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Expression analysis of the porcine *Homeobox A11* gene and its association with litter size in Large White pigs

R. Lin, H. Jia, S. Chen, S. Zhao

Key Laboratory of Agricultural Animal Genetics, Breeding, and Reproduction of Ministry of Education, The Cooperative Innovation Center for Sustainable Pig Production, Huazhong Agricultural University, Wuhan, P.R. China

ABSTRACT: The *homeobox A11* (*HOXA11*) gene, a well-known transcriptional regulator, plays a critical role in normal development of the uterus. In this study, the porcine *HOXA11* gene, including its promoter region, was cloned and sequenced (GenBank Accession No. KF724933). Differential expression levels of *HOXA11* in the endometrium at one stage of the estrous cycle and three stages of gestation for Meishan and Large White pigs were investigated. Association between the single nucleotide polymorphism (SNP) and litter size was conducted. Results suggested that expression of the *HOXA11* gene was the highest on day 15 and decreased on day 26 of gestation, reaching a minimum level of expression on day 50 of gestation in Large White pigs. Expression of the *HOXA11* gene tended to be consistent between day 15 of the estrous cycle and day 26 of gestation, and decreased on day 50 of gestation in Meishan pigs. Series deletion analysis and reporter expression assays identified a promoter region of the *HOXA11* gene which possessed a five times higher promoter activity than the pGL3-Basic vector. Using comparative sequencing, a candidate SNP (KF724933:g.2325C>A) was identified. In a Large White pig population, this polymorphism was associated with the number of piglets. In a combined analysis containing all parity groups, sows with the *AC* genotype had greater total number of born (1.80; $P < 0.05$) and number of born alive (1.82; $P < 0.05$) piglets compared with the *CC* genotype sows. These results indicate *HOXA11* may play a role in the regulation of embryo implantation in pigs.

Keywords: endometrium; *HOXA11*; qPCR; single nucleotide polymorphism; litter size

INTRODUCTION

Litter size of sows is one of the main determinants of efficiency in pig production, with embryonic loss being one of the major obstacles in obtaining a large litter size (Spotter and Distl 2006). However, it is difficult to improve litter size effectively using only traditional genetic selection methods. Considerable advances in molecular technologies provide opportunities to improve litter size more efficiently, including the use of transgenic technology or marker-assisted selection (MAS). In order to make use of such methods, however, it is necessary to identify the main genes associated with litter size.

Homeobox (*Hox*) genes, which encode transcription factors, have long been recognized as important regulators of embryonic development through regulation of downstream target gene expression (Soshnikova 2014). In mammals, this 39-gene complex is located on different chromosomes, designated A, B, C, and D. The *Hoxa* genes regulate the development of the urogenital tract (Krumlauf 1994; Taylor et al. 1997).

Early embryonic death (day 10–30 of gestation) is an important cause of the decline in litter size

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of pigs (Geisert and Schmitt 2002). It is therefore important to examine the genes related to embryo implantation. Studies have shown that *HOXA11* is expressed in the primordia of the lower uterine segment and cervix (Taylor 2000). *HOXA11* expression reportedly depends upon the menstrual cycle stage, increasing dramatically at the time of implantation during the midluteal phase (Taylor 2002). In mice, *Hoxa11* gene expression peaks in uterine stromal cells during metestrus (Gendron et al. 1997). The expression of *HOXA11* is regulated by sex steroid hormones, such as estrogen and progesterone (Daftary and Taylor 2000), and altered *Hox* expression has been found to affect implantation rates (Taylor et al. 1999a).

In women, *HOXA11* is upregulated in the midsecretory endometrium during implantation (Taylor et al. 1999b). *Hoxa11*$^{-/-}$ female mice are sterile and studies have shown that the development of uteri stromal and glandular cells are deficient in *Hoxa11*-mutant mice (Hsieh-Li et al. 1995; Gendron et al. 1997). Moreover, *Hoxa11*homozygous mutant male mice suffer from serious complications in gametogenesis (Hsieh-Li et al. 1995). These findings suggest *HOXA11* is essential for endometrial development, implantation, and maintenance of pregnancy.

The aim of our study was to investigate the possibility that *HOXA11* plays a role in porcine reproductive traits. DNA from the porcine *HOXA11* gene and its promoter region were cloned, and expression levels in the endometrium at different developmental stages in Meishan and Large White pigs were analyzed. A SNP (KF724933:g.2325C>A) was identified and its association with litter size was assessed in a Large White pig population.

MATERIAL AND METHODS

Animals and tissues. All animal procedures were performed according to the protocols approved by the Biological Studies Animal Care and Use Committee of Hubei Province, P.R. China.

Large White and Meishan sows (12 of each breed) were mated with a boar from the corresponding breed. The sows were euthanized at a commercial slaughterhouse on days 15, 26 or 50 of gestation, or day 15 of the estrous cycle (three sows at each stage for each breed). The uteri were removed immediately, and endometrium collected for differential *HOXA11* gene expression analysis. All tissues were washed briefly with PBS, rapidly frozen in liquid nitrogen, and stored at –80°C.

Animals from six different populations were used to investigate the allele frequency, including 147 Large White pigs, 35 Duroc pigs, and 4 indigenous pig populations (34 Meishan, 38 Tongcheng, 51 Enshi black, and 133 Luchuan). Ear tissues were sampled and immersed in 75% ethanol. Association analyses were conducted in the Large White pig population. Large White pigs were obtained from Guangdong Wens Animal Breeding Company Ltd., and rearing conditions were standardized for all sows. The total numbers of piglets born (TNB) and piglets born alive (NBA) were recorded for 479 litters, from 2004 to 2008.

Genomic DNA was extracted from the samples according to a standard phenol/chloroform procedure.

Cloning of the porcine HOXA11 gene. Expressed sequence tags (ESTs) of the porcine *HOXA11* gene were found by comparing all sequences available in the pig EST databases to the human *HOXA11* mRNA sequence (GenBank Acc. No. NM_005523) and the mouse *Hoxa11* mRNA sequence (GenBank Acc. No. NM_010450), using the BLAST algorithm. The genomic sequence of porcine *HOXA11* containing all intron and exon regions was amplified using primers indicated in Table 1 (HOXA11-DNA-F/R).

RNA isolation and reverse transcription. Total RNA was extracted from frozen tissues (approximately 0.1 g) using TRIzol reagent kits (Invitrogen, Carlsbad, USA). The concentration and quality of the extracted RNA was determined using a Nanodrop ND2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and denatured gel electrophoresis. Reverse transcription polymerase chain reaction (RT-PCR) was conducted using RevertAid First Strand cDNA Synthesis kits (Thermo Fisher Scientific), containing oligo (dT), random primers, and DNaseI. After treating 2 µl RNA with DNaseI, the reverse transcription reaction was carried out at 25°C for 5 min, 42°C for 60 min, and 70°C for 5 min.

Quantitative real-time PCR analysis. Quantitative real-time PCR (qPCR) analysis was used to detect differential *HOXA11* gene expression in the endometrium of Meishan and Large White pigs during the four stages. The qPCR reactions were carried out in a LightCycler 480 II (Roche, Penzberg, Germany) using 2X SYBR Green Re-

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Table 1. Primer pairs used for PCR amplification

Primer name	GenBank Acc. No.	Primer sequences (5'→3')	Annealing temperature (°C)	Size (bp)
HOXA11-DNA-F	NC_010460.3	TCACATGACCAGCACCTCCC	56	2595
HOXA11-DNA-R		GGCAAAATCTGCATAGAATCTCCT		
HOXA11-cDNA-F	CF175969.1, EW502001.2	TGTTAAGCTCGGCTACTGC	56	2512
HOXA11-cDNA-R		TGTATGCGGTGGGTGAGA		
HOXA11-promoter-2kb-F	NC_010460.3	TGATTTGTGATACGCAACCCTT	58	2034
HOXA11-promoter-2kb-R		CACGCTCATCAAAATCCATCAT		
HOXA11-promoter-1.5kb-F		TCTCCCAAACATTGTTCAGTCAG	59	1546
HOXA11-promoter-1.5kb-R		CCACGCTCATCAAAATCCAT		
HOXA11-promoter-1kb-F		GGTTGTTGGCGGTTTAGGGA	60	905
HOXA11-promoter-1kb-R		AGGGAGGCTGGAGAAATCTGG		
HOXA11-SNP-F	KF724933.1	CCTATCTGGGGAGCTGGCTTTT	55.8	466
HOXA11-SNP-R		CTTAGAGGAGTGGGTTGGCTGAGTA		
HOXA11-sus-F	KF724933.1	GGAGCGGGAGTTCTTCTTCAGT	60	173
HOXA11-sus-R		AGAGGAGTGGGTTGGCTGAGTA		
GAPDH-sus-F	AF017079.1	CGTCCCTGAGACACGATGGT	60	194
GAPDH-sus-R		GCCTTGACTGTGCCGTGGAAT		

altime PCR Master Mix (Toyobo, Osaka, Japan), which consisted of 0.2μM of each primer and 0.5 μl template cDNA in a final 20 μl volume. The PCR conditions were as follows: 95°C for 3 min; 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s (for 40 cycles). The sequence of qPCR detection primers can be found in Table 1. All PCRs were performed in triplicate and gene expression levels were quantified relative to the expression of *GAPDH* by employing the $2^{-\Delta\Delta Ct}$ value method (Livak and Schmittgen 2001). The Student's *t*-test was used to identify statistical differences in the mean values between two groups.

Luciferase reporter assays. The three contiguous segments (primers listed in Table 1) of the 5'-flanking region were inserted into the *Kpn*I/*Nhe*I sites of the pGL3-Basic vector to generate the pGL3-Pro luciferase reporter vector. *HOXA11* promoter/firefly luciferase reporter constructs were transiently transfected into cell lines. Ishikawa, PK15, and C2C12 cell lines were maintained in our laboratory, and dual-luciferase reporter assay kits were obtained from Promega (Madison, USA). Cells were lysed and assayed for promoter functionality using the dual-luciferase reporter assay system, between 24 h and 48 h after transfection. The enzymatic activity of luciferase was measured with a luminometer VICTOR X2 (Perkin-Elmer, Waltham, USA). To normalize transfection efficiency, the cells were co-transfected with a Renilla luciferase reporter construct (pRL-CMV vector). All results were presented as the mean ± SD. Statistical significance was defined as $P < 0.05$.

SNP detection of the HOXA11 gene. Specific primers (HOXA11-SNP-F/R, Table 1) were designed to detect SNPs contained within exon 2 of the porcine *HOXA11* gene. PCR reactions contained 12.5 μl of 2X GC buffer II, 100 ng of genomic DNA, 0.3μM of each primer, 150μM dNTPs, 1.5mM $MgCl_2$, and 1 Unit LA Taq DNA polymerase (TaKaRa, Dalian, China) in a final volume of 25 μl. The PCR cycles were as follows: initial denaturation for 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 62°C, 30 s at 72°C, and a final extension cycle of 10 min at 72°C. Five randomly selected PCR products from each pig breed were sequenced by a commercial service (Sangon, Shanghai, China). For PCR-RFLP profiles, 6 μl of PCR products were digested with 3 Units *Afe*I (New England Biolabs, Ipswich, USA) for 4 h at 37°C, and then separated by electrophoresis (120 V, 30 min) on a 2% agarose gel with GelRed (Biotium, Hayward, USA).

Statistical analysis. The association between *HOXA11* genotypes and litter size of Large White pigs ($n = 147$) was evaluated with the General Linear Model procedure of SAS software (Statistical Analysis System, Version 8.0, 1999). The model was as follows:

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$$Y_{ijk} = \mu + P_i + S_j + G_k + e_{ijk}$$

where:

Y_{ijk} = reproductive trait (total number of born piglets or number of piglets born alive)

μ = Least Squares Mean

P_i = effect of the i^{th} parity (i = 1, 2, 3, and ≥ 4)

S_j = effect of the j^{th} season

G_k = effect of genotype

e_{ijk} = random residual

RESULTS

Cloning and sequencing of porcine HOXA11 gene. We generated a 2595 bp DNA fragment of the *HOXA11* gene, which contained 721 bp of exon 1, 1425 bp of the intron, and 233 bp of exon 2. The genomic structure of porcine *HOXA11* was predicted by comparing with the human *HOXA11* and confirmed using cDNA sequencing of porcine *HOXA11* (primer: HOXA11-cDNA-F/R). The sequence of porcine *HOXA11* was submitted to GenBank (Accession No. KF724933).

Differential expression of the porcine HOXA11 gene in the endometrium during various gestation stages between the two breeds. Semi-quantitative RT-PCR was used to confirm that the porcine *HOXA11* was specifically expressed in endometrium (data not shown). This result was reported previously (http://www.biogps.org/). To detect the *HOXA11* gene expression patterns in porcine endometrium, we performed qPCR analysis in four stages of two breeds (Figure 1). In the endometrium of Meishan pigs, qPCR results indicated that the *HOXA11* expression was the highest at ed15, in comparison to other stages (Figure 1A), and the expression at gd50 was the lowest. However no significant changes were found between ed15, gd15, and gd26 in Meishan pigs. Significant differences in trends were observed in Large White pigs however (Figure 1B). *HOXA11* gene expression in the endometrium of Large White pigs was significantly higher at gd15 than at ed15 (P = 0.02), and lower at gd26 (P = 0.037). *HOXA11* gene expression was the lowest at gd50 in both Meishan and Large White pigs.

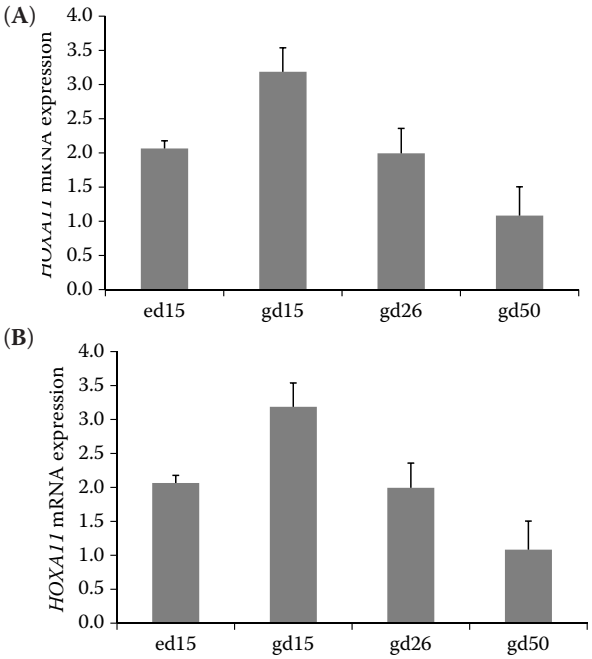

Figure 1. Temporal expression patterns of the porcine *HOXA11* gene in the endometrium of two breeds

mRNA levels of the porcine *HOXA11* gene in Meishan (**A**) and Large White (**B**) endometrium at ed15, gd15, gd26, and gd50. The *x*-axis represents the developmental stage and the *y*-axis shows the fold change in expression
data are expressed as mean ± SD (n = 3)

Construction of the series deletion of the porcine HOXA11 5' flanking region and detection of the promoter activity in cells. We cloned the 5' flanking region sequence of the porcine *HOXA11* gene, and constructed a 5' terminal deletion promoter vector. Results from the dual-luciferase activity assay indicated that the 2 kb promoter vector of *HOXA11* gene had a five times higher promoter activity than the pGL3-Basic vector (Figure 2A). After analyzing

Table 2. Allele frequencies of SNP g.2325C>A of *HOXA11* gene in six pig breeds

Breed	Number of pigs	Genotype distribution			Allele frequencies	
		AA	*AC*	*CC*	*A*	*C*
Large White	147	0	26	121	0.09	0.91
Duroc	35	0	4	31	0.06	0.94
Meishan	34	4	8	22	0.24	0.76
Tongcheng	38	1	14	23	0.21	0.79
Enshi black	51	15	21	15	0.50	0.50
Luchuan	133	3	43	87	0.18	0.82

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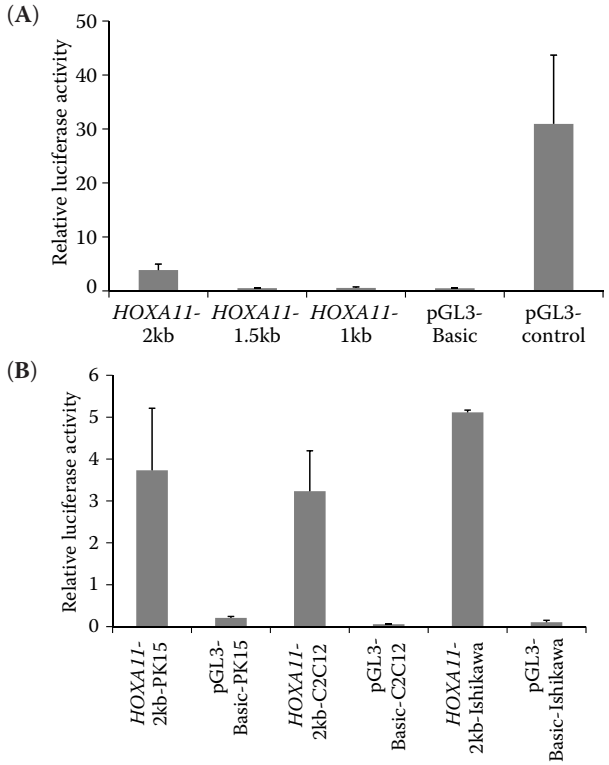

Figure 2. Characterization of the porcine *HOXA11* gene promoter and identification of the effective regulatory promoter region

(**A**) analysis of promoter activity in a series of deletion constructs of the *HOXA11* 5' flanking region in Ishikawa cells, (**B**) reporter analysis of the porcine *HOXA11* gene 2 kb promoter in different cells. The resulting firefly luciferase activity was then normalized to Renilla luciferase activity and the relative values are presented as the fold-increase over the activity of the promoter less pGL3-basic vector
values represent the mean ± SD (n = 3)

the *HOXA11* gene promoter sequence, we predicted the promoter may have a higher binding activity with transcription factors AP-1, c-JUN, OCT-1, and GATA-3. The vector was transfected into the PK15, C2C12, and Ishikawa cell lines, and the promoter was expressed much higher in Ishikawa cell lines than in the other two cell lines (Figure 2B).

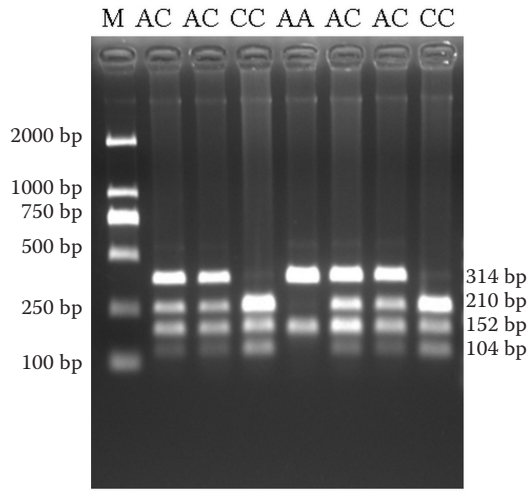

Figure 3. SNP g.2325C>A results of *HOXA11* gene

M = DL2,000 DNA Marker; *AC*, *CC*, *AA* = genotypes

HOXA11 SNP detection and association analysis with litter size. By comparative sequencing of the *HOXA11* gene in six pig breeds, we identified a g.2325C>A polymorphism at 26 bp in exon 2 (Accession No. KF724933) detectable using PCR-*Afe*I-RFLP. The polymorphism did not alter the amino acid residue. To detect the SNP using the RFLP analysis, 466 bp PCR products containing the aforementioned SNP position in exon 2 were digested using *Afe*I to form two fragments for the *A* allele (314 and 152 bp), or three fragments for the *C* allele (210, 152, and 104 bp) (Figure 3). These differences were detected using gel electrophoresis.

Allele frequencies for the SNP g.2325C>A were studied in 438 unrelated pigs from six different populations (Table 2). At this locus, genotype *AA* was only detected in Chinese indigenous pig

Table 3. Association between *HOXA11* SNP g.2325C>A genotypes and litter size in Large White sows

Traits		Genotype (mean ± SE)		
		AA	*AC*	*CC*
1st parity	*n*	0	25	105
	TNB	–	11.477 ± 0.642	10.483 ± 0.309
	NBA	–	10.295 ± 0.704	9.114 ± 0.339
All parities	*n*	0	63	416
	TNB	–	13.125 ± 0.600[a]	11.327 ± 0.596[b]
	NBA	–	12.328 ± 0.623[a]	10.511 ± 0.619[b]

n = number of investigated litters, TNB = total number of born piglets, NBA = number of piglets born alive
[a,b]Least Squares Means values with different letters are significantly different (P < 0.05)

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populations, not in Large White and Duroc pigs. Least Squares Means and allele substitution effects for litter size are shown in Table 3. In Large White pigs, no significant differences were detected for litter size in first parity sows. However, when all parity types were combined, *AC* genotypes differed from homozygous *CC* sows for TNB and NBA. TNB and NBA for genotype *AC* were by 1.80 ($P < 0.05$) and 1.82 ($P < 0.05$) greater per litter size respectively, compared to those of the *CC* genotype.

DISCUSSION

Hox genes, encoding evolutionarily conserved transcription factors, are dynamically expressed in endometrium and are essential for embryonic development, endometrial development, and receptivity (Cakmak and Taylor 2010). As transcription factors, they regulate a vast number of downstream target genes that affect the development of endometrium for embryo implantation. Although *Hoxa11*-null mice can generate a reasonable number of embryos capable of implantation in wild-type surrogate mice, embryos from the wild-type mice cannot be implanted in *Hoxa11*-null mice, suggesting that the implantation failure is caused by uterine factor infertility (Hsieh-Li et al. 1995).

Meishan pigs are prolific breeders, averaging three to five more pigs per litter than the European breeds, even though their ovulation rates are similar (Ford and Youngs 1993; Haley et al. 1995; Vallet et al. 1998). Gene expression in the endometrium of different breeds may relate to reproduction traits. However, expression of the porcine *HOXA11* gene in various breeds at different gestation stages, particularly after implantation, has not been investigated. In this study, we investigated the differential expression profiles of *HOXA11* in the endometrium of Meishan and Large White pigs at four developmental stages. Higher persistent expression levels of endometrial *HOXA11* gene were detected from ed15 to gd26 in Meishan pigs. However, endometrial expression of *HOXA11* reached a peak during the window of implantation (gd15) and reduced sharply at gd26 in Large White pigs. Most fetal mortality in pig breeds occurs in days 12–18 of gestation (Spotter and Distl 2006). We assumed that the dramatic change in *HOXA11* gene expression level during gd15 to gd26 might cause more embryonic deaths in Large White pigs compared with Meishan pigs.

To investigate the regulation of *HOXA11* gene expression, we cloned the 5' promoter region of porcine *HOXA11* gene and analyzed the promoter activity in Ishikawa cells. When we transfected the vector into the PK15, C2C12, and Ishikawa cell lines, we found that the promoter was highly expressed in Ishikawa cells compared with the other two cell lines. However, these differences were not statistically significant. These results were unexpected since the promoter region should theoretically have specific activity in human endometrial adenocarcinoma (Ishikawa) cells. The cause of its widespread expression may relate to the lack of a specific factor involved in transcriptional regulation *in vitro*, or the region may not have contained the complete DNA sequence and therefore lacked components of tissue specific expression for the porcine *HOXA11* gene.

Due to the low heritability of litter size (0.1–0.15), marker-assisted selection (MAS) may be particularly promising for increasing litter size. Some candidate genes have reportedly been associated with litter size in pigs, such as the estrogen receptor (*ESR*) (Rothschild et al. 1996; Short et al. 1997), follicle-stimulating hormone β (*FSHβ*) (Zhao et al. 1998; Li et al. 2008), and retinol-binding protein 4 (*RBP4*) (Rothschild et al. 2000). About 30% of embryos will not survive embryo implantation (Spencer et al. 2004). If it is possible to reduce the embryonic death rate, there will be more piglets per litter. *HOXA11* plays an important role in embryo implantation and development. In this study, SNP (g.2325C>A) in exon 2 of *HOXA11* appeared to be associated with litter size. Our results indicated that sows with the *AC* genotype had significantly more ($P < 0.05$) TNB and NBA than sows with the *CC* genotype, both in the "first parity" and "all parity" groups.

CONCLUSION

HOXA11, an important member of the *Hoxa* gene family, appears to play a key role in embryo implantation in pigs. We identified a polymorphic site at exon 2 of the *HOXA11* gene which was significantly associated with litter size. Our findings suggest the *HOXA11* gene is a candidate gene for litter size in pigs.

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Corresponding Author

Prof. Shuhong Zhao, Ph.D., Huazhong Agricultural University, Key Laboratory of Agricultural Animal Genetics, Breeding, and Reproduction of Ministry of Education, No. 1 Shizishan Street, Hongshan District, Wuhan 430070, P.R. China
Phone: +86 27 87 387 480, e-mail: shzhao@mail.hzau.edu.cn