Identification and characterization of circRNAs in the deposition of intramuscular fat in Aohan fine-wool sheep

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Abstract

Background

Aohan fine-wool sheep (AFWS) is a high-quality fine-wool sheep breed that supplies both wool and meat. The quality of its meat is affected by many factors. Research is needed on the molecular mechanism of intramuscular fat (IMF) growth, which greatly improves mutton quality. The widely expressed non-coding RNA is used in roles such as competitive endogenous RNAs (ceRNAs), including microRNAs (miRNAs). Although circular RNA (circRNA) was studied in many fields, little research was devoted to IMF in sheep. We used RNA-Seq to analyze tissues associated with IMF in 2-month-old and 12-month-old AFWS rams to understand the role of circRNA in the growth and development of sheep IMF.

Results

A total of 11,565 candidate circRNAs were identified, of which 104 were differentially expressed in the two age groups. We analyzed these differentially expressed circRNAs. Enrichment analysis was performed using Gene Ontology and the Kyoto Encyclopedia of Genes and Genomes. The enriched pathways included lipid transport (GO:0006869), negative regulation of canonical Wnt signaling pathway (GO:0090090), fat digestion and absorption (ko04975), and sphingolipid metabolism (ko00600). We used the TargetScan and miRanda software programs for interaction analysis, and a network diagram was created. Six circRNAs were randomly selected and verified the RNA-Seq results by quantitative real-time PCR.

Conclusion

This study provides more information on circRNA regulation in AFWS, and is a useful resource for further research on this sheep breed.

Background

In recent years, with the improvement in the standard of living, the demand for meat products has increased. The consumption of lamb, as low-cholesterol meat, is increasing. The quality of mutton sheep is affected by genetics and the environment [1, 2], with the genetic part determining the selection progress between generations. Therefore, only by clarifying the factors that affect meat quality and gene regulation mechanisms can we make effective choices. Mutton's quality is particularly affected by the IMF deposition [3]. The associated reaction mechanism is an intricate biological process, jointly regulated by epigenetic modifications and related gene expression [4, 5]. Therefore, the research on gene regulation of IMF is of great significance. Current research shows that a variety of molecules are involved in IMF regulation. For example, a functional regulatory sequence variant was identified in pig *MYH3*, providing new insights into the regulation mechanism of IMF in pigs [6]. *PPARG*, *LPL*, *FABP4*, *THRSP*, *RBP7*, *PLIN*,

and *LDLR* are responsible for IMF deposition in chicken [7]. There are many studies on IMF regulatory pathways, such as the sphingolipid signaling pathway (KEGG ssc04071) [8]. The peroxisome proliferator activated-receptors (PPAR) signaling pathway was reported to be directly involved in fat deposition in the pectoralis major of the chicken [9]. Glycerolipid metabolism and the fatty acid degradation pathway might have a contribution to the differences in IMF deposition between strains [10].

Many studies have recently shown that non-coding RNAs, including microRNAs (miRNA), circular RNA (circRNA), and long non-coding RNA (lncRNA), play an important regulatory role in IMF. lncRNA can promote IMF cell differentiation by acting as miRNA sponge [11]. Studies have also found that lncRNA can inhibit lipolysis by reducing autophagy and promoting intramuscular fat formation [12]. CircRNA has also been shown to adjust the content of IMF and improve meat quality [13]. CircRNAs are endogenous covalently closed circular non-coding RNAs expressed in eukaryotic cells. They are formed by back-splicing of mRNA precursors (pre-mRNA) [14–16]. A covalently closed circRNA was first discovered in 1976 in plant viruses [17]. It is different from the standard linear RNA, as its 3' and 5' ends are joined together to form a covalently closed loop structure [18], which makes them more stable and conservative [19], and resistant to digestion by RNase R [20, 21]. In the early days, circRNA was assumed to have no function. After in-depth research, it was found that circRNAs can perform their biological functions in various ways [22]. CircRNA can bind to RNA binding protein to regulate gene expression and protein translation, act as a miRNA sponge, encode functional proteins, and participate in cell communication and signal transduction [23]. circRNA can be classified into the following four types according to their sources: 1. All-exon circRNA [24]; 2. Exon-intron circRNAs (EI circRNAs) with a combination of introns and exons [25]; 3. Lasso-type, composed of circular intronic RNAs (ciRNAs) [26]; 4. circRNA produced by the circularization of viral RNA genome, transfer RNA (tRNA), ribosomal RNA (rRNA), small nuclear RNA (snRNA), etc. [27]. This last type belongs to the intergenic circRNA. Different types of circRNA play different biological functions.

Recent studies reported that circRNA plays a significant role in human visceral fat cells [28]. circRNA has also been studied extensively in mice and has been reported to be associated with fat [29]. Some researchers have identified many differentially expressed genes (DEGs) and circRNAs in the adipose tissue of buffaloes aged six and 30 months, and revealed the existence of circRNA-miRNA association [30]. In research on adipocyte differentiation in the yak, several competitive endogenous RNAs (ceRNAs) and many circRNAs related to adipocyte differentiation were identified [31]. In addition, the researchers studied 0.5 and 2.5-year-old yaks, identifying mRNAs and circRNAs potentially involved in IMF deposition, and constructed a ceRNA network, laying the foundation for subsequent research [32]. Nevertheless, there are few studies on the involvement of circRNA in IMF deposition in sheep.

Aohan fine-wool sheep (AFWS) is a fine-wool sheep breed that is bred independently in China. The AFWS make a significant contribution to mutton production in China, especially in Inner Mongolia [25, 33]. The research on its genes is particularly important, as it can help improve its meat quality. In previous studies on the development of sheep IMF, it was found that the IMF content differs between time points during development. Researchers have found that IMF content of some sheep might increase with growth at a

certain age [34]. Preliminary laboratory experiments showed that IMF content differs greatly between 2-month-old (Mth-2) and 12-month-old (Mth-12) AFWS [35].

We have extensively studied IMF in sheep [36, 37]. In this study, we tested circRNA expression in IMF-related tissues of Mth-2 and Mth-12 AFWS to study the association between circRNA and IMF changes at different developmental stages. Our results indicate that circRNA is important in regulating sheep IMF deposition.

Results

Determination of IMF content at Mth-2 and Mth-12 sheep

We measured the IMF in the longissimus dorsi of Mth-2 and Mth-12 sheep. The results show that IMF content in Mth-12 sheep ($11.2 \pm 0.9\%$) was significantly higher than in Mth-2 sheep ($2.2 \pm 0.006\%$).

Sequencing and localization of the circRNA in sheep muscle

The preliminary filtered data are listed in Additional file 1 (Table S1). The main types identified in this study were all-exon circRNA type (Mth-2: 89.36%; Mth-12: 88.56%), introns composed of lasso-type circRNA (Mth-2: 4.42%; Mth-12: 5.03 %), and circRNA in the intergenic type (Mth-2: 6.22%; Mth-12: 6.41%) (Fig. 1a and 1b). circRNA usually consists of one to four exons (Fig. 1c). It has been reported that the length of circRNA with only one exon is longer than that of circRNA with multiple exons [38]. In this study, we also found this conclusion. We list the 20 longest circRNAs in Additional file 2 (Table S2). All these are composed of one exon, most belong to the intergenic type, and are distributed on different chromosomes. A different number of circRNAs were detected on different chromosomes, with most on chromosomes 1, 2, and 3 (Fig. 1d).

Expression analysis of circRNA in muscle tissue

A total of 11,565 candidate circRNAs were identified. Mth-2 samples expressed 8,186 circRNAs, and Mth-12 samples expressed 6,683. Of these, 3,304 circRNAs were expressed in both age groups (Fig. 2a). The 30 circRNAs with the highest expression levels are listed, and the type of circRNA is marked in Table 1. Only a few belonged to ciRNA and intergenic RNAs regions. The expression of the circRNAs is shown in Fig. 2b. It can be seen from Fig. 2c that the peak gene density of circRNA expression is between 0.75 and 1.5.

Identification of differentially expressed circRNAs

Among the 104 differentially expressed circRNAs detected (Fig. 3a), 38 were upregulated, and 66 were downregulated. The overall distribution of the differentially expressed circRNAs is visually presented as a scatter plot in Fig. 3b and as a cluster heat map in Fig. 3c. The related data are listed in Additional file 3 (Table S3). Among the differentially expressed circRNAs, the source gene of circRNA3117, circRNA7648, circRNA4474, circRNA153, circRNA1817, and circRNA3096 is *Titin* (*TTN*), and the source gene of

circRNA2639 is *USP34*. We found that the expression of circRNA829 in Mth-12 was significantly higher than in Mth-2. These circRNAs may be related to IMF, and we will study them further in the future.

Differentially expressed circRNA-hosting genes based on GO enrichment analysis

After Gene Ontology (GO) enrichment analysis, 251 groups were enriched in the biological process classification. The most significantly enriched group was regulation of transcription, DNA-templated (GO: 0006355), followed by positive regulation of GTPase activity (GO: 0043547), signal transduction (GO: 0007165), positive regulation of transcription by RNA polymerase II (GO: 0045944), and protein phosphorylation (GO: 0006468). In our research, some biological process classification GO terms related to fat metabolism were enriched, including lipid transport (GO: 0006869), negative regulation of canonical Wnt signaling pathway (GO: 0090090), lipid metabolic process (GO: 0006629), and other processes. Eighty-seven groups were significantly enriched in the cellular component classification. The most significantly enriched group was the nucleus (GO: 0005634), followed by integral component of membrane (GO: 0016021), cytosol (GO:0005829), and membrane (GO:0016020). In the molecular function classification, 114 groups were significantly enriched. The most significantly enriched group was protein binding (GO: 0005515), followed by metal ion binding (GO:0046872), ATP binding (GO:0005524), and nucleotide binding (GO:0000166). We sorted the classifications in descending order according to the number of differentially expressed genes annotated with each GO term (S gene number), and selected the top 15 in all three classification categories to draw a histogram (Fig. 4a). We also took the top 20 GO terms according to the significance of enrichment (p -value) to draw a scatter plot (Fig. 4b).

Differentially expressed circRNA-hosting genes based on the KEGG enrichment analysis

After the KEGG enrichment analysis, 95 pathways were significantly enriched. We took the top 20 KEGG terms according to the significance of enrichment (p -value) and drew a scatter plot (Fig. 5). More genes were enriched in Fc gamma R-mediated phagocytosis (ko04666), adherens junction (ko04520), and thyroid hormone signaling pathway (ko04919).

According to the KEGG enrichment analysis, we identified pathways with more enriched genes and pathways that might be related to IMF growth and development. These included Fc gamma R-mediated phagocytosis (ko04666), adherens junction (ko04520), thyroid hormone signaling pathway (ko04919), fat digestion and absorption (ko04975), Notch signaling pathway (ko04330), sphingolipid metabolism (ko00600), ether lipid metabolism (ko00565), glycerolipid metabolism (ko00561), glycerophospholipid metabolism (ko00564), glutamatergic synapse (ko04724), mitogen-activated protein kinases (MAPK) signaling pathway (ko04010), and sphingolipid signaling pathway (ko04071). These 12 pathways involve ten circRNAs and their ten corresponding genes (Additional file 4: Table S4). The association between these genes and circRNAs is presented in Table 2.

Analysis of the interaction between circRNA and miRNA

CircRNA can act as a miRNA sponge, thus inhibiting miRNA from binding to target genes [39]. We selected 12 circRNAs and 18 miRNAs to predict their target relationship using TargetScan score ≥ 90 and miRanda Energy ≤ -40 (Additional file 5: Table S5), and then constructed a network diagram based on the results (Fig. 6). Among them, one circRNA could bind to multiple miRNAs, and one miRNA could bind to multiple circRNAs. We found that oar-miR-487a-5p is targeted by circRNA2748, circRNA7711, circRNA4736, and circRNA9161. We also found that circRNA7771 targets oar-miR-432_1ss23GT, oar-miR-541-5p, oar-miR-412-5p_R+2, oar-miR-1197-3p, oar-miR-323c, oar-miR-487b-5p_L-1R+1, and oar-miR-181a.

Verification of circRNA expression level

We randomly selected six differentially expressed circRNAs and detected their expression levels by quantitative real-time PCR (RT-qPCR) (Fig. 7a). The results were consistent with the trends observed in the RNA-Seq data (Fig. 7b). This shows that RNA-Seq analysis is reliable. As shown in Figure 7c, the selected circRNAs could resist digestion by RNase R, while the linear RNA (GAPDH) in the samples could not. RT-qPCR performed after RNase R digestion showed that the relative expression level of the six circRNAs did not decrease significantly. Instead, in most it has actually increased. The results show that circRNA can resist digestion by RNase R, while linear RNA cannot.

Discussion

Lamb meat quality and its IMF content have a great influence on its taste [40]. Therefore, it is necessary to study the molecular mechanism behind IMF deposition. For the first time, we studied the expression of circRNAs in sheep IMF in two different age groups, detected circRNAs that might have a regulatory relationship with IMF, and constructed a regulatory network based on this information.

In the analysis of differentially expressed circRNAs, we identified 11,565 candidate circRNAs, of which 3,304 were expressed in both age groups, 4,882 only in Mth-2 rams, and 3,379 only in Mth-12 rams. We detected 104 differentially expressed circRNAs. We then performed GO and KEGG enrichment analyses to study the regulatory mechanism of circRNAs. Previous studies have shown that there are many pathways related to the growth and development of fat. The Wnt signaling pathway is related to bovine longissimus dorsi muscle IMF content [41], and is a negative regulator of adipogenesis [42]. According to relevant research reports, glycolipid catabolic process (GO: 19377), calcium ion homeostasis (GO: 55074), lipid metabolic process (GO: 6629), and others are enrichment GO terms significantly related to IMF characteristics [43]. The KEGG is a set of databases and related software used to extract genomic information [44]. It integrates information about molecules, genes, proteins, and biochemical compounds and reactions [45]. Our research found that fat digestion and absorption (ko04975), sphingolipid metabolism (ko00600), ether lipid metabolism (ko00565), glycerolipid metabolism (ko00561), glycerophospholipid metabolism (ko00564), sphingolipid signaling pathway (ko04071), and other KEGG terms might be related to fat metabolism. We analyzed the interaction between circRNA, miRNA, and mRNA in IMF regulation. In the past few years, much research was done on IMF-related mRNA in sheep. For example, researchers found that heart fatty acid-binding protein (*H-FABP*) and peroxisome

proliferator-activated receptor-gamma (*PPARG*) mRNAs have a certain effect on IMF content in sheep [46]. Another study found that the adipocyte fatty acid-binding protein (*A-FABP*) gene affects fat deposition and fatty acid metabolism in chickens [47]. So far, studies done on IMF in pigs [48], cattle [49], sheep [50], chickens [51], and other animals, have established a solid foundation for our subsequent research. It has been reported that acetyl-CoA acyltransferase 2 (*ACAA2*) can promote the differentiation of sheep preadipocytes into adipocytes [52]. Chloride channel, voltage-sensitive 3 (*CLCN3*) might be associated with preadipocytes [53], and its deficiency can improve obesity and the production of macrophages in the adipose tissue of mice fed a high-fat diet [54]. The source gene of circRNA7051, *SMC6*, has been demonstrated to be associated with fat metabolism in humans [55]. The *Titin* is the source gene of circRNA3117, circRNA7648, circRNA4474, circRNA153, circRNA1817, and circRNA3096, and is reported to be related to the growth and development of intermuscular fat and fat deposition regulation [56, 57]. The source gene of circRNA2639 is Ubiquitin-specific protease 34 (*USP34*), which was reported to play a key role in the Wnt signaling pathway [58]. The source gene of circRNA829 is Sorbin and SH3 domain containing 1 (*SORBS1*). Studies have reported that *SORBS1* is related to fat deposition in muscles [59]. We speculate that circRNA829 plays a role in the growth and development of IMF. The source gene of circRNA7445 is K (lysine) acetyltransferase 2B (*KAT2B*), and the source gene of circRNA2997 is spermatogenesis-associated protein-7 (*SPATA7*). These source genes have been reported to be related to fat deposition [60, 61]. Like the circRNAs above, other circRNAs might also be related to IMF. In the future, we intend to study their role in IMF regulation.

We speculate that circRNA2997 and circRNA7051 are involved in regulating the growth and development of IMF in sheep. CircRNA can act as a miRNA sponge [62]. circRNA2959, circRNA4736, circRNA4795, and circRNA7831 target Let-7e, and Let-7e-5p is involved in adipose stem-cell differentiation into epithelial cells [63]. Another study reported that miR-877-5p was identified as a new lipid deposition regulator in bovine IMF [64]. In our study, miR-877 was associated with ciRNA440, ciRNA455, circRNA2959, circRNA4770, circRNA4773, circRNA7754, and circRNA9172. They have a target relationship. We assume that circRNA2959, circRNA4736, circRNA4795, circRNA7831, ciRNA440, ciRNA455, circRNA2959, circRNA4770, circRNA4773, circRNA7754, and circRNA9172 might be regulating fat development in sheep. circRNA2748, circRNA7711, circRNA4736, and circRNA9161 were found to target oar-miR-487a-5p. According to reports, miR-487a is associated with human obesity [65]. In addition, some circRNAs found in this study might be regulating IMF growth and development in AFWS. We will study this association in future experiments.

In summary, we investigated the expression of circRNAs in IMF-related tissues in AFWS. The KEGG and GO enrichment analyses were carried out and made certain predictions on the target relationship between circRNA, miRNA, and mRNA. It is speculated that some circRNAs might have a regulatory relationship with IMF. Based on our results, further work is needed to reveal the potential molecular mechanism of circRNA in AFWS IMF.

Conclusions

We measured and analyzed circRNAs expression in 2-month-old and 12-month-old AFWS. In this study, 104 differentially expressed circRNAs were identified. In GO and KEGG enrichment analysis, negative regulation of the canonical Wnt signaling pathway (GO: 0090090) and fat digestion and absorption (ko04975) were enriched pathways that might be related to the growth and development of fat cells. We used the TargetScan and miRanda software programs to predict the targeting relationship between a selected sample of 12 circRNAs and 18 miRNAs, and constructed a diagram accordingly. This study provides some clues to the genetic basis of sheep IMF deposition, but further research on its regulatory mechanism is necessary.

Methods

Sample collection and preparation

RNA extraction and quality assessment

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from the six samples. We then used Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), NanoDrop ND-2000 (Thermo Fisher Scientific, Waltham, MA, USA), and 1% agarose gel electrophoresis to determine the RNA quality and quantity in the samples. The RNA integrity number (RIN) value, based on A260/A280 and 28S/18S ratios, had to meet the high-throughput sequencing requirements to be used for subsequent sequencing analysis. The required A260/A280 range was 1.8-2.0. If the 28S and 18S bands were clear and there were no other impurities, it indicated that the RNA quality of the sample was qualified. If the level of 28S is about twice that of 18S, indicating that the sample RNA conforms to the standard of library construction. After appraisal, all samples met the high-throughput sequencing requirements.

Library construction and circRNA sequencing

After total RNA extraction, rRNA depletion was used to construct a chain-specific library. After the library was qualified, Illumina HiSeq4000 (LC Sciences, Houston, TX, USA) was used for sequencing, with a double-ended sequencing read length of 2×150 bp (PE150). First-strand complementary DNA (cDNA) was synthesized using random hexamer primers and M-MuLV reverse transcriptase (RNase H-). Second-strand cDNA was then synthesized with dNTPs, DNA polymerase I, and RNase H. Next, T4 and Klenow DNA polymerases were used to repair and modify the ends. The cDNA products were then purified using AMPure XP beads (Beckman Coulter, Brea, CA, USA). Finally, uracil DNA glycosylase (NEB, Ipswich, MA, USA) was used to degrade the U-containing chains to remove the second-strand cDNA. The purified first-strand cDNA was enriched by PCR to obtain a cDNA library.

Statistics and quality control of sequencing data

We filter out unqualified sequences, removing reads with adapters, reads that contained > 5% N (N stands for undetermined base information), and low-quality reads (alkali with a quality value of Q < 10). For

bases account for over 20% of the entire read, we quantified the original sequencing, effective sequencing, Q20, Q30, and GC content, and conducted a comprehensive evaluation.

circRNA differential expression analysis

The expression level of circRNA was normalized, using spliced reads per billion mapping (SRPBM). We used three biological replicates for each age group. The screening conditions used when analyzing the identified circRNAs differential expression were $|\log_2\text{FoldChange}| \geq 1$ and $p\text{-value} \leq 0.05$. During analysis, multiple screening conditions were comprehensively set to obtain the number of up- and downregulated circRNAs.

GO and KEGG enrichment analyses

Gene Ontology (GO) (<http://www.geneontology.org>) has three major categories: molecular function, cell composition, and biological process [68]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) (<http://www.genome.ad.jp/kegg/>) can be used to query metabolic pathways [69], enzymes (or code enzyme genes) [70], and metabolites [71]. The differentially expressed circRNAs were annotated using GO and the KEGG pathway analyses. GO function analysis was performed by the Blast2GO method [72], and statistical enrichment of differential gene expression in the KEGG pathway analysis was detected by the KOBAS software [73].

Interaction analysis of circRNA and miRNA

Two software programs, TargetScan (<http://www.targetscan.org/>) and miRanda (<http://www.microrna.org/microrna/home.do>) were used to predict the targeting relationship between circRNA and miRNA. TargetScan performs miRNA target prediction based on the seed region, while miRanda is based mainly on the free energy of circRNA and miRNA. The lower the free energy, the stronger the binding capacity of the two. Therefore, we chose TargetScan and miRanda to predict the target relationship between the circRNA and miRNA.

Experimental verification by RT-qPCR

We randomly selected six circRNAs and verified their expression levels by RT-qPCR. We used Evo M-MLV RT Kit with gDNA Clean for qPCR II (AG, Changsha, Hunan, China) to convert the total RNA to cDNA. The RT-qPCR analysis was carried out in triplicate on Bio-Rad CFX96 (Bio-Rad, CA, USA). GAPDH was used as internal reference. A reaction volume of 20 μL contained 10 μL SYBR Green Premix Pro Taq HS qPCR Kit (AG), 1.6 μL cDNA, 7.6 μL ddH₂O, and 0.4 μL each of the forward and reverse primers. Primer sequences are listed in Additional file 6: Table S6. The RT-qPCR reaction conditions were set as follows: 95°C for 30 s; 40 cycles of 95°C for 5 s, 58°C for 30 s; 65°C for 30 s; the final stage was at 95°C for 5 min. The $2^{-\Delta\Delta\text{Ct}}$ method was used to analyze the relative expression levels of the selected circRNAs.

Total RNA and RNase R (Genesee Biotech, Guangzhou, Guangdong, China) were mixed to determine the resistance of the selected six circRNAs to RNase R digestion. The mix was incubated at 37°C for 15 min.

cDNA was then synthesized, and RT-qPCR assessed the expression level of the selected circRNAs.

Statistical analysis

Data on IMF content are expressed as means $\pm$ SEM. SPSS software Version 17.0 was used to analyze the experimental results with one-way analysis of variance. The RNA-seq results were analyzed using SPSS 17.0 and R software.

Abbreviations

AFWS

Aohan fine-wool sheep

IMF

Intramuscular fat

ceRNAs

Competitive endogenous RNAs

pre-mRNA

mRNA precursors

tRNA

Transfer RNA

rRNA

Ribosomal RNA

snRNA

Small nuclear RNA

EI circRNAs

Exon-intron circular RNAs

ciRNAs

Circular intronic RNAs

RT-qPCR

Quantitative real-time PCR

Mth-2

2-month-old

Mth-12

12-month-old

miRNA

Micro RNA

lncRNA

Long non-coding RNA

circRNA

Circular RNA

GO

Gene Ontology

KEGG

The Kyoto Encyclopedia of Genes and Genomes

DEG

Differentially expressed gene

PPARG

Peroxisome proliferator-activated receptor gamma

LPL

Lipoprotein lipase gene

FABP4

Fatty acid binding protein 4

THRSP

Thyroid hormone responsive

RBP7

Retinol binding protein 7

H-FABP

Heart fatty acid-binding protein

A-FABP

Adipocyte fatty acid-binding protein

ACAA2

Acetyl-CoA acyltransferase 2

CLCN3

Chloride channel, voltage-sensitive 3

USP34

Ubiquitin-specific protease 34

SORBS1

Sorbin and SH3 domain containing 1

KAT2B

K (lysine) acetyltransferase 2B

SPATA7

Spermatogenesis-associated protein-7

MMP9

Matrix metalloproteinase 9

RIN

RNA integrity number

SRPBM

Spliced reads per billion mapping

Declarations

Ethics approval and consent to participate

All experimental operations conformed with the Guidelines for Experimental Animals of the Ministry of Science and Technology (Beijing, China) and were approved by the Experimental Animal Ethics Committee of Qingdao Agricultural University. The managements of the laboratory animals was in keeping with "Laboratory Animal-Requirements of Environment and Housing Facilities"(GB 14925-2001). Written informed consent to participate was obtained from the AFWS Stud Farm in Inner Mongolia Autonomous Region. All efforts were made to minimize suffering.

Consent for publication

Not applicable.

Availability of data and materials

Additional data can be found in the supplementary files. The RNA-Seq data is will be uploaded to a database before publication.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LZ, NL, JNH, and LSZ designed this study; LZ, FHH, RRZ, LRL, XMD, and WL participated in sample collection; LZ, NL, LSZ, JNH, and JNH analyzed the RNA-Seq and prepared the figures and tables.; LZ wrote the manuscript with contributions from NL, JNH, and LSZ. All authors reviewed and approved the final manuscript.

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Tables

Table 1. The top 30 expressed circRNAs in Mth-2 and Mth-12 rams

Mth-2		Mth-12	
Rank	circRNA	Rank	circRNA
1	circRNA_000001	1	circRNA_000001
2	circRNA_000002	2	circRNA_000002
3	circRNA_000003	3	circRNA_000003
4	circRNA_000004	4	circRNA_000004
5	circRNA_000005	5	circRNA_000005
6	circRNA_000006	6	circRNA_000006
7	circRNA_000007	7	circRNA_000007
8	circRNA_000008	8	circRNA_000008
9	circRNA_000009	9	circRNA_000009
10	circRNA_000010	10	circRNA_000010
11	circRNA_000011	11	circRNA_000011
12	circRNA_000012	12	circRNA_000012
13	circRNA_000013	13	circRNA_000013
14	circRNA_000014	14	circRNA_000014
15	circRNA_000015	15	circRNA_000015
16	circRNA_000016	16	circRNA_000016
17	circRNA_000017	17	circRNA_000017
18	circRNA_000018	18	circRNA_000018
19	circRNA_000019	19	circRNA_000019
20	circRNA_000020	20	circRNA_000020
21	circRNA_000021	21	circRNA_000021
22	circRNA_000022	22	circRNA_000022
23	circRNA_000023	23	circRNA_000023
24	circRNA_000024	24	circRNA_000024
25	circRNA_000025	25	circRNA_000025
26	circRNA_000026	26	circRNA_000026
27	circRNA_000027	27	circRNA_000027
28	circRNA_000028	28	circRNA_000028
29	circRNA_000029	29	circRNA_000029
30	circRNA_000030	30	circRNA_000030

circRNA ID	Mth-2	Mth-12	circType
circRNA2800	1	1	intergenic
circRNA2441	2	2	circRNA
circRNA328	3	4	circRNA
ciRNA67	4	3	ciRNA
circRNA2463	5	6	circRNA
circRNA2591	7	5	circRNA
circRNA2198	6	10	circRNA
circRNA1237	8	8	circRNA
circRNA2242	17	7	circRNA
circRNA2773	9	14	intergenic
circRNA931	10	16	circRNA
circRNA2171	23	13	circRNA
ciRNA121	27	12	ciRNA
circRNA3041	14	17	circRNA
circRNA2541	11	25	circRNA
circRNA3040	15	23	circRNA
ciRNA268	30	9	ciRNA
circRNA1540	28	11	circRNA
circRNA2546	16	26	circRNA
circRNA4635	21	18	circRNA
circRNA3122	18	19	circRNA
circRNA1071	19	21	circRNA
circRNA1761	12	29	circRNA
circRNA2375	22	20	circRNA
circRNA1933	24	24	circRNA
circRNA1028	26	22	circRNA
circRNA1736	13	30	circRNA
circRNA2030	20	28	circRNA

circRNA1828	29	15	circRNA
circRNA1337	25	27	circRNA

The 30 circular RNAs (circRNAs) with the largest difference in expression level are listed. Green means higher expression, and yellow means lower expression. The overrepresentation of green and yellow goes from higher to lower. The numbers from 1 to 30 in each column represent the descending order of circRNA expression levels. The circType indicates the type to which each circRNA belongs. The ciRNAs is circular intronic RNAs. The circRNA is all-exon circRNA. The intergenic is produced by the circularization of viral RNA genome, tRNA, rRNA, snRNA, etc.

Table 2. The ten circular RNAs (circRNAs) and their corresponding gene names

circRNA	Gene name
circRNA9086	<i>ENSOARG000000014894</i>
ciRNA455	<i>ENSOARG000000007720</i>
circRNA2440	<i>ENSOARG000000015818</i>
circRNA3668	<i>PTPRM</i>
circRNA829	<i>SORBS1</i>
circRNA634	<i>RAF1</i>
circRNA7445	<i>KAT2B</i>
circRNA7586	<i>ENSOARG000000010177</i>
circRNA1241	<i>ENSOARG000000009936</i>
circRNA6978	<i>RASA1</i>

The ten circRNAs and ten corresponding source genes are presented. According to KEGG enrichment analysis, we selected 12 pathways that may be related to IMF growth and development. Among them, ten circRNAs and ten corresponding source genes were involved.

Note: *PTPRM*: Protein tyrosine phosphatase receptor type M; *SORBS1*: Sorbin and SH3 domain containing 1; *RAF1*: Rapidly accelerated fibrosarcoma 1; *KAT2B*: K (lysine) acetyltransferase 2B; *RASA1*: RAS p21 GTPase-activating protein 1.

Figures

Figure 1

General characteristics of Aohan fine-wool sheep (AFWS) circular RNAs (circRNAs). a. The types of circRNAs in 2-month-old (Mth-2) rams; b. The types of circRNAs in 12-month-old (Mth-12) rams; c. The number of exons; d. Distribution of circRNAs on the chromosomes.

Figure 2

Circular RNAs (circRNAs) expression. a. Venn diagram, drawn using the number of circRNAs in the two age groups; b. circRNAs expression; c. The peak density of circRNAs expression.

Figure 3

The differentially expressed circular RNAs (circRNAs). a. Histogram of differentially expressed circRNAs; b. Scatter plot of differentially expressed circRNAs; c. Cluster heat map of differentially expressed circRNAs. The abscissa is the sample, and the ordinate is the differentially expressed screened out genes. Different colors indicate different gene expression levels. The color changes from blue to white to red, with red indicating highly expressed genes, and blue indicating genes with low expression level.

Figure 4

Gene Ontology (GO) enrichment analysis of differentially expressed circular RNA (circRNA)-hosting genes. a. Histogram of differentially expressed circRNA-hosting genes' enrichment analysis; b. Scatter plot of differentially expressed circRNA-hosting genes' enrichment analysis. The abscissa indicates the Rich factor, representing the number of differentially expressed genes located in the GO divided by the total number of genes located in the GO (Rich factor = S gene number / B gene number). The larger the Rich factor, the higher the degree of GO enrichment. The ordinate is the GO term or GO function annotation. In the scatter diagram, the size of the dot represents the number of genes with significant differences that match the S gene number of a single GO term, and the color of the dot represents the p-value of the enrichment analysis, that is, the enrichment significance.

Figure 5

Scatter plot of the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differentially expressed circRNA-hosting genes. The abscissa is the Rich factor, representing the number of differentially expressed genes identified in the KEGG analysis divided by the total number of genes in the KEGG ($\text{Rich factor} = \text{S gene number} / \text{B gene number}$). The larger the Rich factor, the higher the degree of KEGG enrichment. The ordinate is the KEGG metabolic pathway term. The dot size represents the

number of genes with significant differences matching the S gene number in a single KEGG term, and the color of the dot represents the p-value of the enrichment analysis, that is, the enrichment significance.

Figure 6

Interaction analysis network diagram of circular RNAs (circRNAs) and micro RNAs (miRNAs). The circRNA-miRNA network diagram was constructed based on 12 circRNAs and 18 miRNAs.

Figure 7

Verification of circular RNAs (circRNAs) and resistance to RNase R. a. Sequencing results of six circRNAs; b. Quantitative real time PCR (RT-qPCR) verification results of six circRNAs; c. The expression levels of circRNAs, and GAPDH after RNase R treatment.

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