Identification of Long Non-Coding RNAs Involved in Fat Deposition in Pigs Using Two High-Throughput Sequencing Methods

Yibing Liu

China Agricultural University

Hong Ao

Chinese Academy of Agricultural Sciences

Fengxia Zhang

China Agricultural University

Xitong Zhao

China Agricultural University

Huatao Liu

China Agricultural University

Yong Shi

China Agricultural University

Kai Xing (✉ xk181986@163.com)

Beijing University of Agriculture

Ying Yu

China Agricultural University

Chuduan Wang

China Agricultural University

Research

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2 **deposition in pigs using two high-throughput sequencing**
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5 Yibing Liu¹, Hong Ao³, Fengxia Zhang¹, Xitong Zhao¹, Huatao Liu¹, Yong Shi¹, Kai Xing^{2*}, Ying
6 Yu^{1*}, Chuduan Wang^{1*}

7 1. Key Laboratory of Animal Genetics, Breeding and Reproduction, Ministry of Agriculture,
8 National Engineering Laboratory for Animal Breeding, College of Animal Science and
9 Technology, China Agricultural University, Beijing, China

10 2. Animal Science and Technology College, Beijing University of Agriculture, Beijing, China

11 3. Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing, China)

12 Email addresses:

13 Yibing Liu, 279547013@qq.com

14 Kai Xing, xk181986@163.com

15 Hong Ao, aohong@caas.cn

16 Fengxia Zhang, 895315294@qq.com

17 Xitong Zhao, zhaoxitong0348@163.com

18 Huatao Liu, 925457179@qq.com

19 Yong Shi, 2273190789@qq.com

20 Ying Yu, yuying@cau.edu.cn

21 Chuduan Wang, cdwang@cau.edu.cn

22

23 **Abstract:**

24 **Background:** Adipose is an important body tissue in pigs, and fatty traits are critical in pig
25 production. The function of long non-coding RNA (lncRNA) in fat deposition and metabolism has
26 been proven in previous studies. In this study, we focused on lncRNAs associated with fattening
27 traits in pigs. The adipose tissue of six Landrace pigs with either extremely-high or -low backfat
28 thickness ($n_{\text{high}} = 3$, $n_{\text{low}} = 3$) was collected, after which we performed strand-specific RNA
29 sequencing using biological replicates and pooling methods.

30 **Results:** A total of 19,631 genes and 2,013 lncRNAs were identified using the coding potential
31 calculator, coding-non-coding index, and Pfam database, including 334 known transcripts and
32 1,679 novel transcripts. Using edgeR, we determined that 220 lncRNAs and 1,512 genes were
33 differentially expressed ($|\text{Fold Change}| > 2$ and false discovery rate < 0.05) between the two
34 groups in biological replicate RNA sequencing (RNA-seq), and 127 lncRNAs and 2,240 genes
35 were differently expressed in pooling RNA-seq. Further Kyoto Encyclopedia of Genes and
36 Genomes and Gene Ontology enrichment analysis of the differentially expressed genes found that
37 some of the genes were involved in several key pathways related to fat development. After
38 targeting gene prediction, we determined that some cis-target genes of the differentially expressed
39 lncRNAs play an important role in fat deposition. For example, *ACSL3* is cis-targeted by lncRNA
40 TCONS-00052400, and it can activate the conversion of long-chain fatty acids. In addition,
41 lncRNA TCONS_00041740 was up-regulated in the high backfat thickness group, and its
42 cis-target gene *ACACB* was also up-regulated in this group. It has been reported that *ACACB* is the
43 rate-limiting enzyme in fatty acid oxidation.

44 **Conclusions:** Since these genes have necessary functions in fat metabolism, the results imply that

the lncRNAs detected in our study may affect fat deposition in pigs through regulation of their target genes. In summary, our study explored the regulation of lncRNA and their target genes on fat deposition in pigs and provided new insights for further investigation of the biological functions of lncRNA.

Keywords: lncRNA, fat deposition, pig, RNA-seq

Introduction

Fat deposition is an important biological process in pig growth. Fat traits are closely related to pork quality as well as the production efficiency and reproductive traits of pigs ^[1]. The fat content of pigs also affects consumers' choice of pork ^[2]. Furthermore, pigs are an ideal animal model for human disease research ^[3] on various diseases such as type 2 diabetes ^[4], hypertension, and coronary heart disease ^[5]. Determining the molecular markers that affect subcutaneous fat deposition in pigs is an important way to effectively accelerate its genetic improvement. Therefore, it is of great significance to explore the molecular regulation mechanisms of fat deposition in pigs. Fat deposition is a complex process influenced by multiple genes and epigenetic regulators including long non-coding RNA (lncRNA). LncRNA is a type of non-coding RNA with a transcript length greater than 200 nt, and accounts for approximately 80% of mammalian genomic transcription products ^[6]. LncRNA can regulate the expression of target genes through a variety of methods and plays an important role in complex biological processes in the body. Many studies have shown that lncRNA can activate gene transcription at the transcriptional level ^[7], participate in translation substitution at the post-transcriptional level ^[8], and produce imprinted genes at the epigenetic modification level ^[9].

With the rapid development of high-throughput sequencing technology, transcriptome sequencing has been widely used in animal studies. In recent years, there have been an increasing number of studies on lncRNA function using high-throughput sequencing technology. The regulation of lncRNA on fat deposition has been reported in various species, including humans, mice, and cattle. For example, Alvarez used de novo RNA sequencing to compare the transcriptomes of different adipose tissues in mice and identified 127 lncRNAs from brown adipose tissue. In addition, they identified lnc-BATE1, which can regulate the growth and thermogenesis of brown adipocytes^[10]. Li sequenced preadipocytes and mature adipocytes of Qinchuan cattle and discovered 16 differentially expressed lncRNAs. Among them, lncRNA-ADNCR can inhibit the differentiation of adipocytes by competitively binding to miR-204 with SIRT1^[11]. However, there are few studies that focus on the regulation of lncRNA on backfat deposition in pigs, and the molecular regulation mechanism for this process is still unclear.

In this study, we performed biological replicates and two pools using high/low phenotype strand-specific library RNA sequencing (RNA-seq) on six Landrace pigs with either extremely-high or -low backfat thickness. We identified the differentially expressed genes and lncRNAs, combining two-part results to obtain the key genes and lncRNAs that affect fat deposition in Landrace pigs (Figure 1). In addition, we explored the crucial genes and lncRNAs involved in molecular regulation mechanisms to provide information for improving fat deposition in pigs.

Materials and Methods

Experimental animals and sample collection

A Landrace female pig resource population was used in this study. We used real-time B-mode ultrasonography (HS1500 convex scanner; Honda Electronics, Toyohashi, Japan) to measure the backfat thickness between the last 3rd and 4th ribs. Combined with pedigree information, we selected three full-sib Landrace pairs with extreme backfat thickness from 132 female Landrace pigs. Three of the pigs with extremely-high backfat thickness formed the high group (BH, 8.88 ± 0.80 mm) and three with extremely-low backfat thickness formed the low group (BL, 3.58 ± 0.39 mm). The experimental population phenotype and selection criteria were the same as those previously reported^[12, 13]. The subcutaneous fat tissues of these individuals were collected after slaughter and then immediately stored in liquid nitrogen until use.

RNA extraction and sequencing

We used a total RNA extraction kit (Bioteke Corporation, Wuxi, China) to extract the total RNAs of all the samples according to the manufacturer's recommendations, and the quality of the extract was checked by a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). After quality control, a library was prepared using the total RNAs. The sequencing library was prepared by random fragmentation of the cDNA samples, followed by 5' and 3' adapter ligation. The adapter-ligated fragments were then PCR-amplified and gel-purified. For cluster generation, the library was loaded into a flow cell where fragments were captured on a lawn of surface-bound oligos complementary to the library adapters. Each fragment was amplified into distinct clonal clusters through bridge amplification. After cluster generation, the samples were sequenced in an Illumina Hiseq 2500 sequencer (Illumina, Inc., San Diego, CA, USA). The sequencing was performed by Biomarker Technologies, Inc. (Rohnert Park, CA, USA). The raw data obtained from the sequencing were trimmed using Trimmomatic software^[14]. Clean data were achieved

after deleting the adapter and low quality reads (Phred quality score ≤ 10). Using FastQC (v 0.11.8)^[15] to check the quality of the paired-end reads of all the six samples. We then used a NGSQC Toolkit (v 2.3.3)^[16] to perform quality inspection and data filtering of the high-throughput sequencing data. Only good quality trimmed reads were considered for downstream analysis.

Mapping and assembly of sequenced RNA reads

The pig reference genome (Sus scrofa 11.1) and annotation files were downloaded from the Ensembl database (http://asia.ensembl.org/Sus_scrofa/Info/Index) and indexed with Bowtie2 (v 2.3.0)^[17]. Clean reads were aligned with the reference genome using TopHat2 (v 2.1.1)^[18] alignment program. Software Cufflinks (v 2.2.1)^[19] was used to assemble the mapped reads and determine their abundance in all the samples, keeping all parameters as default. Cuffmerge, a Cufflinks suite tool, was used to merge samples and obtain the merged gene transfer format (gtf) file for further downstream analysis.

LncRNA filtering pipeline

High stringency was used to filter out putative lncRNAs from the RNA-Seq assembled transcripts. Only non-coding classcodes (such as 'j,' 'i,' 'o,' 'u,' 'x,' and '=') were retained from the Cuffmerge results; all others were removed. Transcripts with less than 200 nucleotides and less than two exon numbers were also deleted. Filtered transcripts were subjected to three of the most frequently used tools for identifying long non-coding RNAs, two of which were the Coding Potential Calculator (CPC)^[20] and Coding-Non-Coding-Index (CNCI)^[21]. Common transcripts from the results of these two tools were then scanned against the Pfam database to discover any probable protein domains^[22]. Similar protocols have been used in previous studies to identify

lncRNAs^[9, 23].

Identification of differentially and exclusively expressed lncRNA and mRNA

We used htseq software (v 0.7.2)^[24] to count the read counts of genes and lncRNAs, and further screened genes by their expression level in different groups. EdgeR^[25], an R package, was used to determine the expression difference between the two groups using an overdispersed Poisson model. We used false discovery rate (FDR) ≤ 0.05 , and |Fold Change| > 2 as the threshold to identify significantly differentially expressed lncRNAs (DELs) and differentially expressed genes (DEGs). Heatmap clustering analysis of all differentially expressed lncRNAs and messenger RNAs (mRNAs) was calculated using the pheatmap R^[26] package. Moreover, if the transcripts were expressed in more than two samples in one group and were not expressed in all samples of the other group, we considered them to be exclusively expressed lncRNAs and exclusively expressed genes.

Gene ontology and KEGG pathway functional annotations

Gene Ontology (GO, <http://www.geneontology.org>^[27]) is the international standard classification of gene function. It classifies the function of genes along three aspects, including molecular function, biological process, and cellular component. Gene Ontology (GO) provides a framework and set of concepts for describing the functions of gene products from all organisms. The Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg>^[28]) can systematically analyze gene function and is a genomic information database that helps researchers to study genes and expression information as a whole network. The functional annotation of the DEGs in this study based on GO and KEGG was completed using DAVID (v 6.8)^[29] and KOBAS 3.0^[30], respectively. The GO terms and KEGG pathways with p values < 0.05 were considered as

significantly enriched, and the results were plotted using the ggplot2^[31] R package.

Target genes prediction and functional annotations

To investigate the function of the differentially expressed lncRNAs, we searched their nearby genes and considered them as potential targets of lncRNAs. It has been reported that the main function of lncRNA is to regulate protein-coding genes through cis- and trans-regulation^[32, 33].

Bedtools (v2.25.0)^[34] was used to search neighborhood genes around 100 kb upstream and downstream of the DELs. In addition, we examined the Spearman's rank correlation between the expression levels of the DELs and DEGs to predict their targeting relationship.

Statistical Analysis

All data were reported as mean $\pm$ standard error of mean. The functions glmFit() and glmLRT() of the edgeR package were used to perform a negative binomial generalized log-linear model fitting and significance test, respectively, and we used the FDR method to adjust the p values. An FDR < 0.05 was considered significant.

Results

Overview of the lncRNA sequencing data

In the biological replicate RNA-seq, a total of 16.24, 16.50, 16.20, 16.32, 16.07, and 17.34 Gb of clean data were generated in the six samples, respectively. Furthermore, the results of the pooling RNA-seq were 16.45 Gb and 15.16 Gb in the extremely-high (HIGH) and extremely-low (LOW) backfat thickness groups, respectively. The average of the guanine-cytosine (GC) content was between 49.37% and 55.55%. The Q30 ranged from 92.53% to 94.27%. These results show that

177 the quality of our eight libraries was suitable for subsequent data analysis.

178 Next, the clean reads were aligned to the reference genome (*Sus scrofa* 11.1) using Tophat v2.1.1.

179 In each sample, more than 77.82% of the clean reads were uniq mapped. The summary of the

180 sequencing data is shown in Table 1.

Table 1 Summary of reads after quality control and mapping to the reference genome

Sample ID	Biological Replicate RNA sequencing						Pooling RNA sequencing	
	H22511	H23712	H31210	L22509	L23709	L31208	HIGH	LOW
Clean Data	16202102282	16317045132	17343934922	16244957328	16074210300	16498461484	15526646169	14298214979
GC(%)	50.71	54.34	55.24	50.89	55.55	51.63	51.38	49.37
Q30(%)	94.27	93.32	93.71	93.76	93.84	93.76	92.53	92.62
Total Reads	108162730	109081084	115964182	109091500	107499706	110216006	106068646	97723146
Mapped Reads	103261178	99528016	106878278	101789248	96471336	103862050	84998524	79213694
	(95.47%)	(91.24%)	(92.16%)	(93.31%)	(89.74%)	(94.23%)	(80.14%)	(81.06%)
Uniq Mapped Reads	96976910	93090379	97146560	97935676	92128163	98096189	82541284	77406164
	(89.66%)	(85.34%)	(83.77%)	(89.77%)	(85.70%)	(89.00%)	(77.82%)	(79.21%)
Multiple Mapped Reads	6284268	6437637	9731718	3853572	4343173	5765861	2457240	1807530
	(5.81%)	(5.90%)	(8.39%)	(3.53%)	(4.04%)	(5.23%)	(2.32%)	(1.85%)

Guanine-cytosine (GC) (%) is the percentage of G and C bases in the total nucleotides; Q30 (%) is the percentage of the bases' mass greater than or equal to Q30 in the clean data; Total Reads is the number of clean reads; Uniq Mapped Reads is the number and percentage of reads that mapped to a unique position in the reference genome in the clean reads; Multiple Mapped Reads is the number and percentage of reads that mapped to multiple positions in the reference genome in the clean reads.

1 **Identification and feature analysis of putative lncRNAs in Landrace pig backfat**

2 We followed stringent criteria and created a pipeline to identify lncRNAs, as shown in Figure 2A.

3 All mapped reads were assembled using Cufflinks. In addition, Cuffmerge was used to merge all

4 the GTF files from the biological replicate and pooling samples to obtain a combined GTF file

5 containing 146,397 transcripts. The information in the assembled transcripts also included the

6 classcode of each transcript, such as '=', '.', 'c', 'j', 'e', 'i', 'o', 'p', 'r', 'u', 'x', and 's.' The

7 transcripts with non-coding potential classcodes of 'j', 'i', 'o', 'u', 'x', and '=' were reserved^[35],

8 after which we filtered them with the length and exon numbers. Next, we predicted the coding

9 potential by CNCI and CPC and compared the transcripts to the Pfam protein database. Finally,

10 we identified 2,013 lncRNAs (Figure 2B). Among them, 334 were known transcripts and 1,679

11 were new transcripts, including 1,250 lincRNAs (62.10%), 506 anti-sense lncRNAs (25.14%), 154

12 sense lncRNAs (7.65%), and 103 intronic lncRNAs (5.12%) (Figure 2C). These 2,013 lncRNAs

13 were distributed throughout all pig chromosomes, but chromosome 1 contained the most lncRNAs

14 (Figure 2D). The majority of the lncRNA lengths were between 601 and 800 nt, and lncRNA with

15 two exons were the most common, as shown in Figures 2E and F.

16

17 **Expression analysis of differentially and exclusively expressed lncRNAs and genes in**

18 **extremely-high or -low backfat pigs**

19 Studies have shown that the expression level of lncRNAs is usually lower than that of

20 protein-coding genes^[36]. In the present study, we compared the expression of the putative

21 lncRNAs with the mRNAs. We found that the lncRNAs tended to be expressed at a lower level

22 than the protein-coding genes in both the biological replicate and pooling RNA-seq (Figures 3A

and 4A). In the biological replicate RNA-seq, 1,512 genes and 220 lncRNAs (Additional File 2) were differentially expressed between the two groups, of which 820 genes were up-regulated in the BH group compared to those in the BL group, and 692 were down-regulated (Figures 3B and D). The expression of 116 lncRNAs was up-regulated in the BH group, and 104 lncRNAs were down-regulated (Figure 3G). Next, we used the 1,512 and 220 differentially expressed mRNAs and lncRNAs, respectively, to perform a clustering analysis. As shown in the heatmap, the expression patterns of the samples in the BH group were distinguished from their expression patterns in the BL group, both for the DEGs (Figure 3E) and DELs (Figure 3H).

In the pooling RNA-seq, we carried out the same analysis as that used for the biological replicate RNA-seq. We found that 2,240 genes and 127 lncRNAs (Additional File 2) were differentially expressed in the two groups, of which 1,206 genes were up-regulated in the HIGH group compared to those in the LOW group, and 1,034 were down-regulated (Figures 4B and E). There were 83 lncRNAs with up-regulated expression levels in the HIGH group, and 44 lncRNAs were down-regulated (Figure 4F).

In addition, to further explore the difference in gene expression between the high and low backfat thickness groups, we analyzed the lncRNAs and genes exclusively expressed in the two groups. There were 620 genes and 133 lncRNAs exclusively expressed in the BH group and 607 genes and 118 lncRNAs exclusively expressed in the BL group (Figures 3C and F). Moreover, based on the pooling RNA-seq results, we identified 643 genes and 136 lncRNAs exclusively expressed in the HIGH group and 892 genes and 177 lncRNAs exclusively expressed in the LOW group (Figures 4C and D).

45

46 **GO and KEGG functional enrichment analysis of DEGs**

47 We used the differentially expressed genes detected in the two sequencing methods to perform a
48 functional enrichment analysis. Figures 5A and C show the ten most significant (FDR < 0.05)
49 terms in the Biological Process (BP) class and the five most significant terms in the Cellular
50 Component (CC) and Molecular Function (MF) classes. In the biological replicate RNA-seq, a
51 total of 977 DEGs were annotated in GO terms. We found many terms directly related to fat
52 development and metabolism, especially in the BP class, including ‘tricarboxylic acid cycle,’
53 ‘triglyceride homeostasis,’ and ‘triglyceride catabolic process.’ Furthermore, there were four
54 mRNAs enriched to ‘positive regulation of triglyceride catabolic process’. This GO term was also
55 significant but not within the top ten; so, it is not displayed in Figure 5. In addition, DEGs were
56 also enriched in some glucose metabolism pathways such as ‘gluconeogenesis’ and ‘glycolytic
57 process.’ The results of the pooling RNA-seq are shown in Figure 5C. We focused on several
58 terms, including ‘positive regulation of fatty acid biosynthetic process,’ ‘cholesterol biosynthetic
59 process,’ ‘lipoprotein metabolic process,’ and ‘carbohydrate metabolic process.’ We also found
60 terms such as ‘gluconeogenesis’ and ‘glycolytic process,’ which were the same as those in the
61 biological replicate RNA-seq. All the terms that were considered important are highlighted by a
62 red frame in Figure 5.

63 Meanwhile, these DEGs also underwent a KEGG analysis to annotate their functions. Figures 5B
64 and D show the most significant 20 pathways in the KEGG results. In the biological replicate
65 RNA-seq, the pathways related to glucose and lipid metabolism, including ‘Glycolysis /
66 Gluconeogenesis,’ ‘Citrate cycle (TCA cycle),’ ‘Pyruvate metabolism,’ ‘Fat digestion and

absorption,' and 'Starch and sucrose metabolism,' were significantly enriched. Moreover, in the pooling RNA-seq, we also found two new pathways that were not previously enriched, 'Synthesis and degradation of ketone bodies' and 'Pentose phosphate pathway,' both of which are important in the glucose metabolism program. We also circled the important pathways with a red frame in Figure 5.

We used the exclusively expressed genes from the biological replicate RNA-seq to perform the functional enrichment analysis. However, there are only a few KEGG pathways related to glucose and lipid metabolism, most of which involved the exclusively expressed genes in the BH group. These pathways include 'Fat digestion and absorption,' 'Synthesis and degradation of ketone bodies,' and 'Pentose phosphate pathway.' Therefore, we believe that the other GO terms and KEGG pathways are not as necessary. These results are shown in Additional File 1.

Target gene prediction of differentially expressed lncRNAs

To further understand the potential function of lncRNA in fat deposition, we predicted the target genes of the lncRNAs. The general method of predicting lncRNA target genes is to search upstream or downstream to identify nearby protein-coding regions, termed as cis-regulating target genes^[37]. It has been reported that lncRNA can regulate coding genes around 10 to 500 kb up and downstream^[38]. We searched the nearby coding genes around 100 kb up and downstream from the 39 DELs, which were common in the two sequencing results. We found 76 pairs of cis-regulatory relationships between 39 DELs and 67 genes (Additional File 3).

If the expression patterns of lncRNA and mRNA show a highly positive or negative correlation, their functions may be highly correlated^[39]. Therefore, we conducted a Spearman's rank

correlation analysis between the expression of the DELs and DEGs. The expression levels of 116 DEGs were significantly correlated with the 39 DELs (correlation coefficient > 0.934 , $P < 0.05$). They were also considered as potential target genes of these DELs. We used 39 DELs and their target genes to draw a picture of the regulatory network to investigate the relationship between them (Figure 6). We highlighted the lncRNAs and their target genes for further analysis with yellow nodes and red labels.

Comparison of biological replicate and pooling RNA-seq

We utilized fragments per kilobase of transcript per million mapped reads (FPKM) ≥ 0.1 as the criteria to ensure expression in all samples, then tested the expression levels of the lncRNAs and genes in each sample using biological replicate and pooling RNA-seq. A total of 1,604 lncRNAs and 16,002 genes were identified in both sequencing results. We also found 266 unique lncRNAs and 2,505 unique genes in the biological replicate RNA-seq; however, only 14 lncRNAs and 1,124 genes were uniquely detected in the pooling RNA-seq (Figures 7A and B). Next, GO and KEGG functional enrichment analysis were performed again with the unique genes of the two sequencing methods. As shown in Figures 7C-F, none of the GO terms or KEGG pathways were related to glucose and lipid metabolism in this analysis.

Discussion

In our study, six full-sib Landrace pigs with either extremely-high or -low backfat thickness were taken as paired samples, and their back subcutaneous tissues were collected. We compared the transcriptome data of the two groups using two methods of RNA-seq, identified the differentially

expressed lncRNAs and genes, then analyzed their function in fat development. The results of our study provide a basis for the selection of pigs for breeding using lncRNAs and their target genes to improve the fat deposition traits of pigs.

In this study, we identified a total of 19,631 genes and 2,013 lncRNAs. In accordance with the results of a previous study ^[36], we found that the expression of lncRNA was much lower than that of mRNA. Our results are similar to those in several previously published articles on adipose tissue transcriptome sequencing. For example, Miao identified 4,910 lncRNAs, 119 of which were differentially expressed in the intramuscular fat tissue of Jinhua and Changbai pigs ^[40]. The results for the functional enrichment of the DEGs showed that some genes were enriched to pathways related to glucose and lipid metabolism. In addition, these pathways were confirmed to be related to lipid metabolism in previous studies, including *APOA1* ^[41] and *STARD3* ^[42]. Chen identified 581 putative lincRNAs related to pig muscle growth and fat deposition, and their target genes were involved in fat deposition-related processes such as the lipid metabolic process and fatty acid degradation ^[43]. The KEGG results showed that the meaningful pathways were mostly concentrated on glucose metabolism. Some of these pathways, such as ‘Glycolysis / Gluconeogenesis,’ were also found in a previous study by our team ^[44]. Although they are not directly related to lipid synthesis and metabolism, glucose and lipids can change into each other and participate together in the tricarboxylic acid cycle. Therefore, the process of glucose metabolism can have an indirect effect on lipid metabolism.

As a kind of non-coding RNA, the main role of lncRNA is to regulate their target genes: cis-regulating nearby protein-coding genes and trans-regulating distant protein-coding genes. We determined 67 cis-target genes and 116 trans-target genes regulated by DELs. The two target

genes we focused on were *ACACB* and *ACSL3*, both of which have been found to be related to lipid metabolism in other studies. *ACSL3* (acyl-CoA synthetase long chain family member 3) is located 64 kb downstream of TCONS-00052400. At the beginning of fatty acid metabolism, long-chain acyl-CoA synthetase (*ACSL*) can activate the conversion of long-chain fatty acids to fatty acyl-CoA^[45]. The *ACSL* family contains five different isoforms, including *ACSL3*, which play different roles in lipid metabolism. Some previous studies showed that knockdown of *ACSL3* may significantly reduce the activity of several lipid-producing transcription factors like peroxisome proliferator-activated receptor- γ and sugar response element binding protein, and then regulate the fat production process in the liver^[46]. Furthermore, *ACSL3* can also affect the secretion of very low-density lipoproteins (VLDL) by promoting the synthesis of lecithin^[47].

ACACB (acetyl-CoA carboxylase beta) is a differentially expressed gene that is cis-regulated by the lncRNA TCONS-00041740, which is located on the antisense strand of *ACACB*. Moreover, in the KEGG enrichment analysis, *ACACB* was involved in the 'pyruvate metabolism' pathway. Based on previous studies, we knew that *ACACB* is the rate-limiting enzyme in fatty acid oxidation^[48]. Moreover, in *ACACB* knockout mice, continuous fatty acid oxidation increases insulin sensitivity, and feeding them a high fat/high carbohydrate diet is more likely to cause obesity and diabetes^[49]. In a previous study by Li, *ACACB* was shown to be a marker gene for childhood obesity^[50].

Some genes were exclusively expressed in one of the two groups. For example, *PLCB2* (phospholipase C beta 2) was exclusively expressed in the BL group. Phospholipase C is a class of glycerol phospholipid hydrolases, hydrolyzing the glycerol phosphate C3 site^[51]. The protein encoded by *PLCB2* is a phosphodiesterase that catalyzes the hydrolysis of phosphatidylinositol 4,

5-bisphosphate to the secondary messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol.

In addition, the gene *PLA2G12B* was exclusively expressed in the BH group. *PLA2G12B* (phospholipase A2 group XIIB) is encoded by this gene and belongs to the phospholipase A2 (*PLA2*) group of enzymes, which plays a role in lipid hydrolysis by releasing free fatty acids and lysophospholipids ^[52]. Studies have shown that a reduction of *PLA2G12B* decreases the amount of serum triglyceride (TG)-rich VLDL particles secreted by the liver, resulting in a reduction in TG content ^[53]. *PLA2G12B* can also participate in the pathogenesis of idiopathic membranous nephropathy (iMN) by regulating lipid metabolism ^[54]. In a previous study by Guan, *PLA2G12B*-null mice had obvious accumulation of large lipid droplets in the liver, displaying the fatty liver phenotype ^[55]. These results indicate that the genes exclusively expressed in the high or low backfat groups may also have a certain regulatory effect on lipid metabolism.

Biological replicate and pooling RNA-seq are two conventional methods used in RNA-seq experiments. In this study, biological replicate RNA-seq detected more lncRNAs and genes than did pooling RNA-seq. Few unique lncRNAs were identified in the latter. However, in the function annotation, the unique genes of both sequencing methods did not enrich the GO terms and KEGG pathways related to glucose and lipid metabolism. To a certain extent, this indicates that the unique genes have no direct influence on fat deposition in Landrace pigs. These results illustrate that when RNA-seq is used to excavate key genes, biological replicate RNA-seq is superior in ‘quantity’, but there is no obvious ‘qualitative’ difference between the two methods. We propose that when the experimental conditions are constrained, such as if the cost of sequencing is too high or the samples are difficult to obtain, replacing biological replicate RNA-seq with pooling RNA-seq can also achieve important results. However, using RNA-seq to determine important

lncRNAs, microRNAs, or other noncoding RNAs via pooling RNA-seq only may cause some important information to be masked.

During pig growth, the excessive development of adipose tissue leads to an excessive accumulation of lipids, which affects the carcass quality of pigs. Overweight pigs show low lean meat rates, low feed conversion rates, and slow growth^[56], while overweight sows may experience dystocia and postpartum disease^[57]. Numerous studies have shown that pigs have many similarities with humans in anatomical structure, physiological metabolism, and disease mechanisms^[58, 59]. Therefore, pigs have many advantages as an animal model for human disease research, especially related to obesity^[60]. For example, after feeding China Guizhou miniature pigs a high-fat, high-sugar diet for 6 months, Xi^[61] found that the pigs developed insulin resistance, mild diabetes, and atherosclerotic lesions. Therefore, this model pig may be a suitable animal model for studying diabetic dyslipidemia complicated with atherosclerosis. Pig backfat thickness can directly reflect their body fat content. Our study obtained differentially expressed lncRNAs between pigs with extremely-high and -low backfat thickness, providing a novel approach for the use of lncRNAs and their target genes to screen low backfat pigs in future breeding work, thereby further improving fat deposition traits in pigs.

Conclusions

In this study, we identified a total of 307 DELs and 3,034 DEGs between pigs with extremely-high and -low fat deposition traits. After the analysis of potential cis- and trans- target genes, we found 183 genes that could be regulated by DELs. Through further functional analysis, we found that two potential targeting relationships may have an important regulatory effect on fat deposition:

TCONS-00041740 to *ACACB*, and TCONS-00052400 to *ACSL3*. These results can provide useful information for understanding the regulation of fat deposition by lncRNA in pigs. However, further genetic experiments are still needed to validate the association of the lncRNA and mRNA functions presented in this study.

Declarations

Ethics approval and consent to participate: All protocols and procedures involving animals were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China) and were approved by the Animal Welfare Committee of China Agricultural University (permit number: DK996).

Consent for publication: Not applicable.

Availability of data and material: The deep sequencing data of total RNA were submitted to NCBI Sequence Read Archive (SRA) with accession number Bioproject: PRJNA6600160.

Competing Interests: The authors declare no competing interests.

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Authors' contributions: Y.L., Y.Y., and C.W. designed the study. Y.L., K.X., F.Z., X.Z., and H.L. performed the experiments and collected samples. K.X., Y.S. and H.A. analyzed the data. Y.L. and K.X. wrote the paper. Y.Y. and C.W. revised the manuscript. All authors read and approved the final manuscript.

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Figure Captions

Figure 1 Technical route of the current study

Figure 2 Summary of putative long non-coding RNA (lncRNA) characteristics in Landrace

pig backfat

(A) Identification pipeline of lncRNAs; (B) Venn diagram of identified lncRNAs from the Coding

Potential Calculator (CPC), Coding-Non-Coding Index (CNCI), and Pfam database; (C) Type and

number of putative lncRNAs; (D) Chromosome distribution of lncRNAs; (E) Length distribution of lncRNAs; (F) Exon number distribution of lncRNAs.

Figure 3 Expression of long non-coding RNAs (lncRNAs) and genes in biological replicate

RNA sequencing (RNA-seq) in extremely-high and -low backfat pigs

(A) Comparison of the expression levels between the lncRNAs and messenger RNAs in six samples. The value of $\log_{10}(\text{Fragments per kilobase of transcript per million mapped reads [FPKM]} + 1)$ is plotted on the Y-axis; (B) Number of differentially expressed lncRNAs (DELs) and differentially expressed genes (DEGs) between the high and low groups; (C) Venn diagram of genes from each group; (D) Volcano plot of DEGs. The Y-axis is the value of $-\log_{10}(\text{false discovery rate[FDR]})$ and the X-axis is the value of $\log_2(\text{FC})$. The two threshold lines show the standard of $\text{FDR} = 0.05$ and $\text{FC} = 2$; (E) Heatmap of genes in six samples. The color scale indicates the FPKM values. Orange indicates high expression and purple indicates low expression; (F) Venn diagram of lncRNAs from each group; (G) Volcano plot of DELs; (H) Heatmap of lncRNAs in six samples.

Figure 4 Expression of long non-coding RNAs (lncRNAs) and genes based on the pooling

RNA sequencing results for extremely-high and -low backfat pigs

(A) Comparison of expression levels between the lncRNAs and messenger RNAs in two groups; (B) Number of differentially expressed lncRNAs (DELs) and differentially expressed genes (DEGs) between the HIGH and LOW groups; (C) Venn diagram of the genes from each group; (D) Venn diagram of the lncRNAs from each group; (E) Volcano plot of the DEGs; (F) Volcano plot of the DELs.

Figure 5 Functional enrichment analysis of differentially expressed genes (DEGs)

(A) Gene Ontology (GO) annotation of DEGs in biological replicate RNA sequencing (RNA-seq); (B) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of DEGs in biological replicate RNA-seq; (C) GO annotation of DEGs in pooling RNA-seq; (D) KEGG pathway analysis of DEGs in pooling RNA-seq. The red boxes indicate the GO terms and KEGG pathways related to glucose and lipid metabolism.

Figure 6 Network of regulatory relationships between differentially expressed long

non-coding RNAs (DELs) and their target genes

DELs are indicated in red ovals; target genes are indicated in blue diamonds; the important long non-coding RNAs and genes are highlighted with yellow nodes and red labels.

Figure 7 Analysis of unique genes in biological replicate and pooling RNA sequencing

(RNA-seq)

(A) Venn diagram of unique genes in two sequencing methods; (B) Venn diagram of unique long non-coding RNAs (lncRNAs) in two sequencing methods; (C) Gene Ontology (GO) annotation of unique genes in biological replicate RNA-seq; (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of unique genes in biological replicate RNA-seq; (E) GO annotation of unique genes in pooling RNA-seq; (F) KEGG pathway analysis of unique genes in pooling RNA-seq.

Figures

Figure 1

Figure 1

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Figure 2

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Figure 7

Figure 7

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