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Transcriptome Profiling of lncRNA Related to Fat Tissues of Qinchuan Cattle

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Abstract:

Qinchuan cattle is one of the five yellow cattle breeds in China with good performance of meat. The proliferation and differentiation level of muscle and fat are closely related to the growth and development of the organism and are the key factors affecting the quality of meat. In order to study the effect of lncRNA on the fat tissues of Qinchuan cattle, six calf and adult bovine adipose tissues were selected for high-throughput sequencing. We obtained 3,716 lncRNA candidates from calves and adult cattle fat samples, among them 789 lncRNA were annotated and 2,927 lncRNA were novel lncRNA. A number of lncRNAs were highly abundant, and 119 lncRNA were differentially expressed between two developmental stages. We further validated several differentially expressed lncRNAs using qPCR, and the results were consistent with the sequencing data. Therefore, we conclude that lncRNA may play an important role in adipose tissue in different age groups of cattle.

Abbreviations:

lncRNAs: long noncoding RNAs
RNA-seq: Ribo-Zero RNA sequencing
CNCI: Coding- non-coding -Index
CPC: Coding Potential Calculator
PhyloCSF: phylogenetic codon substitution frequency
TUCP: transcripts of uncertain coding potential

Key words: RNA-seq; lncRNA; fat tissue; Qinchuan Cattle

Introduction:

Fat is synthesized from glycerin and fatty acids and is an important part of the organism. As the core of the nutritional value of meat, it has a positive effect on the appearance, texture, flavor, hardness and shelf life of the meat[1]. However, the excessive deposition of fat not only affects meat quality, but also risks to human health. Therefore, clarifying the regulation mechanism of lipogenesis is of great significance for improving meat quality. Lipogenesis is a complex and elaborate procedural regulation process involving a series of transcription factors[2]. At present, more and more researchers have reported on the biological processes of fat synthesis.[3, 4] Numerous studies have shown that the peroxisome proliferator-activated receptor (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPα) play a key role in this process. However, lipogenesis involves multiple gene expression, signal transduction, and network regulation. The process is complex and new regulatory factors are constantly being identified. Recent studies have shown that long noncoding RNAs (lncRNAs) also have important regulatory effects on adipogenesis, but there are few studies in cattle.

Long non-coding RNA (LncRNA) with few protein coding functions is a class of non-coding RNA molecules with a transcript length of more than 200 nt, which are located in the nucleus or cytoplasm and are generally transcribed in eukaryotic cells[5]. LncRNA initially was considered as by-products of RNA polymerase II transcription, and have few biological function. However, many studies in recent years have shown that LncRNA is involved in regulating the expression of protein-coding genes at various levels, including epigenetic regulation[6, 7, 8], transcriptional regulation[9] and post-

transcriptional regulation[10, 11], which influences various biological processes, such as dose effect, gene imprinting, cell cycle, development and gamete formation.

Qinchuan cattle is one of the best cattle breeds in China, its good meat performance has attracted much attention[12]. As early as 2016, Li et al. have studied the bovine preadipocytes and differentiated adipocytes, and found that 16 lncRNAs are differentially expressed during adipocyte differentiation. Among these lncRNAs, they found that the expression of lncRNR ADNCR was significantly higher in preadipocytes than differentiated adipocytes and suppresses adipogenic differentiation by targeting miR-204[13]. The aim of our study was to identify lncRNAs with potential roles in calf and adult Qinchuan cattle fat tissues. We hope that our study can provide some references for Qinchuan cattle genetic breeding and promote the improvement of beef cattle breeding in China.

Materials and methods

Sample collection

In this study, subcutaneous adipose tissue from three calves (6 months old) and three adult Qinchuan cattle (24 months old) groin sites were collected from a local slaughterhouse in Xi'An, China. The fresh tissues were immediately frozen in liquid nitrogen, and then stored at -80^{o}C until use. All experiments performed in this study were approved by the International Animal Care and Use Committee of the Northwest A&F University (IACUC–NWAFU). Furthermore, the care and use of animals complied with the local animal welfare laws, guidelines, and policies.

Library preparation, clustering and sequencing

Total RNA was extracted from cells using TRizol reagent (Life Technologies, United States) according to the instructions. Quality was monitored by NanoDrop ND-1000 and Agilent Bioanalyzer 2100 (7≤RIN values≥8). A total amount of 3 μg RNA per sample was used as input material for the RNA sample preparations. Ribosomal RNA was removed by Epicentre Ribo-zero™ rRNA Removal Kit (Epicentre, USA), and rRNA free residue was cleaned up by ethanol precipitation. Subsequently, sequencing libraries were generated using the rRNA-depleted RNA by NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer's instructions. After cluster generation, the libraries were sequenced on an Illumina Hiseq 4000 platform and 150 bp paired-end reads were generated.

Sequencing quality control and reads mapping

Raw data (raw reads) of fastq format were firstly processed through in-house perl-scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads on containing ploy-N and low quality reads from raw data. Then the clean reads were mapped to the Bos taurus genome (https://www.ncbi.nlm.nih.gov/genome/82?genome_assembly_id=371813) using Tophat with three base mismatches allowed. Data was normalized by calculating the FPKM for each gene. The mapping results were compared to the known genes recorded in the database using Cufflinks compare.[14]

Prediction of lncRNA

LncRNAs were predicted by the following steps. Firstly, choosing the transcription which exon number > = 2 and the length > 200 bp. Secondly, the spliced exonic regions transcripts in this study were annotated based on their mapping to lncRNAs in the database. Expressions of each transcript were calculated by Cuffquant, and transcripts with FPKM >= 0.5 were selected. Finally, coding potential of transcripts was predicted by CNCI (Coding- non-coding -Index), CPC (Coding Potential Calculator), Pfam Scan (v1.3), and PhyloCSF (phylogenetic codon substitution frequency). Transcripts that were identified as having coding potential by at least one coding potential predictor software as TUCP (transcripts of uncertain coding potential) [15]. The raw sequencing dataset supporting the results of this study was deposited in the NCBI GEO database (https://www.ncbi.nlm.nih.gov/geo/). The data are accessible through GEO: GSE133735 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE133735).

Target Gene Prediction

The candidate lncRNAs were selected for target prediction via cis- or trans-regulatory effects. For the cis pathway target gene prediction, the genes transcribed within a 10kb window upstream or downstream of lncRNAs were considered as cis target gene. For trans-acting target genes prediction, we clustered the genes from different samples with WGCNA to search common expression modules, the screening conditions were such that the correlation coefficient of lncRNA to mRNA expression was greater than 0.95. Then analyzed their function through functional enrichment

analysis.

Quantification of gene expression level and differential expression analysis

Cuffdiff (v2.1.1) was used to calculate FPKMs of both lncRNAs and coding genes in each sample[16]. Gene FPKMs were computed by summing the FPKMs of transcripts in each gene group. Differentially expressed transcripts were analyzed by Cuffdiff to calculate the q-value and fold change, transcripts with a P-adjust <0.05 were assigned as differentially expressed.

GO and KEGG enrichment analysis

We used GO and KEGG to analyze the main function of the genes and lncRNAs. GO enrichment analysis of differentially expressed genes or lncRNA target genes were implemented by the GO seq R package, in which gene length bias was corrected[17] (P < 0.05). KEGG is a database resource for understanding high-level functions and utilities of the biological system[18] (http://www.genome.jp/kegg/). We used KOBAS software to test the statistical enrichment of differential expression genes or lncRNA target genes in KEGG pathways[19].

Validation of RNA-seq results by qPCR

We conducted qPCR to validate the results from our RNA sequencing approach, 6 differentially expressed lncRNAs were randomly selected to test their expression levels. Total RNA samples were reverse transcribed to cDNA using the PrimeSriptTM RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) according to the manufacturer's recommendations. qPCR was performed using the SYBR R Premix Ex TaqTM kit (TaKaRa, Dalian, China) on the Bio-Rad CFX96 Real-Time PCR system

(Hercules, CA, United States). All the primers are presented in Table S1. The qRT-PCR amplification program was as follows: pre-denaturation at 95ºC for 30 s, followed by 39 cycles of 95ºC for 5 s, 60ºC for 30 s. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative expression levels of lncRNAs.

Statistical analysis

Data were analyzed using Student's t test in GraphPad Prism 6 software. The results were presented as mean ± SEM, and *P* values of < 0.05 were considered statistically significant.

Results

Profile of lncRNA Expression in Cattle fat tissues

In order to determine the expression of lncRNA in adipose tissue of Qinchuan cattle, we selected 3 calves and 3 adult cattle for high-throughput sequencing. To test the reliability of this experiment and the rationality of sample selection, we conducted the correlation test of expression level between samples (Figure 1A). In total, we acquired 89~102 and 91~118 million unique mapped clean reads from the calf and adult stage libraries, respectively (Table S2). A total of 3,716 lncRNAs were identified, including 789 annotated lncRNAs and 2,927 novel lncRNAs. LncRNA can be divided into intergenic lncRNA (lincRNA, 75.9%), intronic lncRNA, anti-sense lncRNA (24.1%), sense lncRNA, bidirectional lncRNA and so on, here the first three types of screening are mainly carried out (Figure 1B). To study the basic features of lncRNAs in fat tissues of Qinchan cattle, the lncRNAs were identified and compared with mRNA. We found that the number of lncRNA exons and the length of lncRNA were relatively

concentrated compared with mRNA (Figure 1C, 1E). But most of the lncRNAs lack ORFs, while mRNA have and the length of the open reading frame is mostly concentrated at 0-1000 bp (Figure 1D). Considering that transcripts of uncertain coding potential (TUCP) might contain portions of lncRNA, our analysis of the expression levels of the three found that the expression levels of lncRNA and TUCP were significantly lower than mRNA (Figure 1F).

Differential expression analysis of lncRNA

We found 119 differentially expressed lncRNA ($P < 0.05$) in the sequencing results of calves and adult cattle, and 7 lncRNAs ($P < 0.05$; $Q < 0.05$) are shown in Table 1. To further identify the potential roles of these lncRNA, see the clustered heatmap in Figure 2A. Among these 7 lncRNAs, 2 were significantly up-regulated and 5 were significantly down-regulated in adult cattle. In order to explore the relationship between the fold change and the significance, we used volcano plot assays to show the lncRNA expression during different stage development, we can see that from calf stage to adult stage, many lncRNAs showed expression variation (Figure 2B). As lncRNAs could exert effects through *cis*- or *trans*-acting target genes, we then demonstrated the differentially expressed mRNA and TUCP expression (Figure 2C-F), and hope to found out the relationship between those different expression lncRNAs and mRNAs.

GO and KEGG enrichment analysis

After the differentially expressed lncRNA transcripts were obtained, 1047 functional groups were found to be significantly enriched in GO enrichment of the nearby mRNA of differentially expressed lncRNAs ($P < 0.05$), and 239 are shown in

Table S3 ($P < 0.05$, $Q < 0.05$). The top 20 of the three major categories of GO enrichment are shown in Figure 3A. In addition, our data showed that 29 pathways were significantly enriched ($P < 0.05$), and 19 are shown in Table S4 ($P < 0.05$, $Q < 0.05$). We can see that the Oxidative phosphorylation had the highest level of significance with 79 annotated genes, followed by Parkinson's disease. The top 20 enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways are present in Figure 3B ($P < 0.05$). In addition, we found that this has many similar functions to the enrichment of the mRNAs (Figure 3C-D). And the results indicate that these pathways may contribute significantly to metabolism and fat. So the potential function of lncRNAs in this study was predicted using the annotated co-expressed mRNA function.

The validation of RNA-seq data by qPCR

To confirm the accuracy of the RNA-seq data, six differentially expressed lncRNAs related to fatty acid transport and adipocyte associated were validated by qPCR. In the RNA-seq data, we can see that the expression of two lncRNAs in adult bovine adipose tissue is higher than that of calf, and the expression of four lncRNAs is higher in calf than in adult cattle (Figure 4A). In the qPCR experiment, we got the same results (Figure 4B). The qPCR results of the differentially expressed lncRNAs were in agreement with the RNA-Seq data, indicating that the two results validated each other, and our sequencing data is reliable.

Discussion

Genomic DNA transcription produces two RNAs, one that can be converted to protein-encoding RNA and another that does not have or has low coding ability

(ncRNA). With the development of high-throughput sequencing technology, more non-coding RNAs have been discovered and played an important role in the development of biology. In recent years, lncRNA has attracted more and more attention similar to mRNA, most lncRNA were transcribed by RNA polymerase II[20], with structure of 5′ to 3′polarity and polyadenylated tail, and there are alternative splicing. However, different from mRNA, lncRNA has low expression abundance, poor conservation among species, and specific expression in tissues and cells. Most lncRNAs are gradually revealed by their diverse functional mechanisms[21].

Fat is an important tissue of body, which can be derived from mesenchymal stem cells. The development of muscle and fat will affect the growth rate, meat yield and meat quality of livestock[22], and will also lead to obesity and other diseases in humans. Qinchuan cattle is one of the five yellow cattle breeds in China with good performance of meat. Therefore, it is necessary to identify lncRNA expressions in Qinchuan cattle at different development stages. In our study, using an RNA-seq method, 3,716 lncRNAs were identificated in Qinchuan cattle fat tissues. 789 of them has been annotated, 2,927 lncRNAs were novel lncRNA. Of all these lncRNAs, 119 were differently expression, and we chose 8 lncRNA which were differently expression to validate the validity of our sequencing. In the process of lncRNA prediction, we could not determine some transcripts' coding ability, and speculated that they might contain some lncRNAs with coding potential, so we took this part as TUCP (transcripts of uncertain coding potential) for analysis. By comparing lncRNA and mRNA, we showed the comparison of the transcript length, exon number and ORF length of lncRNA and

mRNA, and found that the predicted lncRNA basically conforms to the general characteristics. Although lncRNA can regulate the expression of mRNA in different ways[11], the analysis showed that the expression and characteristics of the two were different. GO analysis found that lncRNA and its target genes are mostly related to metabolic process and intracellular part, KEGG pathway found that they are involved in fatty acid metabolism and degradation (Table S3, S4), indicating that lncRNAs may play an important role in fat development.

With the continuous development of biotechnology, more and more research has shown that lncRNA is involved in the proliferation and differentiation of fat.[13, 23, 24, 25] Whether it is diabetes, cancer or other muscle development and fat deposition studies, all indicated that lncRNA plays an important role in fat development. Although some progress has been made in the study of lncRNA, relatively few studies have been conducted on its molecular regulatory mechanism and functions in the development of muscle and fat in livestock and poultry. In-depth exploration of the molecular structure of lncRNA and its role in physiological and pathological processes will help to further understand the regulatory mechanism of fat cell development and provide an important theoretical basis for diagnosis and treatment of diseases, improvement of animal meat quality traits and breeding of new varieties.

Conclusion

In this study, 3,716 lncRNAs were identificated in calf and adult Qinchuan cattle fat tissues by an RNA-seq method. 789 of them has been annotated, 2,927 lncRNAs were novel lncRNA. Of all these lncRNAs, 119 were differently expression, we

randomly selected 8 of them for verification and found that they are consistent with the sequencing results. We believe that this study will provide some reference for the genetic breeding of cattle.

Author contributions

H.C., R.J. and H.L. designed the study. R.J. and H.L. performed the experiments and drafted the manuscript. R.J., H.L., X.L. and C.L. helped perform the experiments and analyzed the data. H.L., Y.H., and H.C. helped collect tissue samples.

Conflicts of interest

The authors declare no conflict of interest.

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Table 1 Significant differentially expressed lncRNA

LncRNA ID	Calf (FPKM)	Adult (FPKM)	log2 (Adult/Calf)	P-Value
ALDBBTAT0000006609	0.1549	2.1044	3.7634	2.19E-04
ALDBBTAT0000001776	2.3016	0.4129	-2.4786	7.02E-04
ALDBBTAT0000005143	10.4463	22.8363	1.1283	1.61E-03
LNC_002855	1.7096	0.0454	-5.2342	4.03E-04
LNC_000411	5.3698	0.1096	-5.6141	8.93E-04
LNC_002845	8.8792	0.9018	-3.2996	1.065E-03
LNC_002844	2.8071	0.3347	-3.0681	1.498E-03

Table S1 primers for qPCR

Name	Primer pairs sequences (5′–3′)	Amplificationlength (bp)
ALDBBTAT0000000877	F: TATGATGACAAGGGCTGGGA	193
	R: AGGAAGCCCATTCCTCTGCCT	193
LNC_001481	F: CGCCTCCTGCATCTCTGAAA	164
	R: CCAACACCATCACGATTCCG	164
LNC_000411	F: TTACAAGCCTGGTGTGTGAGT	191
	R: TGTGCATTCCAGAGTTACACG	191
LNC_002845	F: ATAAGGGGTTGTGCAATGGGG	295
	R: AAAAGTCCAGGTGTGCTCCAA	295
LNC_002844	F: GGATTCACGTTTCTAAGTTGGTGT	172
	R: TTGCCCTATTCCTTTACAGAGGG	172
ALDBBTAT0000007079	F: GAAGCAGCACAGGCTCCATTT	148
	R: TACCCCCGCCTTTCCTTTGTA	148

Table S2 Summary of reads mapping to the Bos taurus reference genome

Samples	Adult 1	Adult 2	Adult 3	Calf 1	Calf 2	Calf 3
Raw reads	94367280	119126430	109773454	105005806	91779236	93247554
Clean reads	90859922	117571292	105144278	101605688	88865576	89537724
Mapped reads	83050441	110136200	98211003	96538809	84670910	84669040
Mapping ratio	91.4%	93.68%	93.41%	95.01%	95.28%	94.56%
Uniquely mapped reads	69609751	100240434	89608961	88815771	77779872	77656770
Unique mapping ratio	76.61%	85.26%	85.22%	87.41%	87.53%	86.73%

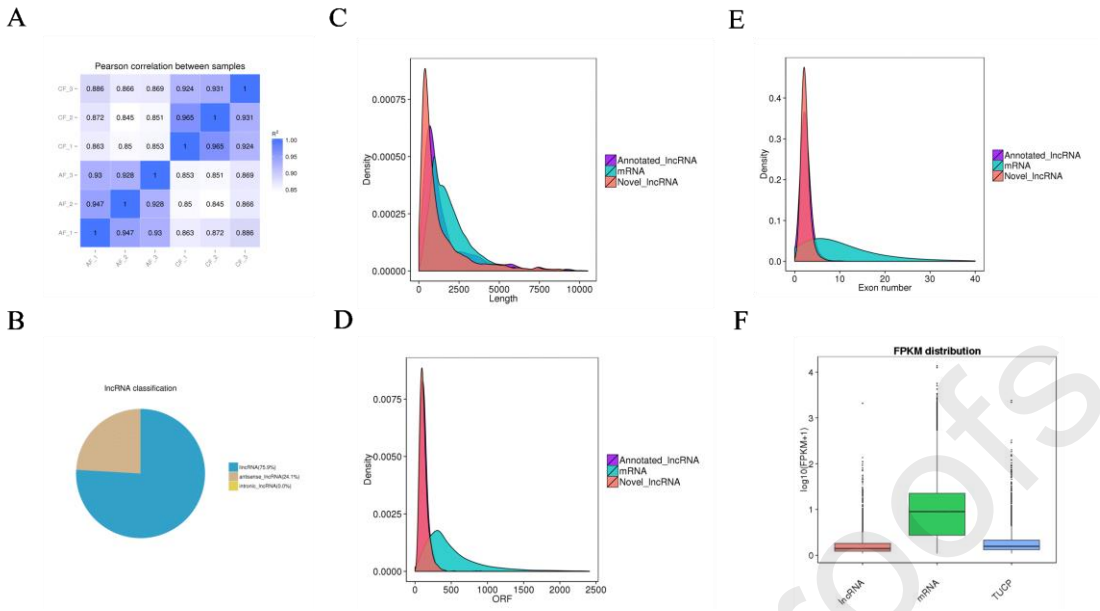

Figure 1. Profile of lncRNA Expression in Cattle fat tissues. (A) the Pearson correlation between samples. (B) the types of lncRNAs. (C-E) the comparation of exons, length and number of open reading frame between lncRNA and mRNA. (F) the expression levels of lncRNA and mRNA.

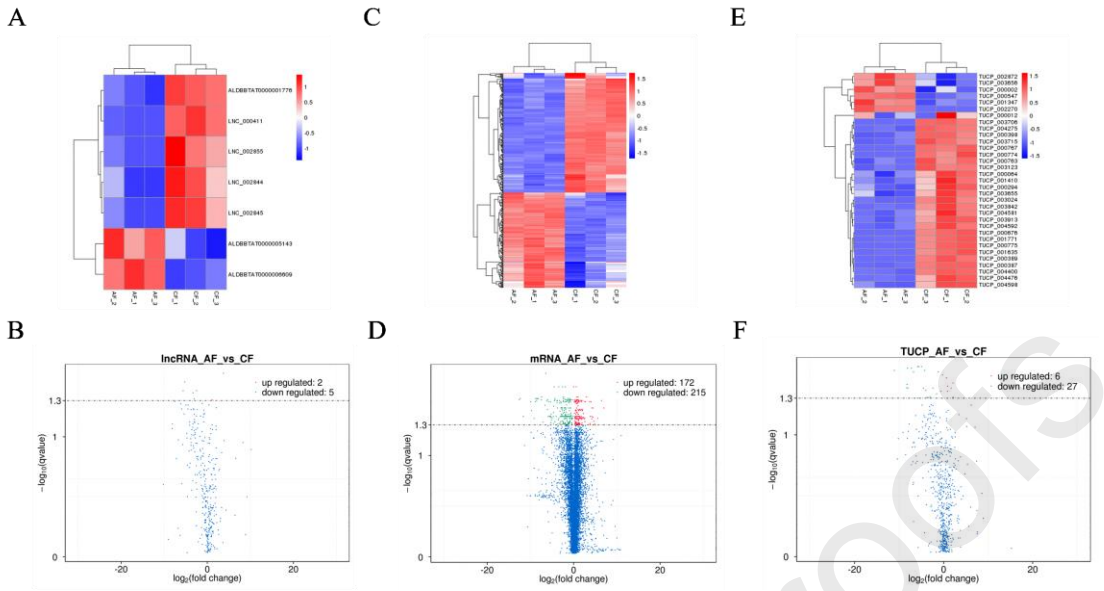

Figure 2. Differential expression analysis of lncRNA. (A) the clustered heatmap of differentially expressed lncRNA. (B) the volcano plot of differentially expressed lncRNA. (C-D) the clustered heatmap and volcano plot of differentially expressed mRNA. (E-F) the clustered heatmap and volcano plot of differentially expressed TUCP.

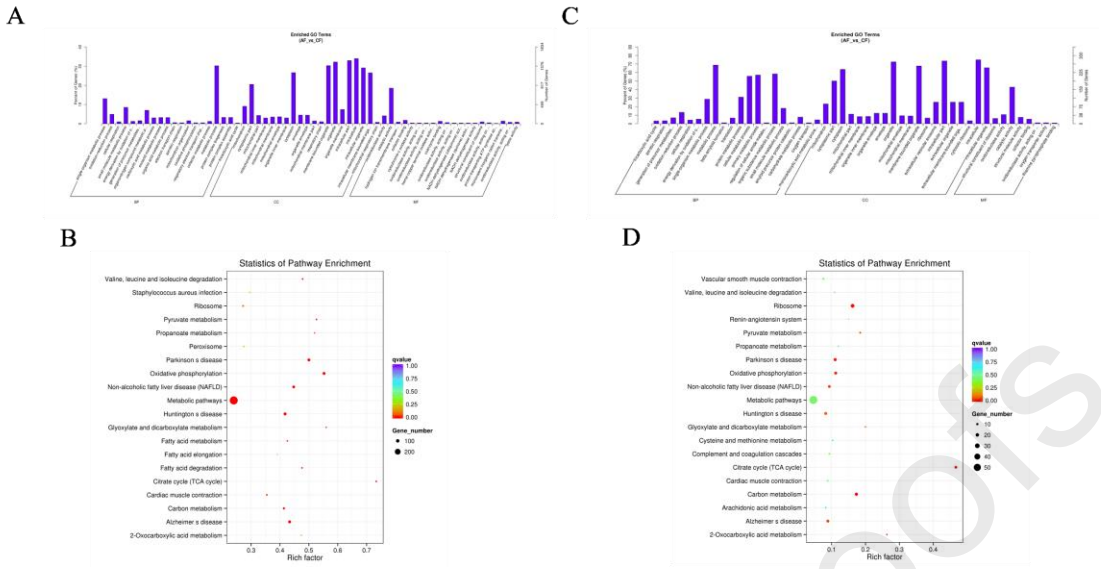

Figure 3. GO and KEGG enrichment analysis. (A) The GO enrichment of the nearby mRNA of differentially expressed lncRNAs. (B) Top 20 of KEGG pathways enrichment for the nearby mRNA of differentially expressed lncRNAs. (C) The GO enrichment of mRNA. (D) Top 20 of KEGG pathways enrichment for mRNA.

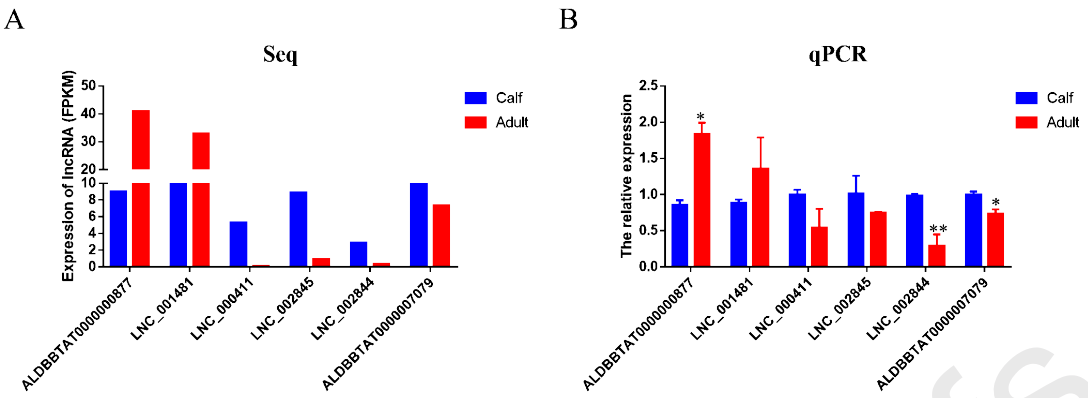

Figure 4. The validation of RNA-Seq data by qPCR. (A) Sequencing FPKM values of six lncRNAs. (B) The lncRNAs were validated by qPCR assays. z

1. Our research object Qinchuan cattle is one of the five yellow cattle breeds in China, and its meat performance is good.

2. For the first time, we performed high-throughput sequencing of LncRNA from Qinchuan cattle calves and adult bovine adipose tissue, and screened 3,716 lncRNA candidates.

3. We screened 119 differentially expressed lncRNAs in calf and adult cattle, and quantified 6 of them.

4. We performed GO and KEGG functional enrichment analysis of differentially expressed lncRNAs to reveal potential functions and potential effects on fat development.

Author contributions

H.C., R.J. and H.L. designed the study. R.J. and H.L. performed the experiments and drafted the manuscript. R.J., H.L., X.L. and C.L. helped perform the experiments and analyzed the data. H.L., Y.H., and H.C. helped collect tissue samples.