Transcriptome Analysis Reveals that Long Noncoding RNAs Contribute to Development Differences of Medium-sized Ovarian Follicle between Meishan and Duroc Sows

Mengxun Li
Shihezi University

Yi Liu
Shihezi University

Lipeng Ma
Shihezi University

Zhichao Zhao
Shihezi University

Su Xie
Shihezi University

Hongbin Gong
Shihezi University

Yishan Sun
Shihezi University

Tao Huang (✉ 2009tao@shzu.edu.cn)
College of Animal Science and Technology, Shihezi University

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Abstract

Background: Ovulation rate is an extremely important factor of litter size in sows. It differs greatly among pig breeds of different genetic backgrounds. Long non-coding RNAs (lncRNAs) can regulate follicle development, granulosa cell (GC) growth and hormone secretion, which in turn affects sow litter size.

Results: In our research, we identified 3554 lncRNAs and 25491 mRNAs in M2 follicle from Meishan and Duroc pigs. lncRNAs sequence and open reading frame (ORF) length is shorter than mRNAs, and it has fewer exons, lower abundance and conserved than protein-coding RNAs. Furthermore, 201 lncRNAs were differentially expressed in breeds, and quantitative trait loci (QTL) analysis of differential expression (DE) lncRNAs were performed. 127 DE lncRNAs are located in 119 reproduction trait-related loci. In addition, the lncRNAs potential target genes (PTGs) in cis or trans were predicted. Gene ontology (GO) and KEGG pathway analysis revealed that some PTGs were include some follicular development and hormone secretion-related biological processes or pathways, such as regulation of progesterone biosynthetic process, oestrogen metabolic process and ovarian steroidogenesis and PI3K-Akt signalling pathway. Furthermore, we also screened 19 differentially expressed lncRNAs of PI3K-Akt signalling pathway as candidates.

Conclusions: This study provided a new significance on the roles of lncRNAs in follicular growth and development and porcine reproduction.

Background

Ovulation rate is an important limiting factor of litter size in pig reproductive performance[1]. Meishan sows ovulate 25–29 eggs per oestrus, whereas Duroc sows ovulate only 12.3[2]. The difference in ovulation rates causes the difference in litter size between the two breeds. Therefore, these two breeds can be compared as prolific and ordinary sows.

Ovarian follicles are the basic functional unit in oocyte development, and they are round, vesicular and located in the ovarian cortex. Many studies on the regulating mechanisms of animal follicle development focus on protein-coding RNA and microRNA (miRNA) in molecular genetic level. Lee et al. found that retinoblastoma (Rb) protein was closely related to the growth state of follicles; the expression of Rb protein was significantly reduced, and follicles underwent rapid atresia[3]. The growth differentiation factor-9 (GDF-9) gene promoted the follicles growth and differentiation, knocking GDF-9 out caused infertility[4]. Inhibition of miR-145 expression reduced the primordial follicles proportion or number and increased growing follicles numbers[5]. MiR-92a inhibits the apoptosis of follicular granulosa cells by binding to its target gene drosophila mothers against decapentaplegic 7 (Smad 7)[6]. However, studies on the effect of lncRNAs on follicle growth and development are still few.

In recent years, numerous lncRNAs have been identified, and some of it have important functions in different biological processes, such as regulation on gene expression [7], genomic imprinting[8] and individual growth regulation[9, 10]. However, lncRNAs identified in pig are relatively few compared with those identified in humans and mice[11, 12], and lncRNAs that affect follicle development in pig are yet to be elucidated.

In this research, RNA samples from medium follicles (M2) of Duroc and Meishan sows were used to perform in high-throughput RNA-Seq. Plenty of lncRNA and mRNA transcripts were identified: differentially expressed lncRNAs and mRNAs between the Meishan and Duroc sows were detected. GO and KEGG analysis were carried in the potential target genes (PTGs) of lncRNAs. Some PTGs significantly take part in the follicle growth-related biological processes and signalling pathways. Combined with the results of DE genes analysis, we found that most of the DE lncRNAs can regulate the expression of their PTGs positively. This study gives the way to future research to explore the functions of specific lncRNAs that may be involved in follicular growth and development.

Results

Overview of RNA sequencing

We analysed the RNA data of six libraries of M2 follicles from three Meishan and three Duroc pigs, and obtained 111,275,282 to 142,313,048 raw reads and 101,760,644 to 138,324,398 clean reads from per sample, respectively. The clean reads were used in the after discarding transcripts with adapters, the low quality reads or other possible contaminants. Tophat2 software was used to perform reference genomic alignment analysis on Clean reads. The results showed that the six sequencing libraries’ mapping ratio were all greater than 70%, indicating that the sequencing accuracy is high and can be used for subsequent analysis (Table 1).
Table 1
The six library simples information.

| Sample name | Raw reads | Clean reads | Clean bases | Mapped Reads | Mapping Ratio | Error rate(%) | Q20(%) | Q30(%) | GC content(%) |
|-------------|-----------|-------------|-------------|--------------|---------------|---------------|--------|--------|---------------|
| MFM2DY4_1   | 111656928 | 107238580   | 16.09G      | 80514662     | 75.08%        | 0.02          | 96.59  | 91.73  | 47.14         |
| MFM2DY4_2   | 121457850 | 116315790   | 17.45G      | 88556504     | 76.13%        | 0.02          | 96.78  | 92.03  | 52.18         |
| MFM2DY4_3   | 111275282 | 106746570   | 16.01G      | 82037293     | 76.85%        | 0.02          | 96.75  | 91.96  | 50.09         |
| DFM2DY4_1   | 118575510 | 111359682   | 16.7G       | 92421276     | 82.99%        | 0.02          | 96.51  | 91.79  | 56.43         |
| DFM2DY4_2   | 107494708 | 101760644   | 15.26G      | 82733818     | 81.3%         | 0.02          | 96.70  | 92.14  | 49.84         |
| DFM2DY4_3   | 142313048 | 138324398   | 20.75G      | 115239405    | 83.3%         | 0.02          | 96.52  | 91.55  | 53.96         |

Identification and characterisation of IncRNA

All transcripts were screened in five steps. Finally, 3,554 IncRNAs were obtained, including 1,997 known IncRNAs and 1,557 novel IncRNAs (Figures 1A and B). Among the IncRNAs, IncRNA and antisense- IncRNA accounted for 89.3% and 10.7%, respectively (Figure 1C). In addition, we obtained 25,491 mRNAs. The lengths of IncRNA sequences were shorter than those of mRNAs. The IncRNA transcripts average length was 1417 bp, while the mRNAs was 2423 bp (Figure 1D). IncRNAs had fewer exons. The exon number of IncRNAs ranged from 1 to 10, whereas that of mRNAs from 1 to 30 (Figure 1E). The IncRNA have shorter ORF than mRNA. The ORF lengths of IncRNAs mostly ranged from 1 bp to 500 bp, whereas those of the mRNAs were mostly from 1 bp to 2000 bp (Figure 1F). The expression abundance of IncRNA were lower than mRNA (Figure 1G). The IncRNA were less conserved compared with the protein coding genes, as revealed through a phastCon analysis (Figure 1H). The structural feature comparative analysis of selected IncRNAs confirmed the accuracy of selection.

DE Analysis of IncRNAs and mRNAs

We use cufflinks to normalize transcript expression to FPKM value, and performed a differential expression transcript analysis between the Meishan and Duroc medium follicle samples. A total of 201 differentially expressed IncRNAs (DEIs) [p value<0.05 and |log10(foldchange)| > 1.3] between the two breeds were detected, in which 117 DEIs were downregulated, 84 were upregulated in Meishan follicles (Figure 2 A). Moreover, 675 DE protein-coding genes were detected, and 265 DEGs were upregulated and 410 were downregulated in Meishan follicles (Figure 2 B). DE IncRNAs and mRNAs were widely distributed on chromosomes, with larger numbers on chromosomes 1, 6 and 13 (Figures 2 A and B). To verify the accuracy of sequencing, 6 DE-IncRNAs (ENSSSCT00000018610, ALDBSSCT0000001721, ALDBSSCT0000000051, LNC_000116, ALDBSSCT0000011300 and ALDBSSCT0000006152) and 6 DE-mRNAs (COL3A1, LRP8, ENSSSCT00000009222, SEPP1, COL5A2 and CYP19A1) were selected randomly, and their relative expression levels in the DFM2DAY4 and MFM2DAY4 groups were detected using qPCR. The expression analysis of the six lncRNA and six mRNA are displayed in the Figure 3, which are consistent with the RNA-Seq analysis results both in IncRNA and mRNA (Figure 3).

QTL Analysis of DE IncRNAs

To explore the preliminary DELs function, we mapped the differential expressions(DE) of IncRNAs transcripts to the QTL database, and performed QTL analysis. The result showed that a total of 1446 QTLs that 145 DELs were located in, the 127 DE IncRNAs are located in 119 reproduction trait-related loci (Figure 4A). By studying the distribution of QTLs on the chromosome, 119 QTLs related to reproduction deposition were found to be distributed in chromosomes 1, 2, 3, 4, 7, 8, 13, 15 and 18 (Figure 4B). The 127 IncRNAs associated with reproduction QTLs could affect corpus luteum number (53), litter size (18), androstosterone (23), total number born alive (4), number of stillborn (3), FSH concentration (3) and number of viable embryos (2) (Figure 4C).

Prediction of IncRNA potential target genes

In order to further explore the regulatory functions of DE IncRNAs, we predicted their PTGs of IncRNAs in 2 ways-cis and trans[13, 14]. In our study, we found that IncRNA may regulate multiple coding genes. For PTGs regulated by IncRNAs in cis, we searched protein-coding transcripts located in 100 kb upstream or downstream of the DELs as its cis-regulatory target genes. We obtained a total of 320 co-localised
target genes of 118 DELs. In trans way analysis, we obtained 2930 PTGs of 127 DELs. We only showed the PTGs of 24 DELs. The PTGs numbers for each DEL were varied. For example, the maximum number among the IncRNA is LNC_000179, and it had 77 PTGs, the second is LNC_000715 with 69 target genes. Some IncRNAs, such as LNC_000718 and LNC_000802, only had 2 target genes (Table 2).

Table 2
Differentially expressed IncRNAs (DELs) and their target genes (PTGs).

| Up DELs                  | Numbers of PTGs |                   | Down DELs | Numbers of PTGs |                   |
|-------------------------|-----------------|------------------|-----------|-----------------|------------------|
|                         | PTGs            | Up Regulated     | Down Regulated | PTGs          | Up Regulated     | Down Regulated |
| ALDBSSCT0000002624      | 4               | 4                | 0          | LNC_000179      | 77               | 0               | 77          |
| LNC_000811              | 15              | 13               | 2          | LNC_000715      | 69               | 68              | 1           |
| ALDBSSCT0000004325      | 21              | 18               | 3          | LNC_001511      | 29               | 1               | 28          |
| LNC_001167              | 48              | 48               | 0          | LNC_000846      | 8                | 2               | 6           |
| LNC_000252              | 36              | 35               | 1          | LNC_001333      | 5                | 1               | 4           |
| ALDBSSCT0000009309      | 26              | 26               | 0          | LNC_000802      | 2                | 0               | 2           |
| LNC_001490              | 26              | 10               | 16         | ALDBSSCT0000010765 | 29               | 2               | 27          |
| LNC_000718              | 2               | 2                | 0          | ALDBSSCT0000010764 | 17               | 0               | 17          |
| LNC_000460              | 20              | 19               | 1          | LNC_000116      | 49               | 7               | 42          |
| LNC_001307              | 17              | 10               | 7          | ALDBSSCT000001721 | 11               | 0               | 11          |

Function Enrichment Analysis for IncRNAs

To study the regulatory role of DE IncRNAs in Meishan and Duroc M2 follicles, we predicted the function of DE target genes by using GO and KEGG to speculate the IncRNA functions. The GO and KEGG analysis results revealed that PTGs participated in 1063 biological processes and 111 pathways, significantly. Many biological processes were involved in follicular development and ovulation, such as the regulation of progesterone biosynthetic, oestrogen metabolism, negative regulation of cell proliferation, ITGA3-ITGB1-THBS1 complex, cellular response to transforming growth factor beta stimulus, meiotic cell cycle and steroid catabolic process (p < 0.05) (Figure 5A). The KEGG pathway analysis showed the PTGs were significantly involved in ovarian steroidogenesis, PI3K-Akt, MAPK, Wnt, BMP and TNF signalling pathway (p < 0.05) (Figure 5B).

Some PTGs that participated in oestrogen metabolic process and ovarian steroidogenesis signalling pathway are highlighted, such as CYP1A1, CYP19A1 and HSD3B1. A gene that participated in oestrogen metabolic process and ovarian steroidogenesis signalling pathway was CYP3A7. HSD17B8 participated in oestrogen metabolic process. ALOX5, LHCG, IGF1, GNAS, CYP2J2 and CYP17A1 participated in ovarian steroidogenesis signalling pathway. In addition, one protein-coding transcripts may be regulated by multiple IncRNAs, such as DE-IncRNA ALDBSSCT0000001929, ALDBSSCT0000006256 and ALDBSSCT0000002430, which were correlated with their target gene CYP1A1 significantly (p<0.05). They were all downregulated in Meishan compared with Duroc sows. LNC_000644 and ALDBSSCT0000006057 were correlated with CYP1A1 significantly (p<0.05) and were unregulated in Meishan. ALDBSSCT0000000051 was correlated with its target gene HSD17B8 significantly (p<0.05). ALDBSSCT0000000051 was downregulated, whereas HSD17B8 was unregulated (Figure 5C). Therefore, we speculated that some DE-IncRNAs participate in the development of porcine follicles by positively or negatively regulating their target genes, which are related to hormone secretion and metabolism.

Screening of potential reproduction-related IncRNAs in PI3K-AKT signalling pathway in pig ovarian follicle

The PI3K-AKT signalling pathway is very important in porcine follicular development. In order to explore the regulatory roles of the DE-IncRNAs involved in ovarian follicle growth and the relationship between these IncRNAs and PI3K-AKT signalling pathway, we analysed the expression of IncRNAs and their PTGs in PI3K-AKT signalling pathway. We found 12 protein coding genes, namely, THBS1, ITGA3, ITGA6, ITGB1, ITGB4, ITGB7, PIK3C2B, AKT2, CREB3L3, IKBKG and TP53 in the PI3K-AKT signalling pathway that were regulated by 19 DE-IncRNAs. Some DE-IncRNAs and their PTGs showed significant positive correlation (p<0.05), as follows: extracellular matrix (ECM) THBS1 and
In our research, the newly identified lncRNAs in pig ovarian follicle have many obvious characteristics, it has shorter and fewer exons, longer exon length and lower abundance, and they are less conserved than mRNAs. The characteristics of these lncRNAs were compliance with those observed in other studies[18, 19]. This current research is a meaningful resource for further studies on the functional lncRNAs and mRNA in pig ovarian follicles.

We obtained 145 differentially expressed lncRNAs QTL analysis, and the result showed that 127 DE lncRNAs were located in known QTLs of corpus luteum number, litter size, androstenone, total number born alive, number of stillborn, FSH concentration and number of viable embryos. The previous studies showed that QTLs controlling ovulation rate are located in pig chromosome 8[20, 21]. QTLs of luteum and ovulation rate are in chromosome 3[22]. The QTLs of ovulation rate are in chromosomes 4, 7, 13 and 15. These QTLs are closely related to pig reproduction[23, 24]. In this study, we found that the QTLs enriched most of the differential lncRNAs, i.e., ovarian follicle lncRNAs, distributed in chromosomes 3, 7, 8, 13 and 15. The results indicated that the DE lncRNAs may participate in the regulation of porcine reproductive traits and may be related to the follicular development in the two pig groups.

LncRNAs have a low expression abundance, and some lncRNAs are still unclearly. Thus, understanding the function of lncRNA is difficult. We obtained the target genes of DELs, and functional analysis found that these target genes participate in multiple biological processes and molecular signalling. CYP19A1 is the target gene of novel lncRNA-LNC_000076, it’s expressed in granulosa cells(GCs) and is rate-limiting enzyme for oestrogen production. It can catalyse the conversion of androgens to oestrogens, thereby increasing the expression level of oestrogen, knockdown of CYP19A1 can result in the inability to ovulate and loss of corpus luteum in female mice[25]. CYP1A1, an important metabolic enzyme in the oestrogen metabolic process and ovarian steroidogenesis signalling pathway, can reduce the level of oestrogen and lead to the acceleration of follicular atresia[26, 27]. DE-lncRNA ALDBSSCT0000001929, ALDBSSCT0000006256 and ALDBSSCT0000002430 targeted and were positively correlated (p<0.05) with CYP1A1. The expression level of CYP1A1 was lower in Meishan, whereas that of CYP19A1 was higher in Meishan compared with Duroc. These results may indicate that the level of oestrogen in the M2 follicles of Meishan sows was higher than that in Duroc. In addition, IGFI and HSD3B1 can stimulate and promote granulosa cells proliferation and promote follicular development[28, 29]. We also found HSD17B8, ALOX5, LHCGR, GNAS, CYP2J2 and CYP17A1 participate in ovarian steroidogenesis signalling pathway. These protein-coding genes may be candidate genes for porcine follicle development and are targeted by one or more lncRNAs (Figure 5C). Therefore, we speculated that lncRNAs may participate in porcine follicular development by regulating their target genes.

Studies have confirmed that the PI3K-AKT signalling pathway plays an major roles in the ovarian function regulation and follicular development[30-32]. PI3K-AKT signalling pathway regulates the primordial follicle maintenance and activation of s and promotes apoptosis of ovarian granulosa cells in humans and mice[33, 34]. KEGG analysis revealed that some target mRNAs of the DE lncRNAs were related to the PI3K-AKT signalling pathways. The THBS1-ITGA-ITGB complex members belong to the TGF-β family. TGF-β signalling can regulate the PI3K-Akt signalling pathway. The lncRNA-ALDBSSCT0000004 244 targets THBS1, which is an ECM. The expression of THBS1 is driven by oestradiol (E2), and its abnormal expression causes the apoptosis of granulosa cells and the acceleration of follicular atresia[35]. ITGA3 is the target gene of novel lncRNA LNC_000857 and LNC_001052. The expression of ITGA3 in M2 follicles of Meishan sows was higher than in those of Duroc. However, at present, no reports of ITGA3 related to animal reproduction exist. ALDBSSCT0000010443 and ALDBSSCT000009356 target ITGB1, which may be participate in the regulation of cumulus expansion and oocyte maturation[36]. ITGB1,
ITGB4 and ITGB7 are the target genes of LNC_001556, LNC_000536 and ALDBSSCT0000004232, respectively. ITGB1 is associated with the apoptosis process[37] and is regulated by progesterone and oestrogen[38]. The expressions of PI3K and AKT is the golden standard for the activation of PI3K-AKT signalling pathway. We found that ENSSSCT0000036444 and ALDBSSCT000000805 target PIK3CA and PIK3C2B, and LNC_000751, LNC_000819 and LNC_000058 target AKT2. PIK3CA and PIK3C2B are the catalytic subunits of PI3K, and the knockout of PIK3CA and PIK3C2B causes early embryo death in mice[39]. PI3K can react with a variety of growth factors, thereby phosphorylating PIPI2 to PIPI3, activating PDK1, and then indirectly or directly activating Akt[40]. AKT2 is a subtype of AKT, and a downstream signalling core molecule of the PI3K-AKT classic signalling pathway[41, 42]. Activated AKT2 can phosphorylate its downstream signalling molecules and produces the cAMP that can activate CREB, TP53 and IKB protein for nuclear gene transcription and expression, thereby regulating cell proliferation[43, 44]. ALDBSSCT0000011847 and ALDBSSCT0000008900 target CREB3L3. ALDBSSCT0000004325 and LNC_001172 target the ILBKG, TP53 and ALDBSSCT0000002268 and were both significantly positively correlated in pairs (p<0.05), LNC_000076 and ALDBSSCT0000002268 target TP53. CREB3L3 is a transcription factor of CREB, and its function is similar to that of CREB. Its abnormal expression can attenuate the up-regulation of Egr1 by GnRH receptor activation, which in turn affects the expression level of LHβ and the growth of granular cells[45]. IKK can induce phosphorylation of IKB (inhibitory protein of NF-kB), dissociate the NF-kB/IkB dimer, activate NF-Kb, initiate the inhibition of apoptosis pathway and maintain porcine follicular development[46]. TP53 is a critical factor for cell survival, and suppression of p53 in oocytes can promote follicular growth and development [47]. In this study, we screened 19 DE-lncRNA candidates and their 12 target gene in the PI3K-AKT signalling pathway. We suggest that IncRNA may participate in follicular growth and development by regulating their target genes, but the specific functions still require systematic research.

Conclusions

We identified lncRNA and mRNA expressed in M2 follicles in Meishan and Duroc sows and found that some IncRNAs participate in the follicular development by regulating their target genes. These findings in transcriptome provide a valuable resource for follicular development and reproduction-related transcripts. The interactions between DE-lncRNAs and their PTGs and enriched pathways provide clues for further research on the role of follicular growth and development in pig. However, the IncRNAs function and molecular mechanism in follicular development remain unclear. Nevertheless, our study provides new insights into IncRNAs associated with follicular development and reproduction in pig.

Methods

Animals and sample collection

Three multiparous Meishan cyclic sows were maintained at the Agricultural Experimental Station of Yangzhou University. Three multiparous Duroc cyclic sows were raised at the Animal Experimental Station of the Shihezi University. Observe the sows every day and determine natural oestrous cycle, day 0 was the first oestrus day). Sows were injected with PGF2α according to the pigs’ weight analogue (cloprostenol, Ningbo Second Hormone Factory) on the 14th day of the oestrous cycle to induce luteal regression and to synchronise the follicular growth phase. 4 days later, the sows were electric stunning and quick bleeding, then ovaries and medium follicles (follicles diameter: 5.0–6.9 mm, M2) were harvested, snap-frozen in LN2 and then stored at -80 °C refrigerator.

RNA-Seq preparation and sequencing analysis

Total RNAs of all the follicle samples were extracted using TRizol reagent (Invitrogen). RNA degradation and contamination was monitored on 1% agarose gels. The total RNA concentration, integrity and purity were detected using qubit RNA assay kit in Qubit 2.0 fluorometer (Life Technologies), RNA Nano 6000 assay kit by a bioanalyzer 2100 (Agilent Technologies) and NanoPhotometer spectrophotometer (IMPLEN). The ribosomal RNAs of all samples were removed using the Ribo-zero rRNA Removal Kit (Epicentre). 6 strand-specific RNA-Seq pools for the M2 follicles of the six sows were constructed according to the manufacturer’s instructions. Purify the samples library fragments using the AMPure XP system (Beckman Coulter, USA) to remove the preferred cDNA fragments of 150-200 bp. The blunt end cDNA fragments were augmented with A base and ligated to the sequencing adapter. The final products were purified (AMPure XP System), and the quality of library was assessed by the system Agilent Bioanalyzer 2100. The samples pools were then analysed using one lane of 100-200nt paired-end HiSeq 4000 platform. Quality control (QC) of RNA-Seq reads was performed using Fast Q C.

Transcriptome assembly
We filtering reads with adapter and low-quality, poly-N reads from raw data through inhouse perl scripts, the clean reads were obtained. Clean data with high quality is the basis for all subsequent analyses. Reference genome files were downloaded from the Ensembl (Sus scrofa 10.2). Pigs reference genome annotation index was built using Bowtie v2.0.6[48]. Appropriate parameters were set using Tophat2 v 2.0.9[49]. The Scripture and Cufflinks [50] were used to assemble and splice the aligned sequences, which can be as small as possible. The transcript set, Cufflinks has specific parameters for the chain-specific library, and the directional information of the transcript chain were accurately provided.

**IncRNA Identification**

The following steps to identify IncRNAs from the nonredundant transcriptome. (1) Transcripts has single exon or two exons were filtered out. (2) Transcripts length with less than 200 bp were removed. (3) Any transcript with the FPKM (a fragment per kilobase of transcript per million mapped read) score lower than 0.5 in every pools was discarded. (4) The remaining transcripts were blasted in pig known annotation IncRNA database – ALDB database [51] using Cuffcompare. Only the transcript of IncRNAs whose splice sites were congruent between our results and those in ALDB were immediately brought in as a known IncRNA. (5) Transcripts of any known protein-coding were discarded, and transcripts that belonged to pre-miRNA, snRNA, rRNA, snoRNA and pseudogenes were removed. (6) The CNCI, CPC, and phyloCSF tool were used to calculate the transcripts that has coding potential. Transcript with a CNCI score of <0[52], CPC score of <0[53], Pfam-scan E-value of <0.001[54] and phyloCSF Max-score of ≤100[55], as well as the intersection result of each tools were defined as novel found IncRNA transcript.

**Differentially Expressed IncRNAs and mRNA Analysis.**

Cufflinks package was used to conduct DE analysis between six follicle libraries of the Meishan and Drouc sows. The fold changes(FC) value were calculated in log2 (FPKM_MFM2/FPKM_DFM2) (FPKM_MFM2: FPKM of Meishan M2 follicle; FPKM_DFM2: FPKM of group Duroc M2 follicle). Transcripts with p-values of less than 0.05 would be identified as differentially expressed.

**qRT-PCR Verification**

Total RNAs were extracted using TRIzol (Invitrogen, CA, USA) and cDNA was synthesized using a RT-PCR kit (TaKaRa, Japan). qPCR reactions were performed using SYBR Green (TaKaRa Biotech, Dalian, China) according to the manufacturer's protocol. The reaction was conducted by combining 12.5 µl of 2x Real Master Mix (TaKaRa Biotechnology), 2 µl of cDNA, 1 µl each of the upstream and downstream primers, and 8.5 µl of RNase-free ddH2O water. Specific primers were designed using the Primer Premier 5.0 program (Table 3) and confirmed with BLAST. The expression levels of gene were normalized to linear GAPDH levels using 2^(-ΔΔCt) method[56] and the statistical difference was analysed using SPSS17.0. The correlation between the results of RNA-Seq and qPCR was calculated using a correlation test.
Table 3
The specific primers for qPCR.

| Transcript type | Transcript name | Forward primer  | Reverse primer  | Product size (bp) |
|-----------------|-----------------|-----------------|-----------------|------------------|
| LncRNA          | ENSSSCT00000018610 | TGGTCTGCTCTAAACCTGGACT | CTTCAGACAGGCTCAAGGGG | 297              |
|                 | ALDBSSCT00000001721 | ACTCTTACGTGAGCTGACAA | TGGTCAATTCTCCCTGGGATTG | 81               |
|                 | ALDBSSCT00000000051 | AAGACAGAGCAGAAAAAG | CTACGCCACTCCAGAAAG | 128              |
|                 | LNC_000116 | GCCCTCTCTTGGCTTGGTT | TCGGTGGCTTCCGGAGTTTT | 134              |
|                 | ALDBSSCT00000011300 | CAGGGGGTCAATTTTGC | CACGGCTTGAATGCGTTT | 122              |
|                 | ALDBSSCT0000006152 | CAAGGGGGTCAATTTTGCC | CAGGGGGTCAATTTTGC | 122              |
| mRNA            | COL3A1 | ATCGCTGTTGTGGAGGT | GAAGTCATAATCTTGTGTGT | 100              |
|                 | LRP8   | CCAATCGCATCTACTGGTGTGAC | GGAGAGTGCAGCTGCTCATCAAT | 115              |
|                 | ENSSSCT0000009222 | ATGCCTTCAATGGGACAACG | CAGTGCGCTGGGTAAGTCAA | 262              |
|                 | SEPP1  | CCTTCATTGACCTCACTAC | GTTGTCATACTTCTCATGCTTTC | 320              |
|                 | COL5A2 | GGGACATTTGGGAAACCTGCC | GGGAGTATGAGGGTCAGCA | 114              |
|                 | CYP19A1 | CCAGCATTACCAGAACCC | TGTGCCTCCATTACCCGAG | 92               |
|                 | GAPDH  | TTCCAGATAGATCCACCCAG | TCGGCAAGGGGGCGAGAT | 242              |

QTL analysis of Differentially Expressed IncRNAs

To predict the functions of DELs, QTLs analysis was performed. The location information of DELs was obtained from the transcriptome file and the QTL data of pig were downloaded from Animal QTLdb. Next, BEDTools and the "intersectBed" command were used for QTL analysis[57].

Prediction of PTGs of IncRNAs

Two methods were used to predict the PTGs of IncRNAs. We identified the PTGs regulated by IncRNAs in cis, which were defined as protein-coding genes located at 100 Kb upstream and downstream of the IncRNA, by BEDTools 2.17.0[58]. The trans regulation of a IncRNA and its PTG was identified by the expression level correlation analysis of each pair of IncRNA and PTG. According to the Pearson's correlation coefficients (|r| > 0.95), the PTGs were selected to construct a IncRNA–mRNA co-expression network.

GO and KEGG Pathway Enrichment Analysis

The prediction of the IncRNA and PTGs’ function such as gene enrichment and pathway analysis were performed using gene ontology (GO) (http://www.geneontology.org/) [59] and Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg) [60]. To further explore the main biological functions of differentially expressed genes, KOBAS software was used to detect the statistical enrichment of IncRNA PTGs or DEGs in KEGG pathways. The enrichment findings with a q-value of less than or equal to 0.05 was considered statistically significant.

Declarations

Ethics approval and consent to participate

All animal experiments in our study were carried out in accordance with the rules of Medical Ethics Committee, First Affiliated Hospital, Medical College, Shihezi University (2014-073-01, 5 March 2014).

Consent to publish
Availability of data and materials

The datasets and supporting conclusions are included within this manuscript or its supporting files. The datasets generated during this study are available from the corresponding author upon request.

Competing interests

No potential conflicts of interest were disclosed.

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Authors' contributions

TH conceived and designed the experiments. MXL and YL analyzed main content of the data and wrote the paper with the help of TH. HB G and SX mainly collects follicle samples. ZCZ performed the RNA extraction. LPM and YSS performed the qPCR experiment and data analysis.

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Identification and characterization IncRNAs and mRNAs in M2 follicles. (A) 157,846 transcripts were assembled; (B) Identification of non-coding IncRNAs by using four tools-CPC, PFAM, phyloCSF and CNCI; (C) IncRNAs classification; (D) The length of IncRNAs and mRNAs; (E) The IncRNAs and mRNAs exon number; (F) Open reading frame (ORF) length distribution of mRNAs and IncRNAs; (G) Violin plot of expression abundance (showed in log10 (FPKM + 1)) for mRNAs and IncRNAs. (H) Conservation compare with mRNAs and IncRNAs by using phasCon software.
Figure 2

Differential Expression (DE) Analysis of lncRNA and mRNA in two pig breeds. (A) DE lncRNAs between the Meishan and Duroc M2 follicle. Loop heat map from inside to outside, 1: MFM2DY4_1, 2: MFM2DY4_2, 3: MFM2DY4_3, 4: DFM2DY4_1, 2: DFM2DY4_2, 3: DFM2DY4_3. (B) DE protein-coding genes between the Meishan and Duroc follicle. Loop heat map from inside to outside, 1: MFM2DY4_1, 2: MFM2DY4_2, 3: MFM2DY4_3, 4: DFM2DY4_1, 2: DFM2DY4_2, 3: DFM2DY4_3.
Figure 3

Validation of the expression levels of lncRNAs and mRNAs. (A, C) The qPCR verification of the 6 DE-mRNAs in DFM2DAY4 and MFM2DAY4. (B, D) The RNA-seq results between DFM2DAY4 and MFM2DAY4. The expression of transcripts was normalized by GAPDH. The results were expressed as mean±SE, * represents P<0.05, ** represents P<0.01.
Figure 4

QTLs analysis of DELs. (A) The number distribution of QTLs associated with reproduction and all of the QTLs. (B) The chromosome distribution of QTLs associated with reproduction. (C) The IncRNA number of QTLs associated with reproduction QTLs.
Figure 5

GO and KEGG pathway analysis of the PTGs of differentially expressed IncRNAs (DEls) (A) Biological processes of PTGs of DELs. (B) Pathways of PTGs of DELs. (C) The interaction analysis of PTGs and DELs in estrogen metabolic process and ovarian steroidogenesis signaling pathway. Triangles represent IncRNAs, circles represent mRNA, and red is up-regulated genes, purple is down-regulated genes.
Figure 6

The regulatory network analysis and differentially expressed transcripts involved in PI3K-AKT signaling pathway in our study (RNA-Seq data). The red arrow represents positive correlation, the black arrow represents negative correlation.