Comparative analyses of long non-coding RNA in lean and obese pigs

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ABSTRACT

Objectives: Current studies have revealed that long non-coding RNA plays a crucial role in fat metabolism. However, the difference of IncRNA between lean (Duroc) and obese (Luchuan) pig remain undefined. Here, we investigated the expressional profile of IncRNA in these two pigs and discussed the relationship between IncRNA and fat deposition.

Materials and Methods: The Chinese Luchuan pig has a dramatic differences in backfat thickness as compared with Duroc pig. In this study, 4868 IncRNA transcripts (including 3235 novel transcripts) were identified. We determined that patterns of differently expressed IncRNAs and mRNAs are strongly tissue-specific. The differentially expressed IncRNAs in adipose tissue have 794 potential target genes, which are involved in adipocytokine signaling pathways, the PI3k-Akt signaling pathway, and calcium signaling pathways. In addition, differentially expressed IncRNAs were located to 13 adipose-related quantitative trait loci which include 65 QTL_ID. Subsequently, IncRNA and mRNA in the same QTL_ID were analyzed and their co-expression in two QTL_ID were confirmed by qPCR.

Conclusions: Our study provides an insight into mechanism behind the fat metabolic differences between the two breeds and lays an important groundwork for further research regarding the regulatory role of IncRNA in obesity development.

INTRODUCTION

Obesity has become a major health concern around the world and is the main risk factor for non-alcoholic fatty liver disease (NAFLD), type 2 diabetes and cardiovascular diseases (CVDs) [1]. Therefore, the study of fat deposition and its mechanism is of great benefit for prevention and treatment of obesity and its related diseases. The Luchuan pig is a typical obese breed as it has higher intramuscular fat and backfat thickness compared with the Duroc breed. They are good models to investigate the regulatory mechanism of fat metabolism.

Long non-coding RNAs (lncRNAs) are defined as non-coding RNAs of at least 200 nucleotides. In the past, lncRNAs were considered to be “evolutionary junk” or transcriptional “noise” along with other non-coding RNAs [2, 3]. However, in recent years, as the rapid development of technologies has facilitated analysis of the “transcriptome”, there is increasing evidence that lncRNAs play a crucial role in many biological processes [4, 5], such as telomere homeostasis and chromosome replication [6–8], control of nuclear architecture and translation [9], X-chromosome inactivation [10], regulation of epigenetic modifications [11], control of mRNA and protein stability [12, 13], and regulation of miRNA activity [14, 15]. As research into lncRNA increased, many databases were established and included lncRNA data for both domesticated animals and poultry. At the time of publication, a total of 12,103 pig lncRNAs, 8,923 chicken lncRNAs and 8,250 cow lncRNAs are included in the...
ALDB database [16]. While analyzing IncRNA expression in pig, Zhao et al. developed a systematic protocol for the identification and characterization of IncRNAs in fetal porcine skeletal muscle [17]. Moreover, an antisense IncRNA of the PU.1 gene was identified, which can form a sense-antisense RNA duplex to promote adipogenesis [18]. Wang et al. investigated the IncRNAs in porcine endometrial tissue samples using RNA-seq [19]. Currently, porcine fat deposition is less well understood. Toward that end, in order to compare the IncRNA expression differences between the lean and obese breeds, IncRNA sequences were obtained from three different types of tissues (liver, muscle and fat) of Luchuan and Duroc pigs.

In this study, we identified differentially expressed IncRNA molecules and predicted their target genes. Moreover, the correlation between the identified IncRNA molecules and QTL were investigated. These results will provide a useful resource to further explore the role of IncRNAs in fat deposition.

**RESULTS**

**Overview of IncRNA sequencing data**

After 180 days of identical feeding conditions, the average backfat thickness of Luchuan pigs was 35.33 ± 0.57 mm while that of the Duroc pigs was 12 ± 1.00 mm. Next, three tissue samples (liver, muscle and fat) were collected from three animals from each porcine breed: L-liver (Luchuan liver), D-liver (Duroc liver), L-muscle (Luchuan muscle), D-muscle (Duroc muscle), L-fat (Luchuan fat) and D-fat (Duroc fat). Total RNA from each sample was sequenced by using the Illumina HiSeq 2500 platform. A total of 16.84 G, 14.82 G, 13.37 G, 12.26 G, 15.56 G and 14.00 G clean data were generated in the L-liver, D-liver, L-muscle, D-muscle, L-fat and D-fat samples, respectively. The GC content averaged between 51.84 and 56.77% while the Q30 ranged between 95.08 and 96.16%. These results show that the quality of our six libraries was good and suitable for subsequent analysis. Next, the clean reads were aligned to the reference genome (Sus scrofa 10.2) using TopHat v2.1.0 (Table 1). More than 68.54% of clean reads were uniq mapped in each sample. An average of 40.68% of clean reads were mapped to sense strand and 39.43% of clean reads were mapped to antisense strand. A high quality library is a necessary for IncRNA sequencing; therefore, the data from each read’s relative position in gene (5′–3′) were analyzed to ensure the quality of these six samples. Obviously, the vast majority of reads were evenly distributed throughout the gene by random sampling, which indicates that the quality and homogeneity of the samples was good (Figure 1A). Moreover, the ratio of reads corresponding to exon, intron, and intergenic regions was different (Figure 1B), suggesting that the RNA expression profiles were tissue specific.

All mapped reads of the six libraries were assembled using Cufflinks [20]; then, the assembled transcripts were filtered using a rigorous method. Transcripts which were >200 bp in length, contained two exons, had at least three reads coverage, and had a 0.1 FPKM value were retained. As IncRNA do not encode proteins, the protein coding potential of the remaining transcripts was determined using four separate protocols: CPC, CNCI, CPAT and pafm. Finally, 4868 IncRNA transcripts were identified (Figure 1C), including 2403 lincRNAs (49.36%), 252 anti-sense IncRNAs (5.18%), 216 intronic IncRNAs (4.44%), and 1997 sense IncRNAs (41.02%) (Figure 1D). Moreover, 3235 novel IncRNA transcripts were revealed by blasting their sequences in NONCODE and IncRAdb.

These 4868 IncRNA transcripts were distributed throughout all chromosomes found in pig, although chromosome 1 contained the greatest number of IncRNAs (Figure 2A). On the whole, there were fewer alternatively spliced isoforms per IncRNA molecule as compared with mRNA molecules (Figure 2B). Lengths between 600–1200 bp and ≥3000 bp from both IncRNA and mRNA molecules were most common (Figure 2C–2D). All expression information of IncRNAs and genes are shown in Supplementary Tables 1–2. We noticed that all the IncRNAs tended to be expressed at a lower level than the protein-coding genes (Figure 3A). Then, all of differently expressed transcripts were filtered to include only those transcripts with a false discovery rate (FDR) >0.05 and fold change ≥2 or ≤0.5; the FDR is obtained by from adjusting the p-value, and the fold change was obtain from gene expression of Luchuan / gene expression of Duroc pig. In adipose tissue, 503 IncRNA and 2173 mRNA molecules were detected (Figure 3B). All of the differentially expressed IncRNAs and mRNAs are shown in Supplementary Tables 3–4. Two Venn diagrams depict the differentially expressed IncRNA and mRNA molecules in each of the three tissue types (Figure 3C–3D). A total of 386, 349 and 336 differentially expressed IncRNAs appear to be specific for the liver, muscle and fat, respectively. Tissue specific IncRNAs were a major proportion of the differentially expressed IncRNAs. Similarly, 1123, 800 and 1513 differently expressed mRNAs appear to be specific for the liver, muscle and fat, respectively.

Next, 1286 and 4271 unique and differentially expressed IncRNAs and mRNA were used to perform a tissue-specific clustering analysis. As shown in the heat map, the up-regulated IncRNAs were divided into six clusters. The expression pattern of Duroc IncRNAs (D-liver, D-muscle and D-fat) was distinct from the expression of Luchuan IncRNAs (L-liver, L-muscle and L-fat) (Figure 4A). In contrast, according to the heat map of differentially expressed mRNA transcripts, the
Figure 1: Overview of lncRNA sequencing data. (A) The distribution of mapped reads on mRNA (5′–3′). Data in reflects the percentage of mapped reads assigned to all regions of mRNA. The location of the normalized mRNA is on the horizontal (x) axis; the percentage of reads as compared to total mapped reads for the position is on the vertical (y) axis. As the reference mRNA is different in length, each mRNA is divided into 100 intervals by length. (B) Reads mapped to different regions of the genome. (C) Venn diagrams show the result of four computational approaches. 4868 candidate lncRNAs were identified from an intersection results from the CNCI (coding-non-coding index), CPC (coding potential calculator), Pfam (protein folding domain database), and CPAT (coding potential assessing tool). (D) The type and number of predicted long non-coding RNAs (lncRNAs). Intronic lncRNA: lncRNA transcript from the intron region of gene; Antisense lncRNA: lncRNA has opposite transcriptional direction compared to adjacent mRNA; Sense lncRNA: lncRNA has the same transcriptional direction as the adjacent mRNA; Intergenic lncRNA: lncRNA transcribed from a position between two genes.
cluster of up-regulated mRNA molecules was similar between the Luchuan liver and Duroc liver samples as well as between the Luchuan muscle and Duroc muscle samples. However, the Luchuan fat and Duroc fat samples expressed distinct mRNA molecules (Figure 4B). In order to further understand the potential function of IncRNA in fat deposition, the target genes of the IncRNAs were predicted. A portion of some IncRNA molecules and their target gene are presented in Table 2. All of the target genes are listed in Supplementary Table 5.

**Gene ontology (GO) and KEGG pathway enrichment analysis**

Considering the adipose tissue had the greatest number (2173) of differentially expressed mRNAs and is a major organ for fat deposition, a gene ontology (GO) analysis was performed for the adipose tissues. The most enriched GO terms are shown in Figure 5A; complete information is listed in Supplementary Table 6. We primarily focused on genes involved in electron carrier activity and antioxidant activity as these functions may be involved in the regulation of fat deposition. Meanwhile, these target genes also underwent a KEGG analysis to determine the potential biological function of the identified IncRNAs. The data showed that some pathways related to fat metabolism and energy metabolism were significantly enriched, such as signaling pathways associated with adipocytokines, calcium signaling, MAPK, FOXO and PI3k/Akt (Figure 5B and Supplementary Table 7). To our surprise, there were 16 target genes that function as part of the PI3k-Akt signaling pathway, which is closely related to insulin signaling pathway [21].

**QTL location analysis of DE-IncRNAs and quantitative validation**

Due to the tight connection between QTL and traits, and to further explore the role of differentially expressed IncRNA molecules on fat deposition, a correlation analysis was performed between IncRNA and fat-associated QTL by mapping differentially expressed IncRNAs onto pig QTL regions. This analysis indicated that 275 differentially expressed IncRNAs are located in 13 fat-associated QTL (Supplementary Table 8). At the same time, differentially expressed mRNAs were mapped onto these 13 QTL and 498 mRNAs were found to localize to the 13 QTL as well. Genes associated with the trait “abdominal fat weight” had the greatest number of associated differentially expressed IncRNAs (138) and mRNAs (513). The trait, “average backfat thickness”, had the second highest number of associated differentially expressed IncRNAs (89) and mRNAs (306) (Table 3).

Next, we tried to identify which QTL play a crucial role in fat deposition. The number of differentially expressed IncRNAs in each QTL_ID was analyzed; 65 QTL_ID containing differentially expressed IncRNAs were identified. The score for each QTL_ID were sorted (score = the number of differentially expressed IncRNAs / the span length of each QTL_ID) (Supplementary Table 9). The top 20 QTL_ID are listed in Table 4. More than half of the top 20 QTL_ID are associated with the “average

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**Figure 2: Features of IncRNAs and mRNAs in the genome.** (A) LncRNAs distribution by chromosome. (B) Alternatively spliced isoforms per IncRNA and mRNA molecule. (C&D) Distribution of IncRNA and mRNA molecules by length.
backfat thickness” trait. Next, the differentially expressed mRNAs were assigned to 65 QTL_ID. This analysis indicated that gene ELOVL6 is located in the same region of QTL_ID 21252 as lncRNA TCONS_00185144 and TCONS_00181156. Moreover, the gene STEAP4 as found in QTL_ID 21259 along with lncRNA TCONS_00199412 and TCONS_00197271. Subsequently, quantitative real-time PCR confirmed their co-expression relationship (Figure 6A–6B). These results suggest that lncRNA may participate in regulations of genes in the same QTL_ID.

**DISCUSSION**

Fat deposition is a complex metabolic process involving many genes. Although many groups have studied genes related to backfat thickness [22–24], until now, the relationship between fat deposition and lncRNAs is not very clear. In this study, the expression of lncRNA and mRNA molecules in the adipose tissue of Luchuan and Duroc pigs were investigated and the potential regulatory role of lncRNA was analyzed.

A total of 4,868 differentially expressed lncRNA transcripts and 8843 differentially expressed mRNA transcripts were obtained from three tissues (liver, muscle, and fat). A significantly greater number of lncRNAs were found on chromosome 1 as compared to other chromosomes. In addition, the number of alternatively spliced isoforms per lncRNA molecule was significantly less than the number per mRNA molecule. These observations are in agreement with Shen et al. [25].

![Figure 3: Differential expression of lncRNAs and mRNAs by tissue.](image)
(A) The number of differentially expressed lncRNAs and mRNAs by tissue. Red bar represents up-regulated transcripts and the green bar represent down-regulate transcripts. (B) Expression profiles of lncRNA and mRNA in each tissue category. We used log10(FPKM) as the final data to indicate expression level. (C, D) Tissue-specific expression of lncRNAs and mRNA.
addition, lncRNA molecules were of lower abundance as compared with mRNA molecules (Figure 3A). Both lncRNA and mRNA expression were strongly tissue-specific (Figure 3C–3D), which is also apparently indicated by the heat maps (Figure 4A–4B). All of results indicated that the lncRNA identified in this study have strong tissue-specific expression. Adipose tissue was found to have the greatest number of up-regulated lncRNA molecules and differentially expressed mRNA molecules. Adipose tissue data were used to perform the KEGG analysis and

### Table 2: Differentially expressed lncRNAs and their target mRNA in each tissue

| Tissue | IncRNA_ID   | Fold change | Regulated | Gene_ID  | Gene name | Fold change | Regulated |
|--------|-------------|-------------|-----------|----------|-----------|-------------|-----------|
| liver  | TCONS_00109066 | 222.8439    | up        | ENSSSCG000000014368 | –         | 25.75714    | up        |
| liver  | TCONS_00156770 | 175.7342    | up        | ENSSSCG000000003967 | ZMYND12   | 0.235277    | down      |
| liver  | TCONS_00176571 | 79.80171    | up        | ENSSSCG000000002627 | GSTA4     | 2.524848    | up        |
| liver  | TCONS_00130158 | 0.007197    | down      | ENSSSCG000000006155 | ZBTB10    | 0.277276    | down      |
| liver  | TCONS_00062308 | 0.005966    | down      | ENSSSCG000000024537 | –         | 5.06822     | up        |
| liver  | TCONS_00143452 | 0.00467     | down      | ENSSSCG00000000878 | DEPDC4    | 0.321732    | down      |
| muscle | TCONS_00013468 | 123.2883    | up        | ENSSSCG000000005087 | SIX1      | 0.428436    | down      |
| muscle | TCONS_00108745 | 93.70243    | up        | ENSSSCG000000014083 | ANKDD1B   | 2.762261    | up        |
| muscle | TCONS_00136478 | 81.86808    | up        | ENSSSCG000000006506 | SYT11     | 0.325533    | down      |
| muscle | TCONS_00024736 | 0.006558    | down      | ENSSSCG000000023031 | TNNT2     | 7.496831    | up        |
| muscle | TCONS_00018030 | 0.004239    | down      | ENSSSCG000000005087 | SIX1      | 0.428436    | down      |
| muscle | TCONS_00008591 | 0.002903    | down      | ENSSSCG000000004602 | TEX9      | 0.160484    | down      |
| fat    | TCONS_00027769 | 167.275     | up        | ENSSSCG000000010837 | FAM177B   | 63.01933    | up        |
| fat    | TCONS_00181629 | 149.6883    | up        | ENSSSCG000000027340 | –         | 112.1       | up        |
| fat    | TCONS_00109934 | 73.74533    | up        | ENSSSCG000000013148 | GLYATL2   | 59.994      | up        |
| fat    | TCONS_00066375 | 0.01899     | down      | ENSSSCG000000010069 | DERL3     | 18.56326    | up        |
| fat    | TCONS_00194244 | 0.017954    | down      | ENSSSCG000000014861 | MOGAT2    | 0.156778    | down      |
| fat    | TCONS_00174306 | 0.007341    | down      | ENSSSCG00000001826 | CCDC37    | 13.31454    | up        |

The top three up regulated and down regulated lncRNAs with its target mRNA in each tissue.

### Table 3: Differentially expressed IncRNA and mRNA molecules (L-fat VS D-fat) assigned into QTL trait regions

| Trait                        | Number of QTL_ID | All DE-lncRNAs | Up-lncRNA | Down-lncRNA | All DE-mRNAs | Up-mRNA | Down-mRNA |
|------------------------------|------------------|----------------|-----------|-------------|---------------|---------|-----------|
| Abdominal fat percentage     | 2                | 4              | 3         | 1           | 8             | 5       | 3         |
| Abdominal fat weight         | 17               | 138            | 79        | 59          | 513           | 308     | 205       |
| Adipocyte diameter           | 6                | 23             | 13        | 10          | 97            | 53      | 44        |
| Arachidic acid content       | 2                | 3              | 1         | 2           | 3             | 2       | 1         |
| Arachidonate acid content    | 1                | 3              | 2         | 1           | 1             | 1       | 0         |
| Average backfat thickness    | 28               | 89             | 60        | 29          | 306           | 167     | 139       |
| backfat above muscle dorsi   | 2                | 5              | 2         | 3           | 82            | 53      | 29        |
| backfat at last rib          | 1                | 2              | 0         | 2           | 17            | 5       | 12        |
| Backfat at rump              | 1                | 1              | 0         | 1           | 8             | 4       | 4         |
| Backfat between 6th and 7th ribs | 1          | 2              | 0         | 2           | 8             | 5       | 3         |
| Backfat weight               | 2                | 3              | 2         | 1           | 15            | 5       | 10        |
| Loin fat percentage          | 1                | 1              | 1         | 0           | 0             | 0       | 0         |
| Percentage of backfat and leaf fat in carcass | 1 | 1 | 1 | 0 | 7 | 3 | 4 |

Up-lncRNA: up regulated lncRNA number; Down-lncRNA: down regulated lncRNA number; Up-mRNA: up regulated mRNA number; Down-mRNA: down regulated mRNA number.
enrichment. A total of 794 lncRNA target genes were assigned to 226 functional signaling pathways. We focused on those target genes associated with calcium signaling.

Calcium is a key intracellular signal responsible for regulating numerous cellular processes. As an extracellular Ca^{2+} sensor, CaSR activation in the visceral white adipose tissue is associated with increase of adipose progenitor cells proliferation and elevate of adipocyte differentiation [26]. Another study reported that Seipin promotes fat storage in adipose tissue by regulating

| QTL_ID | DE-lncRNAs | Chrome | Trait Name | Start | End | Score  |
|--------|------------|--------|------------|-------|-----|--------|
| 31542  | 3          | 14     | Arachidonic acid content | FA-C20:4 | 138365048 | 138414114 | 6.11E-05 |
| 658    | 1          | 10     | Average backfat thickness | BFT | 11227321 | 11306620 | 1.26E-05 |
| 12712  | 1          | 1      | Abdominal fat weight | ABDF | 294607712 | 294736101 | 7.79E-06 |
| 21436  | 1          | 16     | Loin fat percentage | LOINFP | 83843383 | 84125107 | 3.55E-06 |
| 735    | 1          | 1      | Average backfat thickness | BFT | 307398864 | 307784034 | 2.6E-06 |
| 17773  | 1          | 1      | Average backfat thickness | BFT | 140716292 | 141412550 | 1.44E-06 |
| 12713  | 1          | 1      | Abdominal fat weight | ABDF | 245011782 | 245772300 | 1.31E-06 |
| 22290  | 1          | X      | Average backfat thickness | BFT | 113078996 | 113962590 | 1.13E-06 |
| 736    | 1          | 4      | Average backfat thickness | BFT | 140987596 | 142372300 | 7.22E-07 |
| 7293   | 3          | 4      | Abdominal fat percentage | ABDFP | 81983315 | 87016757 | 5.96E-07 |
| 22480  | 2          | 5      | Arachidic acid content | FA-C20:0 | 56004411 | 59682626 | 5.44E-07 |
| 22509  | 1          | 10     | Arachidic acid content | FA-C20:0 | 56004411 | 59682626 | 2.72E-07 |
| 5435   | 1          | 10     | Average backfat thickness | BFT | 28168636 | 32088890 | 2.55E-07 |
| 3000   | 2          | 10     | Average backfat thickness | BFT | 32088890 | 41334738 | 2.16E-07 |
| 7530   | 1          | 3      | Average backfat thickness | BFT | 12295139 | 12692663 | 2.16E-07 |
| 18001  | 1          | 9      | Average backfat thickness | BFT | 145703416 | 151394450 | 1.76E-07 |
| 17803  | 1          | 8      | Average backfat thickness | BFT | 811090 | 6651169 | 1.71E-07 |
| 23307  | 1          | 6      | Backfat at rump | BFTR | 152297333 | 158443390 | 1.63E-07 |
| 849    | 1          | 1      | Abdominal fat weight | ABDF | 226764071 | 233806417 | 1.42E-07 |
| 2923   | 1          | 13     | Average backfat thickness | BFT | 208227233 | 215641489 | 1.35E-07 |

Figure 4: Heat-map of differently expression lncRNAs and mRNAs. (A) Cluster heat-map of differentially expressed lncRNAs from each sample. (B) Cluster heat-map of differentially expressed mRNAs from each sample.
intracellular calcium homeostasis [27]. Both our data and that of others suggest that fat deposition is regulated by calcium signaling. Further study in this field may provide new strategies to control fat deposition.

The common method of predicting lncRNA target genes is to search a 100 kb upstream or downstream region to identify nearby protein coding regions. In order to obtain more reliable target gene information for lncRNA

![Figure 5: GO and KEGG analysis of target genes in adipose tissue.](image)

(A) Gene Ontology analysis of target genes of differentially expressed lncRNAs from adipose tissue (L-fat vs D-fat). DEG Unigene: differentially expressed genes number in all annotation Biological Process GO term. All Unigene: Unigene number in all annotation Biological Process GO term. (B) KEGG pathway enrichment analysis of target genes of differentially expressed lncRNAs from adipose tissue (L-fat vs D-fat).
molecules, target genes were predicted using QTL_ID regions. We found that lncRNA TCONS_00199412 and TCONS_00197271 were co-expressed with gene STEAP4 in the QTL_ID 21259 region. In addition, lncRNA TCONS_00185144 and TCONS_00181156 were co-expressed with ELOVL6 (elongation of long chain fatty acids family member 6) and all three transcripts were localized to the QTL_ID 21252 region. Subsequently, their co-expression was confirmed using quantitative real-time PCR (Figure 6B). ELOVL6 is believed to be involved in insulin resistance, lipogenesis, and obesity [28]. These results indicate the feasibility of using the QTL_ID region to predict the lncRNA target genes.

In conclusion, our data provide further basic knowledge of pig’s lncRNAs. These results lay important groundwork for the further investigation of the regulatory role of lncRNA in fat deposition.

MATERIALS AND METHODS

Animals and samples collection

Three Luchuan and three Duroc boars were used in this study. The animals were allowed access to feed and water ad libitum and were housed under identical conditions. All pigs were sacrificed at 180 days of age, they had been performed overnight fasting before sacrificed. Three types of tissue samples (liver, muscle, and fat) were collected from three animals of each porcine breed. All animal experiments were performed under approval of the Institutional Animal Care and Use Committee (IACUC) of Guangxi University.

RNA quantification and qualification

A total of 1.5 μg RNA per sample were obtained using TRIzol reagent (Invitrogen, USA); rRNA was removed using a Ribo-Zero rRNA Removal Kit (Epicentre, Madison, WI, USA). 1.5% agarose gels were used for monitoring RNA degradation and DNA contamination. RNA concentration and purity were analyzed using a NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific, Wilmington, DE). RNA integrity was assessed using an RNA Nano 6000 Assay Kit and Agilent Bioanalyzer 2100 System (Agilent Technologies, CA, USA).

Preparation of the IncRNA-Seq libraries

Six cDNA libraries were generated using NEBNext® UltraTM Directional RNA Library Prep Kit. First strand cDNA was synthesized using reverse transcriptase with random hexamer primers. Subsequently, DNA polymerase I and RNase H were used for second strand cDNA synthesis. The exonuclease/polymerase activities can convert overhangs into blunt ends. After adenylation was completed, ligation was performed with NEBNext Adaptor. The final 150–200 bp fragments were selected by filtration with AMPure XP Beads (Beckman Coulter, Beverly, USA). Then 3 μl USER Enzyme (NEB, USA) was used with size-selected and adapter-ligated cDNA at 37°C for 15 min before PCR. At last, PCR products were generated using Phusion High-Fidelity DNA polymerase and purified using the AMPure XP system. An Agilent Bioanalyzer 2100 and qPCR were used to assess the quality of the libraries.

IncRNA identification and analysis

Cufflinks software [20] was used to assemble the transcriptome. The resulting sequence was based on the reads mapped to the reference genome (Sus scrofa 10.2). Then, the assembled transcripts were annotated using the Cuffcompare program (a Cufflinks package). The

Figure 6: Co-expression of transcripts validation via quantitative real-time PCR. (A) IGV diagram indicates the location of co-expressed transcripts in the same QTL_ID. (B) Quantitative real-time PCR validation of lncRNAs and genes in the same QTL_ID region.
unknown transcripts were retained and screened for putative lncRNAs. Four computational approaches (CPC/CNCI/Pfam/cpat) were used to screen the putative lncRNAs for protein coding ability. Transcripts >200 nt and > two exons were retained as lncRNA candidates and were further screened by CPC/CNCI/Pfam/cpat that to ensure every transcript is a long non-coding RNA. At last, Cuffcompare was used to categorize the lncRNA transcripts as lincRNA, intronic lncRNA, anti-sense lncRNA and sense lncRNA.

**Quantification of expression levels and differential expression analysis**

The FPKM (fragments per kilo-base of exon per million fragments mapped, calculated based on the length of the fragments and reads count mapped to the fragment) of both lncRNAs and coding genes were calculated using Cuffdiff (v2.1.1) [20] for each sample. Gene FPKMs were computed by summing the FPKMs of transcripts in each group. The \( P \) value was adjusted using Q value [29]. A threshold for significantly different expression was set as a Q value <0.01 and |log2(fold change)| >1.

**GO enrichment analysis and KEGG pathway enrichment analysis**

Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) and the target genes of differentially expressed lncRNAs was implemented using the topGO-R software packages. KOBAS [30] software was used to test the statistical enrichment of differentially expressed genes in KEGG pathways.

**Quantitative real-time PCR**

Quantitative real-time PCR (qPCR) was used to validate RNA-seq results. Three replicate tissue samples from each pig were obtained and three pigs were used from each breed. Total RNA from these samples was extracted using TRIzol reagent (Invitrogen, USA); then, total RNA was purified using RNase-free DNase I (GeneStar, Beijing, China). One μg total RNA was reverse transcribed using M-MLV RNAse H-negative reverse transcriptase (Takara, Dalian, China). Finally, quantitative real-time PCR was performed on a qTOWER 3.0 real-time PCR System (Analytik-jena) with 2 × Realstar Green Fast Mixture (GeneStar, Beijing, China). The quantitative real-time PCR primer pairs used in this study are listed in Supplementary Table 10. The reaction conditions were: denaturation for 30 s at 95°C followed by 40 cycles of 95°C 15 s and 60°C 1 min. Relative gene expression levels were calculated from the Ct value and analyzed using the 2-ΔΔCT method. Samples were analyzed in triplicate to ensure their statistical significance.

**Abbreviations**

- lncRNA: long non-coding RNA
- DE-lncRNAs: differentially expressed long non-coding RNAs
- DE-mRNAs: differentially expressed mRNAs
- QTL: quantitative trait loci
- DEG: differentially expressed gene
- GO: Gene Ontology
- KEGG: Kyoto Encyclopedia of Genes and Genomes

**Authors’ contributions**

Lin Yu, Yixing Li and Lei Zhou conceived the project and design the protocol; Lin Yu, Lina Tai, Lifang Zhang and Yi Chu performed the experiments; Lin Yu, Yixing Li and Lei Zhou wrote the manuscript. All authors read and approved the final manuscript.

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**CONFLICTS OF INTEREST**

The authors have declared that no competing interests exist.

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