Association of rs81471943 and reproductive traits reveals EXOC4 as a novel molecular breeding marker in pigs

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Abstract

Background: In mammals, the exocyst complex component 4 (EXOC4) gene has often been reported to be involved in vesicle transport. The rs81471943 (C/T) locates at the intron of porcine EXOC4, and six quantitative trait loci (QTLs) within 5-10 Mb around EXOC4 are associated with ovary weight, teat number, total born alive, and corpus luteum number. However, the molecular mechanisms between EXOC4 and reproductive performance of pigs remains incompletely elucidated.

Results: In this study, rs81471943 was genotyped from a total of 994 Duroc sows, and the genotype and allele frequency of rs81471943 (C/T) were statistically analyzed. Then the associations between rs81471943 and four reproductive traits including number of piglets born alive (NBA), litter weight at birth (LWB), number of piglets weaned (NW), and litter weight at weaning (LWW) were determined. Besides, the sanger sequencing and PCR-restriction fragment length polymorphism (PCR-RFLP) were utilized to identify genotype of rs81471943. We found that the genotype frequency of CC was significantly higher than that of CT and TT, and TT was the favorable genotype on NW (P=0.01) and LWW (P<0.01). Moreover, 5'-deletion and luciferase assays identified a positive transcription regulatory element in -1826/-1551 of EXOC4. After exploring -1826/-1551 fragment, -1781G/A linked with rs81471943 (C/T) were identified by analysis the transcription activity of the haplotypes, and -1781 G/A might influence the potential binding of P53, ETS transcription factor (ELK1), and myeloid zinc finger 1 (MZF1).

Conclusions: In 994 Duroc pigs, rs81471943 of TT was the favorable genotype on NW (P=0.01) and LWW (P<0.01), and the haplotypes of -1781G/A and rs81471943 significantly affect the transcription activity of EXOC4, which might influence the binding of potential cis-acting elements P53, ELK1
and/or MZF1. These findings provide useful information for identifying the molecular marker of

EXOC4-assisted selection in pig breeding.

Keywords: Commercial pig; Reproductive traits; EXOC4; Gene polymorphism; Promoter
Background

Pigs have experienced a particularly long history of haplotype changes through artificial selection from domestication to modern breeding practices [1, 2]. In porcine production, improvements in reproductive performance of sows are slow due to the low heritability of reproductive traits [3]. The recent development of sequencing and genotyping technology for pigs has enabled the exploration of genomic evidence of selection and the detection of candidate genes associated with some target traits. Studies have found that the positive selections of Duroc are associated with specific genes related to lactation [4], reproduction[5], meat quality [6], and growth traits [7]. Other studies have reported that the reproductive traits such as number of piglets born alive (NBA) [8], lactation capacity, litter weight at weaning (LWW) [9], and number of piglets weaned (NW) [10] all have been genetically improved through phenotypic or selective signatures.

Most of porcine reproduction performance is evaluated using quantitative traits, which are mainly affected by minor genes. The minor genes can be identified by quantitative trait locus (QTLs) mapping [11, 12]. According to PigQTLdb (https://www.animalgenome.org/cgi-bin/QTLdb/SS/index), there are 29,865 QTLs associated with 688 different traits. On chromosome 18, there are three, three, two and five QTLs significantly associated with ovary weight [13], teat number, total born alive, and corpus luteum number traits [14-16], respectively. In the commercial single-nucleotide polymorphism (SNP) array, one SNP (rs81471943) locates on the exons of exocyst complex component 4 (EXOC4) gene, and six QTLs associated with reproduction traits are identified within 5-10 Mb around EXOC4 on chromosome 18, suggesting that EXOC4 may be highly associated with reproductive traits. EXOC4 belongs to the exocyst complex gene family, which connects vesicles to the cell membrane and
participates in vesicular-mediated transport. EXOC4 has been reported to express in many human
tissues, with slightly higher expression in the ovary, skeletal muscle, spleen, and hypothalamus[17].
However, the underlying relationships between rs81471943 and reproductive traits as well as
transcription mechanism of EXOC4 are still unclear in pigs.
To explore the relationship between rs81471943 and reproduction traits, in this study, we acquired the
genotype frequencies of rs81471943 in Durocs, and the associations between rs81471943 and
reproductive traits such as NW, LWW, NBA, and litter weight at birth (LWB) were determined. Then
5'-deletion and luciferase assay were utilized to identify whether rs81471943 associates with the
transcription of EXOC4. The study contributes to the understanding of the regulatory mechanisms of
the EXOC4 and the molecular marker-assisted selection in pig breeding.

**Materials and Methods**

**Animals**

The ear samples of 994 Duroc sows prepared for SNPs genotyping were obtained from a breeding herd
in Fujian China and were collected from 2009 to 2017 and were taken from a previous study [18]. The
ear samples were collected into 75% alcohol immediately and stored at -20 °C. TaKaRa MinBEST
Universal Genomic DNA Extraction Kit (Ver 4.0) was applied to extracted the genomic DNA was from
ear tissues. The A260/280 ratios of DNA samples were determined with NanoDrop 2000 (Thermo
Scientific), when the DNA samples with A260/280 ratio from 1.7 to 2.0 were genotyped using Illumina
PorcineSNP60 BeadChip (Illumina, San Diego, CA, USA). Four reproductive traits, including NBA,
LWB, NW, and LWW were recorded. In which, NBA and LWB were measured in 24 hours after
delivery, and LWW and NW were recorded after weaned.
To determine the polymorphic loci rs81471943 (C/T) of porcine EXOC4 gene, 8 Duroc pigs were sequenced by Sanger sequenced, 10 Duroc pigs were genotyped by PCR-restriction fragment length polymorphism (PCR-RFLP).

**Polymorphism Identification and Genotype with PCR-Restriction PCR-RFLP**

DNA extracted from the ear samples of 10 pigs was set as the template. The fragment containing rs81471943 (Chromosome 18: 16,079,412) of EXOC4 to a length of 640 bp (Chromosome 18: 16,078,898-16,079,537) was amplified using designed PCR primer using rTaq (Vazyme, Piscataway, USA). The primers are listed in Table 1. Then, based on PCR-RFLP, PCR products were digested by BsrB I (identification sequence: CCGCTC) restriction endonuclease (NEB, Ipswich, United Kingdom) and determined using agarose gel electrophoresis. PCR products with two digested fragments were CC genotype (565 bp + 75 bp), with three digested fragments were CT genotype (640 bp + 565 bp + 75bp), and with one digested fragment was TT genotype (640 bp).

**Construction of EXOC4 5′Deletion Fragment Vectors and Luciferase Assay**

According to promoter sequences of porcine EXOC4 (ENSSSCG00000016543, Chromosome 18: 16,057,927-16,346,564) from Ensembl genome browser (release 89), we indicted that there was no leader exon present in EXOC4. PCR was performed to obtain the EXOC4 promoter of 2791 bp by PrimerSTAR® (TaKaRa, Dalian, Liaoning, China). The primers are listed in Table 1. Then, PCR products were purified by gelatinization and the addition of an “AAA” tail for ligation with PMD-18T vector. After, the recombinant vector was transformed into DH5α competent cells and inoculated on ampicillin-containing lysogeny broth plates at 37 °C for 12 h. Monoclonal bacteria were picked and
cultivated in ampicillin with lysogeny broth medium for incubation for 6 h at 37 °C shaker. The plasmids were extracted from bacteria and correct plasmids for sequencing were named, respectively. Then, we designed 10 pairs of primers to amplify deletion fragments of EXOC4 promoter. The transcription start site of EXOC4 gene located at Chromosome 18:16,346,564, and the longest 5’ deletion fragment was named P0 (-2657/+134), and other longer fragments were named P1 (-2204/+134), P2 (-1914/+134), P3 (-1682/+134), P4 (-1323/+134), P5 (-886/+134) and P6 (-518/+134). The shorter 5’ deletion fragments were named P3A (-1826/+134), P4A (-1551/+134), and P4B (-1225/+134). Primers are listed in Table 1. Simultaneously, the PCR product was obtained by PrimerSTAR® high fidelity enzyme (TaKaRa, Dalian, Liaoning, China). Primers are presented in Table 1. PCR products were purified by gelatinization, and the addition of “AAA” tail to ligated with PMD-18T, then sequenced by Sanger Sequencing (Beijing Aoboxingke, China). The DNA sequences of PCR products were determined by using DNASTAR software. Each deletion fragment digested with Hind III and MIu I was cloned into the eukaryotic expression vector pGL3-vector, which was also digested with Hind III and MIu I restriction endonuclease. Then, according to the manufacturer’s instructions for the dual-luciferase reporter assay kit (Promega, Madison, WI, USA), we used the BioTek Synergy 2 multifunctional microplate reader (BioTek, Winooski, VT, USA) for fluorescence detection. The ratio expression of firefly luciferase to renilla luciferase of each deletion fragment was calculated.

**Culture of porcine Granulosa Cells (GCs) in Vitro**

Porcine GCs were cultured according to previous studies [19]. Porcine ovaries were collected from local slaughterhouse in Guangzhou, China. The ovaries were stored in phosphate-buffered saline (PBS)
containing penicillin (100 IU/mL) and streptomycin (100 μg/mL; Invitrogen, Shanghai, China) at
37 °C and transported to the laboratory quickly. Then, 5-7 mm follicles were punctured, and GCs in
follicular fluid were collected using 1-mL syringe. After washing the isolated GCs twice with PBS, the
GCs were seeded into 75-cm2 flasks and cultured at 37 °C under 5% CO2 in DMEM (Hyclone, Logan,
UT, USA) containing 10% fetal bovine serum(100 IU/mL penicillin, and 100 μg/mL streptomycin;
Hyclone, Logan, UT, USA).

Identification of SNP and transcription binding sites
The DNA of porcine ears was extracted and used as a template for PCR amplification. PCR was
performed by PrimerSTAR® high fidelity enzyme (TaKaRa, Dalian, Liaoning, China) to obtain the
fragment containing rs81471943 of EXOC4 to a length of 640 bp; the primers are listed in Table 1
under EXOC4-SNP. Then, the PCR product was linked to the T-vector and sequenced by Sanger
Sequencing (Aoboxingke, Beijing, China). The DNA sequences of PCR products were determined
using DNASTAR software. To identify the SNP, the alignment sequence and reference sequence were
aligned using BLAST. Then, the potential transcription factor binding region was predicted by
TFBIND [21], and Jaspar [22].

Statistical analysis
The phenotypic data of Duroc pigs were used for the association analysis of rs81471943 and
reproductive traits, including NBA, LWB, LWW, and NW. Estimated breeding values (EBVs) of all
pigs and the reliabilities of EBVs were imputed using animal model best linear unbiased prediction
[20] and obtained from the in-farm genetic evaluation software Herdsman swine management platform.
The association between SNP and phenotypes in this research were tested by a single marker regression mixed linear model via SAS software. The molecular experiment data were expressed as mean ± standard deviation (SD) of repeated experiments. Each experiment was repeated at least three times independently. The significance of differences in means between two groups was analyzed using Student’s t-test (two-tailed) for molecular experiments. * indicates $P < 0.05$; ** indicates $P < 0.01$.

Results

Polymorphisms of rs81471943

The genotype frequencies of rs81471943 on EXOC4 gene of the 994 Duroc pigs were calculated and counted (Table 2). EXOC4 contained three genotypes, whereas CC was the dominant genotype with genotype frequency 0.715, which was higher than that of CT (0.258) and TT (0.027). The dominant allele was C, whose allele frequency was 0.844, which significantly higher than that of T allele (0.156). The $\chi^2$ valued 0.001 ($\chi^20.05 (2) =5.99$, $P > 0.05$) confirmed that the frequency distribution of rs81471943 was in accordance with the Hardy Weinberg equilibrium law in the selected Duroc pig population.

Association Between rs81471943 and Reproduction Traits

The description statistics for phenotypes of 994 Duroc sows were provided in Table 3 as well as EBVs shown in Table 4. The relationship between the genotype of rs81471943 and reproductive traits was further explored and analyzed. The NBA, LWW, and NW of individuals with TT was higher than that with CC and CT, and the LWB of individuals with CC was higher than that with CT and TT (Table 3).
Moreover, the EBVs of NBA of individuals with TT was higher than that with CT and CC, there was no significant difference among the three genotypes. The EBVs of LWB (P=0.02) of individuals with CC was significantly higher than that with CT and TT. The EBVs of NW (P=0.01) and LWW (P<0.01) of individuals with TT was significantly higher than that with CC and CT (Table 4).

These observations suggested that TT was the favorable genotype on NW (P=0.01) and LWW (P<0.01), while CC was the favorable genotype on LWB (P=0.02).

**Isolation of rs81471943 on EXOC4**

Target fragments of EXOC4 contained rs81471943 were amplified by extracted DNA from eight Duroc pigs (Figure 1A) and identified by Sanger sequencing (Figure 1B, C, D). Compared with the sequence of EXOC4 in the Ensembl genome browser (release 89), a polymorphic mutation base C/T located on EXOC4 (Chromosome 18: 16,079,412), which is in line with rs81471943 in the commercial SNP array.

To determine the polymorphic loci rs81471943 of EXOC4, 10 pigs were used in PCR-RFLP detection. As shown in Figure 2, three genotypes CC, CT, and TT were identified by restriction endonuclease BsrBI.

**Transcription Activity Analysis of the EXOC4 Promoter**

To investigate effects of rs81471943 on the expression of EXOC4, we tried to explore whether there is any SNP maker, which links with rs81471943, at the promoter of EXOC4. 5’-deletion and a luciferase assay were first used to identify regulatory elements on EXOC4 promoter. Six fragments with 5’-deletion of EXOC4 promoter were amplified (Figure 3A) and cloned into pGL3-vector (Figure 3B). Compared with EXOC4-P2 (-1914/+134), the relative luciferase activity of EXOC4-P1 (-2204/+134),
EXOC4-P3 (-1682/+134), and EXOC4-P4 (-1323/+134) were all significantly decreased (Figure 3C), indicating that P1-P2 (-2204/-1914) region might harbor the negative control elements, and P2-P4 (-1914/-1323) region might harbor the positive transcription regulatory elements.

To further investigate positively regulatory elements of EXOC4, a smaller deletion fragment was operated on this region. The shorter 5’ deletion fragments of P3A (-1826/+134), P4A (-1551/+134), and P4B (-1225/+134) were amplified (Figure 4A) and cloned into the eukaryotic expression vector pGL3-vector (Figure 4B). As shown in Figure 4C, compared with EXOC4-P3A, the relative luciferase activity of EXOC4-P3 and EXOC4-P4A were all significantly decreased. Similarly, compared with EXOC4-P3, the relative luciferase activity of EXOC4-P4A was significantly decreased. These results indicate that positive transcription regulatory elements might exist in P3A-P4A (-1826/-1551) of EXOC4.

Transcription activity analysis of different haplotypes on EXOC4 gene

To further determine the relationship between rs81471943 and transcription activity of EXOC4 in pigs, the fragment -1826/-1551 of EXOC4 was amplified and sequenced on Duroc pigs. Interestingly, after comparing the sequences published on Ensembl genome browser (release 89), one SNP was identified and located on -1781G/A of EXOC4, where also was the potential transcription factor-binding fragment. As the linkage between -1781G/A and rs81471943, four haplotypes were defined as HA-1(GC), HA-2(AC), HA-3(GT), and HA-4(AT). The 5’ deletion fragments EXOC4-P2 (-1914/+134) of four haplotypes were amplified and cloned into the eukaryotic expression vector pGL3-vector. Luciferase assays were used to detect the effect of different haplotypes on transcription of EXOC4. As shown in Figure 5, we found that the luciferase activity of HA-1 was significantly higher than that of HA-2 (P<
0.05), HA-3 was significantly higher than that of HA-4 ($P < 0.05$). These observations suggested that rs81471943 might link with -1781G/A, and -1781G showed a favorable to affect the expression of \textit{EXOC4}. Moreover, many potential binding sites of transcription factors were predicted on -1781G/A of \textit{EXOC4} (Table 6), and \textit{P53} and \textit{ETS} transcription factor (ELK1) might bind at -1781A, and myeloid zinc finger 1 (\textit{MZF1}) might bind at -1781G.

**Discussion**

\textit{EXOC4} is essential for the growth and development of human and animals, and is a component of an exocyst complex and is associated with various phenomena, such as cell migration, endophoria formation, cytokinesis, glucose uptake and neural development in mammal. One study indicates that the mutation of specific SNPs loci in \textit{EXOC4} leads to an increase in the malformation rate of human newborns [21], and \textit{EXOC4} also involves in insulin-stimulated glucose transport [22, 23]. Jiao et al investigated the interactions between \textit{EXOC4}-1q23.1 and body mass index in a European-American adult female cohort via genome-wide interaction analyses, and results suggest \textit{EXOC4}-related pathways may contribute to the development of obesity [24]. Similarly, after \textit{EXOC4} knockout in embryos, mice can form gastrointestinal embryos normally, but are unable to progress beyond the primitive streak stage and die shortly [25]. In addition, the genome-wide association studies in chicken also report that three QTLs are located on \textit{ECOX4} and associated with growth trait of 49-56 day-old chicken [26], bodyweight of 63 day-old chicken [27] and pectoralis weight of 70 day-old chicken [28], respectively. These observations suggested that \textit{ECOX4} might be important for economic traits in livestock.

In this study, we found rs81471943 (C/T) located in the seventh intron of the \textit{EXOC4} gene. After
analyzing the relationship between genotype of the EXOC4 and reproductive traits, we found some correlation between reproductive traits and EXOC4 genotype in Duroc pigs (Table 3). There were three genotypes (CC, CT, and TT) exist in the Duroc populations, while C was the beneficial allele. Moreover, TT was the favorable genotype on NW ($P=0.01$) and LWW ($P<0.01$), while CC was the favorable genotype on LWB ($P=0.02$).

Then association between SNP and phenotypes confirmed that TT was the favorable genotype on NW ($P=0.01$) and LWW ($P<0.01$) in Duroc pigs (Table 3 and Table 4). Previous studies have shown that there are three QTLs were significantly associated with teat number [14-16] on chromosome 18, which indicated that EXOC4 could affect the lactation performance of commercial pig. In this study, we also found TT was significantly associated with NW and LWW, but not NBA and LWB. In addition, previous studies demonstrated that the reproductive traits were highly correlated with each other, such as NBA is highly genetically correlated with LWB [29]. In this study, we found that while CC was the favorable genotype on LWB ($P=0.02$), but not NBA. This observation might be caused by the limited population size used in this study, and it was likely that CC will be significantly associated with NBA with large population size [30]. Collectively, although the results were got from the small population, to the certain extent, the association of rs81471943 and lactation capacity NW and LWW could provide useful information for EXOC4-mediated reproduction in pigs. [31-33].

To explore effects of rs81471943 on the expression of EXOC4, the SNP makers which link with rs81471943 at the promoter of EXOC4 were further investigated. In this study, regulatory elements of EXOC4 promoter were first identified (Figure 4,5). 5’-deletions and the gene reporter assays were constructed for EXOC4, which covered the 2657 bp sequence before the upstream of transcription start
site and the 134 bp sequence of the first exon. We found the positive regulatory elements might localize in the P3A-P4A (-1826/-1551) region (Figure 4), and the negative control element might localize in the P1-P2 (-2204/-1914) region (Figure 3). After exploring the SNP of P3A-P4A (-1826/-1551) region containing positive transcription regulatory elements, one SNP was found located on -1781. As the linkage between -1781G/A and rs81471943, four haplotypes were defined as HA-1 (GC), HA-2 (AC), HA-3 (GT), and HA-4 (AT) (Table 5). Then we further analyzed the transcription activity of four haplotypes, and results showed that the transcription activity of HA-1 (GC) is significantly higher than HA-2 (AC) (Figure 6), and the transcription activity of HA-3 (GT) was significantly higher than HA-4 (AT) (Figure 6). These results indicated that rs81471943 might link with -1781G/A to affect the expression of EXOC4.

-1781G/A might regulate the expression of EXOC4 by affecting the binding of transcription factors [31-33]. In this study, we also predicted that transcription factors P53, ELK1, and MZF1 would be located on the -1826/-1551 region of the porcine EXOC4 promoter. P53 participates in maintaining cell growth [34]. P53 can enhance their stability through phosphorylation and the activation or inhibition of the transcription of downstream genes, thus inducing cell cycle arrest and apoptosis [35, 36]. ELK1 is a transcription factor belonging to the ETS oncogene family and induces hormone-resistant or metastatic prostate cancers [37], and plays an important role in breast and ovarian cancers [38]. As a bifunctional transcription factor, MZF1 belongs to the zinc finger protein Kruppel transcription factor family, which regulate downstream gene expression by binding to the cis-acting element TGGGA” on gene promoter [39, 40]. Results in Table 6 and Figure 5 indicated that the mutation of -1781G/A might be the cause for the difference in transcriptional. Taken together,
rs81471943 that was significantly associated with NW and LWW might link with -1781G/A, localizing at the positively regulatory elements of EXOC4 promoter, to affect the expression of EXOC4 of Duroc pigs in genetics.

Conclusions

In Duroc pigs, we found that the CC genotype frequency was significantly higher than that of CT and TT in rs81471943, and TT was the favorable genotype on NW \( (P=0.01) \) and LWW \( (P<0.01) \). -1826/-1551 of EXOC4 holds a positive regulatory element, and the haplotypes of -1781G/A and rs81471943 might significantly affect the transcription activity of EXOC4. Moreover, -1781G/A might influence the binding of potential cis-acting elements \( P53, \) \( ELK1 \) and/or \( MZF1 \). These findings provide useful information for further investigation of EXOC4-mediated lactation traits in pigs.

List of abbreviations

\( EXOC4 \): the exocyst complex component 4; QTL: quantitative trait loci; NBA: number of piglets born alive; LWB: litter weight at birth; NW: number of piglets weaned; LWW: litter weight at weaning; PCR-RFLP: PCR-restriction fragment length polymorphism; SNP: single-nucleotide polymorphism; \( MZF1 \): myeloid zinc finger 1; \( ELK1 \): ETS transcription factor; GCs: granulosa cells; EBVs: Estimated breeding values; SD: standard deviation; SE: standard error.

Declarations

Ethics approval and consent to participate

Animal care and all experiments were conducted according to the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, revised June
2004) and approved by the Animal Care and Use Committee of the South China Agricultural University, Guangzhou, China (approval number SCAU#2013-10).

**Consent for publication**

Not applicable.

**Availability of data and materials**

The SNPs genotyping data were taken from a previous study [18]. The EBVs of all pigs and the reliabilities of EBVs were imputed using animal model best linear unbiased prediction [20] and obtained from the in-farm genetic evaluation software Herdsman swine management platform (S & S Programming, Lafayette, IN, USA).

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

Y. T. He performed the research and wrote the original manuscript; X. F. Zhou. and X. L. Yuan reviewed and edited the manuscript; B. Hu, R. R. Zheng, Y Jiang, and Z. X. Yao supervised the research; Z. Zhang administrated the project; J. Q. Li managed the funding. The authors reviewed and approved
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Figure 1 Mapping of SNP rs81471943 (C/T) on EXOC4 (A) PCR products contained SNP rs81471943 of EXOC4 promoter (640 bp). rs81471943 (C/T) polymorphism locus located at (Chromosome 18: 16,079,412), and the three genotypes were CT (B), CC (C), and TT (D) respectively. M1000: DNA markers of 1000 bp.

Figure 2 PCR-RFLP detection of rs81471943 in Duroc pigs. M1000: DNA markers of 1000 bp. Three genotypes were identified by restriction endonuclease BsrB I. PCR products with two digested fragments were CC genotype (565 bp + 75 bp), with three digested fragments were CT genotype (640 bp + 565 bp + 75bp), and with one digested fragment was TT genotype (640 bp).

Figure 3 Transcription activity of 5' deletion fragment of EXOC4 promoter. (A) PCR products of 5’ deletion fragments from promoter of EXOC4; (B) Enzyme digestion identification of pGL3-vector with deletion fragment of EXOC4 promoter by restriction enzyme digestion; (C) The relative luciferase activity of 5’ deletion fragment on EXOC4 promoter. M5000: DNA markers of 5000 bp. * indicates $P < 0.05$; ** indicates $P < 0.01$. Data were represented as means ± SD.
Figure 4  Transcription activity of P2-P4 of EXOC4. (A) PCR products of smaller 5’ deletion fragments from promoter of EXOC4; (B) Enzyme digestion identification of pGL3-vector with further deletion fragment of EXOC4 promoter; (C) The relative luciferase activity of smaller 5’ deletion fragment on EXOC4 promoter. M5000: DNA markers of 5000 bp. * indicates $P < 0.05$; ** indicates $P < 0.01$. Data were represented as means ± SD.

Figure 5  Transcriptional Activity of Different Haplotypes at Binding Region The relative luciferase activity of EXOC4 with different haplotypes HA-1 (GC), HA-2 (AC), HA-3 (GT), and HA-4 (GT). * indicates $P < 0.05$; ** indicates $P < 0.01$. Data were represented as means ± SD.
**Table 1** Primers used in this study

| Name               | Primer Sequence                  | Length(bp) |
|--------------------|----------------------------------|------------|
| EXOC4-SNP          | F: ACAGCCTCGGCTCCAACCTTA         | 640        |
|                    | R: TGCTTTTACGAAGGGGACA           |            |
| EXOC4-Promoter     | F: GAGCGAGTCTCTGTCTACAGT         | 2791       |
|                    | R: TGCTTTTACGAAGGGGACA           |            |
| P0 (-2657/+134)    | F: CGACGCGTGAGCGAGTCCCTTGTCAGT  | 2791       |
|                    | R: CCCAAGCTTGCGCATTGGGGATTTCTACA|            |
| P1 (-2204/+134)    | F: CGACGCGTGAGCGAGTCCCTTGTCAGT  | 2338       |
| P2 (-1914/+134)    | F: CGACGCGTGAGCGAGTCCCTTGTCAGT  | 2048       |
| P3 (-1682/+134)    | F: CGACGCGTGAGCGAGTCCCTTGTCAGT  | 1816       |
| P4 (-1323/+134)    | F: CGACGCGTGAGCGAGTCCCTTGTCAGT  | 1457       |
| P5 (-886/+134)     | F: CGACGCGTGAGCGAGTCCCTTGTCAGT  | 1020       |
| P6 (-518/+134)     | F: CGACGCGTGAGCGAGTCCCTTGTCAGT  | 652        |
| P3A (-1826/+134)   | F: CGACGCGTGAGCGAGTCCCTTGTCAGT  | 1950       |
|                    | R: CCCAAGCTTGCGCATTGGGGATTTCTACA|            |
| P4A (-1551/+134)   | F: CGACGCGTGAGCGAGTCCCTTGTCAGT  | 1685       |
| P4B (-1225/+134)   | F: CGACGCGTGAGCGAGTCCCTTGTCAGT  | 1359       |

**Table 2** Genotypic Frequency and Genetic Frequency of rs81471943 on EXOC4 Gene in Duroc pig

| Genotype | Sample Quantity | Genotype Frequency | Allele | Allele Frequency | $\chi^2$ |
|----------|-----------------|--------------------|--------|-----------------|---------|
| CC       | 27              | 0.027              | C      | 0.844           | 0.224   |
| CT       | 256             | 0.258              | T      | 0.156           |         |
| TT       | 711             | 0.715              |        |                 |         |

$\chi^2 0.05 (2) = 5.99, \chi^2 0.01 (2) = 9.21.$
### Table 3 Descriptive statistics for phenotypes of Duroc pigs

| Trait       | NBA | LWB | NW | LWW |
|-------------|-----|-----|----|-----|
| Genotype    | CC  | CT  | TT | CC  | CT  | TT | CC  | CT  | TT |
| Number      | 27  | 256 | 771 | 27 | 256 | 771 | 27 | 256 | 771 |
| Least Squares Means | 7.89 | 7.95 | 8.00 | 13.77 | 13.46 | 13.61 | 6.25 | 6.02 | 6.30 |
| S.E.        | 0.13 | 0.16 | 0.35 | 0.22 | 0.27 | 0.59 | 0.14 | 0.17 | 0.38 |
| Min.        | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Max.        | 15.00 | 14.00 | 15.00 | 25.90 | 25.70 | 22.00 | 12.00 | 12.00 | 11.00 |

Notes: NBA - number of piglets born alive, LWB - litter weight at birth, NW - number of piglets weaned, LWW - litter weight at weaning.

### Table 4 Association Analysis of Genotypes and Reproductive Traits for rs81471943 of Duroc pigs

| Genotype | Genotype frequency (number) | NBA EBV | LWB EBV | NW EBV | LWW EBV |
|----------|-----------------------------|---------|---------|--------|---------|
| CC       | 0.721 (775)                 | 0.12±0.07 | 0.38±0.12 ab | 0.53±0.19 ab | 5.35±1.43 ab |
| CT       | 0.252 (271)                 | 0.17±0.09 | 0.12±0.14 a  | 0.36±0.29 a  | 3.66±1.48 a  |
| TT       | 0.028 (29)                  | 0.21±0.19 | 0.24±0.31 b  | 0.61±0.26 b  | 6.06±1.94 b  |

Notes: NBA - number of piglets born alive, LWB - litter weight at birth, NW - number of piglets weaned. The association between SNP and phenotypes were tested by a single marker regression mixed linear model via SAS software (P< 0.05).

### Table 5 Relationship Between Duroc of Different EXOC4 Genotypes with SNP Promoter

| Haplotype | HA-1 | HA-2 | HA-3 | HA-4 |
|-----------|------|------|------|------|
| -1781     | G    | A    | G    | A    |
| rs81471943| C    | C    | T    | T    |

### Table 6. Prediction of potential binding sites on -1781G/A.

| TF     | Nucleotide Location | Chain | Scored | Position  | Sequence Pattern     |
|--------|---------------------|-------|--------|-----------|----------------------|
| P53    | -1786-1777          | -     | 0.796  | -1781A    | AGGAAGGTCA           |
| ELK1   | -1785-1773          | -     | 0.783  | -1781A    | ACGTGAGGAAGGTC       |
| MZF1   | -1787-1775          | +     | 0.856  | -1781G    | TGAGGAGGGTCAT        |

The alphabet in boldface is the mutation site.