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DIFFERENTIAL EXPRESSION OF MIR-145, MIR-429 AND ITS TARGET GENES IN PARTIAL REPRODUCTIVE TISSUES OF SWINE WITH HIGH AND LOW LITTER SIZE*

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Abstract

To justify the function of miRNAs in reproductive regulation in swine, the expression of miR-145, miR-429 and their related genes were studied in reproductive tissues of sows. Wannan black pig and Yorkshire pigs with extremely high (n=6) and low (n=6) litter size were sampled, and real-time quantitative PCR (qPCR) was performed on tissue samples from ovaries, uterus, oviduct, hypothalamus, and pituitary. The results indicated that miR-145, miR-429, and zinc finger E-box binding homeobox 1 gene (ZEB1) were expressed significantly different in Wannan black pig and Yorkshire pigs. In pigs with different fecundity, miR-145 in the uterus was expressed significantly lower in pigs with high litter size, than in pigs with low litter size. The miR-429 expression in the oviduct and pituitary of pigs with high litter size was significantly higher compared with tissues sampled from pigs with low litter size. The ZEB1 expression in the pituitary was lower in pigs with high litter size in comparison to pigs with low litter size, while luteinizing hormone beta subunit (LHβ) showed the opposite pattern of expression. In conclusion, miR-145 and miR-429 were differently expressed in pigs with high and low litter size and might have a role in affecting litter size of sows.

Key words: litter size, miR-429, miR-145, swine, ZEB1

Reproductive traits, particularly litter size, are very important aspects for the economics of the modern pig-raising industry (Chidgey et al., 2015). Reproduction in

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pigs mainly depends on regulation of the hypothalamic-pituitary-gonadal axis, and it is affected by interactions between multiple genes and the environment (Deaver and Bryan, 1999; van Rensburg and Spencer, 2014). MicroRNAs are short non-coding RNAs (about 22 nucleotides long) that play important roles in reproduction and other physiological functions (Wienholds and Plasterk, 2005). Lei et al. (2011) found that a T/C mutation in miR-27a was associated with litter size, and probably could be used as a molecular marker for litter size in pig breeding program. Donadeu et al. (2012) reported that several different miRNAs, miR-224, miR-378, miR-383 and miR-21, were shown to be involved in regulating follicle development and ovulation.

Recent studies have highlighted the role of miR-145 and miR-429 in the biology of cancer, fat metabolism, and cell differentiation (Ye et al., 2015; Cristobal et al., 2014), as well as their regulatory functions in mammalian reproduction (Schauer et al., 2013). Porcine miR-145 is located on chromosome 2 and its sequence is homologous with that of other mammals, such as sheep and mice. Yan et al. (2012) indicated that miR-145 could inhibit mRNA and protein expression of activin A receptor IB (ACVRIB), and interfere with phosphorylation of Smad-2, which results in inhibition of granulosa cell proliferation, leading to disordered ovulation. The miR-429 belongs to the miR-200 family (Jimenez et al., 2015), zinc finger E-box binding homeobox 1 (ZEB1) is the target gene of miR-429 and is a transcriptional repressor with CACCT-binding ability (Sekido et al., 1994). In mouse, miR-429 and miR-200b are higher expressed in the pituitary gland, where they inhibit expression of the transcriptional repressor ZEB1. A knockout of these miRNAs inhibits luteinizing hormone (LH) synthesis by repressing transcription of luteinizing hormone beta subunit (LHβ), resulting in failure of ovulation (Hasuwa et al., 2013). Thus miR-145 and miR-429 may be one of the miRNAs that control the reproductive traits of pigs.

However, studies of miR-429 and miR-145 in pigs have mainly focused on their roles in growth, development of muscle, metabolism and differentiation of adipocytes (Li et al., 2011; Bai et al., 2014). So far the expression of miR-145 and miR-429 in reproductive regulation in pigs was rarely reported. Both Wannan black pig (a Chinese indigenous breed) and Yorkshire (a European commercial breed) pigs are known for their high fertility rate and high litter size (Wang et al., 2014). However, some sows of these two breeds will always have low litter size, which can seriously restrict productivity in a livestock breeding context. In this study, we sampled some reproductive tissues from Wannan black pig and Yorkshire pigs with extremely high and low litter size, and analyzed the expression of miR-145, miR-429 and target genes of miR-429 by using real-time quantitative PCR (qPCR). The aim of this study was to investigate the possible effects of these miRNAs and their target genes on litter size in swine.

Material and methods

Animals and tissue collection

This study was performed according to the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology,

China) and approved by the Institutional Animal Care and Use Committee of the College of Animal Science and Technology, Anhui Agricultural University, Hefei, China (permit No. AHAU20101025). The animals had *ad libitum* access to water and feed and were humanely sacrificed. Wannan black and Yorkshire pigs were obtained from the experimental pig farm at Anhui Agricultural University. We collected total litter sizes in Wannan black and Yorkshire pigs during three recent years. The average total litter size was 12.1 piglets per litter, the 10% lower tail probability was 7.1 piglets per litter and the 10% upper tail probability was 14.2 piglets per litter. Thus, we defined the sample pigs with a high litter size as those having more than 14.2 piglets per litter and pigs with a low litter size as those having fewer than 7.1 piglets per litter. Twelve healthy female pigs in diestrus phase were used in this study, forming two groups: the high litter size group (n=6) including three Wannan black and three Yorkshire pigs; and the low litter size group (n=6) including three Wannan black and three Yorkshire pigs (Table 1), representing pigs with high and low fecundity, respectively. The total number born (TNB) was used as an indication of the high and low litter sizes and based on 4 parities of sows aged 30 months. To reduce the effects of age and parity on litter size, as far as possible, pigs of similar age and parity were used both within and between groups. The ovaries, pituitary, hypothalamus, oviduct and uterus were rapidly harvested from the carcasses and immediately frozen in liquid nitrogen. All tissue samples were stored at −80°C prior to RNA extraction.

Table 1. The total number born of Wannan black and Yorkshire pigs with high and low litter sizes

Breed	Group	Number	TNB
Wannan black	high litter size	1	14.6±0.8
		2	14.5±0.4
		3	15.2±1.3
	low litter size	4	5.2±1.9
		5	4.0±0.4
		6	5.0 ± 1.6
Yorkshire	high litter size	1	16.5±1.8
		2	16.5 ± 1.8
		3	17.0±1.1
	low litter size	4	5.2±1.9
		5	5.0±0.7
		6	5.8±2.1

Values are means ± standard error. TNB represents the total number born and was based on 4 parities of sows aged 30 months.

The mRNA quantification

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration of the extracted RNA was measured using a NanoDrop OP-2100 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA), and RNA integrity was confirmed by denaturing agarose electrophoresis. One microgram of total RNA from each sample was reverse-transcribed into cDNA using the Prime Script™ RT Kit (TaKaRa, Tokyo, Japan) at 37°C for 15 min, 85°C for 5 s in a 10 μL reaction mixture, according to the manufacturer's

instructions. All gene sequences obtained were checked against those in the NCBI database, and the β-actin gene was used as an endogenous control gene. All primers used are listed in Table 2. The qPCR was performed using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, CA, USA). Thermal cycling conditions consisted of 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. Three replicates were used for each amplification.

Table 2. Primer sequences for genes and miRNAs

Primer name[1]	Sequence accession[2]	Primer sequences
ssc-miR-145-3p	MIMAT0022919	F-GGATTCCTGGAAATACTGTTCT
ssc-miR-145-5p	MIMAT0002123	F-GTCCAGTTTTCCCAGGAATCCCTT
ssc-miR-429	MIMAT0020591	F-TAATACTGTCTGGTAATGCCGT
LHβ	ENSSSCG00000003151	F-GCTCCAGAGACTGCTGTTGT R-GACAGGGCAAGCCTCATTCT
ZEB1	XM-003482813.2	F-AACAGTGTCCCGTGCTTGCG R-CCTTCCGTGCTGGTGTCTTGC
U6	ENSSSCT00000019750	F-GGCAAGGATGACACGCAAAT
β-Actin	XM-003124280.2	F-CTCTTCCAGCCCTCCTTCC R-GGTCCTTGCGGATGTCG

[1]The -3p and -5p are produced in 3' and 5' end sequences of one miRNA precursor sequence (pre-miR-145).
[2]Sequence accession numbers are obtained from the miRBase 21 (http://www.mirbase.org), GenBank (https://www.ncbi.nlm.nih.gov/genbank) and EMBL (http://www.ebi.ac.uk).

The miRNA quantification

For miRNAs, one microgram of total RNA from each sample was reverse-transcribed into cDNA using the NCode™ EXPRESS SYBR® GreenER™ miRNA qPCR Kit (Invitrogen, CA, USA) at 37°C for 1 h in a 20 μL reaction mixture, according to the manufacturer's instructions. With reference to miRBase 21.0 (http://www.mirbase.org/), we designed miRNA-specific forward primers by poly (A)-tailing of miR-145 and miR-429; the universal reverse primer was provided in the Invitrogen kit. Porcine U6 small nuclear RNA (U6) was used as an endogenous control gene in creating a fluorescence quantitative standard curve (Li et al., 2014). All primers used are listed in Table 2. We actually focused on the miR-145-3p and miR-145-5p that are produced in 3' and 5' end sequences of one precursor sequence of miR-145 (pre-miR-145). The qPCR was performed using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, CA, USA). Thermal cycling conditions consisted of 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. There were three replicates for each amplification.

Statistical analyses

Relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method with Microsoft Excel 2007. The $2^{-\Delta\Delta CT}$ method is a convenient way to analyze the relative changes in gene expression from qPCR experiments. With respect to the ΔΔCT of the $2^{-\Delta\Delta CT}$ method, the first ΔCT is the difference in threshold cycle between the target and reference genes. The ΔΔCT is the difference in ΔCT between the target and reference samples (Stephens and Moley, 2009).

All data were presented as the mean ± SEM. Significance tests were performed using the t-test in SPSS 22.0 for Windows (IBM, Chicago, IL, USA). The t-test is any statistical hypothesis test in which the test statistic follows a Student's t-distribution under the null hypothesis. It can be used to determine if two sets of data are significantly different from each other. The $P<0.05$ was considered as a significant difference between compared groups of animals, whereas $P<0.01$ was considered as a highly significant difference.

Results

Differential expression of miRNAs and genes between Wannan black and Yorkshire pigs

In the ovary and uterus tissue samples, the expression of miR-145-5p and miR-145-3p were lower in Wannan black pig than in Yorkshire (Figure 1 A, B). In the oviduct, miR-145-3p expression was higher in Wannan black pig than in Yorkshire ($P<0.01$) (Figure 1 B). In the uterus, miR-429 was expressed significantly more in Wannan black pig than in Yorkshire ($P<0.05$), but in the oviduct, expression of miR-429 was significantly lower in Wannan black pig than in Yorkshire ($P<0.05$) (Figure 1 C).

In the ovary, ZEB1 expression was significantly higher in Wannan black pig than in Yorkshire ($P<0.01$) (Figure 1 D). The expression of LHβ in all experimental tissues was not significantly different between the two breeds and it had highest expression in the pituitary (Figure 1 E).

Differential expression of miRNAs and genes in pigs with high and low litter size

In Yorkshire, miR-145-3p and miR-145-5p in the uterus were lower expressed in pigs with high litter size than in pigs with low litter size ($P<0.01$) (Figure 2 A, B). The miR-429 had higher expression in the oviduct and pituitary of pigs with high litter size, than in the same tissues in pigs with low litter size ($P<0.01$ and $P<0.05$, respectively) (Figure 2 C). In the pituitary, ZEB1 expression was significantly lower in pigs with high litter size than in pigs with low litter size ($P<0.01$) (Figure 2 D), while LHβ expression was significantly higher in pigs with high litter size than in pigs with low litter size ($P<0.01$) (Figure 2 E).

In Wannan black pig, miR-145-3p and miR-145-5p in the uterus were lower expressed in pigs with high litter size than in pigs with low litter size ($P<0.05$ and $P<0.01$, respectively) (Figure 3 A, B), and miR-145-5p in the oviduct was lower expressed in pigs with higher litter size than in pigs with lower litter size ($P<0.05$) (Figure 3 A). The miR-429 was higher expressed in the oviduct and pituitary of pigs with higher litter size than in pigs with low litter size ($P<0.01$) (Figure 3 C). In the pituitary, ZEB1 expression was lower in pigs with higher litter size than in pigs with low litter size ($P<0.01$) (Figure 3 D), while LHβ expression was significantly higher in pigs with high litter size than in pigs with low litter size ($P<0.01$) (Figure 3 E).

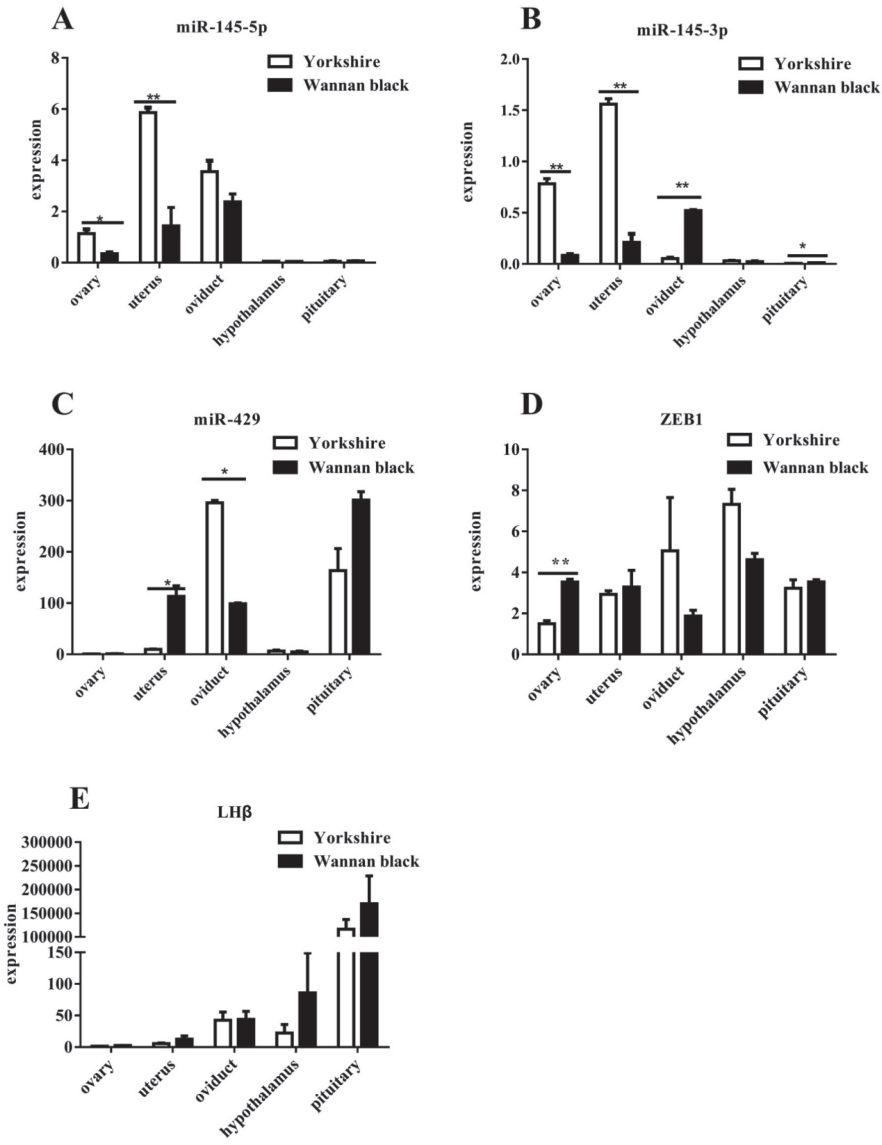

Figure 1. Differential expression of miRNAs and target genes in partial reproductive tissues of Wannan black and Yorkshire pigs. (A) miR-145-5p; (B) miR-145-3p; (C) miR-429; (D) ZEB1; (E) LHß. Significance level is indicated by either one asterisk ($P<0.05$) or two asterisks ($P<0.01$)

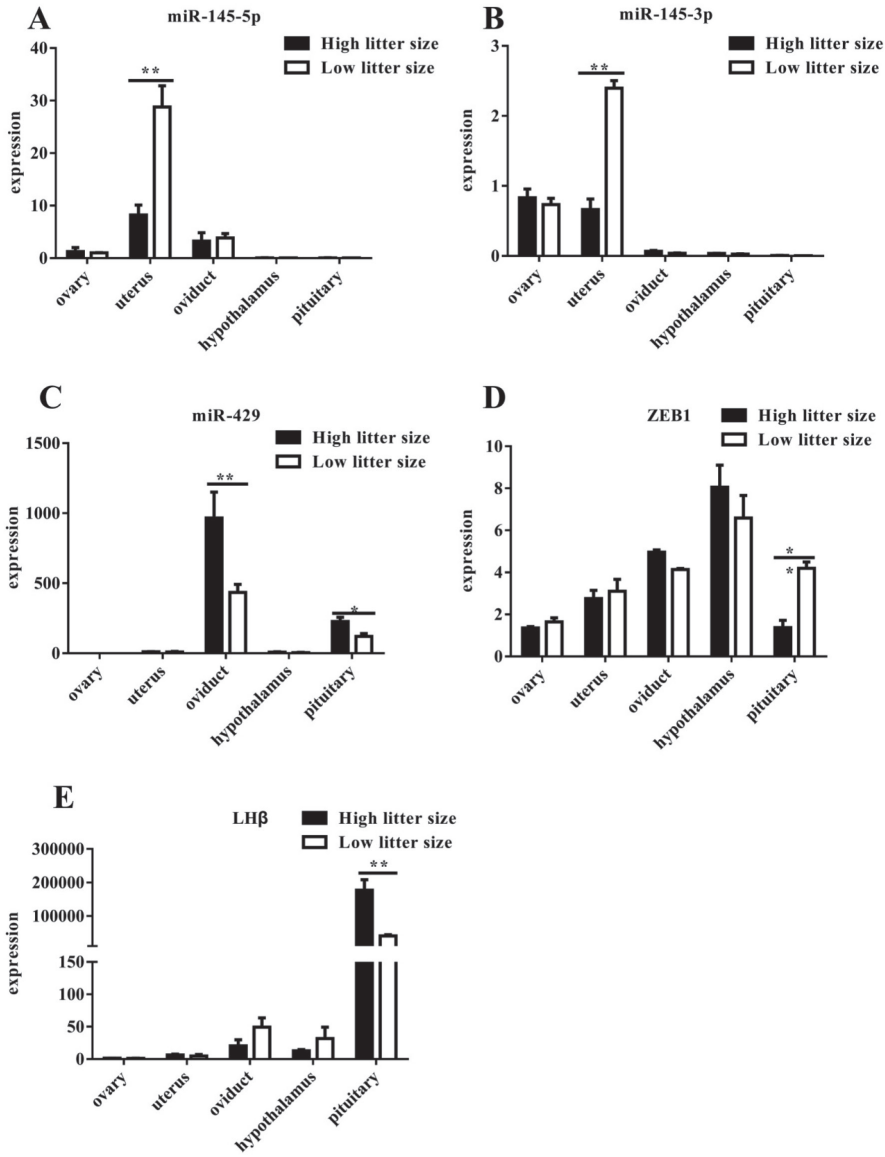

Figure 2. Differential expression of miRNAs and target genes in partial reproductive tissues of Yorkshire pigs with high and low litter size. (A) miR-145-5p; (B) miR-145-3p; (C) miR-429; (D) ZEB1; (E) LHß. Significance level is indicated by either one asterisk ($P<0.05$) or two asterisks ($P<0.01$)

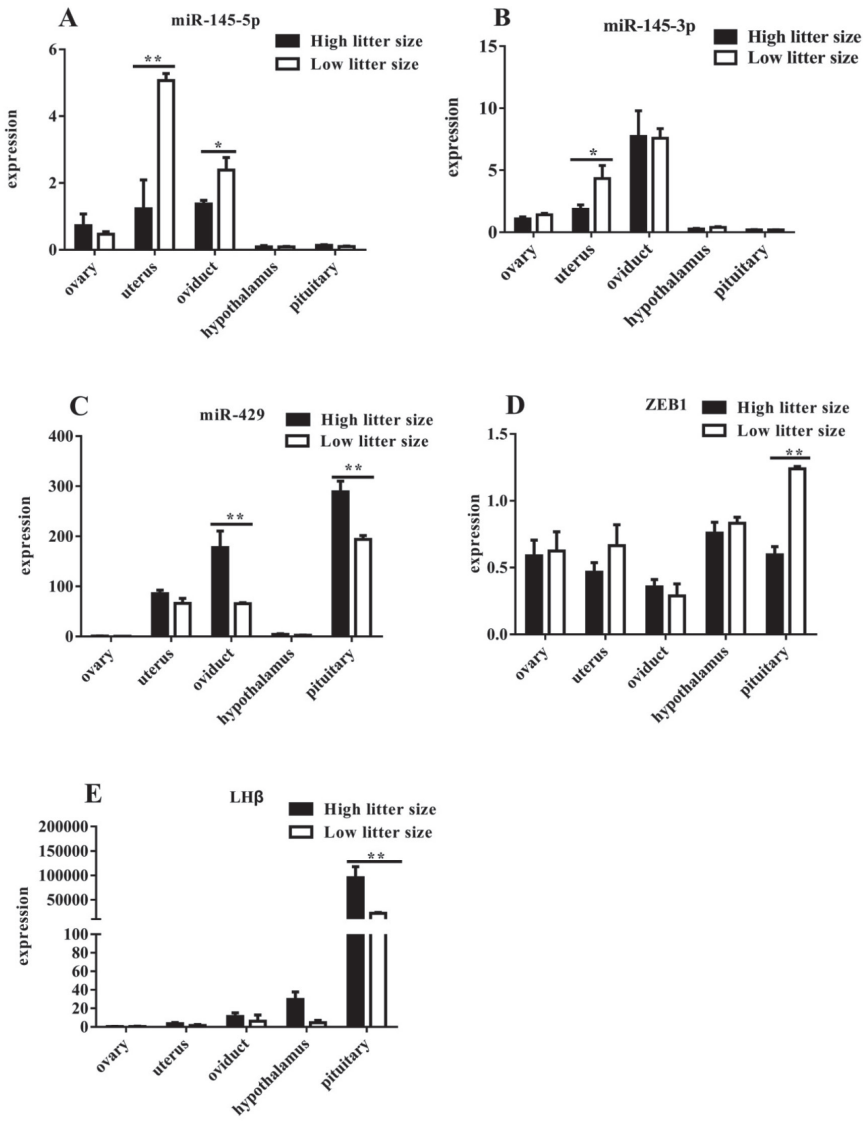

Figure 3. Differential expression of miRNAs and target genes in partial reproductive tissues of Wannan black pig with high and low litter size. (A) miR-145-5p; (B) miR-145-3p; (C) miR-429; (D) ZEB1; (E) LHß. Significance level is indicated by either one asterisk ($P<0.05$) or two asterisks ($P<0.01$)

Discussion

The uterus is a central organ to mammalian reproduction system, menstruation and foetal development (Stephens and Moley, 2009). Through our experimental results, it can be seen that the expression of miR-145 in the uterus was lower in pigs with higher litter size than in pigs with low litter size in both breeds. This suggests that the miR-145 participates in regulation of the uterine cycle and potentially in pathological changes, and the high expression of miR-145 in the uterus may have a negative impact on the litter size of sows. Some studies have pointed out that miR-145 is upregulated in the antiproliferative action of progesterone (P4) on endometrial epithelial cells (Yuan et al., 2015; Li et al., 2011). In a normal uterus, the synthesis of estradiol (E2) at every estrus or menstrual cycle results in the proliferation of endometrial epithelial cells. By contrast, P4 inhibits this estrogen-induced cell proliferation and stimulates epithelial differentiation in the preparation of embryonic implantation (Tong and Pollard, 1999). Therefore, the concentration of miR-145 in mammalian uterus is too high and it would undermine or disrupt the regulation of hormone feedback mechanism, and the uterus cannot provide a suitable environment for the preimplantation of embryonic development, blastocyst implantation, and pregnancy maintenance.

There are multiple CACCT-binding sites with porcine miR-429's seed sequence in the 3'-UTR of ZEB1 gene (Sekido et al., 1994). Also the LHβ 5'upstream region has multiple CACCT sites. In this study, the expression of miR-429 and LHβ in the pituitary was higher expressed in pigs with higher litter size compared to pigs with low litter size, in both breeds, whereas ZEB1 was weakly expressed in pigs with higher litter size. The results of this experiment are in agreement with the experimental results of Hasuwa et al. (2013) in mouse.

The LH is secreted by anterior pituitary basophils and promotes the development of male accessory sex glands and sperm maturation (Pillon et al., 2004), induces oocyte maturation, and plays a prominent role in embryonic development. The LH increases the number of ovulations during the preovulatory stage. The LH is composed of conjugated α and β subunits; its binding specificity is determined by the β subunits (Sherman et al., 2001). The LHβ is a key factor to control ovulation in mammalian menstrual cycle (Woo et al., 2015). In this study, miR-429 was highly expressed in the pituitary of pigs with higher litter size than in pigs with lower litter size. This suggests that miR-429 in the pituitary may have a positive impact on the litter size of sows. However, with the aim to investigate the possible effects of miR-429 and target genes on porcine litter size, further research needs to be undertaken with ovaries in oestrum or specific ovarian cells (e.g., theca cells and granulosa cells) to determine the miRNAs functions, both *in vitro* and *in vivo*.

Conclusion

In conclusion, this study investigated the differential expression of miR-145, miR-429, ZEB1 and LHβ in reproductive tissues of pigs with high and low litter size. We identified that miR-145 and miR-429 were differently expressed in pigs with high and low litter size and might have a role in affecting the litter size of sows.

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