phylogenic trees (Figure 1) of *VEGF*, *VEGF-R1*, *VEGF-R2*, *eNOS*, *CDH5* and *Arrb2* based on the amino acid sequences generated using the DNAMAN program.

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

Genes	Primer	Sequences (5′–3′)	Product size (bp)	T_m value (°C)
VEGF	Forward	GAGACCAGAAACCCCACGAA	138	60
	Reverse	GCACACAGGACGGCTTGAA		
VEGF-R1	Forward	TCATCACTGTGAAGCATCGAAA	162	60
	Reverse	CGGGAGCAAAAGTCATACCATA		
VEGF-R2	Forward	CGGGCTACTTATCCATCGTCA	127	60
	Reverse	CCAAGAGGCTTCCCTAGTTTCA		
eNOS	Forward	AAGCGAGTGAAGGCGACAA	189	60
	Reverse	ATCCCCGTTCCCAAAGGT		
Arrb2	Forward	GGTGCTTGTGGACCCTGACTA	136	60
	Reverse	CTGGTAGGTGGCGATGAACA		
CDH5	Forward	GACAAGGATGTAACACCACGAGA	178	60
	Reverse	TCCCGTTGTCCGAGATGAGTA		
RPL32	Forward	GGGCATGAACCATGAGAAGT	93	60
	Reverse	GTCTTCTGGGTGGCAGTGAT		

VEGF = vascular endothelial growth factor; *eNOS* = endothelial nitric oxide synthase; *Arrb2* = β-arrestin2; *CDH5* = vascular endothelial cadherin; *RPL32* = ribosomal protein L32.

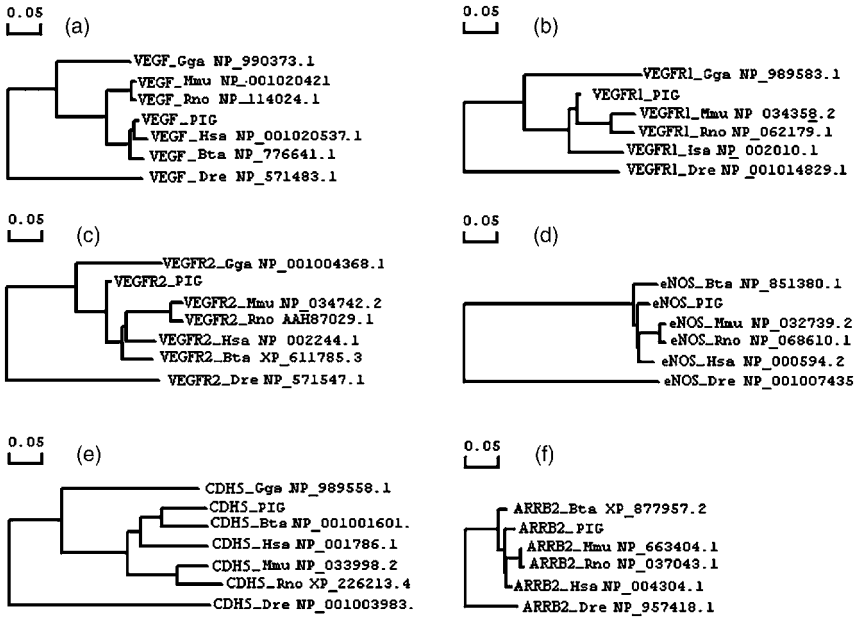


Figure 1 Phylogenetic relationship of vascular endothelial growth factor (*VEGF*) (a), *VEGF-R1* (b), *VEGF-R2* (c), endothelial nitric oxide synthase (*eNOS*) (d), vascular endothelial cadherin (*CDH5*) (e) and β-arrestin2 (*Arrb2*) (f) in different species. Amino acid sequences of the six porcine genes were predicted by our study.

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 placenta; however, their expression was significantly

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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.

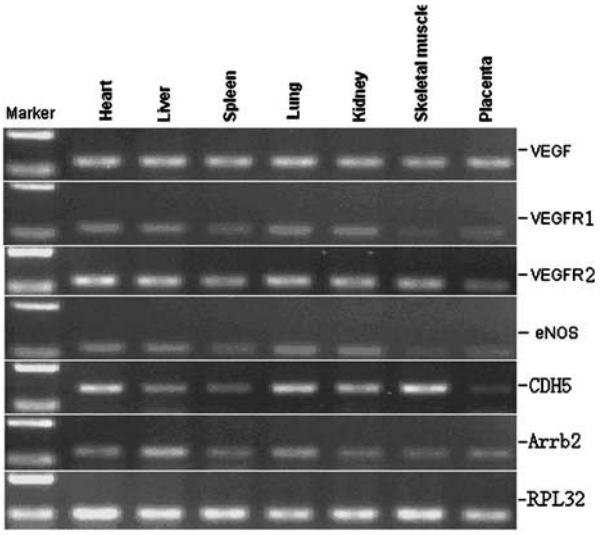


Figure 2 Tissue distribution analysis of porcine *VEGF*, *VEGF-R2*, *eNOS*, *Arrb2* and *CDH5* genes.

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

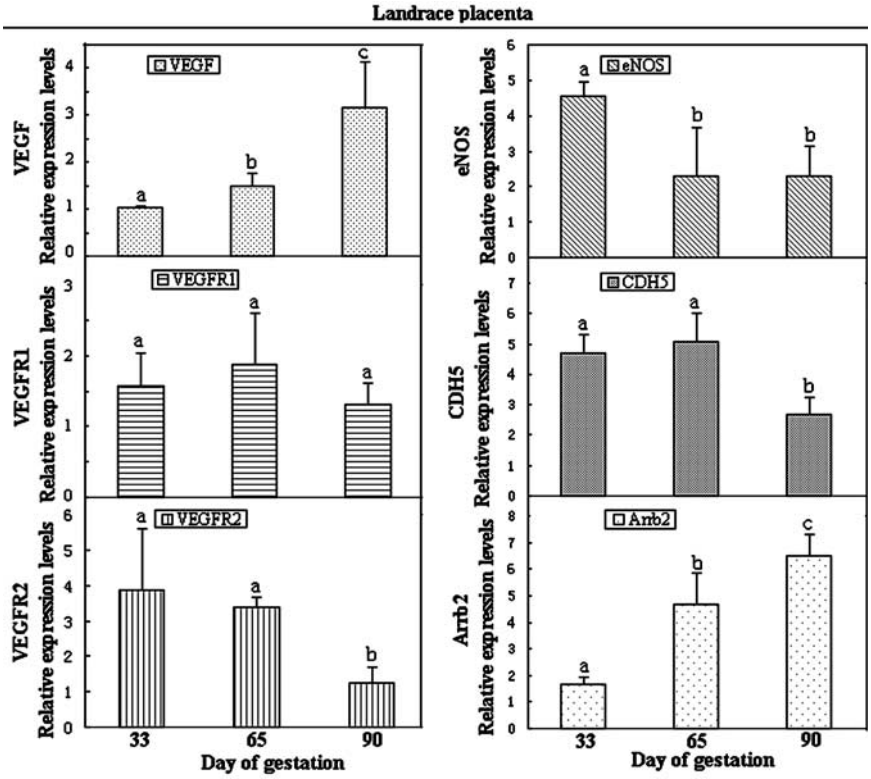


Figure 3 Expression differences in Landrace placenta of 33-, 65- and 90-day gestation of *VEGF*, *VEGF-R1*, *VEGF-R2*, *eNOS*, *CDH5* and *Arrb2* genes. Different letters indicate significant differences ($\Delta Ct \pm$ s.d.) ($P < 0.05$).

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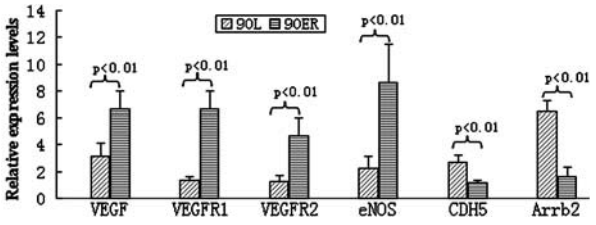


Figure 4 Expression differences between Landrace and Erhualian placentas of the 90-day gestation of *VEGF*, *VEGF-R1*, *VEGF-R2*, *eNOS*, *CDH5* and *Arrb2* genes. 90L represents Landrace pigs, 90ER represents Erhualian pigs.

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 Erhulian 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 to high placental efficiency in the Erhuanlian 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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