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

Yingting He

South China Agricultural University College of Animal Science

Xiaofeng Zhou

South China Agricultural University

Bin Hu

Guangdong Academy of Agricultural Sciences

Rongrong zheng

South China Agricultural University

Yao Jiang

South China Agricultural University

Zhixiang Yao

Guangdong Dexing Food Co., Ltd

Zhe Zhang

South China Agricultural University

Hao Zhang

South China Agricultural University

Xilong Wang

Guangdong Laboratory Animals Monitoring Institute

Jiaqi Li (✉ jqli@scau.edu.cn)

South China Agricultural University

Xiaolong Yuan (✉ yxl@scau.edu.cn)

South China Agricultural University

Research

Keywords: Commercial pig, Reproductive traits, EXOC 4, Gene polymorphism, Promoter

Posted Date: June 10th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-33761/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

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

Yingting He ^{1#}, Xiaofeng Zhou ^{1#}, Bin Hu ^{2#}, Rongrong Zheng ¹, Yao Jiang ¹, Zhixiang Yao ³, Zhe Zhang ¹, Hao Zhang ¹, Xilong Wang ⁴, Jiaqi Li ^{1*} and Xiaolong Yuan ^{1,4*}

¹ National Engineering Research Center for Breeding Swine Industry, Guangdong Provincial Key Lab of Agro-Animal Genomics and Molecular Breeding, College of Animal Science, South China Agricultural University, Guangzhou, Guangdong China

² State Key Laboratory of Livestock and Poultry Breeding, Guangdong Public Laboratory of Animal Breeding and Nutrition, Guangdong Key Laboratory of Animal Breeding and Nutrition, Institute of Animal Science, Guangdong Academy of Agricultural Sciences, Guangzhou 510640, People's Republic of China

³ Guangdong Dexing Food Co., Ltd

⁴ Guangdong Provincial Key Laboratory of Laboratory Animals, Guangdong Laboratory Animals Monitoring Institute, Guangzhou, China

[#]These authors contributed equally to this work.

*Correspondence: Jiaqi Li and Xiaolong Yuan

Email addresses:

Yingting He: 15521059247@163.com;

Xiaofeng Zhou: zxf93715@163.com;

Bin Hu: husanbo@foxmail.com;

21 Rongrong Zheng: 18819266704zrr@sina.com;

22 Yao Jiang: jyao192@163.com;

23 Zhixiang Yao: yao.zx@dexing1996.com;

24 Zhe Zhang: zhezhang@scau.edu.cn;

25 Hao Zhang: zhanghao@scau.edu.cn;

26 Xilong Wang: wangxilonggd@163.com;

27 Jiaqi Li: jqli@scau.edu.cn;

28 Xiaolong Yuan: yxl@scau.edu.cn.

29 Abstract

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

35 **Results:** In this study, rs81471943 was genotyped from a total of 994 Duroc sows, and the genotype
36 and allele frequency of rs81471943 (C/T) were statistically analyzed. Then the associations between
37 rs81471943 and four reproductive traits including number of piglets born alive (NBA), litter weight at
38 birth (LWB), number of piglets weaned (NW), and litter weight at weaning (LWW) were determined.
39 Besides, the sanger sequencing and PCR-restriction fragment length polymorphism (PCR-RFLP) were
40 utilized to identify genotype of rs81471943. We found that the genotype frequency of CC was
41 significantly higher than that of CT and TT, and TT was the favorable genotype on NW ($P=0.01$) and
42 LWW ($P<0.01$). Moreover, 5'-deletion and luciferase assays identified a positive transcription
43 regulatory element in -1826/-1551 of *EXOC4*. After exploring -1826/-1551 fragment, -1781G/A linked
44 with rs81471943 (C/T) were identified by analysis the transcription activity of the haplotypes, and -
45 1781 G/A might influence the potential binding of *P53*, ETS transcription factor (*ELK1*), and myeloid
46 zinc finger 1 (*MZFI*).

47 **Conclusions:** In 994 Duroc pigs, rs81471943 of TT was the favorable genotype on NW ($P=0.01$) and
48 LWW ($P<0.01$), and the haplotypes of -1781G/A and rs81471943 significantly affect the transcription
49 activity of *EXOC4*, which might influence the binding of potential cis-acting elements *P53*, *ELK1*

50 and/or *MZF1*. These findings provide useful information for identifying the molecular marker of
51 *EXOC4*-assisted selection in pig breeding.

52 **Keywords:** Commercial pig; Reproductive traits; *EXOC4*; Gene polymorphism; Promoter

53 Background

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

64 Most of porcine reproduction performance is evaluated using quantitative traits, which are mainly
65 affected by minor genes. The minor genes can be identified by quantitative trait locus (QTLs) mapping
66 [11, 12]. According to PigQTLdb (<https://www.animalgenome.org/cgi-bin/QTLdb/SS/index>), there are
67 29,865 QTLs associated with 688 different traits. On chromosome 18, there are three, three, two and
68 five QTLs significantly associated with ovary weight [13], teat number, total born alive, and corpus
69 luteum number traits [14-16], respectively. In the commercial single-nucleotide polymorphism (SNP)
70 array, one SNP (rs81471943) locates on the exons of exocyst complex component 4 (*EXOC4*) gene,
71 and six QTLs associated with reproduction traits are identified within 5-10 Mb around *EXOC4* on
72 chromosome 18, suggesting that *EXOC4* may be highly associated with reproductive traits. *EXOC4*
73 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 MiniBEST 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.

95 To determine the polymorphic loci rs81471943 (C/T) of porcine *EXOC4* gene, 8 Duroc pigs were
 96 sequenced by Sanger sequenced, 10 Duroc pigs were genotyped by PCR-restriction fragment length
 97 polymorphism (PCR-RFLP).

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

99 DNA extracted from the ear samples of 10 pigs was set as the template. The fragment containing
 100 rs81471943 (Chromosome 18: 16,079,412) of *EXOC4* to a length of 640 bp (Chromosome 18:
 101 16,078,898-16,079,537) was amplified using designed PCR primer using rTaq (Vazyme, Piscataway,
 102 USA). The primers are listed in Table 1. Then, based on PCR-RFLP, PCR products were digested by
 103 BsrB I (identification sequence: CCGCTC) restriction endonuclease (NEB, Ipswich, United Kingdom)
 104 and determined using agarose gel electrophoresis. PCR products with two digested fragments were CC
 105 genotype (565 bp + 75 bp), with three digested fragments were CT genotype (640 bp + 565 bp + 75bp),
 106 and with one digested fragment was TT genotype (640 bp).

107 ***Construction of EXOC4 5'Deletion Fragment Vectors and Luciferase Assay***

108 According to promoter sequences of porcine *EXOC4* (ENSSSCG00000016543, Chromosome 18:
 109 16,057,927-16,346,564) from Ensembl genome browser (release 89), we indicted that there was no
 110 leader exon present in *EXOC4*. PCR was performed to obtain the *EXOC4* promoter of 2791 bp by
 111 PrimerSTAR® (TaKaRa, Dalian, Liaoning, China). The primers are listed in Table 1. Then, PCR
 112 products were purified by gelatinization and the addition of an “AAA” tail for ligation with PMD-18T
 113 vector. After, the recombinant vector was transformed into DH5α competent cells and inoculated on
 114 ampicillin-containing lysogeny broth plates at 37 °C for 12 h. Monoclonal bacteria were picked and

115 cultivated in ampicillin with lysogeny broth medium for incubation for 6 h at 37 °C shaker. The
 116 plasmids were extracted from bacteria and correct plasmids for sequencing were named, respectively.
 117 Then, we designed 10 pairs of primers to amplify deletion fragments of *EXOC4* promoter. The
 118 transcription start site of *EXOC4* gene located at Chromosome 18:16,346,564, and the longest 5'
 119 deletion fragment was named P0 (-2657/+134), and other longer fragments were named P1 (-
 120 2204/+134), P2 (-1914/+134), P3 (-1682/+134), P4 (-1323/+134), P5 (-886/+134) and P6 (-518/+134).
 121 The shorter 5' deletion fragments were named P3A (-1826/+134), P4A (-1551/+134), and P4B (-
 122 1225/+134). Primers are listed in Table 1. Simultaneously, the PCR product was obtained by
 123 PrimerSTAR® high fidelity enzyme (TaKaRa, Dalian, Liaoning, China). Primers are presented in
 124 Table 1. PCR products were purified by gelatinization, and the addition of “AAA” tail to ligated with
 125 PMD-18T, then sequenced by Sanger Sequencing (Beijing Aoboxingke, China). The DNA sequences
 126 of PCR products were determined by using DNASTAR software. Each deletion fragment digested with
 127 Hind III and Mlu I was cloned into the eukaryotic expression vector pGL3-vector, which was also
 128 digested with Hind III and Mlu I restriction endonuclease. Then, according to the manufacturer's
 129 instructions for the dual-luciferase reporter assay kit (Promega, Madison, WI, USA), we used the
 130 BioTek Synergy 2 multifunctional microplate reader (BioTek, Winooski, VT, USA) for fluorescence
 131 detection. The ratio expression of firefly luciferase to renilla luciferase of each deletion fragment was
 132 calculated.

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

134 Porcine GCs were cultured according to previous studies [19]. Porcine ovaries were collected from
 135 local slaughterhouse in Guangzhou, China. The ovaries were stored in phosphate-buffered saline (PBS)

136 containing penicillin (100 IU/mL) and streptomycin (100 µg/mL; Invitrogen, Shanghai, China) at
 137 37 °C and transported to the laboratory quickly. Then, 5-7 mm follicles were punctured, and GCs in
 138 follicular fluid were collected using 1-mL syringe. After washing the isolated GCs twice with PBS, the
 139 GCs were seeded into 75-cm² flasks and cultured at 37 °C under 5% CO₂ in DMEM (Hyclone, Logan,
 140 UT, USA) containing 10% fetal bovine serum (100 IU/mL penicillin, and 100 µg/mL streptomycin;
 141 Hyclone, Logan, UT, USA).

142 *Identification of SNP and transcription binding sites*

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

151 *Statistical analysis*

152 The phenotypic data of Duroc pigs were used for the association analysis of rs81471943 and
 153 reproductive traits, including NBA, LWB, LWW, and NW. Estimated breeding values (EBVs) of all
 154 pigs and the reliabilities of EBVs were imputed using animal model best linear unbiased prediction
 155 [20] and obtained from the in-farm genetic evaluation software Herdsman swine management platform

(S & S Programming, Lafayette, IN, USA). 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 $\pm$ 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 χ^2 valued 0.001 ($\chi^2_{0.05}(2) = 5.99$, $P > 0.05$) 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 ($P=0.02$).

182 ***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 BsrB I.

190 ***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 *EXOC4*. Moreover, many potential binding sites of transcription factors were predicted on -1781G/A of *EXOC4* (Table 6), and P53 and ETS transcription factor (ELK1) might bind at -1781A, and myeloid zinc finger 1 (*MZFI*) might bind at -1781G.

Discussion

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 *EXOC4* leads to an increase in the malformation rate of human newborns [21], and *EXOC4* also involves in insulin-stimulated glucose transport [22, 23]. Jiao et al investigated the interactions between *EXOC4*-1q23.1 and body mass index in a European-American adult female cohort via genome-wide interaction analyses, and results suggest *EXOC4*-related pathways may contribute to the development of obesity [24]. Similarly, after *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 *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 *ECOX4* might be important for economic traits in livestock.

In this study, we found rs81471943 (C/T) located in the seventh intron of the *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

259 site and the 134 bp sequence of the first exon. We found the positive regulatory elements might localize
 260 in the P3A-P4A (-1826/-1551) region (Figure 4), and the negative control element might localize in
 261 the P1-P2 (-2204/-1914) region (Figure 3). After exploring the SNP of P3A-P4A (-1826/-1551) region
 262 containing positive transcription regulatory elements, one SNP was found located on -1781. As the
 263 linkage between -1781G/A and rs81471943, four haplotypes were defined as HA-1 (GC), HA-2 (AC),
 264 HA-3 (GT), and HA-4 (AT) (Table 5). Then we further analyzed the transcription activity of four
 265 haplotypes, and results showed that the transcription activity of HA-1 (GC) is significantly higher than
 266 HA-2 (AC) (Figure 6), and the transcription activity of HA-3 (GT) was significantly higher than HA-
 267 4 (AT) (Figure 6). These results indicated that rs81471943 might link with -1781G/A to affect the
 268 expression of *EXOC4*.

269 -1781G/A might regulate the expression of *EXOC4* by affecting the binding of transcription factors
 270 [31-33]. In this study, we also predicted that transcription factors *P53*, *ELK1*, and *MZF1* would be
 271 located on the -1826/-1551 region of the porcine *EXOC4* promotor. *P53* participates in maintaining
 272 cell growth [34]. *P53* can enhance their stability through phosphorylation and the activation or
 273 inhibition of the transcription of downstream genes, thus inducing cell cycle arrest and apoptosis [35,
 274 36]. *ELK1* is a transcription factor belonging to the ETS oncogene family and induces hormone-
 275 resistant or metastatic prostate cancers [37], and plays an important role in breast and ovarian cancers
 276 [38]. As a bifunctional transcription factor, *MZF1* belongs to the zinc finger protein Kruppel
 277 transcription factor family, which regulate downstream gene expression by binding to the cis-acting
 278 element TGGGA" on gene promoter [39, 40]. Results in Table 6 and Figure 5 indicated that the
 279 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.

Funding

This work was supported by the National Natural Science Foundation of China (31902131), Youth Innovative fund of Guangdong Education Department (2018KQNCX019), Natural Science Foundation of Guangdong Province (2019A1515010676), earmarked fund for China Agriculture Research System (CARS-35) and the Key R&D Program of Guangdong Province (2018B020203003).

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

the final manuscript.

Acknowledgements

The authors would like to thank Fujian Yongcheng farming and animal husbandry Co., Ltd for providing the data and offering the opportunity to conduct this study. We gratefully thank Prof. H. Zhang for his help in this work. Also, we are grateful to the editors and the anonymous reviewers.

Reference

1. Larson G, Albarella U, Dobney K, Rowley-Conwy P, Schibler J, Tresset A, Vigne JD, Edwards CJ, Schlumbaum A, Dinu A *et al*: **Ancient DNA, pig domestication, and the spread of the Neolithic into Europe.** *P Natl Acad Sci USA* 2007, **104**(39):15276-15281.
2. Wilkinson S, Lu ZH, Megens HJ, Archibald AL, Haley C, Jackson IJ, Groenen MA, Crooijmans RP, Ogden R, Wiener P: **Signatures of diversifying selection in European pig breeds.** *PLoS Genet* 2013, **9**(4):e1003453.
3. Goddard ME, Hayes BJ: **Mapping genes for complex traits in domestic animals and their use in breeding programmes.** *Nat Rev Genet* 2009, **10**(6):381-391.
4. Thekkoot DM, Young JM, Rothschild MF, Dekkers JCM: **Genomewide association analysis of sow lactation performance traits in lines of Yorkshire pigs divergently selected for residual feed intake during grow-finish phase.** *Journal of animal science* 2016, **94**(6):2317-2331.
5. Bjerre D, Madsen LB, Mark T, Cirera S, Larsen K, Jorgensen CB, Fredholm M: **Potential Role of the Porcine Superoxide Dismutase 1 (SOD1) Gene in Pig Reproduction.** *Anim Biotechnol* 2013, **24**(1):1-9.
6. Fonseca S, Wilson IJ, Horgan GW, Maltin CA: **Slow fiber cluster pattern in pig longissimus thoracis muscle: Implications for myogenesis.** *Journal of animal science* 2003, **81**(4):973-983.
7. Ji J, Yan G, Chen D, Xiao S, Gao J, Zhang Z: **An association study using imputed whole-genome sequence data identifies novel significant loci for growth-related traits in a Duroc x Erhualian F2 population.** *J Anim Breed Genet* 2019, **136**(3):217-228.

- 345 8. Wang D, Ning C, Xiang H, Zheng X, Kong M, Yin T, Liu J, Zhao X: **Polymorphism of**
 346 **mitochondrial tRNA genes associated with the number of pigs born alive.** *J Anim Sci*
 347 *Biotechnol* 2018, **9**:86.
- 348 9. Craig A, Gordon A, Magowan E: **Understanding the drivers of improved pig weaning weight**
 349 **by investigation of colostrum intake, sow lactation feed intake, or lactation diet specification.**
 350 *J Anim Sci* 2017, **95**(10):4499-4509.
- 351 10. Wilson ME, Biensen NJ, Ford SP: **Novel insight into the control of litter size in pigs, using**
 352 **placental efficiency as a selection tool.** *Journal of animal science* 1999, **77**(7):1654-1658.
- 353 11. Andersson L: **Genetic dissection of phenotypic diversity in farm animals.** *Nature Reviews*
 354 *Genetics* 2001, **2**(2):130-138.
- 355 12. Andersson L, Georges M: **Domestic-animal genomics: deciphering the genetics of complex**
 356 **traits.** *Nat Rev Genet* 2004, **5**(3):202-212.
- 357 13. Rosendo A, Iannuccelli N, Gilbert H, Riquet J, Billon Y, Amigues Y, Milan D, Bidanel JP:
 358 **Microsatellite mapping of quantitative trait loci affecting female reproductive tract**
 359 **characteristics in Meishan x Large White F₂ pigs.** *J Anim Sci* 2012, **90**(1):37-44.
- 360 14. Hernandez SC, Finlayson HA, Ashworth CJ, Haley CS, Archibald AL: **A genome-wide linkage**
 361 **analysis for reproductive traits in F₂ Large White × Meishan cross gilts.** *Animal Genetics*
 362 2014, **45**(2):191-197.
- 363 15. Tribout T, Iannuccelli N, Druet T, Gilbert H, Riquet J, Gueblez R, Mercat MJ, Bidanel JP, Milan
 364 D, Le Roy P: **Detection of quantitative trait loci for reproduction and production traits in**
 365 **Large White and French Landrace pig populations.** *Genetics, selection, evolution : GSE* 2008,
 366 **40**(1):61-78.
- 367 16. Duijvesteijn N, Veltmaat JM, Knol EF, Harlizius B: **High-resolution association mapping of**
 368 **number of teats in pigs reveals regions controlling vertebral development.** *BMC Genomics*
 369 2014, **15**(1):542.
- 370 17. Nagase T, Kikuno R, Hattori A, Kondo Y, Okumura K, Ohara O: **Prediction of the coding**
 371 **sequences of unidentified human genes. XIX. The complete sequences of 100 new cDNA**
 372 **clones from brain which code for large proteins in vitro.** *DNA Research* 2000, **7**(6):347-355.

- 373 18. Zhang Z, Chen Z, Ye S, He Y, Huang S, Yuan X, Chen Z, Zhang H, Li J: **Genome-Wide**
 374 **Association Study for Reproductive Traits in a Duroc Pig Population.** *Animals (Basel)* 2019,
 375 **9(10).**
- 376 19. Yuan X, Zhou X, He Y, Zhong Y, Zhang A, Zhang Z, Zhang H, Li J: **C/EBPbeta Promotes**
 377 **STAT3 Expression and Affects Cell Apoptosis and Proliferation in Porcine Ovarian**
 378 **Granulosa Cells.** *Genes (Basel)* 2018, **9(6).**
- 379 20. Henderson CR: **Best linear unbiased estimation and prediction under a selection model.**
 380 *Biometrics* 1975, **31(2):423-447.**
- 381 21. Barkefors I, Fuchs PF, Heldin J, Bergstrom T, Forsberg-Nilsson K, Kreuger J: **Exocyst complex**
 382 **component 3-like 2 (EXOC3L2) associates with the exocyst complex and mediates**
 383 **directional migration of endothelial cells.** *J Biol Chem* 2011, **286(27):24189-24199.**
- 384 22. Laramie JM, Wilk JB, Williamson SL, Nagle MW, Latourelle JC, Tobin JE, Province MA,
 385 Borecki IB, Myers RH: **Polymorphisms near EXOC4 and LRGUK on chromosome 7q32 are**
 386 **associated with Type 2 Diabetes and fasting glucose; the NHLBI Family Heart Study.** *BMC*
 387 *Med Genet* 2008, **9:46-55.**
- 388 23. Nagase T, Ishikawa K-i, Miyajima N, Tanaka A, Kotani H, Nomura N, Ohara O: **Prediction of**
 389 **the Coding Sequences of Unidentified Human Genes. IX. The Complete Sequences of 100**
 390 **New cDNA Clones from Brain Which Can Code for Large Proteins in vitro.** *DNA Research*
 391 1998, **5(1):31-39.**
- 392 24. Jiao H, Zang Y, Zhang M, Zhang Y, Wang Y, Wang K, Price RA, Li WD: **Genome-Wide**
 393 **Interaction and Pathway Association Studies for Body Mass Index.** *Front Genet* 2019, **10:404.**
- 394 25. Tanaka T, Goto K, Iino M: **Sec8 modulates TGF-beta induced EMT by controlling N-cadherin**
 395 **via regulation of Smad3/4.** *Cell Signal* 2017, **29:115-126.**
- 396 26. Gao Y, Hu XX, Du ZQ, Deng XM, Huang YH, Fei J, Feng JD, Liu ZL, Da Y, Li N: **A genome**
 397 **scan for quantitative trait loci associated with body weight at different developmental stages**
 398 **in chickens.** *Animal Genetics* 2006, **37(3):276-278.**
- 399 27. Nadaf J, Pitel F, Gilbert H, Duclos MJ, Vignoles F, Beaumont C, Vignal A, Porter TE, Cogburn
 400 LA, Aggrey SE *et al*: **QTL for several metabolic traits map to loci controlling growth and**

- body composition in an F2 intercross between high- and low-growth chicken lines. *Physiol Genomics* 2009, **38**(3):241-249.
28. Park HB, Jacobsson L, Wahlberg P, Siegel PB, Andersson L: **QTL analysis of body composition and metabolic traits in an intercross between chicken lines divergently selected for growth.** *Physiol Genomics* 2006, **25**(2):216-223.
29. Ogawa S, Konta A, Kimata M, Ishii K, Uemoto Y, Satoh M: **Estimation of genetic parameters for farrowing traits in purebred Landrace and Large White pigs.** *Anim Sci J* 2019, **90**(1):23-28.
30. Charlesworth B: **Fundamental concepts in genetics: effective population size and patterns of molecular evolution and variation.** *Nat Rev Genet* 2009, **10**(3):195-205.
31. Fan Y, Chen H, Li A, Shi Y, Zhang Y, Feng Q, Sun Y, Zheng H, He Y: **A promoter polymorphism (rs17222919, -1316T/G) of *ALOX5AP* gene is associated with decreased risk of ischemic stroke in two independent Chinese populations.** *PLoS One* 2015, **10**(3):e0122393.
32. Rubattu S, Giusti B, Lotta LA, Peyvandi F, Cotugno M, Stanzione R, Marchitti S, Palombella AM, Di Castro S, Rasura M *et al*: **Association of a single nucleotide polymorphism of the *NPR3* gene promoter with early onset ischemic stroke in an Italian cohort.** *European Journal of Internal Medicine* 2013, **24**(1):80-82.
33. Xiao M, Guo L, Wang T, Zhu T, Jia L, Chen L, Wen F: **Interleukin-1B-31T/C promoter polymorphism and chronic obstructive pulmonary disease risk: a meta-analysis.** *Arch Med Sci* 2014, **10**(3):434-438.
34. Vousden KH, Lu X: **Live or let die: the cell's response to p53.** *Nat Rev Cancer* 2002, **2**(8):594-604.
35. Purvis JE, Karhohs KW, Mock C, Batchelor E, Loewer A, Lahav G: **p53 dynamics control cell fate.** *Science* 2012, **336**(6087):1440-1444.
36. Vousden KH, Prives C: **Blinded by the Light: The Growing Complexity of p53.** *Cell* 2009, **137**(3):413-431.
37. Patki M, Chari V, Sivakumaran S, Gonit M, Trumbly R, Ratnam M: **The ETS domain transcription factor ELK1 directs a critical component of growth signaling by the androgen**

- 429 **receptor in prostate cancer cells.** *J Biol Chem* 2013, **288**(16):11047-11065.
- 430 38. Goncharenko-Khaider N, Matte I, Lane D, Rancourt C, Piche A: **Ovarian cancer ascites**
431 **increase Mcl-1 expression in tumor cells through ERK1/2-Elk-1 signaling to attenuate**
432 **TRAIL-induced apoptosis.** *Mol Cancer* 2012, **11**:84.
- 433 39. Morris JF, Hromas R, Rauscher FJ: **Characterization of the DNA-binding properties of the**
434 **myeloid zinc finger protein MZF1: two independent DNA-binding domains recognize two**
435 **DNA consensus sequences with a common G-rich core.** 1994, **14**(3):1786-1795.
- 436 40. Deng Y, Wang J, Wang G, Jin Y, Luo X, Xia X, Gong J, Hu J: **p55PIK transcriptionally activated**
437 **by MZF1 promotes colorectal cancer cell proliferation.** *Biomed Research International* 2013,
438 **2013**:868131.
- 439
- 440

Figure 1 Mapping of SNP rs81471943 (C/T) on *EXOC4* (A) PCR products contained SNP rs81471943 of *EXOC4* promotor (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 $\pm$ 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 $\pm$ 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 $\pm$ SD.

Table 1 Primers used in this study

Name	Primer Sequence	Length(bp)
EXOC4-SNP	F: ACAGCCTCGGCTCAACCTTA R: TGCTTTTACGAAGGGGGACA	640
EXOC4-Promoter	F: GAGCGAGTCCTTGTCTACAGT R: TGCTTTTACGAAGGGGGACA	2791
P0 (-2657/+134)	F: CGACGCGTGAGCGAGTCCTTGTCTACAGT R: CCCAAGCTTGCGCATTGGGGATTCTTACA	2791
P1 (-2204/+134)	F: CGACGCGTGGGAACCTCGTTCTTTCCCCC	2338
P2 (-1914/+134)	F: CGACGCGTCCACTCGGACTGTCATCAGC	2048
P3 (-1682/+134)	F: CGACGCGTAAGATGGGAGATGGTTCGGG	1816
P4 (-1323/+134)	F: CGACGCGTGGATGGCTGGATTCCACACT	1457
P5 (-886/+134)	F: CGACGCGTTTTTACAGGTTACTGGTCAGACT	1020
P6 (-518/+134)	F: CGACGCGTATTTAATGTGCAGGACCATGCG	652
P3A (-1826/+134)	F: CGACGCGTATGGCTATGTCTAGCCTCCC R: CCCAAGCTTGCGCATTGGGGATTCTTACA	1950
P4A (-1551/+134)	F: CGACGCGTACCAGCCACCTCACTTTTGA	1685
P4B (-1225/+134)	F: CGACGCGTTCATTGGGATGCTACCAGGC	1359

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

Genotype	Sample Quantity	Genotype Frequency	Allele	Allele Frequency	χ^2
CC	27	0.027	C	0.844	0.224
CT	256	0.258	T	0.156	
TT	711	0.715			

χ^2 0.05 (2) =5.99, χ^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	CC	CT	TT
Number	27	256	771	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	49.06	46.68	49.63
S.E.	0.13	0.16	0.35	0.22	0.27	0.59	0.14	0.17	0.38	1.14	1.36	3.00
Min.	0.00	0.00	0.00	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	109.50	100.00	90.50

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

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, LWW- litter weight at weaning, 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
<i>P53</i>	-1786-1777	-	0.796	-1781A	AGGAAGGTCA
<i>ELK1</i>	-1785-1773	-	0.783	-1781A	ACGTGAGGAAGGTC
<i>MZF1</i>	-1787-1775	+	0.856	-1781G	TGAGGAGGGTCAT

The alphabet in boldface is the mutation site.

Figures

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 $\pm$ 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 $\pm$ 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 $\pm$ SD.