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Differential CircRNA Screening and Identification of Subcutaneous Fat Tissue in Plateau-type Yaks under Different Feeding Methods

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ABSTRACT

Background: In order to explore the differential expression of circular RNA (circRNA) in the subcutaneous fat tissue of Qinghai yaks under different feeding methods during the dry grass period and its regulatory mechanism.

Methods: The present study adopted Illumina sequencing technology to sequence the transcriptome of subcutaneous fat tissue of Qinghai yaks naturally grazed and fattened by housed feeding during the dry grass period and used bioinformatics software to screen out the differentially expressed. The circRNAs were screened for differential expression using bioinformatics software. circRNAs related to fat metabolism were screened by GO function and KEGG pathway enrichment analysis of their source genes and three differentially expressed circRNAs were randomly screened and the expression of the circRNAs was verified by real-time fluorescence quantitative PCR (qRT-PCR).

Result: Transcriptome sequencing results showed that a total of 628 differentially expressed circRNAs were obtained, of which 219 were up-regulated and 409 were down-regulated. GO functional enrichment analysis showed that the circRNAs in the free-grazing group originated from genes for major molecular constructs, cellular parts, organs, bioregulation and cellular processes, *etc.* KEGG pathway enrichment analysis showed that the circRNAs could be participate in signaling pathways such as phagosomes, protein digestion and absorption. qRT-PCR results showed that the expression levels of the three circRNAs were consistent with the sequencing results and the sequencing results were accurate and reliable. This experiment provides a molecular basis for further understanding of fat metabolism in yaks.

Key words: CircRNA, GO, KEGG, Source genes, Subcutaneous fat tissue, Yak.

INTRODUCTION

Adipose tissue is not only an important energy storage organ in the organism, but also an important secretion organ (Porta *et al*., 2020). The fat content and distribution of the animal body is one of the important factors affecting the meat production performance and health status of animals. Generally speaking, most of the fat in the animal body is stored in the subcutaneous area, which has the function of storing energy, regulating body temperature and preventing heat dissipation. Subcutaneous fat is very important to the animal body in alpine regions and the study of subcutaneous fat deposition is of great significance to the survival of animals in alpine regions.

CircRNA is a unique class of ncRNA molecules identified in the 1980s, consisting of linear RNAs that are reverse spliced and covalently bonded to form a closed-loop structure, which is involved in the regulation of a variety of biological processes (Li *et al*., 2020). CircRNAs are mainly classified into exonic circRNA (ecircRNA), intron circRNA (ciRNA) and intergenic circular RNA and their biogenesis depends on typical reverse splicing mechanisms and is regulated by specific cis- and trans-acting elements (Chen *et al*., 2021 and regulated by specific cis-acting elements and trans-acting factors (Chen *et al*., 2021). In recent years, it has been found that circRNAs are involved in the regulation of fat deposition as miRNA sponges, which have indispensable roles in lipid metabolism in different animals. One study found that circINSR can promote the formation and proliferation of preadipocytes through circINSR-miR-15/16-CCND1/Bcl-2/FOXO1/EPT1 signaling (Shen *et al*., 2020). In bovine adipocytes, (Wu *et al*., 2022) found that cell differentiation was promoted and cell proliferation and apoptosis were inhibited through the circPPARγ-miR-200b/miR-92a-3p-YY1 signaling pathway. Transcriptome sequencing of bovine preadipocytes, pre-differentiated adipocytes and mature adipocytes by Zhang *et al*. (2021) found that circRNA was abundantly expressed in white fat. (Feng *et al*., 2022) found that circMARK3 promoted adipogenic differentiation of buffalo adipocytes by up-regulating the expression of adipocyte signature genes *PPARG*, *C/EBPα* and *FABP4*.

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circFUT10 promotes bovine adipocyte proliferation and inhibits cell differentiation (Jiang *et al*., 2020).

Yaks are known as the ″Boat of the Plateau″ and the by-products they provide, such as meat, milk, skins and fur, are indispensable to local herders. As one of the five major pastoral areas in China, Qinghai Province ranks first in the country in terms of the number of yaks and the quality of yaks (Zhang *et al*., 2022). Yak meat is characterized by high protein, low fat and rich nutrients, *etc.* In this study, we used RNA-seq technology to identify circRNAs differentially expressed in subcutaneous adipose tissues of plateau yaks under different feeding methods and explored the possible regulatory mechanisms of circRNAs in the lipid metabolism of yaks.

MATERIALS AND METHODS

Experimental animals

In this experiment, 6 plateau-type male yaks of 18 months of age with similar weight and good body condition were selected from the Meilongzhang Livestock Management Specialized Cooperative in Qilian County, Haibei Tibetan Autonomous Prefecture, Qinghai Province, China, under the same feeding conditions as those in the previous period and were randomly divided into 2 groups with 3 replicates in each group. One group (G24 _SF) was naturally grazed for 6 months to 24 months of age; the other group (F24 _SF) was fully housed for 6 months to 24 months of age. The yaks were slaughtered at the end of the experiment in April of 2009 and the subcutaneous fat was collected from their backs and then the samples were put into liquid nitrogen tanks and transported back to the laboratory, where they were stored at -80° for spare use.

Feeding management

The yaks of the natural grazing group grazed in the same pasture (38.30 N, 99.78 E) from 07:00 to 18:00 every day and fed and watered freely during the grazing period. The main dominant species of forage in the grazing pasture were *Stipa capillata* L., *Poa annua* L. and *Carex myosuroides* Vill. The yaks in the full-housed group were fed with total mixed ration (TMR), once a day at 07:00 and once a day at 17:00, with adequate water intake. The concentrate used in this experiment was in pellet form.

RNA extraction and cDNA library construction and transcriptome sequencing

The total RNA was extracted from subcutaneous adipose tissue according to the instructions of total RNA Extraction Kit (sikejie) and the concentration and purity of total RNA were determined by NanoDrop One and the quality of RNA was detected by 2% agarose gel electrophoresis. The extracted total RNA was used as a template to synthesize cDNA according to the instructions of the Reverse Transcription Kit (sikejie) and the cDNA was sequenced on the Illumina Hiseq 2000 platform and stored at -20°C.

circRNA prediction

Based on Back splice junction (BSJ) reads, circRNA prediction was performed using the software CIRI2 and compared with the database circBase (animal).

Screening for differentially expressed circRNAs

The expression levels of circRNAs were quantified using RSEM (http://deweylab.github.io/RSEM/) and the differential expression analysis of circRNAs in different samples was performed using DESeq software with the screening conditions of fold change ≥ 2 and padj < 0.05 and the differential expression of circRNA s were plotted as volcano plots.

Functional analysis of source genes

Differential circRNA-derived genes were enriched and analyzed by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) based on the DAVID database.

Experimental validation of real-time fluorescence quantitative PCR

Three circRNAs were randomly selected and their relative expression levels were verified by real-time fluorescence quantitative PCR (Table 1).

Statistical analysis of data

Data were statistically analyzed using Graph PdPrim 8.0 and one-way ANOVA (One-Way ANOVA) was used to analyze the significance of the differences and the results were expressed as the mean±standard deviation, with $P<0.05$ being considered as a significant difference and $P<0.01$ as a highly significant difference.

Table 1: Sequence specific primers of circRNAs.

Primer name	Primer sequence	Product length
19_30428869_30429917	AACAGCCGCTTCACCTACAG	100
	CGGGAGGTCTTGGTGGTTTT	
23_47332020_47332905	GCATTTACCGGGCAACTTTACA	150
	GGTATATGGATTTAACTTGTCCACAGT	
8_46700648_467014	TGAGGTTTGTGTAAGGCTCCTG	82
	TGCCCTGTGAATACATCAGGAC	
GAPDH	AGTTCAACGGCACAGTCAAGG	83
	ACCACATACTCAGCACCAGCA	

RESULTS AND DISCUSSION

Transcriptome sequencing data analysis

Through circRNA sequencing analysis, raw data quality control and filtering, six cDNA libraries were constructed and a total of 11,187,520,262 to 13,382,8336 clean reads were obtained and all samples had a Q20 value of more than 98%, a Q30 value of more than 94% and a GC content of 47% to 52% (Table 2). The above results indicate that the sequencing data are of high quality and can be used for subsequent experiments.

circRNA prediction

30299 circRNAs were obtained by CIRI2 software analysis, of which 1880 circRNAs originated from exon shearing, 6650 were intron shearing and 3769 were intergenic_ region shearing (Fig 1).

Analysis of differentially expressed circRNAs

The transcriptome sequencing data of G24 and F24 subcutaneous adipose tissue were analyzed by DESeq software and the results showed that a total of 628 differentially expressed circ RNAs were screened compared with G24, of which 219 circRNAs were up-regulated and 409 circRNAs were down-regulated (Fig 2).

Source gene GO annotation analysis and KEGG enrichment analysis

The 98 source genes of differentially expressed circRNAs were analyzed using GO function enrichment and the top 20 significantly enriched GO entries were mainly annotated to cellular components, biological processes and molecular functions, which were mainly related to molecular constructs, cellular parts, organs, bioregulation, cellular processes, *etc* (Fig 3).

KEGG enrichment analysis of differentially expressed circ RNA source genes was performed using KEGG, in which 98 source genes could be KEGG-enriched again with 80 and the top 20 significantly enriched KEGG pathways were mainly phagosomes, AGE-RAGE signaling pathway in diabetic complications, protein digestion and absorption, cellular adhesion molecules, natural killer cell-mediated cytotoxicity and human T-cell leukemia virus infection (Fig 4, Table 3).

Analysis of KEGG-enriched pathways revealed nine pathways associated with adipose: Glycosaminoglycan biosynthesis-heparan sulfate/heparin, Fat digestion and absorption, Glycerolipid metabolism,Glycerophospholipid metabolism, Cholesterol metabolism, PI3K-Akt signaling pathway, PPAR signaling pathway, AMPK signaling pathway and Wnt signaling pathway (Table 4).

Differential expression of circRNA qRT-PCR validation

In order to verify the accuracy of the sequencing results, 19_30428869_30429917, 23_47332020_47332905 and 8_46700648_467014 were randomly selected for qRT-PCR validation, as shown in the figure and the results of the qRT-PCR assay were in agreement with the results of transcriptome sequencing (Fig 5).

Table 2: circRNA-seq quality control statistics.

Sample	Clean reads	Mapped	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)
F24_SF1	121149048	111828271 (92.31%)	0.0246	98.19	94.58	47.96
F24_SF2	125486926	116775300 (93.06%)	0.0246	98.16	94.58	48.31
F24_SF3	116880732	108065445 (92.46%)	0.0246	98.15	94.57	51.36
G24_SF1	133828336	123208623 (92.06%)	0.0244	98.26	94.8	49.91
G24_SF2	125636990	117409963 (93.45%)	0.0247	98.14	94.45	47.78
G24_SF3	111875262	104283247 (93.21%)	0.0245	98.19	94.65	49.5

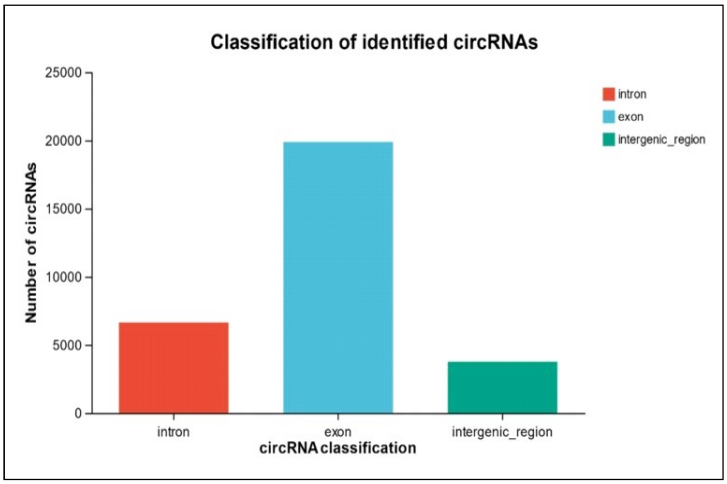


Fig 1: circRNA classification.

Animal meat provides people with rich protein and fat and occupies an important part of their lives. Yak meat is a natural green food, which is characterized by high protein, rich in fatty acids and low fat and provides local residents with daily life needs as well as economic income (Li *et al*., 2023). Due to the high altitude and long dry grass period in

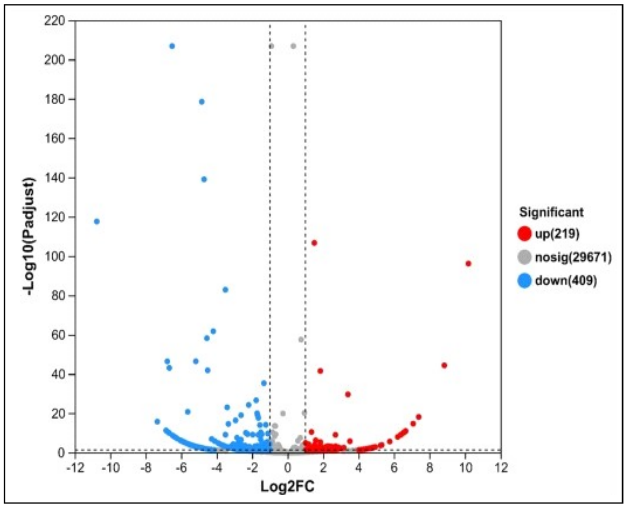


Fig 2: Differential expression analysis of circRNAs.

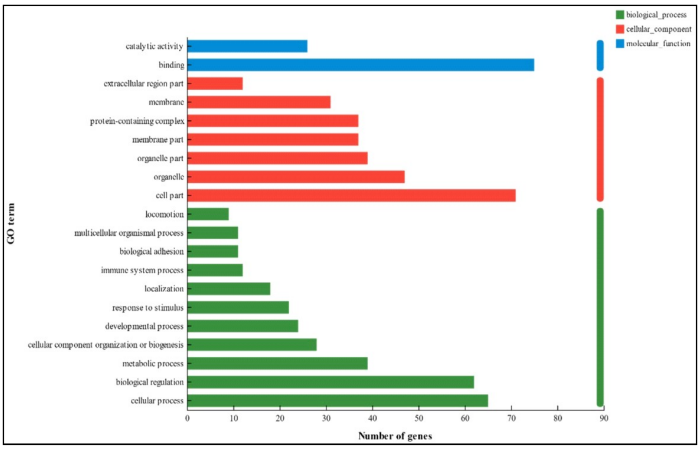


Fig 3: Host gene GO notes.

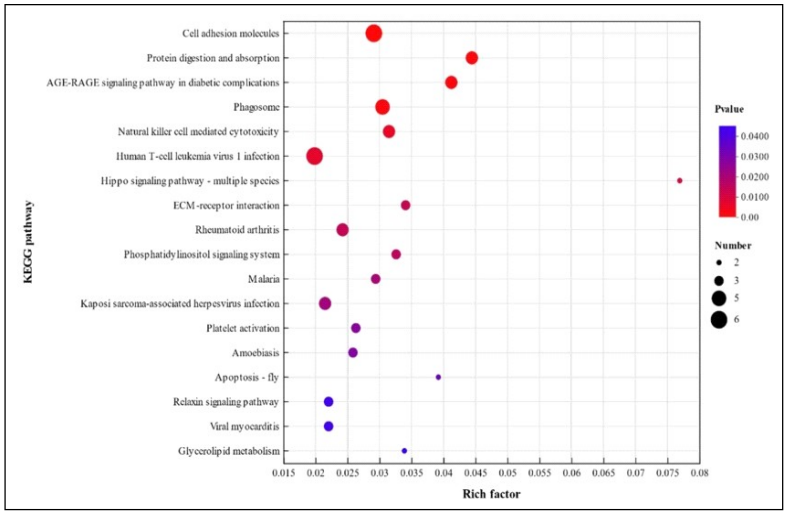


Fig 4: Source gene KEGG enrichment analysis.

Qinghai and other places, after entering the dry grass period, the temperature is low and yaks will consume the fat stored in the previous period to provide the energy they need, so reducing fat deposition and fat conversion is an important challenge for yak industry. Animal fat deposition is regulated by a variety of factors, including age, breed and environment, *etc.* At the molecular level, most studies have focused on genes, mRNAs and miRNAs. In recent years, researchers have found that circRNAs exert some influence on lipid metabolism (Yu *et al*., 2021).

As transcriptome sequencing continues to develop, there are more and more studies on the widespread presence of circRNAs in various organisms, including humans (Tan *et al*., 2017), mice (Gruner *et al*., 2016), cattle (Wei *et al*., 2017), sheep (Zhai *et al*., 2022) and pigs (Li *et al*., 2018). There are few studies on circRNAs in yak. (Zhang *et al*., 2020) performed RNA-Seq sequencing on yak adipocytes at 0 d and 12 d of differentiation and identified a total of 136 differential circRNAs. Huang *et al*. (2021) screened 1057 differential circRNAs in bovine-yak and yak dorsal longest muscle using RNA-Seq sequencing technology. The above studies suggest that circRNAs may play important roles in yak cells and tissues. The study of differential circRNAs in subcutaneous fat tissue of plateau-type yaks has not been reported yet. In this study, six samples of yak subcutaneous fat tissue under different feeding conditions were collected for RNA-Seq sequencing and a total of 628 differentially expressed circRNAs were screened, which could help to reveal the changes of circRNAs expression in yak subcutaneous fat tissue under different feeding conditions at the molecular level and further proved that a large number of circRNAs widely existed in the tissues of living organisms and might play a crucial role. In order to investigate the role of circRNAs in subcutaneous fat tissue of yaks under different feeding conditions, the present study was conducted to analyze the functionality of genes originating from differential circRNAs. GO functional annotation analysis showed that the genes originating from differential circRNAs were annotated in cellular components, biological processes and molecular functions, suggesting that they were involved in these processes. KEGG pathway analysis showed that differential circRNA-derived genes were enriched to a total of 9 pathways related to adiposity. Among them, *CD36* was enriched into 4 pathways, *DGKZ* was enriched into 2 pathways and *PNPLA3* and *COL1A2* were enriched into 1 pathway. Cluster of differentiation 36 (*CD36*) is a class of single-chain transmembrane proteins that act as receptors for the transport of extracellular fatty acids into the intracellular compartment, provide docking sites for fatty acid enzymes such as fatty acid-binding protein 4 (*FABP4*) and promote fatty acid transport in adipocytes, skeletal muscle cells and smooth muscle cells. cells and smooth muscle cells (Abumrad and Goldberg, 2016; Kim and Dyck, 2016). *PNPLA3* is patatin like phospholipase domain containing 3 also known as lipocalin. *PNPLA3* is expressed in a variety of human tissues, with the highest expression in liver tissues (Huang *et al*., 2010; Pirazzi *et al*., 2014). And the highest expression was found in white and brown adipose tissues in mice (Lake *et al*., 2005). It was found

Table 3: KEGG signaling pathway analysis of differentially expressed circRNAs (top 6).

KEGG pathway	Gene number	p-value
Cell adhesion molecules	6	0.0011
Protein digestion and absorption	4	0.0017
AGE-RAGE signaling pathway in diabetic complications	4	0.0023
Phagosome	5	0.0024
Natural killer cell mediated cytotoxicity	4	0.0060
Human T-cell leukemia virus 1 infection	6	0.0075

Table 4: Source gene KEGG enrichment analysis.

Pathway name	Gene name
Glycosaminoglycan biosynthesis-heparan sulfate/heparin	*EXT1*
Fat digestion and absorption	*CD36*
Glycerolipid metabolism	*PNPLA30DGKZ*
Glycerophospholipid metabolism	*GPD20DGKZ*
Cholesterol metabolism	*CD36*
PI3K-Akt signaling pathway	*COL1A2*
PPAR signaling pathway	*CD36*
AMPK signaling pathway	*CD36*
Wnt signaling pathway	*NFATC2*

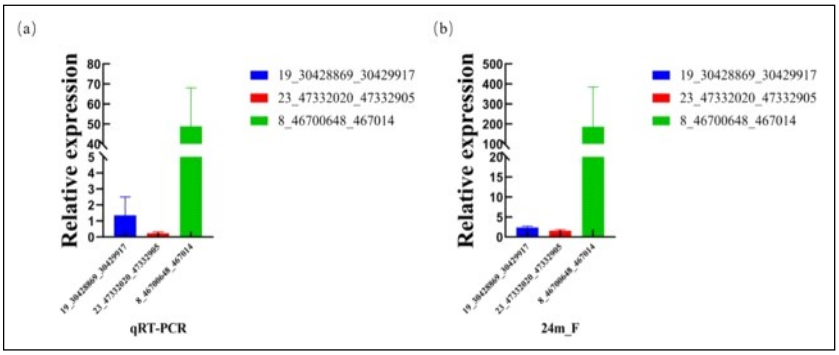


Fig 5: qRT-PCR results.

that overexpression of the human mutant *PNOLA3* in the livers of mice fed with high sucrose caused hepatic steatosis in mice (Li *et al*., 2012; BasuRay *et al*., 2019). The heterotrimer composed of two COL1A1 subunits and one *COL1A2* subunit is type I collagen, which belongs to the major structural proteins that make up skin, bone, *etc* (Viguet-Carrin *et al*., 2006). It was found that *COL1A1* and *COL1A2* were differentially expressed in very high intramuscular adiposity and low intramuscular adiposity in pigs (Pan *et al*., 2023) and in morphological changes in the structural organization of the bovine fetal ovary (Franchi *et al*., 2020), then *COL1A2* may be related to adiposity regulation and the regulatory mechanism of *COL1A2* can be investigated subsequently. NFATC1-5 constitutes a family of nuclear factor of activated T cells (NFAT) transcription factors and it has been found that NFATC1-5 regulates T cell activation and differentiation and also participates in regulating the expression of genes such as cell cycle and death (Shaw *et al*., 1988). NFATC1-5 constitutes a family of nuclear factor of activated T cells (NFAT) transcription factors and it has been found that NFATC1-5 regulates T cell activation and differentiation and is also involved in regulating the expression of genes such as cell cycle and death (Shaw *et al*., 1988; Park *et al*., 2020; Li *et al*., 2023). More and more studies have shown that circRNAs can act as ceRNAs and indirectly regulate the expression of mRNAs by competitively binding to miRNAs (Liu *et al*., 2018) to perform biological functions. GO function and KEGG pathway enrichment analyses of their source genes were performed to screen circRNAs related to fat metabolism and the targeting relationship between circRNA, but the verification has yet to be tested.

Conclusion

In this study, we detected differential circRNAs in subcutaneous fat tissue of yaks under different feeding conditions by RNA-Seq and obtained 628 differentially expressed circRNAs, 98 of their host genes were analyzed by GO annotation and KEGG enrichment and were found to be enriched in 9 adipose-related pathways, including the AMPK signaling pathway. 19_30428869_30429917, 23 _47332020_47332905 and 8_46700648_467014 were verified by qRT-PCR, which were consistent with the sequencing results. This experiment promotes the study of circRNA in the regulation of fat metabolism in yaks and provides research ideas for further understanding of yak fat metabolism.

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Institutional review board statement

The animal handling procedures in this study were approved by the Ethics Committee for the Use of Laboratory Animals, College of Animal Husbandry and Veterinary Science, Qinghai University (Permit No.2023-QHMKY-001).

Informed consent statement

Not applicable.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

The animal welfare statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received.

Conflicts of interest

The authors declared that they had no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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