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Original Article

Identification of candidate genes associated with Xiang pig estrus by genome-wide transcriptome analysis

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Abstract: Oestrus is one of the most important physiological processes especially in pig reproduction and breeding. Oestrus is affected by interactions between multiple genes and the environment. Although recent studies have identified some genes associated with prolificacy in pigs, transcriptomic pattern of specific genes affecting estrus in porcine is unclear. In order to identify candidate genes associated with estrus in swine, we assessed gene expression changes of the ovaries from Xiang pigs within estrus or no-estrus stage using the RNA-Seq method. A total of 432 differentially expressed genes were identified: 204 genes were upregulated and 228 genes were downregulated in oestrous ovary samples when compared with non-oestrous samples. A large number of these genes related to steroid hormone regulation in animal ovaries, including 51 Gene Ontology terms and top 20 Kyoto Encyclopedia of Genes and Genomes pathways involved in steroid biosynthesis and ovarian steroidogenesis. From these differentially expressed genes, we identified a total of 14 genes using a bioinformatics screen that may be associated with oestrus in Xiang pigs, which were *CYP51, EBP, TM7SF2, MSMO1, SQLE, LSS, DHCR24, FDFT1, HMGCS1, FDPS, MVK, IDI1, ACAT2*, and *ACAT1*. These results provide a list of new candidate genes for porcine prolificacy to be further investigated.

Keywords: Oestrus, production, Xiang pig, genome-wide transcriptome analysis

Introduction

As an important part of the national economy, the pig industry is crucial to China's livestock industry. And oestrus is one of the most important physiological processes in pig production and is affected by interactions between multiple genes and the environment. Oestrus is a behavioral performance, as well as an external and visible sign of ovulation. During each oestrus cycle, the ovary undergoes proliferation, differentiation and apoptosis. And these normal physiological changes directly affect and/or determine the ovulation, fertilization rate, the litter size and production of female animals. Detection and regulation of oestrus help us to better understand animal reproduction efficiency, human reproductive medicine and biochemical research. As the rapid development of swine industry, oestrus and ovulation play a crucial role in improving production efficiency. Therefore, the objective of this current study was to investigate the candidate genes related to swine oestrus, with the hope to increase the reproductive ability and create greater economic value.

There has been some recent progress in characterizing the major genes involved in the prolificacy of swine, such as the estrogen receptor (*ESR*) [1], follicle-stimulating hormone beta subunit (*FSH-β*) [2], retinol-binding protein 4 (*RBP4*) [3]. With the recent publication of the pig, more candidate genes or quantitative trait loci (QTLs) have been extensively investigated for their involvement in porcine production [4-6]; but genomic location, function and interaction of these genes requires further research. RNA sequencing (RNA-Seq) can measure genes, both quantitatively and functionally, at the transcriptome level [7]. Up to now, RNA-Seq has been used to study specific ovarian genes of cattle [8, 9], goat [10, 11] and pig [12], but transcriptomic changes of specific genes in pig oestrus are not clear. Therefore, transcriptomic analysis of pig ovaries using RNA-Seq may

Table 1. Primer sequences

Gene Name	Forward (5'-3')	Reverse (5'-3')
β-actin	TGCGTGACATTAAGGAGAAG	GCTCGTAGCTCTTCTCCA
CYP51	GGCTCTTACCAGGCTGGCTT	GTCTGCGTTTCTGGATTGCC
TM7SF2	CAACCCACGCATCTGTTCCT	GTTCTGCCTCCTGCATCAGC
EBP	ATCCTGGCTGGCCTCTTCTC	AGTCTCCGCCAAGTGCTCAG
MSMO1	GCACACCCTCTGGAAACCCT	ATCAAACGAACGGTCACCCA
SQLE	ACCTTTCTCGGCATTGCCAC	AGGAATACCAGCACGCCCAG
LSS	AAAGCCCTAGCCGAGAGCAG	ACGTCATCCCATTCAGAGCG
DHCR24	CTGCCTGTGTTGCCAGAGCT	CAGATGTGCTGGAACAGGCC
FDFT1	GGAATTGGCCTTTCCCGTCT	TTTTCTGCAGGAACAGGCCC
HMGCS1	TACGGCTGCCTTGCATCTGT	CAGAGTGGCAGCCAAACCAG
FDPS	GTTGCCCGACTCAAGGAGGT	CAATACACCAGCCCACGGTC
MVK	ATGCGAGGAGATCCCAAACC	CATGAATCACCCTCTCCCCC
IDI1	GCTTGTTGCAGCCATCCACT	GGAATGCCCAGTTCAGCCTT
ACAT2	AAACATGAGCAAGGCCCCTC	AACGCATCTGTCAGCCCATC
ACAT1	CCGTGCACCACGATAAACAA	ATGCTCTCCATTCCACCTGC

explain heredity of estrus, and be used to identify key genes relating to oestrus.

In this study, to identify the candidate genes that influence pig oestrous, we performed a comparative analysis of the whole transcriptomes of ovary between estrus group and non-estrus group. We identified a series of differentially expressed genes (DEGs) between these two groups, which represent potential candidate genes affecting pig oestrus traits. Using these data, we identified several important GO terms and metabolic pathways associated with pig oestrus. In conclusion, our data provides a solid foundation for identifying the critical genes whose functions affect pig oestrus, and can facilitate studies on the molecular regulatory mechanisms underlying animal oestrus, hoping to increase the yield of domesticated animals.

Materials and methods

Ethics statement

All experimental procedures and sample collection were performed according to the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China; revised in June 2004) and approved by the Biological Studies Animal Care and Use Committee of Guizhou Province, PR China. This report fully adhered to the ARRIVE Guidelines for the reporting of animal research [13]. The animals were fed in the same environment and given the same diet ad libitum during the experimental period. Food was not given to the animals on the night before they were slaughtered.

Animals and ovary collection

A total of six healthy female Xiang pigs used in this study were divided into two groups: the oestrous group (n=3) and the no-oestrous group (n=3). In order to reduce the effects of age on estrus, three pigs of similar age from each group were selected as biological replicates for RNA-Seq. The mean age of the oestrous group was 150±6.0 days, and mean age of the non-oestrous group was 152.0±3 days, which had no statistical difference and been comparable (P>0.05). Their intact ovaries were rapidly harvested from their carcasses and immediately frozen in liquid nitrogen. All tissue samples were stored at -80°C until the total RNA extraction was performed.

Oestrus detection

Oestrus detection was performed once a day. Standing oestrus was determined by the back-pressure test and the degrees of swelling and reddening of the vulva, according to Eliasson method (1989) [14]. The same oestrous detection procedure was performed twice daily in front of a boar [15]. A sow not showing signs of oestrus within 10 days after last ovulation was considered non-oestrous.

mRNA library preparation and sequencing

Total RNA was extracted from ovaries using TRIzol (Invitrogen, Carlsbad, CA, USA) and was purified using DNase I [16, 17]. The quality of the total RNA was checked using the Agilent 2100 Size Bioanalyzer system (Santa Clara, CA, USA). A total amount of 3 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) according to manufacturer's instructions. Briefly, mRNA was extracted from total RNA using oligo (dT) magnet-

Table 2. RNA sequencing results of mRNA from the ovaries of oestrous or non-oestrous groups of Xiang pigs

Sample ID[1]	N1	N2	N3	O1	O2	O3
Clean Reads	52218318	50004320	52994446	49981708	50375286	50368172
Total Base Pairs	7832747700	7500648000	7949166900	4498353720	4533775740	4533135480
Unique Match	26660588	24588262	23679778	32974828	32714413	33256352
Unique Match Rate	51.06%	49.17%	44.68%	65.97%	64.94%	66.03%
Total Mapped Reads	28480570	26308398	25665310	36502081	36259741	36774725
Total Mapped Reads Rate	54.54%	52.61%	48.43%	73.03%	71.98%	73.01%
Expressed Gene	15881	15453	16340	17504	17116	17151
Expressed Transcripts	17579	17134	18163	19669	19326	19295

1. N1, N2, N3 and O1, O2, O3 are replicate from the oestrous and non-oestrous groups.

Table 3. Correlations value between each two samples

Sample ID[1]	N1	N2	N3	O1	O2	O3
N1	1	0.6857844	0.989965	0.6872669	0.6797085	0.7620019
N2	0.6857844	1	0.6752062	0.8937058	0.856389	0.876933
N3	0.989965	0.6752062	1	0.6784261	0.6782016	0.7556047
O1	0.6872669	0.8937058	0.6784261	1	0.9466573	0.9523997
O2	0.6797085	0.856389	0.6782016	0.9466573	1	0.9613884
O3	0.7620019	0.876933	0.7556047	0.9523997	0.9613884	1

1. N1, N2, N3 and O1, O2, O3 are replicate from the oestrous and non-oestrous groups.

ic beads and sheared into short fragments of about 200 bases. These fragmented mRNAs were then used as templates for cDNA synthesis. The cDNAs were amplified by PCR method to complete the library. The cDNA library was sequenced using the Illumina HiSeq™ 2000 platform.

Analysis of RNA-Seq data

Raw RNA-Seq reads were processed through in-house perl scripts. Clean reads were obtained by removing reads containing low quality reads and/or adaptor sequences from raw reads [18], and mapped to the pig genome (*Sus scrofa* 11.1) using TopHat software [19] (Electronic, allowing up to two base mismatches. The exon expression level was then calculated using RSEM [20]. Differences in expression were determined using NOISeq. The results showed that genes with a probility value of ≥8, and a difference of 2 in FPKM value was designated as differently expressed gene to reduce the occurrence of false positives [21].

DEGs lists were submitted to the databases of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) for enrichment analysis of the significant over representation of GO terms and KEGG-pathway categories [22-24]. In all tests, P values were calculated using the Benjamini-corrected modified Fisher's exact test and <0.05 was taken as the difference significantly.

Quantitative PCR analysis

The total RNA from pig tissues was extracted with an RNApure Tissue Kit (CWBIO, China), according to the manufacturer's instructions, and reverse-transcribed into first strand cDNA using the HiFiScript 1st Strand cDNA Synthesis Kit (CWBIO, China). Quantitative PCR was performed with the TransStart Green qPCR SuperMix (Transgen, China) and tested by the DA7600 Real-time Nucleic Acid Amplification Fluorescence Detection System (Bio-Rad) in a 20 μL reaction system. β-actin was tested at the same time to normalize mRNA levels. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative mRNA expression changes. The thermal cycling conditions were 95°C for 10 min, followed by 39 cycles of 95°C for 15 s and 59°C for 1 min. The primers were synthesized by the Beijing Genomics Institute and are listed in **Table 1**.

Comparison of gene expression levels related to estrus was performed by using the student's

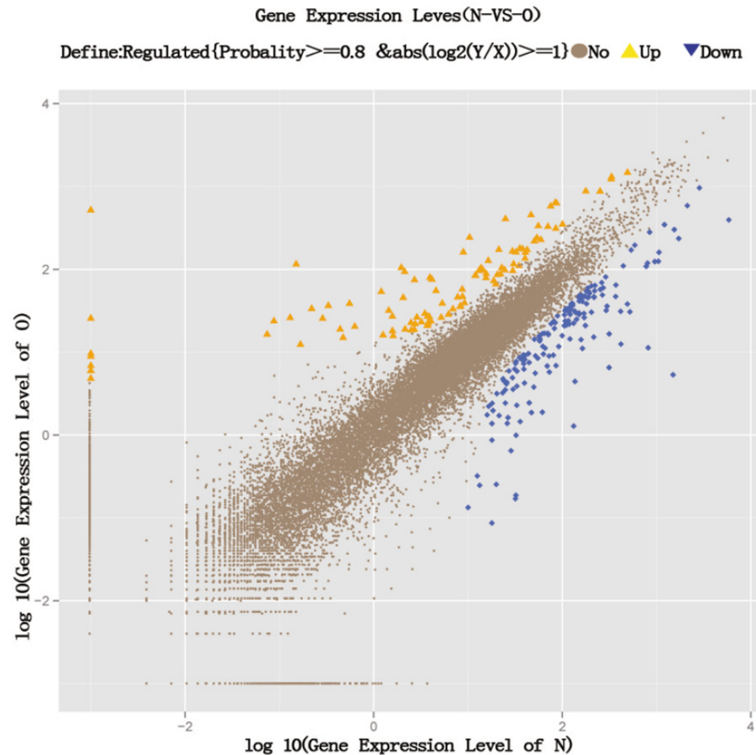

Figure 1. Genes expression level in oestrous and non-ostrous samples (O: oestrous group; N: non-ostrous group).

t test, and correlations between qPCR and RNA-Seq measures were calculated.

Results

Overview of sequencing data

After removing the low quality and adaptor sequences, we obtained approximately 49 to 52 million clean reads for six RNA-Seq libraries, and the percentages of mapped reads ranging from 48.43% to 73.03%, the unique match rate ranging from 44.68% to 66.03% (**Table 2**). These results indicated that our six libraries were of high quality, and had high coverage of the pig genome. This allowed us to compare the ovary transcriptomes from pigs with oestrous or not.

Differentially expressed genes between estrus and no-estrus groups

Subsequently, the present study detected the gene expression levels and identified the differentially expressed genes between oetrous and non-oetrous samples using the RSEM software package. RSEM maximum likelihood abundance was estimated by using the Expectation-Maximization (EM) algorithm of its statistical model, including the modeling of PE and variable-length reads, fragment length distributions and quality scores, to determine which transcripts were isoforms of the same gene. The FPKM method was used to determine the gene expression levels. The analysis contained the majority of the annotated pig genes. The correlation of the gene expression between two samples was evaluated. Results revealed that gene expression levels among two groups were highly correlated, suggesting that the experiments were reliable and the samples selection were reasonable (**Table 3**).

The number of expressed genes in each library was similar among libraries (15,453-17,504) (**Table 2**). A total of 432 genes were differentially expressed between the two groups, in which 204 genes were upregulated and 228 genes were downregulated in the oestrous group (**Figure 1**). The 14 most differentially down expressed genes related to the pigs' production from the total of 432 DEGs identified between the non-oestruos and oestrous samples were: cytochrome P450, family 51 (*CYP-51*), emopamil binding protein (*EBP*), transmembrane 7 superfamily member 2 (*TM7S-F2*), methylsterol monooxygenase 1 (*MSMO1*), squalene epoxidase (*SQLE*), lanosterol synthase (*LSS*), 24-dehydrocholesterol reductase (*DHCR24*), farnesyl-diphosphate farnesyltransferase 1 (*FDFT1*), 3-hydroxy-3-methylglutaryl-CoA synthase 1 (*HMGCS1*), farnesyl diphosphate synthase (*FDPS*), mevalonate kinase (*MVK*), isopentenyl-diphosphate delta isomerase 1 (*IDI1*), acetyl-CoA acetyltransferase 2 (*ACAT2*), acetyl-CoA acetyltransferase 1 (*AC-AT1*) (**Table 4**).

Functional enrichment analysis of differentially expressed genes

To define the biological functions of the 432 DEGs, GO and KEGG analysis were carried out. Fifty-one significantly enriched GO terms (cor-

Table 4. Detailed information on the 14 differentially expressed genes related to oestrus

Gene name	Readcount-N	Readcount-O	Probability	log_2FoldChang (O/N)	Up/down (O/N)	Description
CYP51	147.56	43.69	0.83	-1.76	Down	Cytochrome P450, family 51
EBP	183.17	32.85	0.87	-2.48	Down	Emopamil binding protein
TM7SF2	539.88	19.13	0.94	-4.81	Down	Transmembrane 7 superfamily member 2
MSMO1	312.48	71.83	0.86	-2.12	Down	Methylsterol monooxygenase 1
SQLE	73.06	23.91	0.80	-1.61	Down	Squalene epoxidase
LSS	108.44	17.88	0.87	-2.60	Down	Lanosterol synthase
DHCR24	138.19	31.90	0.85	-2.10	Down	24-dehydrocholesterol reductase
FDFT1	213.75	58.38	0.84	-1.87	Down	Farnesyl-diphosphate farnesyltransferase 1
HMGCS1	229.55	46.98	0.86	-2.28	Down	3-hydroxy-3-methylglutaryl-CoA synthase 1
FDPS	371.10	70.14	0.87	-1.87	Down	Farnesyl diphosphate synthase
MVK	90.66	6.85	0.89	-3.72	Down	Mevalonate kinase
IDI1	840.00	197.02	0.86	-2.09	Down	Isopentenyl-diphosphate delta isomerase 1
ACAT2	157.79	30.84	0.87	-2.35	Down	Acetyl-CoA acetyltransferase 2
ACAT1	146.23	45.69	0.82	-1.67	Down	Acetyl-CoA acetyltransferase 1

N: non-oestrous group; O: oestrous group.

rected $P<0.05$) were identified, including biological regulation, cellular component organization or biogenesis, cellular process, developmental process, regulation of biological process, metabolic process, negative regulation of biological process, positive regulation of biological process, reproduction, reproductive process (**Figure 2**). Meanwhile, 20 significantly enriched KEGG pathways were identified, including metabolic pathways, terpeniod backbone biosynthesis, PPAR signaling pathway, steroid biosynthesis, starch and sucrose metabolism, ribosome, fat digestion and absorption (**Figure 3**). Among these GO terms and KEGG pathways, the steroid biosynthesis, reproduction and reproductive process were the ones related to steroid hormone regulation in animal ovaries and therefore likely to be contributing to osterous. However, as most GO and KEGG assignments and distributions are related to reproduction, growth and development, and metabolism, our results indicate that the DEGs are involved in a wide range of regulatory functions in Xiang pig ovaries.

qRT-PCR validation

The transcript levels of 14 genes (*CYP51, EBP, TM7SF2, MSMO1, SQLE, LSS, DHCR24, FDFT1, HMGCS1, FDPS, MVK, IDI1, ACAT2*, and *ACAT1*) were re-evaluated using qRT-PCR technology. RNA from the same animals assigned to the RNA-Seq analysis was used for the qRT-PCR validation experiment (**Figure 4**). Overall, the results obtained from qRT-PCR were in agreement with the results of RNA-seq.

Discussion

Ovaries are one of the most important animal reproductive organs, which directly regulate ovulation and female hormone secretion and have a significant impact on the fecundity of mammals [25, 26]. Also, animals showing clear visible oestrous signs have high yield [27, 28]. But, until now, rare study investigated the oestrous candidate genes related to the production of Xiang pig. To the best of our knowledge, this is the first report focused on pig oestrous using RNA-seq.

It is well known that there is gene expression specificity in different tissues and cells. The ovaries contain a mixture of different tissue, and the expression of candidate genes may differ between them. We strove to ensure that we obtained intact ovaries and ground them completely for the purpose of RNA extraction, to ensure that the RNA-Seq results were representative of the complete Xiang pig ovarian transcriptome. In order to minimize the effect of age, we selected six pigs with similar age for RNA-Seq. Two sequencing libraries were constructed from the non-oestrous and oestrous samples. High quality transcriptome data was generated (about 52 million clean reads for each sample), which was sufficient for the quantitative analysis of gene expression.

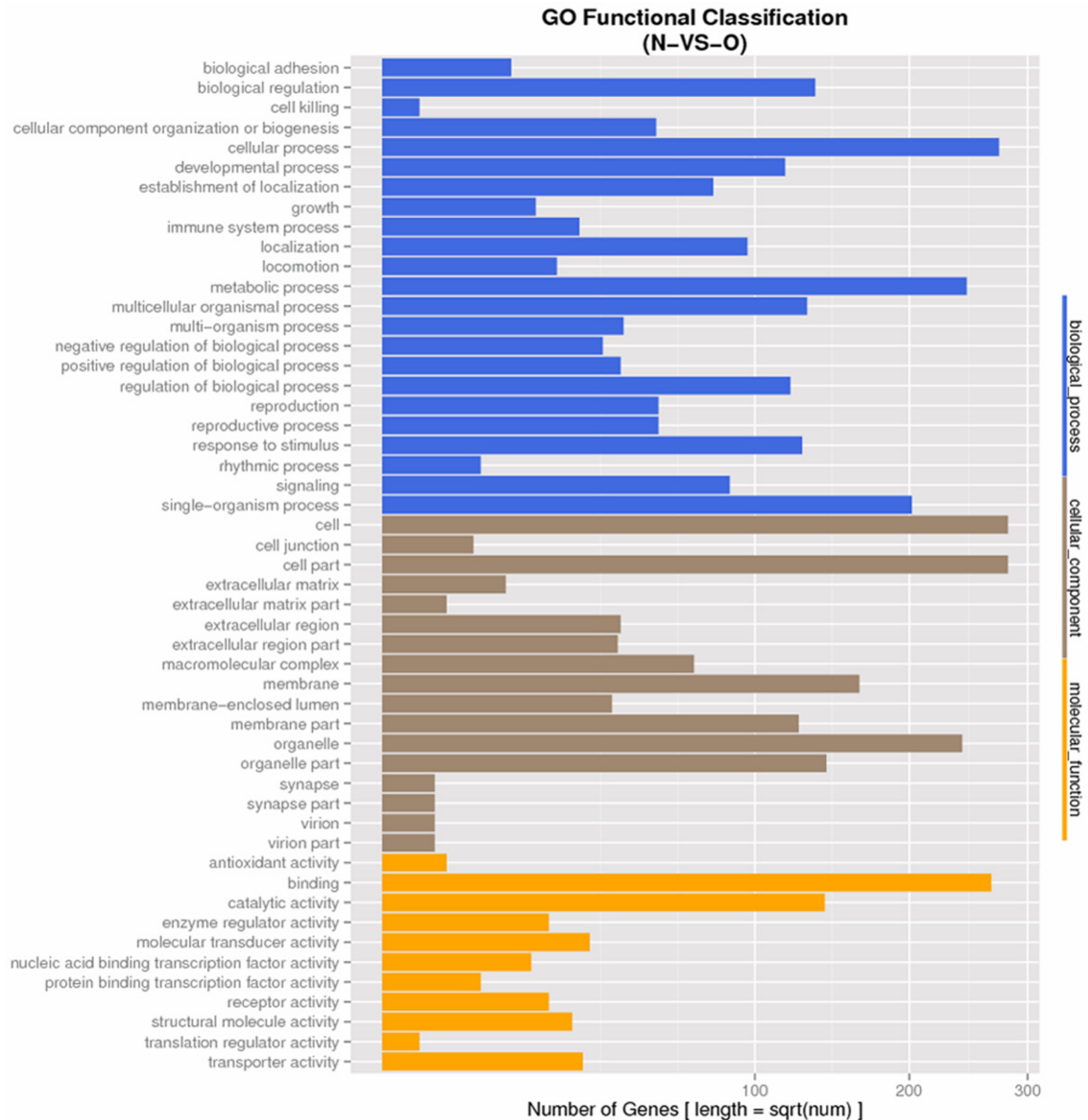

Figure 2. The most enriched top 51 Go terms.

The increased plasma concentrations of oestrogen and luteinizing hormone (LH), and the decreased concentration of progesterone before ovulation can initiate the occurrence of oestrous behavior [29]. Besides, enzymes in ovarian tissue, such as the cytochrome P450 (CYP) family, hydroxy steroid dehydrogenase and catechol-O-methyl-transferaseenzyme, directly catalyze the synthesis and metabolism of estrogen from cholesterol [30]. The expression of key steroidogenic enzymes responsible for the production of oestrogens - oestradiol (E2) and oestrone (E1) as well as testosterone (*T*), namely *CYP17A1* and *CYP19A3* was noted in the porcine uterus during early pregnancy and the oestrous cycle [31, 32]. And in the study, we found 14 candidate genes related to the oestrus of swine: *CYP51, EBP, TM7SF2, MSMO1, SQLE, LSS, DHCR24, FDFT1, HMGCS1, FDPS, MVK, IDI1, ACAT2*, and *ACAT1*. *CYP51* is a member of CYP family, just as descripted, can catalyze the synthesis and metabolism of oestrogen. *TM7SF2* gene has been reported to be involved in cholesterol biosynthesis by encoding the protein 3β-hydroxysterol Δ14-reductase [33], indicating it may involve in the

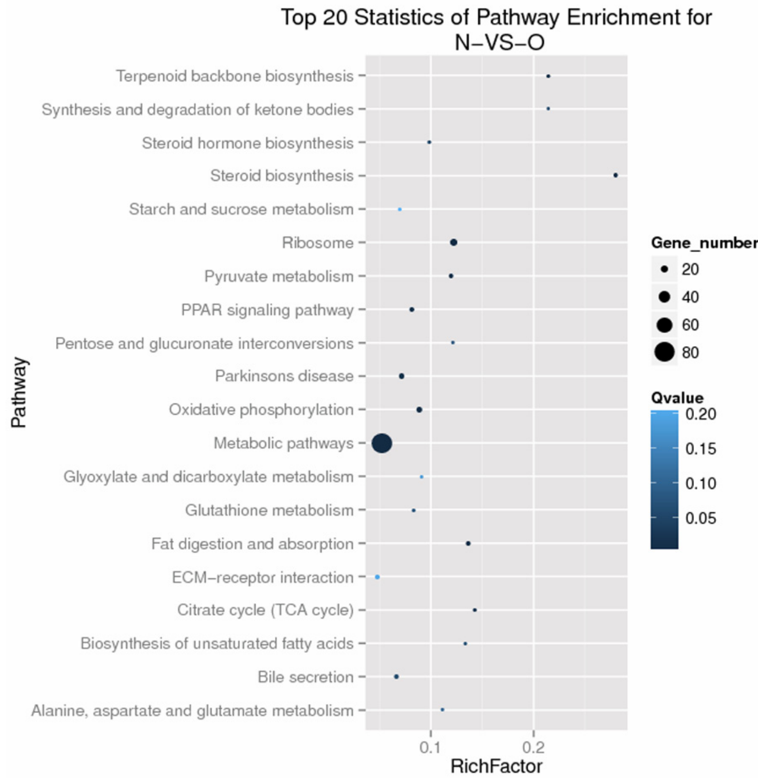

Figure 3. The most enriched 20 pathways. (The size of black circle represents the DEGs number).

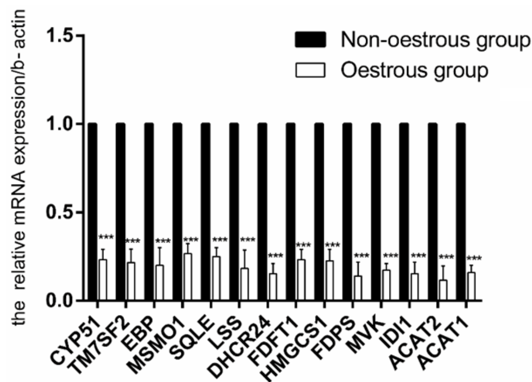

Figure 4. The expression levels of genes related to oestrus were quantified using qRT-PCR. The data are expressed as means ± SD. Statistical significance was calculated using one-way repeated-measures analysis of variance (n=9, per group). ***P<0.001.

process of oestrus. Besides, *SQLE* is also reported to regulate cholesterol synthesis [34], *LSS* has relationship with oestrogen [35] and so on.

To experimentally validate the DEGs identified from the sequencing data, we analyzed 14 DEGs using qPCR. The results confirmed that the expression patterns of these DEGs were consistent with those obtained from transcriptome sequencing data (**Figure 4**). These results indicate that the DEGs identified in the genome-wide transcriptome sequencing data are reliable.

We also performed GO enrichment analysis of DEGs from the DEGseq comparisons N (non-oestroud group) *vs*. O (oestrous group). Notably, from the GO and KEGG analysis, we found that the functions of DEGs between the two groups were mainly including biological regulation, cellular component organization or biogenesis, cellular process, developmental process, regulation of biological process, metabolic process, negative regulation of biological process, positive regulation of biological process, reproduction, reproductive process, metabolic pathways, terpeniod backbone biosynthesis, PPAR signaling pathway, steroid biosynthesis, starch and sucrose metabolism, ribosome, fat digestion and absorption. It is accepted that the molecular regulation of animal traits is very complex and the relationship between genes and traits is often that of "one-to-many" or "many-to-one" [36]. The DEGs were not only enriched in reproduction-related pathways but also in those involved with steroid biosynthesis and fatty metabolism. This suggests that the genes may be associated with both reproduction and fat metabolism. The synthesis of steroid hormones is closely related to fat metabolism. It has been demonstrated that all of steroid hormones are synthesized from cholesterol [37]. The pathways mentioned above are, to a greater or lesser degree, involved in the development of follicular cells and oocytes. Consequently, functional studies should be performed with these DGEs in order to identify key candidate genes influencing reproductive traits in swine.

In conclusion, this study screened for DEGs in the pigs' ovarian tissues with oestrous or non-oestrous character using RNA-Seq. We identi-

fied 204 genes that were upregulated and 228 genes that were downregulated in the oestrous samples compared with the non-oestrous group. After analyzing the function of these genes, we found 14 DEGs that maybe relevant to the prolificacy of pigs and verified these genes by qPCR technology. This new information provides a solid foundation for further studies of the molecular mechanisms underlying porcine prolificacy. In the future, biochemical and physiological analyses of these candidate genes will be conducted.

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Disclosure of conflict of interest

None.

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References

[1] Munoz G, Ovilo C, Estelle J, Silio L, Fernandez A and Rodriguez C. Association with litter size of new polymorphisms on ESR1 and ESR2 genes in a Chinese-European pig line. Genet Sel Evol 2007; 39: 195-206.

[2] Zhao Y, Li N, Xiao L, Cao G, Chen Y, Zhang S, Wu C, Zhang J, Sun S and Xu X. FSHB subunit gene is associated with major gene controlling litter size in commercial pig breeds. Sci China C Life Sci 1998; 41: 664-668.

[3] Sun YX, Zeng YQ, Tang H, Fan XZ, Chen QM, Li H, Qian Y and Song YP. [Relationship of genetic polymorphism of PRLR and RBP4 genes with litter size traits in pig]. Yi Chuan 2009; 31: 63-68.

[4] Martinez-Montes AM, Fernandez A, Perez-Montarelo D, Alves E, Benitez RM, Nunez Y, Ovilo C, Ibanez-Escriche N, Folch JM and Fernandez AI. Using RNA-Seq SNP data to reveal potential causal mutations related to pig production traits and RNA editing. Anim Genet 2017; 48: 151-165.

[5] Rohrer GA, Wise TH and Ford JJ. Deciphering the pig genome to understand gamete production. Soc Reprod Fertil Suppl 2006; 62: 293-301.

[6] Reiner G. [Product quality and genome analysis in pig production-a review]. Dtsch Tierarztl Wochenschr 2006; 113: 65-69.

[7] Marioni JC, Mason CE, Mane SM, Stephens M and Gilad Y. RNA-seq: an assessment of technical reproducibility and comparison with gene expression arrays. Genome Res 2008; 18: 1509-1517.

[8] Gonella-Diaza AM, Andrade SC, Sponchiado M, Pugliesi G, Mesquita FS, Van Hoeck V, Strefezzi Rde F, Gasparin GR, Coutinho LL and Binelli M. Size of the ovulatory follicle dictates spatial differences in the oviductal transcriptome in cattle. PLoS One 2015; 10: e0145321.

[9] McGettigan PA, Browne JA, Carrington SD, Crowe MA, Fair T, Forde N, Loftus BJ, Lohan A, Lonergan P, Pluta K, Mamo S, Murphy A, Roche J, Walsh SW, Creevey CJ, Earley B, Keady S, Kenny DA, Matthews D, McCabe M, Morris D, O'Loughlin A, Waters S, Diskin MG and Evans AC. Fertility and genomics: comparison of gene expression in contrasting reproductive tissues of female cattle. Reprod Fertil Dev 2016; 28: 11-24.

[10] Zi XD, Lu JY and Ma L. Identification and comparative analysis of the ovarian microRNAs of prolific and non-prolific goats during the follicular phase using high-throughput sequencing. Sci Rep 2017; 7: 1921.

[11] Ling YH, Quan Q, Xiang H, Zhu L, Chu MX, Zhang XR and Han CY. Expression profiles of differentially expressed genes affecting fecundity in goat ovarian tissues. Genet Mol Res 2015; 14: 18743-18752.

[12] Zhang X, Huang L, Wu T, Feng Y, Ding Y, Ye P and Yin Z. Transcriptomic analysis of ovaries from pigs with high and low litter size. PLoS One 2015; 10: e0139514.

[13] Kilkenny C, Browne WJ, Cuthi I, Emerson M and Altman DG. Improving bioscience research reporting: the arrive guidelines for reporting animal research. Vet Clin Pathol 2012; 41: 27-31.

[14] Eliasson L. A study on puberty and oestrus in gilts. Zentralbl Veterinarmed A 1989; 36: 46-54.

[15] Hulten F, Wallenbeck A and Rydhmer L. Ovarian activity and oestrous signs among group-housed, lactating sows: influence of behaviour, environment and production. Reprod Domest Anim 2006; 41: 448-454.
[16] Li X, Ye Y, Zhou X, Huang C and Wu M. Atg7 enhances host defense against infection via downregulation of superoxide but upregulation of nitric oxide. J Immunol 2015; 194: 1112-1121.
[17] Li X, Zhou X, Ye Y, Li Y, Li J, Privratsky B, Wu E, Gao H, Huang C and Wu M. Lyn regulates inflammatory responses in Klebsiella pneumoniae infection via the p38/NF-kappaB pathway. Eur J Immunol 2014; 44: 763-773.
[18] Kerpedjiev P, Frellsen J, Lindgreen S and Krogh A. Adaptable probabilistic mapping of short reads using position specific scoring matrices. BMC Bioinformatics 2014; 15: 100.
[19] Kim D and Salzberg SL. TopHat-Fusion: an algorithm for discovery of novel fusion transcripts. Genome Biol 2011; 12: R72.
[20] Mortazavi A, Williams BA, McCue K, Schaeffer L and Wold B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat Methods 2008; 5: 621-628.
[21] Wang Z, Gerstein M and Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet 2009; 10: 57-63.
[22] Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM and Sherlock G. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 2000; 25: 25-29.
[23] Kanehisa M, Araki M, Goto S, Hattori M, Hirakawa M, Itoh M, Katayama T, Kawashima S, Okuda S, Tokimatsu T and Yamanishi Y. KEGG for linking genomes to life and the environment. Nucleic Acids Res 2008; 36: D480-484.
[24] Mao X, Cai T, Olyarchuk JG and Wei L. Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary. Bioinformatics 2005; 21: 3787-3793.
[25] Weiner S, Wright KH and Wallach EE. Studies on the function of the denervated rabbit ovary: human chorionic gonadotropin-induced ovulation. Fertil Steril 1975; 26: 363-368.
[26] Balen A. The effects of ovulation induction with gonadotrophins on the ovary and uterus and implications for assisted reproduction. Hum Reprod 1995; 10: 2233-2237.
[27] Gerritsen R, Soede NM, Hazeleger W, Langendijk P, Dieleman SJ, Taverne MA and Kemp B. Intermittent suckling enables estrus and pregnancy during lactation in sows: effects of stage of lactation and lactation during early pregnancy. Theriogenology 2009; 71: 432-440.
[28] Patterson J, Wellen A, Hahn M, Pasternak A, Lowe J, DeHaas S, Kraus D, Williams N and Foxcroft G. Responses to delayed estrus after weaning in sows using oral progestagen treatment. J Anim Sci 2008; 86: 1996-2004.
[29] Soede NM, Langendijk P and Kemp B. Reproductive cycles in pigs. Anim Reprod Sci 2011; 124: 251-258.
[30] Low YL, Li Y, Humphreys K, Thalamuthu A, Darabi H, Wedren S, Bonnard C, Czene K, Iles MM, Heikkinen T, Aittomaki K, Blomqvist C, Nevanlinna H, Hall P, Liu ET and Liu J. Multi-variant pathway association analysis reveals the importance of genetic determinants of estrogen metabolism in breast and endometrial cancer susceptibility. PLoS Genet 2010; 6: e1001012.
[31] Chen X, Li A, Chen W, Wei J, Fu J and Wang A. Differential gene expression in uterine endometrium during implantation in pigs. Biol Reprod 2015; 92: 52.
[32] Kiezun M, Smolinska N, Dobrzyn K, Szeszko K, Rytelewska E and Kaminski T. The effect of orexin A on CYP17A1 and CYP19A3 expression and on oestradiol, oestrone and testosterone secretion in the porcine uterus during early pregnancy and the oestrous cycle. Theriogenology 2017; 90: 129-140.
[33] Zuleger N, Boyle S, Kelly DA, de las Heras JI, Lazou V, Korfali N, Batrakou DG, Randles KN, Morris GE, Harrison DJ, Bickmore WA and Schirmer EC. Specific nuclear envelope transmembrane proteins can promote the location of chromosomes to and from the nuclear periphery. Genome Biol 2013; 14: R14.
[34] Howe V, Sharpe LJ, Prabhu AV and Brown AJ. New insights into cellular cholesterol acquisition: promoter analysis of human HMGCR and SQLE, two key control enzymes in cholesterol synthesis. Biochim Biophys Acta 2017; 1862: 647-657.
[35] Muth-Kohne E, Westphal-Settele K, Bruckner J, Konradi S, Schiller V, Schafers C, Teigeler M and Fenske M. Linking the response of endocrine regulated genes to adverse effects on sex differentiation improves comprehension of aromatase inhibition in a fish sexual development test. Aquat Toxicol 2016; 176: 116-127.
[36] Guo ZL, Zhu Y, Su XT, Liu J, Yang QX, Nan JY, Zhao BC, Zhang YY, Yu YN, Li B, Xiao HB and Wang Z. DanHong injection dose-dependently varies amino acid metabolites and metabolic pathways in the treatment of rats with cerebral ischemia. Acta Pharmacol Sin 2015; 36: 748-757.
[37] Mouzat K, Baron S, Marceau G, Caira F, Sapin V, Volle DH, Lumbroso S, Lobaccaro JM. Emerging roles for LXRs and LRH-1 in female reproduction. Mol Cell Endocrinol 2013; 368: 47-58.