Identification and characterization of circRNA during muscle development in different breeds of cattle

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Abstract

Background

Black cattle are a new breed of cattle that are developed by applying modern biotechnology, such as somatic cloning, and conventional breeding methods to Luxi cattle. It is very important to study the function and regulatory mechanism of circRNAs in muscle differentiation among different breeds to improve meat quality and meat production performance and to provide new ideas for beef cattle meat quality improvements and new breed development. Therefore, the goal of this study was to sequence and identify circRNAs in muscle tissues of different breeds of cattle. We used RNA-seq to identify circRNAs in the muscles of two breeds of black cattle (Black and Luxi).

Results

We identified 14640 circRNAs and found 655 differentially expressed circRNAs. We also analysed the classification and characteristics of circRNAs in muscle tissue. GO and KEGG analyses were used on the parental genes of circRNAs. They were mainly involved in a variety of biological processes, such as muscle fibre development, smooth muscle cell proliferation, bone system morphogenesis, tight junctions and the MAPK, AMPK and mTOR signalling pathways. In addition, we used miRanda to predict the interactions between 15 circRNAs and 12 miRNAs. Based on the above assays, we identified circRNAs (circ0001048, circ0001103, circ0001159, circ0003719, circ0003794, circ0003721, circ0003720, circ0001519, circ0001530, circ0005060, circ0006589, circ0000181, circ0000190, circ0010558, circ0010577) that may play an important role in the regulation of muscle growth and development.

Conclusion

Our results provide more information about circRNAs regulating muscle development in different breeds of cattle and lay a solid foundation for future experiments.

Background

CircRNA is a unique kind of noncoding RNA that has no 5' terminal cap or 3' terminal poly (a) tail and presents a closed ring structure [1]. It was first discovered in 1976 by Kolakofsky and Sanger in plant viroid and parainfluenza virus particles by electron microscopy that showed closed loop, circular RNAs [2,3]. It was found that most of the circRNAs originate from exons and a few from introns. In terms of function, circRNAs mainly adsorb miRNAs through the "molecular sponge" mechanism, thus inhibiting the regulatory role of miRNAs and enhancing the expression levels of target genes [4 – 6]. In recent years, circRNA has become a new research hotspot in the field of scientific research. It has been found that circRNAs are involved in many biological processes, including growth and development, and diseases, among others. At present, research on circRNAs is mainly focused on cancer. There are almost no reports about the regulation by circRNAs of the development of bovine skeletal muscle. Li found that the circRNAs circFUT1O and circFGFR4 could regulate the proliferation and differentiation of bovine skeletal

muscle cells by absorbing miR-133a and miR-107, respectively [7,8]. Wei found that circLMO7 can regulate the differentiation and apoptosis of bovine skeletal muscle cells by absorbing miR-378a-3p [9]. In conclusion, circRNAs play an important role in the development of bovine muscle.

Black cattle are the first embryo transfer calves in China and are obtained by vitrified frozen somatic cell cloning embryos. After careful cultivation by researchers, Black cattle bulls were used in breeding. Researchers overcame the shortcomings of female Luxi cattle by hybrid breeding combined with molecular marker-assisted breeding, adopted black hair and obtained bull semen, and improved Luxi cattle by hybridization with another breed so that the offspring combined the excellent characteristics of both and improved production performance. In 2015, Black cattle were identified as a new species group by authoritative experts and established a Chinese type base for new breed cultivation. Luxi cattle are one of the five local breeds of beef cattle in China, with high meat production capacity and tender meat quality, and are well known as "five flowers and three layers of meat". We selected Black cattle and Luxi cattle for this research study, identified circRNAs in muscle tissue, analysed their genomic characteristics, expression differences, etc. Through high-throughput sequencing technology combined with functional verification tests, we revealed the function and regulatory mechanism of circRNA in muscle differentiation and combined these findings with the characteristics of different breeds to cultivate fast-growing and high meat production rate beef cattle varieties to increase the speed of beef cattle breeding and provide a theoretical basis for the development of China's beef industry.

Results

Apparent differences in muscle fibres in different breeds of beef cattle

The muscle fibres of the longest muscle in the back of Black cattle and Luxi cattle were significantly different in the apparent observation of paraffin sections stained by HE (Fig. 1). The length of single muscle fibres of Black cattle was significantly longer than that of Luxi cattle, and the number of nuclei in each muscle fibre was also greater. The boundary between the muscle fibres of Black cattle was clearer and rounder than that of Luxi cattle. IPP software analysis showed that there were significant differences in the muscle fibre diameter, length and weight ($P < 0.05$) but no significant differences in other muscle fibre properties ($P > 0.05$) (Table 1).

Table 1
Comparison of muscle fibre characteristics and growth characteristics of different cattles

Characteristics	Luxi cattle	Black cattle
Area (μm^2)	4831.21 ± 255.314	5224.373 ± 442.365
Diameter (μm)	59.339 ± 0.944*	65.473 ± 2.054
Length (μm)	165.965 ± 8.874*	106.722 ± 8.306
Density (EA/μm^2)	1543.777 ± 61.880	1744.158 ± 102.999
Number of muscle fibres (EA)	157.125 ± 5.959	139.833 ± 70618
Weight (kg)	291.063 ± 3.335*	309.500 ± 4.992
Note: In the table, * indicates a significant difference ($P < 0.05$)		

Transcriptome Quantification

To examine the expression profile of circRNAs in the longissimus dorsi muscle of different breeds of beef cattle, we established a cDNA library of six longissimus dorsi muscle samples from Luxi cattle (L) and Black cattle (B) and obtained the raw data after Illumina sequencing. After removing the low-quality reads and the reads with adapter sequences from the RNA-seq raw reads, 223,609,230 clean reads (B: 122,463,284, L: 101,145,946; Table 2) were obtained. Then, we queried the clean reads of the latest reference genome and used TopHat (http://tophat.cbcb.umd.edu/) for mapping. In the B and L samples, 98.08% and 97.53% of the reads were located in the reference genome, and the clean (Q30) base rates were 94.30% and 93.72%, respectively.

Table 2
Read quality and mapping results for RNA-seq

Sample	Total raw reads	Total clean reads	Mapped reads	Mapping rate (%)	Clean (Q30) Base Rate (%)
L1	126,735,736	122,463,284	120,167,801	98.13%	94.00%
L2	125,664,524	119,811,368	117,518,654	98.09%	94.36%
L3	125,900,178	119,637,778	117,281,323	98.03%	94.54%
B1	99,182,298	95,960,692	94,094,172	98.05%	93.75%
B2	105,345,396	101,145,946	97,711,021	96.60%	93.56%
B3	102,435,924	98,666,436	96,633,740	97.94%	93.86%

Table 3
The 30 circRNAs with the highest expression in each group

B-circRNA_ID	B-Source_Gene	L-circRNA_ID	L-Source_Gene
circ0013465	gene 30421	circ0013465	gene 30421
circ0011592	gene 25926	circ0011592	gene 25926
circ0001880	gene 2182	circ0001880	gene 2182
circ0008118	gene 16072	circ0006451	gene 12913
circ0012379	Null	circ0012379	Null
circ0006451	gene 12913	circ0005801	gene 12000
circ0010250	gene 21361	circ0012393	Null
circ0011321	gene 25624	circ0005239	gene 11048
circ0003768	gene 6914	circ0008118	gene 16072
circ0000295	gene 1312	circ0011321	gene 25624
circ0005801	gene 12000	circ0012381	Null
circ0005239	gene 11048	circ0014578	gene 33995
circ0012381	Null	circ0003768	gene 6914
circ0007282	gene 14472	circ0000295	gene 1312
circ0012244	gene 27622	circ0000448	gene 267
circ0003777	gene 6914	circ0012335	gene 27977
circ0000923	gene 2584	circ0005452	gene 11270
circ0013776	gene 31637	circ0013776	gene 31637
circ0009975	gene 20815	circ0002476	gene 2820
circ0000448	gene 267	circ0005082	gene 10664
circ0002476	gene 2820	circ0012389	Null
circ0007065	gene 14054	circ0000923	gene 2584
circ0008388	gene 16629	circ0009975	gene 20815
circ0003798	gene 6918	circ0008388	gene 16629
circ0001875	gene 2179	circ0008946	gene 17970
circ0001112	gene 1570	circ0009532	gene 19466

B-circRNA_ID	B-Source_Gene	L-circRNA_ID	L-Source_Gene
circ0008946	gene 17970	circ0001205	gene 1570
circ0009481	gene 19383	circ0001212	gene 1570
circ0009532	gene 19466	circ0006938	gene 13857
circ0010865	Null	circ0001083	gene 1570

Differential Expression Of Circrnas

In this study, 655 differential circRNAs and 467 parental genes (attachment 2: Table S2) were detected, of which 267 were upregulated and 388 were downregulated (Fig. 3a). circRNAs with significant differential expression are visualized by a red volcano map (Fig. 3b) and cluster heat map (Fig. 3d) to illustrate the distribution of differential circRNAs.

Enrichment analysis by GO, KEGG and SPONG

The function of a circRNA is reflected by its parental genes. To determine the function of these genes, GO analysis was carried out. According to the statistical data of differentially expressed circRNAs and their parental genes, we annotated the parental genes of circRNAs instead of the circRNAs because there is currently no information on the annotation of circRNAs. The parental genes of differentially expressed circRNAs were annotated by 65 different GO terms (Fig. 4a). The most annotated GO terms were cellular process (BP), single organization process (BP), cell part (CC), organelle (CC), binding (MF), and catalytic (MF). To understand the functions of DEGs, goatools (https://pypi.org/project/goatools/) were used to conduct functional enrichment GO analyses. The results showed that 142 differentially expressed parental genes of circRNAs were significantly enriched. The highest enrichment was cell process, regulation of major metabolic processes, intracellular part, intracellular organelle, membrane-bound organelle and egg white matter binding (Fig. 4b-d). The significant enrichment results of the three GO categories are listed in attached document S3. According to the statistical results of the GO functional enrichment analysis (attachment 3: Table S3), we identified 29 terms of 60 different parental genes related to muscle growth and development. See attachment 4: Table S4 for details. According to statistics of the enrichment degrees of the 60 DE parental genes in each term (Fig. 5), it is found that the genes with higher enrichment degrees were gene 1570 (TTN), gene 23041 (MYBPC2), gene 6914 (MYBPC1), gene 1832 (NEB), and gene e22584 (MYH15), while the genes with lower enrichment degrees were gene 7152 (ZBCC9), gene 5917 (ACTR3b), and gene 5819 (EZH2), all of which are involved in the process of muscle growth and development, but the enrichment degree was highly different.

To predict the functions of significantly enriched parental genes, pathway analysis was conducted based on the KEGG pathway database. Among the 36 pathways with significant enrichment, the AMPK

signalling pathway, cellular pathway, and cellular pathway and adaptive signalling in cardiomyocytes were the most significantly enriched (Fig. 5a, attachment 5: Table S5). All the samples were enriched by KO, and the distribution map was made according to the significance of the Q-values of the KO enrichment analysis (Fig. 6). Based on the above enrichment results, we identified 15 pathways (AMPK signalling pathway, adaptive signalling in cardiometrics, osteoblast differentiation, differentiated cardiopathy (DCM), hypertonic cardiopathy (HCM), MAPK signalling pathway, TNF signalling pathway, focal adhesion, and cAMP signalling pathway. These pathways were enriched for 49 parental genes (Table 4). One parental gene was involved in the regulation of multiple pathways. According to the statistics of the pathway enrichment analysis of the 49 parental genes (Fig. 7), the genes with higher enrichment were gene 20196 (AKT3), gene 1084 (PIK3CB), gene 25426 (PIK3R1), gene 31865 (MAPK8), and gene 21361 (MYL2), and the genes with lower enrichment were gene 6914 (MYBPC1), gene 7074 (BNM1L), gene 8206 (TBC1D1), and gene 822 (MECOM), all of which are involved in muscle processes. The pathways related to meat growth and development were also regulated, but the degree of enrichment was different.

Table 4

List of significantly enriched KEGG pathways related to muscle development

KEGG Pathway Name	Map	Candidate_Gene	Count
AMPK signalling pathway	map 04152	gene 1084; gene 1117; gene 20196; gene 21519; gene 23708; gene 25426; gene 33995; gene 5046; gene 5262; gene 5909; gene 8206; gene 8658	12
Adrenergic signalling in cardiomyocytes	map 04261	gene 1117; gene 13101; gene 13654; gene 15015; gene 20196; gene 21361; gene 22584; gene 27250; gene 27526; gene 5262; gene 5380; gene 8658	12
Osteoclast differentiation	map 04380	gene 1084; gene 11438; gene 12516; gene 17507; gene 20196; gene 22083; gene 1832; gene 25426; gene 27157; gene 31865; gene 8026	11
Dilated cardiomyopathy (DCM)	map 05414	gene 13654; gene 1570; gene 21361; gene 22584; gene 24411; gene 27250; gene 27380; gene 34158	8
Hypertrophic cardiomyopathy (HCM)	map 05410	gene 13654; gene 1570; gene 21361; gene 22584; gene 24411; gene 27250; gene 27380; gene 34158	8
MAPK signalling pathway	map 04010	gene 10636; gene 11438; gene 14563; gene 18172; gene 18729; gene 20196; gene 6914; gene 25232; gene 27250; gene 31330; gene 31865; gene 34246; gene 5152; gene 8026; gene 1832	15
TNF signalling pathway	map 04668	gene 1084; gene 17183 gene 20196; gene 25232; gene 25426; gene 31865; gene 5262; gene 7074; gene 8026	9
Focal adhesion	map 04510	gene 1084; gene 12407; gene 15015; gene 15498; gene 20196; gene 21361; gene 25426; gene 28102; gene 30957; gene 31865; gene 5152; gene 6914	12
cAMP signalling pathway	map 04024	gene 1084; gene 1832; gene 15015; gene 18729; gene 20196; gene 22584; gene 25426; gene 27526; gene 31865; gene 34652; gene 5262; gene 5380	12
Regulation of actin cytoskeleton	map 04810	gene 1832; gene 15015; gene 18729; gene 21361; gene 6914; gene 25426; gene 26115; gene 30957; gene 31330	9
Regulation of lipolysis in adipocytes	map 04923	gene 1084; gene 20196; gene 25426	3
mTOR signalling pathway	map 04150	gene 1084; gene 20196; gene 25426; gene 34246; gene 5909	5
Wnt signalling pathway	map 04310	gene 11438; gene 17507; gene 31865; gene 5380	4

KEGG Pathway Name	Map	Candidate_Gene	Count
PPAR signalling pathway	map 03320	gene 6914; gene 5046	2
Vascular smooth muscle contraction	map 04270	gene 15015; gene 6914	2

According to the interaction data and differential expression of miRNAs and circRNAs, the interaction network data files were generated and imported into Cytoscape software. The attributes of the target circRNAs were visualized in the network, and the topological attributes of some networks are marked (Fig. 8). A total of 1799 circRNAs and 653 miRNAs were obtained to generate 5037 pairs of interactions between miRNAs and circRNAs, in which circ0013807 interacts with 37 miRNAs, circ0006152 interacts with 27 miRNAs, miR-11988 interacts with 449 circRNAs, and miR-11986b interacts with 376 circRNAs.

Target miRNAs of differentially expressed circRNAs in different breeds of beef cattle

To further understand the function of circRNAs, we used miRanda (http://www.microrna.org/microrna/home.do) to predict the interactions between circRNAs and miRNAs. A total of 1799 circRNAs and 652 miRNAs were predicted to interact (attachment 6: Table S6). The circRNAs with the most target miRNAs were circ0013807 (37), circ0006152 (27), and circ0008394 (26). According to the above differential expression and GO/KEGG/PPI enrichment analyses, we identified 7 genes of different sources related to muscle growth, corresponding to 15 circRNAs and 12 target miRNAs (Table 5). It was found that there are multiple binding sites of miRNAs in some circRNAs (such as MYBPC1 and miR-11986b, RYR1 and miR-10171-3p) sequences. After a miRNA is adsorbed, it cannot regulate its corresponding target gene, thus a circRNA acts as a miRNA molecular sponge.

Confirmation Of Circrna Expression By Qrt-pcr

To verify the expression level of differentially expressed circRNAs, we randomly selected six highly expressed circRNAs and detected their expression level by qRT-PCR (attachment 7: Table S7). These results were consistent with the trends observed in RNA-seq data (Fig. 9a), with a correlation coefficient R2 = 0.9982 indicating that the RNA-seq results were reliable (Fig. 9b).

Discussion

In this study, we first examined the apparent differences in muscle fibres of different breeds of beef cattle. The results showed that there were obvious differences in the apparent observation of the muscle fibres of HE stained paraffin sections of the longissimus dorsi of Black cattle and Luxi cattle. The length of a

single muscle fibre of Black cattle was significantly longer than that of Luxi cattle, and the number of nuclei in each muscle fibre was also greater. The border between the muscle fibres of Black cattle was clearer and rounder than that of Luxi cattle. IPP software analysis showed that there were significant differences in the diameter and length of muscle fibres ($P < 0.05$) but no significant differences in the number, density or area of other muscle fibres ($P > 0.05$). The occurrence of these differences may be the key factors leading to the differences in meat production performance and meat quality of the two breeds of cattle after birth, which was also the research basis of this study to explore the underlying molecular regulatory mechanism.

We used RNA-seq technology to study the expression of circRNA in the longissimus dorsi muscle of different breeds of beef cattle. A total of 14640 circRNAs and 4201 parental genes were detected. circ0013465 (UBE2D1), circ0011592 (UBE3A) and circ0001880 (MYL1) were the most highly expressed in the two libraries. PROSITE-ProRule annotation of these proteins (UBE2D1 and UBE3A) showed that they are involved in protein ubiquitination, which is part of the protein modifications that regulate cell metabolism within eukaryotes [10]. MYL1 is a crucial protein for adequate skeletal muscle function and belongs to the myosin family [11]. The ubiquitin proteasome system (UPS) is mainly responsible for the increased protein breakdown observed in muscle wasting. The Ube family of E3 ligases is a class of enzymes (i.e., troponin I, myosin heavy chains and actin) that can guide the degradation of major contractile proteins. Their catalytic activity depends on the covalent binding of polyubiquitin chains catalysed by a specific E2 on the substrate [12]. Studies have shown that UPS can control almost any muscle mass and recovery process in catabolism. The muscle-specific E3 ligase UBE family participates in the targeting of actin, myosin, troponin and other major contractile proteins [13], indicating that the high expression of circRNAs plays a certain role in muscle development and redifferentiation.

According to the fold change > 1.5 and $P < 0.05$ criteria, 655 differentially expressed circRNAs were identified, corresponding to 467 parental genes, 267 of which were upregulated and 388 downregulated in Luxi cattle. The function of a circRNA is reflected in its parental gene. Because there is no information about the annotation of circRNAs at present, we annotated the parental genes of differentially expressed circRNAs. As a result, is the genes were annotated in 65 different GO terms, which mainly play a role in biological processes such as regulation of cell process, regulation of metabolic process and part in the cell. We identified 29 related terms. There were 60 different genes associated with muscle growth and development. The genes with higher enrichment were gene 1570 (TTN), gene 23041 (MYBPC2), gene 6914(MYBPC1), gene 1832 (NEB), and gene 22584 (MYH15). All of the above genes participate in muscle growth and development, and their corresponding circRNAs also play a role in this process. The number of circRNA parental genes in the different samples was significantly different. The difference reflects the cumulative effect of circRNAs on expression characteristics. Based on the KEGG pathway database, we further analysed the circRNAs and found that the AMPK signalling pathway, cellular signalling pathway, and cellular signalling and alternative signalling in cardiomyocytes were the most significantly enriched pathways. In our study, based on the above enrichment results, we identified 15 distinct enriched pathways related to muscle growth and development, including the AMPK signalling pathway, MAPK signalling pathway and adaptive signalling in cardiometrics, which influence muscle fibre processes

[14,15]; the MTOR signalling pathway and Wnt signalling pathway, which are involved in the regulation of skeletal muscle development and regeneration [16 – 18]; the PPAR signalling pathway, which is involved in the regulation of intramuscular fat deposition [19] and the cytoskeletal signalling pathway. According to the statistical data, the genes with higher enrichment levels were gene 20196 (AKT3), gene 1084 (PIK3CB), gene 25426 (PIK3R1), gene 31865 (MAPK8), gene 21361 (MYL2), and gene 6914 (MYBPC1), which could indicate that the circRNAs produced by these genes may play a role in the growth and development of muscle through these pathways. Combining these results with the above results, we identified 7 parental genes (TNN, MYBPC1, NEB, MEF2C, MYH7, PPP2R3A and RYR1) and 38 corresponding circRNAs. Comparing these results with previous research results, significant differences were observed in the expression of circRNAs related to the muscle development of different breeds of cattle, suggesting that circRNAs may play an important role in muscle development. Whether these circRNAs have specific functions and what the functional mechanism is need to be studied further.

circRNAs can play important roles by regulating the transcription and expression of their parental genes [20]. At present, there is a relatively limited understanding of the details of the formation of circRNA and its functional mechanism. circRNA can be obtained by transcription of protein-coding genes or intergenic regions [21]. The formation of circRNA from a protein-coding gene is caused by the variable splicing of the parental gene [22]. Therefore, there should be a certain correlation between a circRNA and its parental gene expression. We found that one source gene may produce multiple circRNA subtypes. For example, the MYBPC1 gene can produce nine different circRNA subtypes. We obtained the FPKM value of the two varieties and found that both were differentially expressed. Although one source gene may produce multiple circRNA subtypes at the same time, only 3 or 4 of them have high expression levels, and the rest have low expression levels, which indicates that the cyclization of RNA in muscle is strictly regulated. To further understand the biological function and molecular function of the parental genes of significantly differentially expressed circRNAs, we predicted the interaction between circRNAs and miRNAs and constructed a network from the interaction data. The interaction network showed that a single miRNA may be correlated with multiple differentially expressed circRNAs, and there have been reports that circRNAs can competitively adsorb miRNAs [23]. Based on the high-throughput sequencing results, we selected 15 circRNAs related to muscle development as candidate circRNAs (circ0001048, circ0001103, circ0001159, circ0003719, circ0003794, circ0003721, circ0003720, circ0001519, circ0001530, circ0005060, circ0006589, circ0000181, circ0000190, circ0010558, circ0010577). In addition, the target miRNAs were predicted, the corresponding mRNA targets of the miRNAs were predicted, and the circRNA-miRNA-mRNA network was constructed to further study the regulation of muscle development. We will further verify this network in future experiments, which provides a new basis for the study of muscle development in cattle.

In addition to the above findings, there were pathways that were not found in our study that are known to be important and enriched for many parental genes and some that have been reported in previous studies to regulate muscle growth and development. The miRNAs that have been shown to be expressed specifically or preferentially in muscles are called muscle-specific miRNAs (muscle-specific microRNAs, myomiRs) [24] and include miR-1, miR-206, miR-128, miR-483, miR-2425-5p, miR-181a, miR-208a, miR-

208b and miR-486 [25 – 27]. MiR-206 is specifically expressed only in skeletal muscle, and its targeted circRNAs (circ0001651, circ0010874, circ0010882, circ0010890, circ0010896, circ0012793) were not significantly differentially expressed. Whether these circRNAs have specific functions and what their functional mechanisms are need further study.

Conclusion

In this study, we investigated the expression of circRNAs in muscle tissues of different breeds of beef cattle, obtained 655 differentially expressed circRNAs and 467 parental genes, selected 15 circRNAs related to muscle development as candidate circRNAs, and predicted the target miRNAs. These findings may provide clues for further research on muscle development in different breeds of beef cattle.

Methods

Sample preparation

The animals used in this research institute, Black cattle and Luxi cattle, were selected from Shandong Black Cattle Technology Co., Ltd. and Dadi yellow cattle. Three healthy black cattle and three Luxi cattle animals 18 months old were selected. All experimental cattles were raised in the same farm environment. The cattles were anesthetized with sodium pentobarbital at a dose of 25 mg/kg by intravenous injection The operation of anesthetized following the structions of the literatures [28]. After collecting samples, all eexperimental cattles were released, and the samples were stored in liquid nitrogen to extract total RNA. Muscle tissue samples were fixed in 4% formaldehyde and stained with haematoxylin and eosin (HE) for histological observation. All experimental and surgical procedures involved in this study followed the "guidelines for experimental animals" of the Ministry of Science and Technology (Beijing, China). All procedures and animal care were in line with the recommendations of the European Commission (1997) and were approved by the Experimental Animal Ethics Committee of Qingdao Agricultural University.

HE staining of muscle tissue

Paraffin sections were made from muscle tissue fixed with 4% paraformaldehyde. The HE staining protocol included dewaxing, covering with water, haematoxylin staining, washing with water, 5% acetic acid differentiation, back blue, eosin staining, dehydration, natural drying, sealing and image acquisition. The specific steps have been described previously [29]. HE staining images were used for counting, and the surface area was measured by Image-Pro Plus software. SPASS software was used for statistical analysis to calculate significant differences.

circRNA sequencing

High-throughput full transcriptome sequencing and subsequent bioinformatics analysis were carried out by Annoload Technologies (Beijing, China) as follows. Total RNA was extracted from the longissimus dorsi muscle of Black cattle (B) and Luxi cattle (L) using TRIzol reagent (TIANGEN) according to the manufacturer's instructions, and genomic DNA was removed with DNase I (Takara). RNA quality (RNA integrity number, RIN) was determined by an Agilent 2100 Bioanalyzer, and ND-2000 (NanoDrop) was quantified. Using a Ribo-Zero Gold Kit to remove rRNA from samples, and according to the specifications for the NEBNext Ultra Directional RNA Library Preparation Kit for Illumina (NEB, Ipswich, USA), different index tags were selected to construct the library, and Illumina was used to sequence the constructed library.

Read quality control and mapping

The original paired-end reads were trimmed and filtered for quality Trimmomatic using the default parameters (http://www.usadellab.org/cms/index.php, page = Trimmomatic). Then, TopHat software (http://tophat.cbcb.umd.edu/) was used to align the clean reads with the reference bovine genome (https://www.ncbi.nlm.nih.gov/genome/82?, genome assembly ID = 371813) and obtain the orientation pattern. The software was used to align the RNA sequence reads with the genome to detect gene expression and exon splicing. The genome was constructed on the superfast short read mapper Bowtie 2 for mapping with default parameters.

Identification of differentially expressed circRNAs

We used SRPBM as a standardized method to quantify the expression of circRNA, and DEseq2 was used to analyse the differential expression of circRNA [30]. In pairwise comparisons, circRNAs with $P<0.05$ and absolute multiple change value greater than 1.5 were considered to be significantly differentially expressed, and finally, the number of upregulated and downregulated circRNAs was obtained.

Enrichment analysis of GO, KEGG and PPI pathways

GO analysis and KEGG pathway analysis of the parental genes of differentially expressed circRNAs were used for annotation. The Blast2GO method was used for GO function analysis. KOBAS software was used to test the statistical enrichment of differential gene expression in the KEGG pathway. When $P<0.05$, GO terms and KEGG pathways were considered to be significantly enriched.

Prediction of miRNA targets of circRNAs

To explore the function of circRNAs and predict which circRNA acts as a miRNA sponge, we used miRanda (3.3a) (http://www.microrna.org/microrna/home.do) to predict the target relationship [31]. In

view of the published reports and the extractability of the sequences, we only selected the type of class and antisense circRNAs to predict the miRNA targeting relationship.

Experimental verification of circRNA

Real-time quantitative PCR (qRT-PCR) was used to verify the expression of circRNA. The expression levels of the selected circRNAs were standardized with the levels of the housekeeping gene GAPDH. Primer 3.0 software was used to design primers (additional file 6: S6), which were synthesized by SANGON Biotechnology Co., Ltd. (Shanghai, China). Using the fastquant RT Kit (with gDNase) (Tiangen, China), total RNA was converted to cDNA using random hexamers. The qRT-PCR reaction (20 μL) consisted of 1 μL template cDNA, 5 ml (5 × 1 ml vials) 10 μL upstream and downstream primers, respectively, RNAse-free water. The procedure was as follows: 94 °C for 10 min and 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 40 s. The expression of GAPDH was calculated by the $2^{-\Delta\Delta CT}$ value method.

Abbreviations

"HE staining": haematoxylin and eosin staining; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; PPI: SRPBM: spliced reads per billion mapping; RNA-seq: RNA sequencing technology; qRT-PCR: real-time quantitative PCR; SPONG: the interaction network between miRNA and circRNA.

Declarations

Ethics approval and consent to participate

All experimental designs and procedures in this study were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Qingdao Agricultural University IACUC (Institutional Animal Care and Use Committee).

Consent for publication

Written informed consent to publish was obtained from Qingdao Agricultural University.

Availability of data and materials

Additional data can be found in the supplementary files.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

YJD, XJB, CZX and RLL designed the study. RLL and XXL conducted the experiment. RLL, XXL and XJB performed and collected the data. RLL analysed the data and wrote the manuscript. All authors read and approved the final manuscript.

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Additional Files

Additional file 1: Table S1. Total circRNA detected in Black cattle and Luxi cattle.

Additional file 2: Table S2. Differentially expressed circRNAs in the two comparison groups.

Additional file 3: Table S3. The results of the GO functional enrichment analysis for source genes of circRNAs.

Additional file 4: Table S4. The screened results of GO enrichment analysis.

Additional file 5: Table S5. Detailed results of KEGG pathway analysis for source genes of circRNAs.

Additional file 6: Table S6. Detailed results of predicted the interactions between circRNAs and miRNAs.

Additional file 7: Table S7. Primer sequences for qRT-PCR of randomly selected circRNAs.

Figures

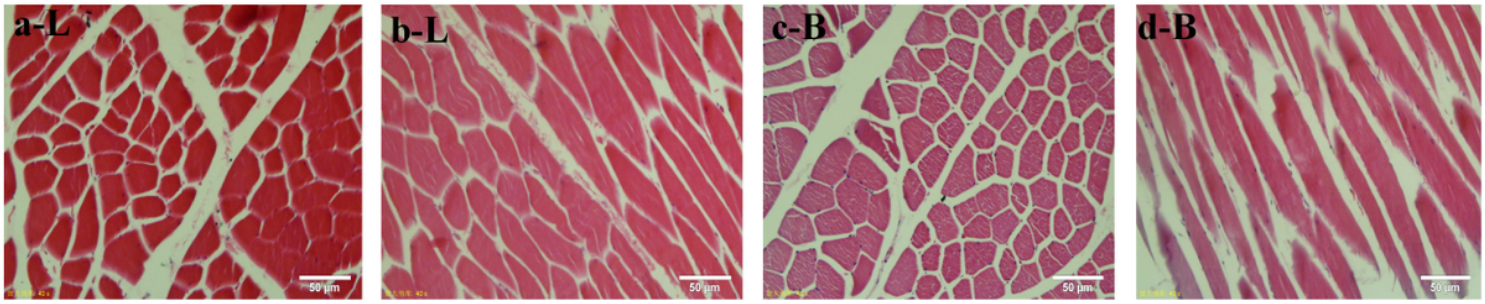

Figure 1

HE staining of muscle tissue paraffin sections.

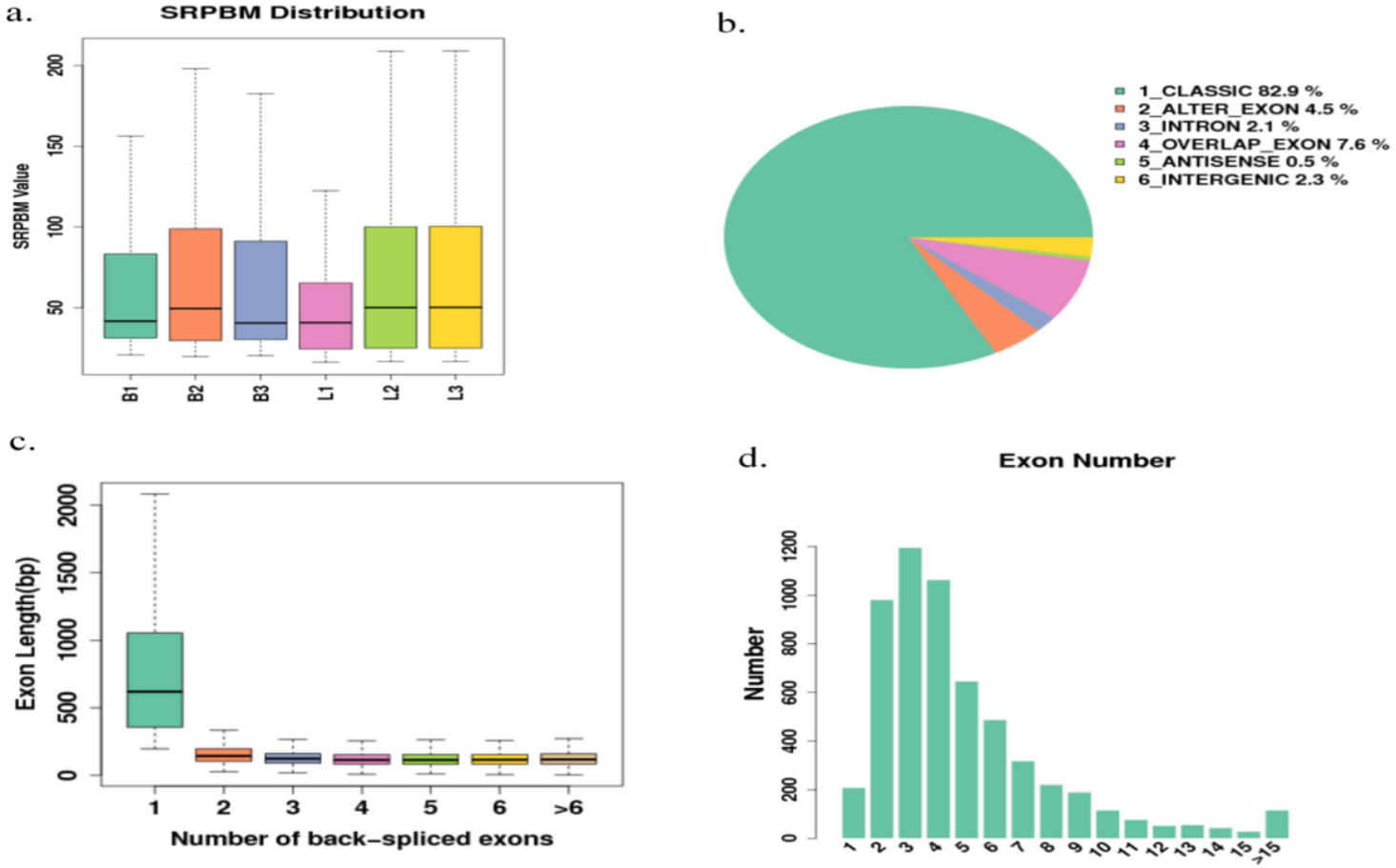

Figure 2

Expression level of sample circRNAs

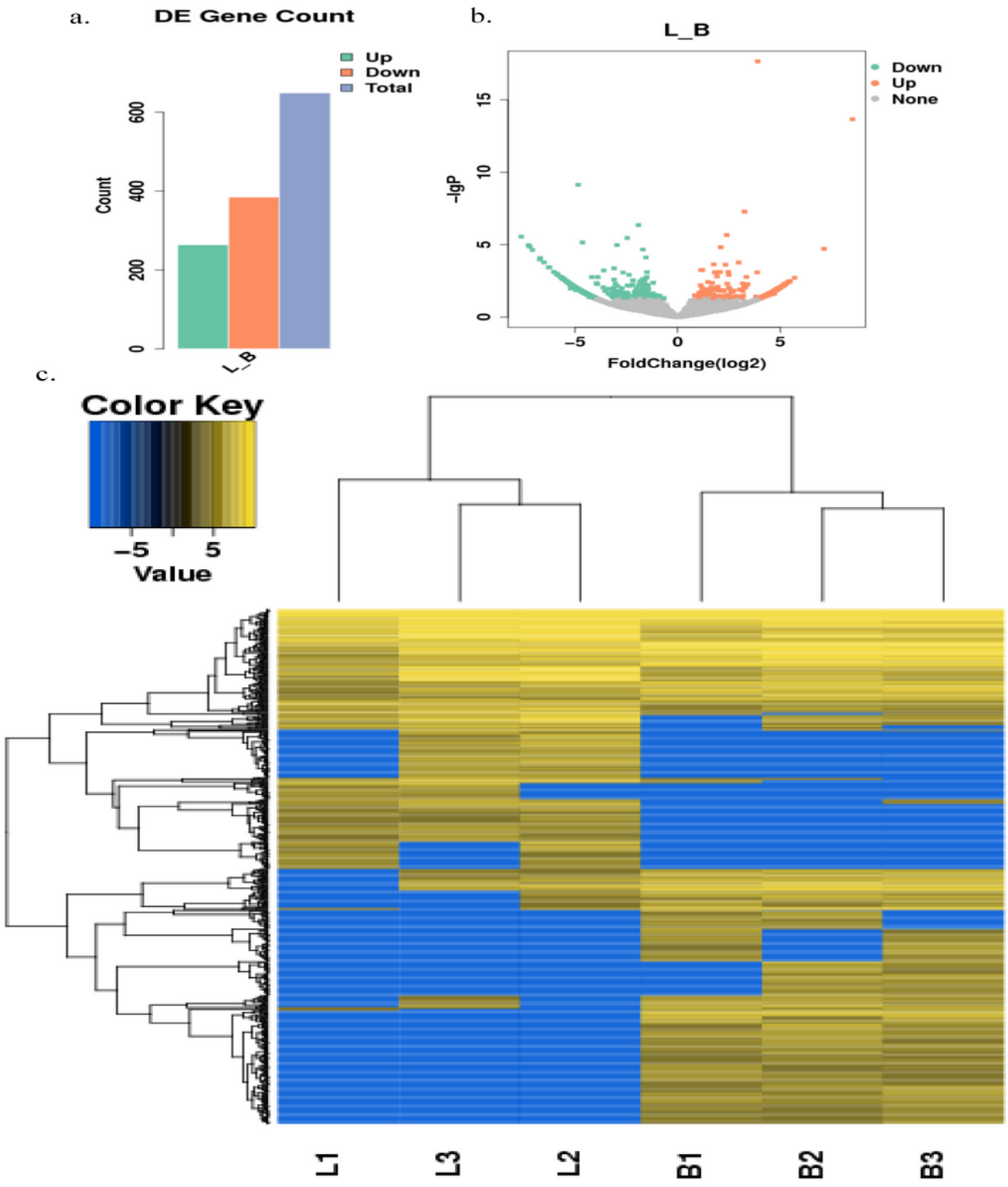

Figure 3

Differential expression of circRNAs in the samples

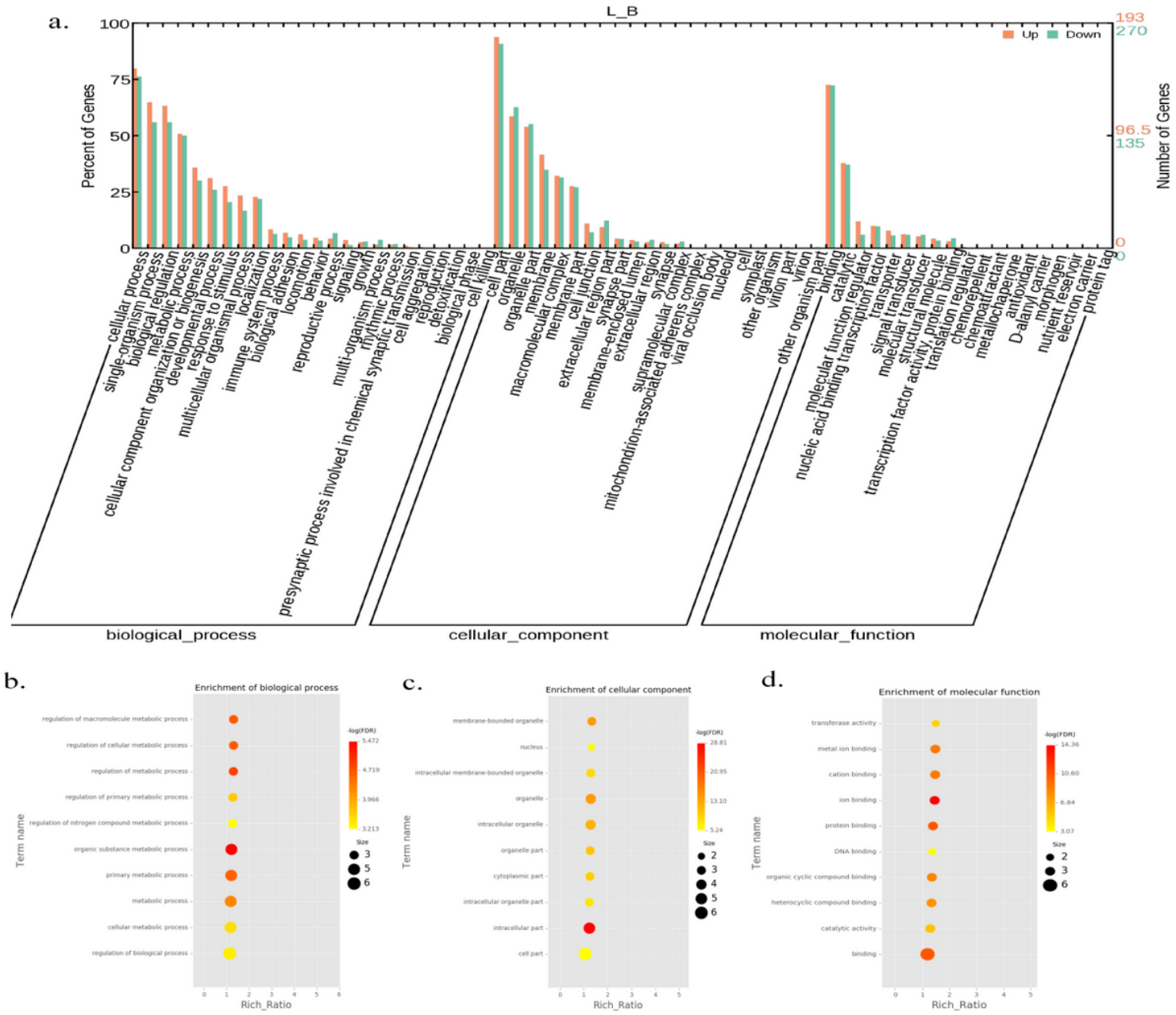

Figure 4

GO annotation of differential circRNAs and enrichment analysis

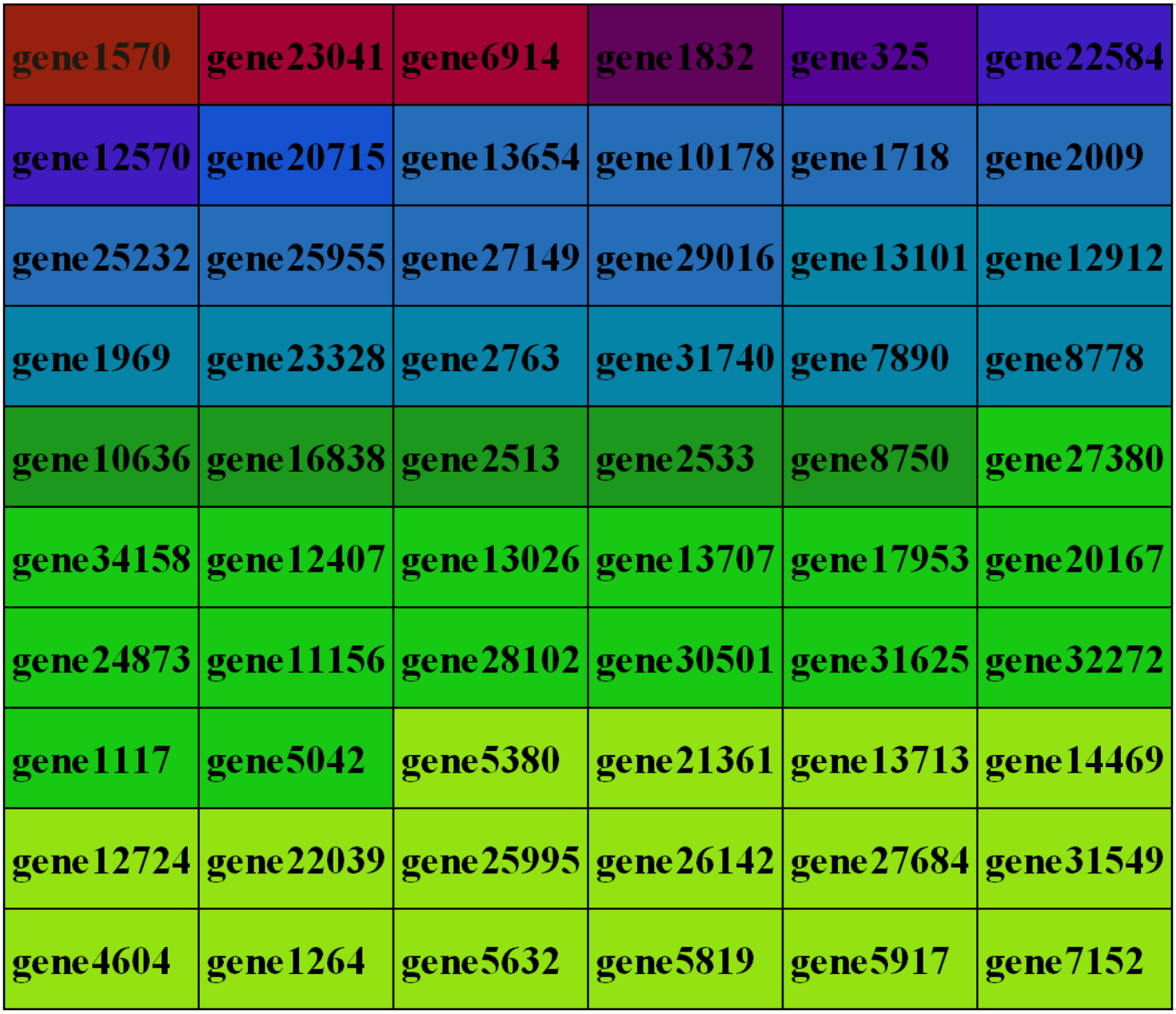

Figure 5

Enrichment degree of the identified parental genes in different GO terms

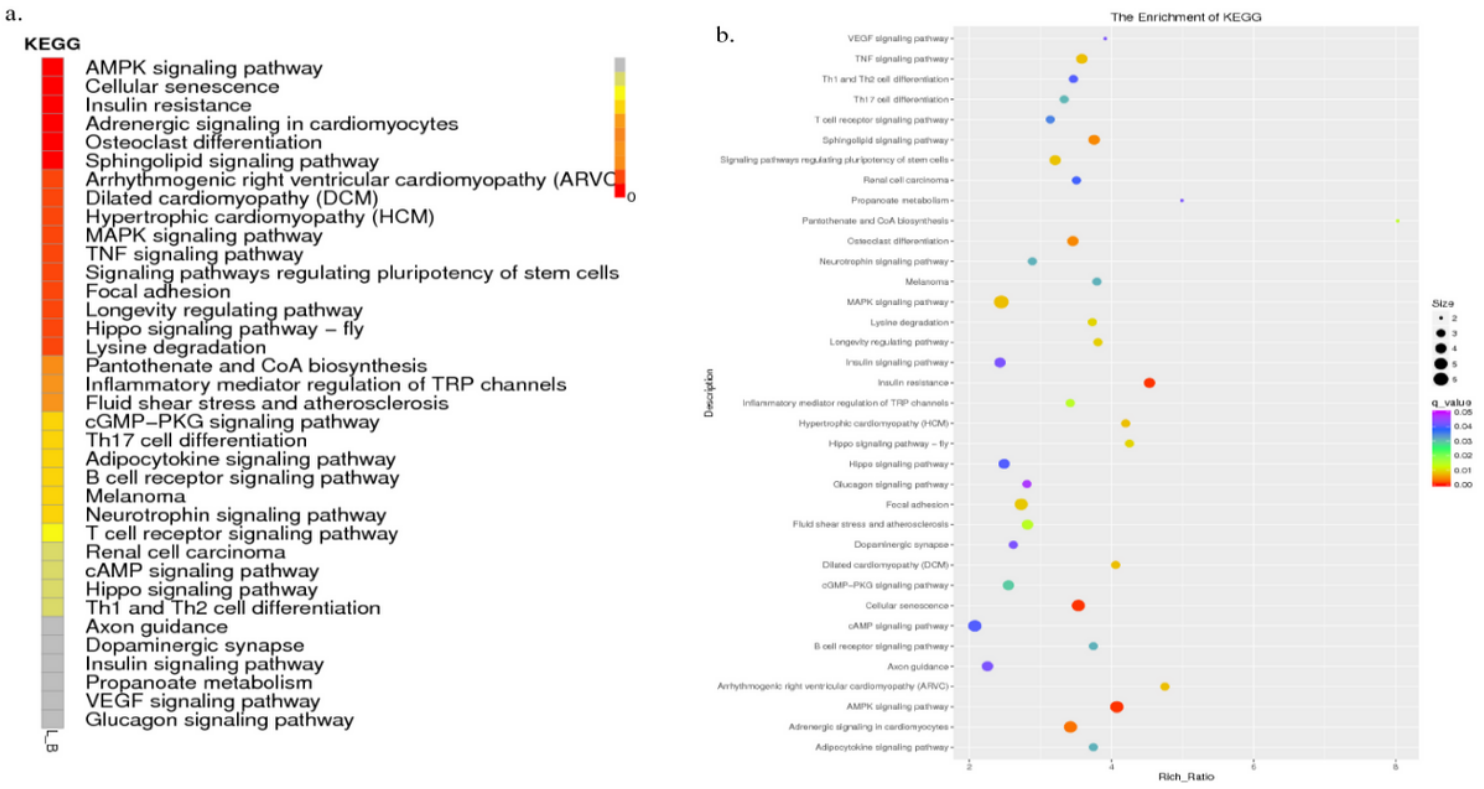

Figure 6

Enrichment results of the KEGG analysis

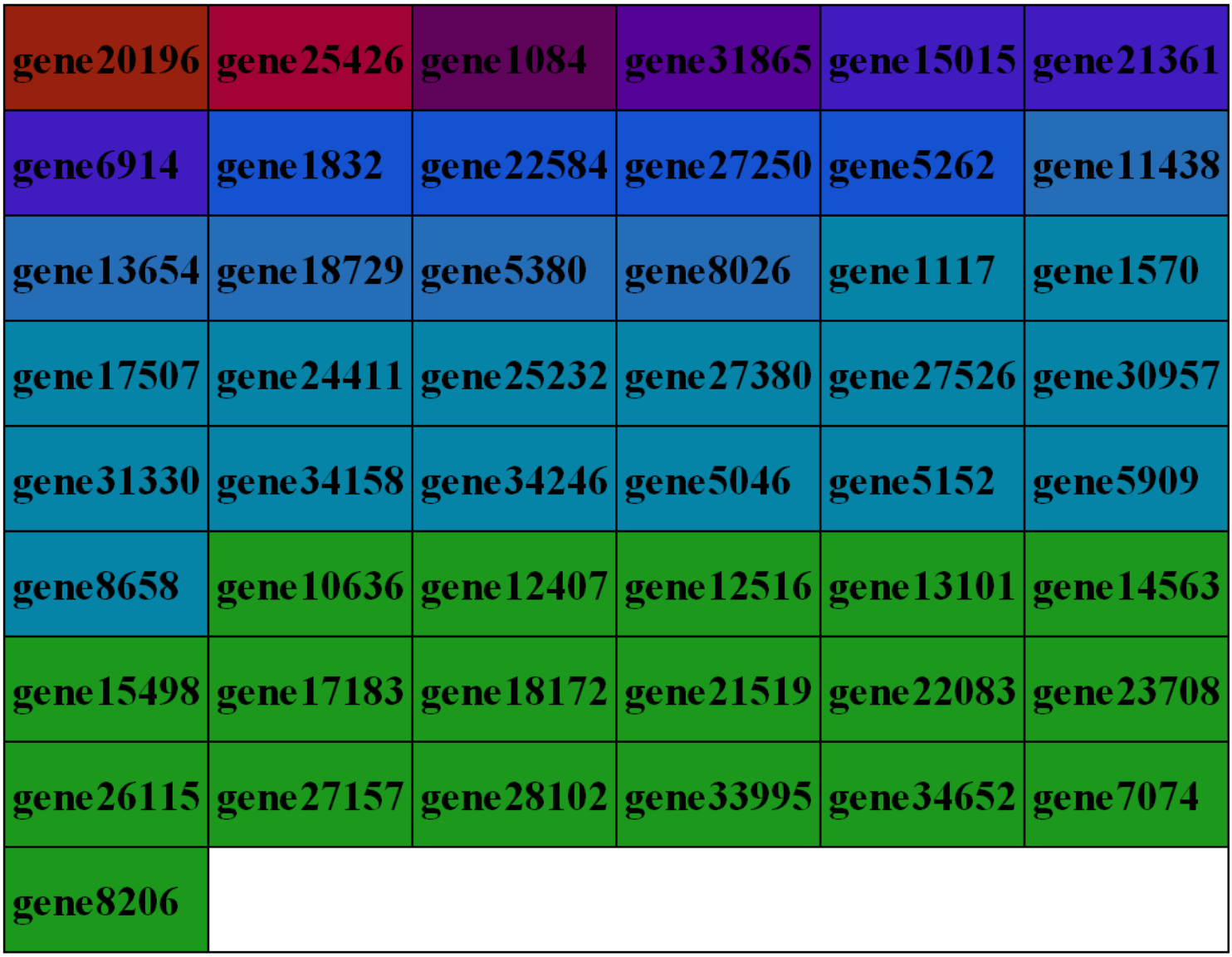

Figure 7

Enrichment degree of identified parental genes in different pathways

Figure 8

Interaction between circRNAs and miRNAs.

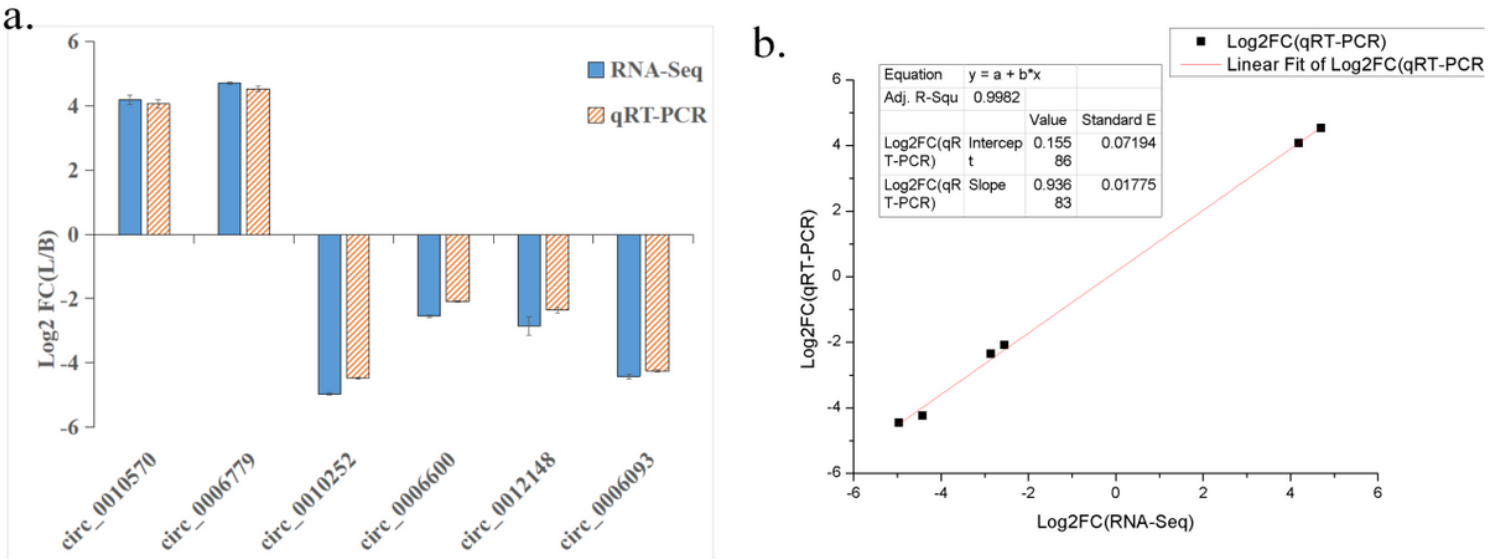

Figure 9

Linear fitting of RNA-seq and qRT-PCR circRNA expression data

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Attachment4.xls
- Attachment2.xls
- Attachment6.xls
- Attachment5.xls
- Attachment3.xls
- Attachment1.xls
- Attachment7.xls

Table 5
Prediction of the target relationships between miRNAs and circRNAs

circRNA	miRNA
circ0001048	miR-11988
circ0001103	miR-2386
circ0001159	miR-1814c
circ0003719	miR-11988
circ0003794	miR-2285
circ0003721	miR-11986b
circ0003720	miR-11988
circ0001519	miR-11986b
circ0001530	miR-181a
circ0005060	miR-2466-5p
circ0006589	miR-2285q
circ0000181	miR-1248
circ0000190	miR-154c
circ0010558	miR-2340
circ0010577	miR-10171-3p