

Identification of Crucial Circrnas in Skeletal Muscle During Chicken Embryonic Development

Genxi Zhang (✉ gxzhang@yzu.edu.cn)

Yangzhou University

Pengfei Wu

Yangzhou University

Kaizhi Zhou

Yangzhou University

Jin Zhang

Yangzhou University

Xuanze Ling

Yangzhou University

Xinchao Zhang

Yangzhou University

Li Zhang

Shanxi Agricultural University

Peifeng Li

Shanxi Agricultural University

Qingyu Wei

Shanxi Agricultural University

Tao Zhang

Yangzhou University

Xinglong Wang

Yangzhou University

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Abstract

Background: Chicken provides humans with a large amount of animal protein every year, in which skeletal muscle plays a leading role. The embryonic skeletal muscle development determines the number of muscle fibers and will affect the muscle production of chickens. CircRNAs are involved in a variety of important biological processes, including muscle development. However, studies on circRNAs in the chicken embryo muscle development are still lacking.

Results: In the study, we collected chicken leg muscles at 14 and 20-day embryonic ages both in the fast- and slow-growing groups for RNA-seq. We identified 245 and 440 differentially expressed (DE) circRNAs in the comparison group F14vsF20 and S14vsS20 respectively. GO enrichment analysis for the host genes of DE circRNAs showed that biological process (BP) terms in the top 20 related to growth in F14vsF20 were found such as positive regulation of transcription involved in G1/S phase of mitotic cell cycle, multicellular organismal macromolecule metabolic process, and multicellular organismal metabolic process. In group S14vsS20, we also found some BP terms associated with growth in the top 20 including actomyosin structure organization, actin cytoskeleton organization and myofibril assembly. A total of 7 significantly enriched pathways were obtained, containing Adherens junction and Tight junction. Further analysis of those pathways found three crucial host genes *MYH9*, *YBX3*, *IGF1R* in both fast- and slow-growing groups, three important host genes *CTNNA3*, *AFDN* and *CREBBP* only in the fast-growing group, and six host genes *FGFR2*, *ACTN2*, *COL1A2*, *CDC42*, *DOCK1* and *MYL3* only in the slow-growing group. In addition, circRNA-miRNA network also revealed some key regulation pairs such as *novel_circ_0007646-miR-1625-5p*, *novel_circ_0007646-miR-1680-5p*, *novel_circ_0008913-miR-148b-5p*, *novel_circ_0008906-miR-148b-5p* and *novel_circ_0001640-miR-1759-3p*.

Conclusions: These findings would guide our next research and it would also lay a theoretical foundation for further improving the development mechanism of embryonic skeletal muscle.

Background

China is rich in local chicken genetic resources, most of which have the advantages of high meat quality and strong stress resistance. However, the growth rate of local chicken resources is usually slow, which seriously limits their industrialization. In recent years, the market share of high-quality broilers has been increasing. Therefore, using local chickens to cultivate high-quality broilers is the development direction of breeding in China in the future. Skeletal muscle is crucial to the broiler industry and it can directly affect production performance [1, 2]. The study on the skeletal muscle mechanism of chickens would make contributions to future breeding work.

At present, a large number of studies have involved the mechanism of skeletal muscle growth and development of genes [3, 4]. In recent years, the diverse mechanisms and functions of non-coding RNAs (mainly including miRNA, lncRNA and circRNA) have attracted much attention. The function of miRNAs in skeletal muscle has been gradually revealed in chicken. Studies have shown that some specific miRNAs

related to skeletal muscle development were labeled myomiRs including *miR-1*, *miR-133* and *miR-206* [5, 6]. With in-depth research, numbers of non-skeletal muscle-specific miRNAs have also been found to be closely related to skeletal muscle growth [7–9]. LncRNA widely exists in eukaryotic organisms and it could modulate transcription, epigenetic modifications, protein/RNA stability, translation, and posttranslational modifications by interacting with DNA, RNAs and/or proteins [10]. In the study of chicken skeletal muscle, it was found that lncRNA-six1 could activate the gene Six1 in cis-acting by encoding a micro peptide of about 7.26 kDa and promoting the proliferation and differentiation of chicken myoblasts [11]. In addition, *lncRNA-six1* was also found to affect the proliferation and differentiation of myoblasts as a ceRNA by adsorbing *miR-1611*[12].

CircRNA, as a class of non-coding RNAs without 5' caps and 3' tails, has also been widely studied in recent years. CircRNA was covalently closed RNA molecules generated by back splicing of mRNA [13] and it could participate in various biological processes through a variety of mechanisms [14]. Shen et al. [15] identified an abundant circular RNA circTMTTC1 through RNA-seq and found it could inhibit chicken skeletal muscle satellite cells (SMSCs) differentiation by sponging *miR-128-3p*. Yin et al. [16] identified a novel circular RNA *circFAM188B*, which encodes a novel protein *circFAM188B-103aa* to promote proliferation and inhibit differentiation in chicken SMSCs. Chen et al. [17] found that circHIPK3 could act as a sponge of *miR-30a-3p* and exert a counteractive effect of *miR-30a-3p* by promoting the proliferation and differentiation of myoblasts.

At present, studies on circRNA regulating skeletal muscle development of chicken at embryonic stages were still limited. Therefore, we collected leg muscles of 14 and 20-day embryos of Bian chickens for RNA-seq and finally identified the key circRNAs of skeletal muscle growth during embryos. This work will provide a theoretical basis for further improving the mechanism of circRNA regulating skeletal muscle growth and development.

Results

Quality control of raw data

The RNA extracted in the experiment is of high quality and fully meets the needs for sequencing (Table S1). The quality control results of raw data were shown in Table S2. Clean bases of samples have 11.95G (S14_1) at least. The percentage of the clean base with Q20 and Q30 was more than 98.00% (F20_4) and 93.99% (F20_4) respectively. The GC content of the samples ranged from 45.26–48.49%. All the further analyses were based on clean data with high quality.

Differential expression analysis

Differential expression analysis showed that 245 differentially expressed (DE) circRNAs were obtained in F14vsF20 with P-value ≤ 0.05 . Compared with 20-day embryo ages, there are 118 up-regulated and 127 down-regulated circRNAs in 14-day embryo (Fig. 1a). In the comparison group S14vsS20, we identified 440 DE circRNAs, including 175 up-regulated and 265-down regulated DE circRNAs (Fig. 1b). Further

analysis showed that there were 121 co-differentially expressed circRNAs in the two comparison groups F14vsF20 and S14vsS20 (Fig. 1c). In addition, a heatmap of DE circRNAs in differentially comparison groups based on transcript per million (TPM) values is shown in Fig. 2. Samples in the same group are clustered together, which shows that the repeatability of samples within the group is reliable.

Functional analysis for host genes of differentially expressed circRNAs

Because most circRNAs are derived from middle exons of protein-coding genes, the processing of circRNAs can affect the splicing of their precursor transcripts, leading to altered gene expression outcomes [18]. GO and KEGG pathway enrichment for the host genes of DE circRNAs was performed in F14vsF20 and S14vsS20 respectively.

In group F14vsF20, there are 17 biological process (BP) entries in the top 20 GO terms, some of which are related to growth and development (Fig. 3a), including positive regulation of transcription involved in G1/S phase of mitotic cell cycle, multicellular organismal macromolecule metabolic process and multicellular organismal metabolic process. In another comparison group, we found that there were 10 BP entries in the first 20 GO terms, and most of them were related to growth and development (Fig. 3b), containing actomyosin structure organization, actin cytoskeleton organization and myofibril assembly.

The top 20 pathways of KEGG enrichment analysis are shown in Fig. 3c and d. In the F14vsF20 group, we found two significantly enriched pathways, Adherens junction and Tight junction, and they were closely related to growth. In addition, pathways MAPK signaling pathway, VEGF signaling pathway and focal adhesion in the top 20 are also important to muscle development. Among the top 20 pathways in S14vsS20 group, we found seven significantly enriched pathways, including adherens junction and tight junction, which were the same as that in group F14vsF20. We selected all significant pathways and their enriched genes for visualization with Cytoscape. The result (Fig. 4) showed that four genes (red nodes) were simultaneously enriched in the significant pathways of the two comparison groups, and three genes (pink nodes) were only significantly enriched pathways of F14vsF20 group and all other genes (green nodes) were significantly enriched in S14vsS20.

Result of validation for DE circRNAs

A total of six DE circRNAs were selected for validation, and agarose gel electrophoresis showed (Fig. 5a) that all the convergent primers (CP) amplified bands both in the genomic DNA (gDNA) and cDNA, and the product sizes were the same. However, divergent primers (DP) only amplified product bands in cDNA. Sanger sequencing (Fig. 5b) for back-spliced junction (BSJ) sites was the same as RNA-seq sequence. Finally, RT-qPCR (Fig. 5c and d) for the 6 DE circRNAs were all consistent with the trends of RNA-seq. The all above results suggested that the sequencing results of circRNA are reliable.

CircRNA-miRNA networks

The main mechanism of circRNAs may act as a miRNA sponge to modulate post-transcriptional regulation [19, 20]. We performed KEGG and GO functional enrichment analysis on miRNAs targeted by circRNA. Some pathways related to amino acid metabolism in the top 20 (Table S3 and S4) are enriched, including Cysteine and methionine metabolism, Arginine and proline metabolism, Biosynthesis of amino acids and Tyrosine metabolism. There are also some pathways associated with mRNA, such as RNA transport, RNA polymerase and mRNA surveillance pathway in the top 20. In addition, we also found Glycolysis / Gluconeogenesis and Fructose and mannose metabolism pathways related to carbohydrate metabolism. In the GO enrichment results, we found 55 biological process (BP) terms related to skeletal muscle both in F14vsF20 and S14vsS20 groups (Table S5 and S6). Based on the genes enriched in these entries, we constructed the DE circRNA-miRNA relationship pairs for the two comparison groups and found 9453 and 16635 pairs respectively (Table S7 and S8). The energy values were shown in the third column of the Table S7 and S8. The lower the energy value, the more reliable the targeted binding relationship is. Finally, we chose the circRNA-miRNA pairs with $\text{energy} \leq -50$ for visualization using the software Cytoscape (Fig. 6).

The NetworkAnalyzer plug-in in Cytoscape software was used to calculate the connection degree. CircRNAs or miRNAs with higher connection degree were identified as highly connected.

Novel_circ_0007646 had the highest degree both in the two groups, and *novel_circ_0007646-miR-1625-5p*, *-miR-1680-5p* and *-miR-6570-3p* pairs were also important in the two groups. The miRNA with the highest connectivity in the two groups was *miR-148b-5p*. *MiR-148b-5p-novel_circ_0008913* and *-novel_circ_0008908* pairs may play an important role in the fast-growing group, while *miR-148b-5p-novel_circ_0008906* and *-novel_circ_0008907* were significant in the slow-growing group. In addition, we also speculated that *miR-1759-3p-novel_circ_0001640* pairs may also significantly regulate the growth and development of skeletal muscle according to their degrees in the slow-growing group.

Discussion

As a source of animal protein, chicken occupies an important share in the market of meat and it has provided the human diet across the globe with large amounts of protein [21]. Improving the quantity and quality of chicken meat has always been the goal of researchers in the past few decades. Many genes closely related to the development of skeletal muscle have also been revealed, such as *MSTN* [22], *MRFs* [23], *MEF2* [24], etc. In recent years, the research of skeletal muscle has gradually transferred to non-coding RNAs. CircRNA, as one of the major non-coding RNAs, has rich and extremely important functions on a variety of biological processes, including skeletal muscle development [19, 25].

In the study, we collected chicken leg muscles of different embryonic ages for transcriptome sequencing and identified 245 and 440 DE circRNAs in comparison group F14vsF20 and S14vsS20. GO enrichment for the host genes of DE circRNAs was performed in F14vsF20 and S14vsS20 respectively, and some BP terms related to skeletal muscle have been identified including myofibril assembly, actomyosin structure organization and protein autophosphorylation. KEGG enrichment analysis showed that two and seven pathways were significantly enriched in F14vsF20 and S14vsS20 respectively (Fig. 4) and they were all

closely related to skeletal muscle development. Fig. 4 showed that red nodes, *MYH9*, *YBX3*, *IGF1R* and *EPB41L3* were simultaneously enriched in the significant pathways of the two comparison groups. Pink nodes, *CTNNA3*, *AFDN* and *CREBBP*, were only significantly enriched pathways of F14vsF20 group. All other genes (green nodes) were significantly enriched in pathways of S14vsS20 and genes such as *FGFR2*, *ACTN2*, *COL1A2*, *CDC42*, *DOCK1* and *MYL3* are closely related to skeletal muscle growth.

The hierarchy of skeletal muscles runs from the top to bottom as muscles, fibre bundles, fibres, myofibrils and sarcomeres [26]. In cross-striated muscle, sarcomeres contained Myosin II (thick) filaments and actin (thin) filaments [27], which were both involved in the regulation of the contraction of striated muscle [28]. The myosin II subfamily is the largest class of myosins and includes skeletal, cardiac and smooth muscle myosins, as well as non-muscle myosin-2 (NM2) isoforms [29]. The class α myosin forms the filaments in muscle and non-muscle cells as a hexameric protein complex, consisting of two myosin heavy chain (*MyHC*) subunits, two regulatory light chains (RLCs) and two essential light chains (ELCs) [30]. Together, the ELCs and the RLCs are named myosin light chains (MLCs), which are important regulators of actin-myosin interactions. Vertebrates hold three paralog genes (*MYH9*, *MYH10* and *MYH14*), which are located in different chromosomes and encode three *NMHC2* isoforms (*NMHC2A*, *2B* and *2C*, respectively) [29]. Before muscle-specific myosin II (MM II) is organized in mature myofibrils to carry out that role, however, non-muscle isoforms of myosin II (NM II) are present in premyofibrils and nascent myofibrils that lead to mature myofibril formation [31]. During embryonic development, from 14 to 20 embryonic ages, we found that *MYH9* was found as the host gene of DE circRNAs in the significantly enriched pathways of both F14vsF20 and S14vsS20 (Fig. 5). The results suggest that *novel_circ_0002968* and *novel_circ_0002969*, and the host gene *MYH9* may play an important role in myofibril formation. In addition, *MYL3* as the host gene of DE *novel_circ_0004825*, was found to be significantly enriched in group S14vsS20 and it was a member of the myosin light chains (MLCs). Study [32] has found that *MYL3* could bind calcium ions, promote muscle development, and participate in the contraction of striated muscles.

YBX3 is a member of Y-box protein family, which contains a conserved cold shock domain (CSD), enabling these proteins to bind to single-stranded nucleic acids [33]. DNA- and RNA-binding capabilities allow members of this family to perform diverse functions, including regulation of transcription, splicing, translation, and mRNA stability [34]. Studies have confirmed that the post-translational phosphorylation of *MSY3* (*YBX3*) by Akt kinase could rescue down-regulation of myogenin caused by binding of *MSY3* in skeletal muscle [35, 36]. *IGF1R* is an *IGF-1* receptor with a transmembrane location that activates *PI3K/Akt* signaling and possesses tyrosine kinase activity, and its expression is significant in terms of myoblast proliferation and normal muscle mass maintenance [37]. *IGF1R*, as the host gene of *novel_circ_0000087*, was significantly enriched in adherens junction and focal adhesion pathways in both F14vsF20 and S14vsS20.

CTNNA3, *AFDN* and *CREBBP* were found to be significantly enriched in pathways of F14vsF20. The study of *CTNNA3* and *AFDN* in skeletal muscle has not been reported, but there are many studies on them in tumor cells, mostly related to tumor cell proliferation and migration [38, 39]. The host gene of DE

novel_circ_0008559 and *novel_circ_0006094* were *CTNNA3* and *AFDN*, respectively. We speculated that they may also regulate the growth of skeletal muscle cells. CREB-binding protein (*CREBBP*, or in short *CBP*) is a kind of lysine (K) acetyltransferases (KAT) belonging to the KAT3 family of proteins known to modify histones, as well as non-histone proteins, thereby regulating chromatin accessibility and transcription [40]. Svensson et al. [41] revealed that *CREBBP* was required for the control and maintenance of contractile function and transcriptional homeostasis in skeletal muscle of adult mice.

In comparison group S12vsS20, we also identified many host genes of DE circRNAs related to skeletal muscle development. Fibroblast Growth Factor Receptor 2 (*FGFR2*) was a member of FGFRs, which are a family of receptor tyrosine kinases expressed on the cell membrane that play crucial roles in both developmental and adult cells [42]. *FGFR2* has been proved to regulate the myogenesis of skeletal muscle [43, 44]. *ACTN2* was highly expressed in muscle where it acted as a major structural component of the contractile apparatus at the Z-line [45]. Sharma et al. [46] found that *col1a2⁺* muscle progenitor cells contributed to new myofibers in normal muscle growth and also during muscle regeneration. *Integrin/FAK* pathway is required for C2C12 myoblast differentiation by regulating the expression of *MyoD* and *CDC42* [47]. *DOCK1* (also known as Dock180) is a prototypical member of a new family of atypical Rho GTPase activators and Laurin et al. [48] have identified *DOCK1* and *DOCK5* as critical regulators of the fusion step during primary myogenesis in mammals.

CircRNAs can serve as miRNA sponges to regulate the expression of mRNA by competing for endogenous RNAs (ceRNAs) mechanisms. Ouyang et al. [49] found that *circSVIL* could promote myoblast proliferation and differentiation by sponging *miR-203* in chicken. Wei et al. [50] revealed that *circFNDC3AL* upregulated *BCL9* expression to promote chicken skeletal muscle satellite cells proliferation and differentiation by binding to *miR-204*. KEGG and GO enrichment analyses were performed for miRNAs targeted by DE circRNAs as a sponge. Cysteine and methionine metabolism, RNA transport and Glycolysis / Gluconeogenesis were found in the top 20 pathways of the two groups. In the GO enrichment results, 55 biological process terms related to skeletal muscle were enriched including skeletal muscle cell differentiation, skeletal muscle satellite cell migration, skeletal muscle tissue regeneration, and so on. We selected the miRNAs in these items and constructed the miRNA-circRNA interaction network (Fig. 6).

CircRNAs generally have more than one miRNA binding site. For example, *ciRS-7* contains over 60 target sites for *miR-7* and can function as a *miR-7* sponge and influence *miR-7* target gene expression [20]. In the study, *novel_circ_0007646* had the highest degree both in the two groups, and the targeted miRNAs include *miR-1625-5p*, *miR-1680-5p* and *miR-6570-3p* pairs, etc. In addition, one miRNA may also bind to multiple circRNAs. The targeted miRNA with the highest connectivity in the two groups was *miR-148b-5p*, and the predicted results showed that it could bind to circRNAs such as *novel_circ_0008913* and *novel_circ_0008908* in the fast-growing group, and *circ_0008906* and *novel_circ_0008907* in the slow-growing group. In the S14vsS20, we also found that the nodes in *miR-1759-3p-novel_circ_0001640* pairs both have a high degree, suggesting their important role in skeletal muscle development.

Conclusion

In the study, we collected the leg muscles of 14 and 20 embryonic Bian chickens for circRNA-seq. We identified 245 and 440 DE circRNAs in the fast- and slow-growing groups, respectively. Functional enrichment analysis for the host genes of DE circRNAs revealed several important candidate genes, such as *MYH9*, *IGF1R*, *YBX3* and *CREBBP*. These host genes and their corresponding DE circRNAs may play a significant role in skeletal muscle development. In addition, the circRNA-miRNA network constructed based on ceRNA mechanism also found some crucial regulatory relationship pairs related to skeletal muscle, including *novel_circ_0007646-miR-1625-5p*, *novel_circ_0007646-miR-1680-5p*, *novel_circ_0008913-miR-148b-5p*, *circ_0008906-miR-148b-5p* and *novel_circ_0001640-miR-1759-3p*. This experiment would lay a foundation for further improving the molecular regulation mechanism of skeletal muscle.

Methods

Animals and tissues

At the age of 300 days, twelve female and one male Bian chickens with the average body weight in different genders were selected from the slow-growing and fast-growing Bian chicken groups. After artificial insemination, the half-sib fertilized eggs were collected respectively in the two groups. They were hatched with a temperature of 37 °C and humidity of 60% until 14 or 20-day embryo ages (14E and 20E). The eggshell was destroyed and the chick embryos were decapitated rapidly. At the same time, a small amount of allantoic fluid at 14E and blood at 20E were collected for sex identification. Then the left leg muscles of female chicken were collected and frozen in liquid nitrogen immediately.

We dissected the chicken embryos and preliminarily determined their gender by gonadal observation because hens had regressed right gonad. And then the *CHD1* gene was amplified using allantoic fluid or the full blood to further identify the sex of chicken embryos. The agarose gel electrophoresis results of hens would show two bands, while cocks showed one band. Finally, the left leg muscles of female Bian chicken embryos were used for RNA-seq and four biological replicates were set in this experiment.

RNA quantification and qualification

Total RNA was extracted with TRIzol reagent. RNA degradation and contamination were monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

The construction of cDNA library and sequencing

The ribosomal RNA was depleted from total RNA using the rRNA Removal Kit following manufacturer's instruction. First strand cDNA was reverse-transcribed using fragmented RNA and dNTPs (dATP, dTTP, dCTP and dGTP). RNA was degraded using RNase H, and second strand cDNA was synthesised using DNA polymerase I and dNTPs (dATP, dUTP, dCTP and dGTP). Remaining overhangs of double-strand cDNA were converted into blunt ends via exonuclease/ polymerase activities. After adenylation of 3'ends

of DNA fragments, sequencing adaptors were ligated to the cDNA. The library fragments were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. Finally, the qualified cDNA libraries were sequenced on the Illumina platform (NovaSeq 6000) and 150 bp paired-end reads were generated.

Data analysis

Clean data (clean reads) were obtained by removing reads containing adapter, reads on containing poly-N and low-quality reads from raw data. At the same time, Q20, Q30 and GC content of the clean data were calculated. All the downstream analyses were based on the clean data with high quality. Clean reads for each sample were mapped to the reference genome GRCg6a with the software Bowtie2 [51].

The circRNA were detected and identified using find_circ [19] and CIRI2 [52]. The raw counts were normalized using TPM [53]. DESeq2 [54] was used for differential expression analysis. Finally, circRNAs with P-value ≤ 0.05 were assigned as differentially expressed.

Gene Ontology (GO) enrichment analysis for host genes of differentially expressed (DE) circRNAs were implemented by the Goseq R package, in which gene length bias was corrected. GO terms with P-value ≤ 0.05 were considered significantly enriched. KEGG is a database resource for understanding high-level functions and utilities of the biological system. We used KOBAS software to test the statistical enrichment of differential expression genes or circRNA host genes in KEGG pathways.

Validation for DE circRNAs

Six DE circRNAs were selected randomly for validation of RNA-seq in the two comparison groups F14vsF20 and S14vsS20, containing five same DE circRNAs. Divergent primers (DP) and convergent primers (CP) were designed with Primer 5.0 to amplify circRNAs back-spliced junction (BSJ) sites and linear mRNAs and they were shown in Table S9.

PCR products of divergent and convergent primers for cDNA and genomic DNA (gDNA) were analyzed by agarose gel electrophoresis. BSJ sites of circRNAs were further validated by sanger sequencing at Sango Biotech Co. Ltd. (Shanghai, China). RT-qPCR were performed on the platform QuantStudio 3 (Applied Biosystems, USA) with reagent Taq Pro Universal SYBR qPCR Master Mix (Q712, Vazyme Biotech Co., Ltd, China) according to the instruction. β -actin were selected as housekeeping gene and the $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression of DE circRNAs.

CircRNA-miRNA interaction analysis

CircRNAs as ceRNA can recruit miRNAs to regulate target gene expression [20], which was also one of the main regulation modes for circRNA. MiRanda software was used to predict the miRNA binding sites of DE circRNA, and 1200 and 1229 targeted miRNAs were obtained for DE circRNA of F14vsF20 and S14vsS20, respectively.

GO and KEGG enrichment analysis of miRNAs were performed using the Novomagic, a free online platform for data analysis (<https://magic.novogene.com>). Finally, we selected miRNAs enriched in GO terms related to "skeletal muscle" and constructed circRNA-miRNA interaction network.

Abbreviations

DE circRNA: Differentially expressed circRNA; 14E: 14-day embryo; 20E: 20-day embryo; F14/F20: 14/20-day embryo of the fast-growing group; S14/S20: S14/20-day embryo of the slow-growing group; GO: Gene Ontology; BP: Biological process; CC: Cell component; MF: Molecular function; KEGG: Kyoto Encyclopedia of Genes and Genomes; TPM: Transcripts Per Million; ceRNA: Competing endogenous RNA; gDNA: Genomic DNA; cDNA: Complementary DNA; SMSCs: Skeletal muscle satellite cells (SMSCs); RT-qPCR: Real Time Quantitative PCR; DP: Divergent primers; CP: convergent primers.

Declarations

Ethics approval and consent to participate

The animal experiments performed in the study were all evaluated and approved by the Animal Ethics Committee of Yangzhou University. We confirm that all methods were performed in accordance with the relevant guidelines and regulations. We also confirm that the study is reported in accordance with ARRIVE guidelines.

Consent for publication

Not applicable

Availability of data and materials

The raw data of the study has been uploaded to the Sequence Read Archive (SRA) database and the accession number is PRJNA773377 (<https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA773377>).

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions

PFW performed the data analysis and drafted the manuscript. KZZ, JZ, XZL and X CZ contributed to laboratory experiments. PFL, LZ and QYW artificially inseminated female chickens and collected eggs for hatching. TZ and XLW revised the manuscript. GXZ designed the study and also revised the manuscript. All authors read and approved the final manuscript.

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Figures

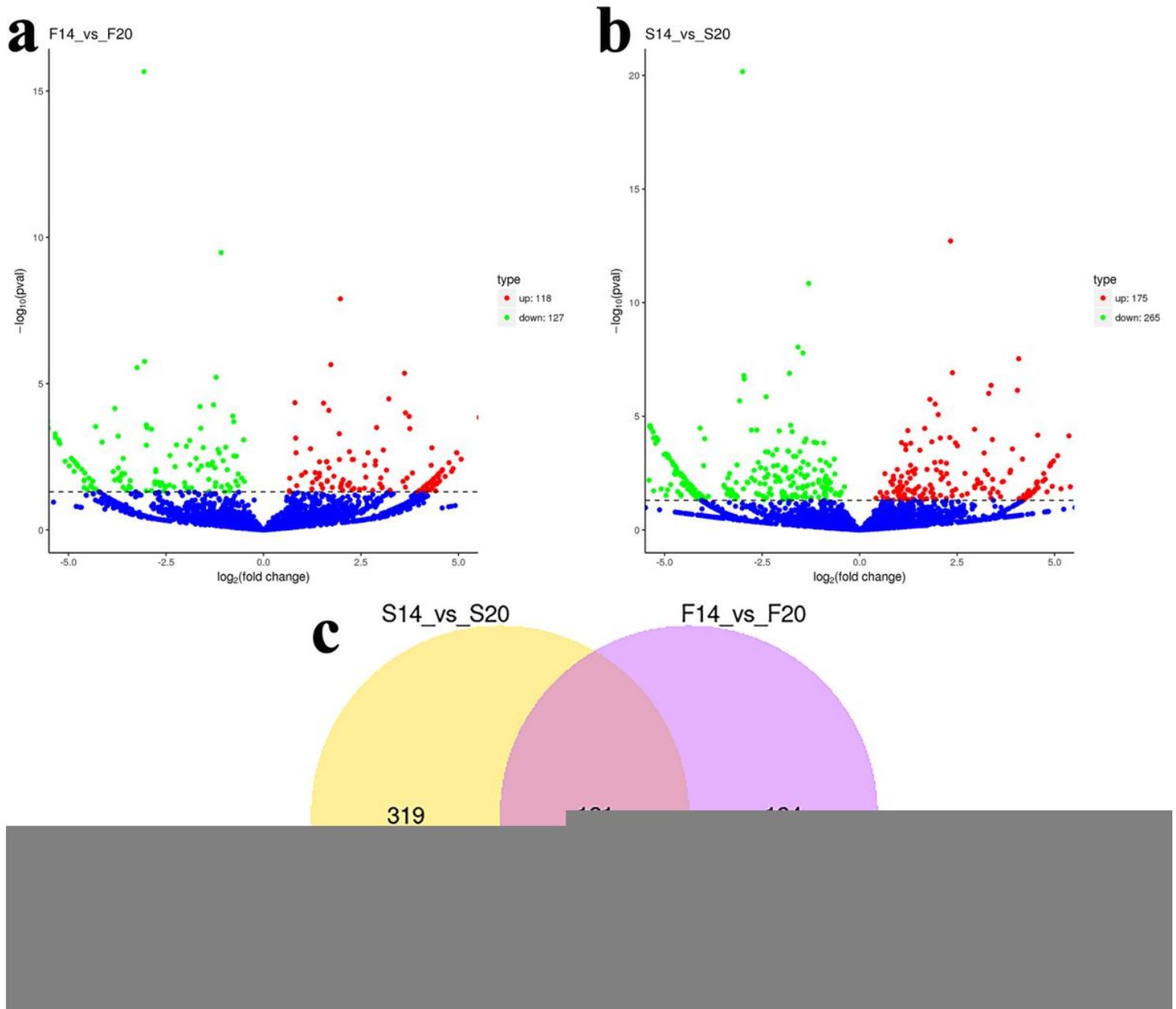


Figure 1

Differentially expressed (DE) circRNA analysis. **(a)** DE circRNAs for F14vsF20; **(b)** DE circRNAs for S14vsS20; **(c)** Venn diagram for DE circRNAs of the fast- and slow-growing groups.

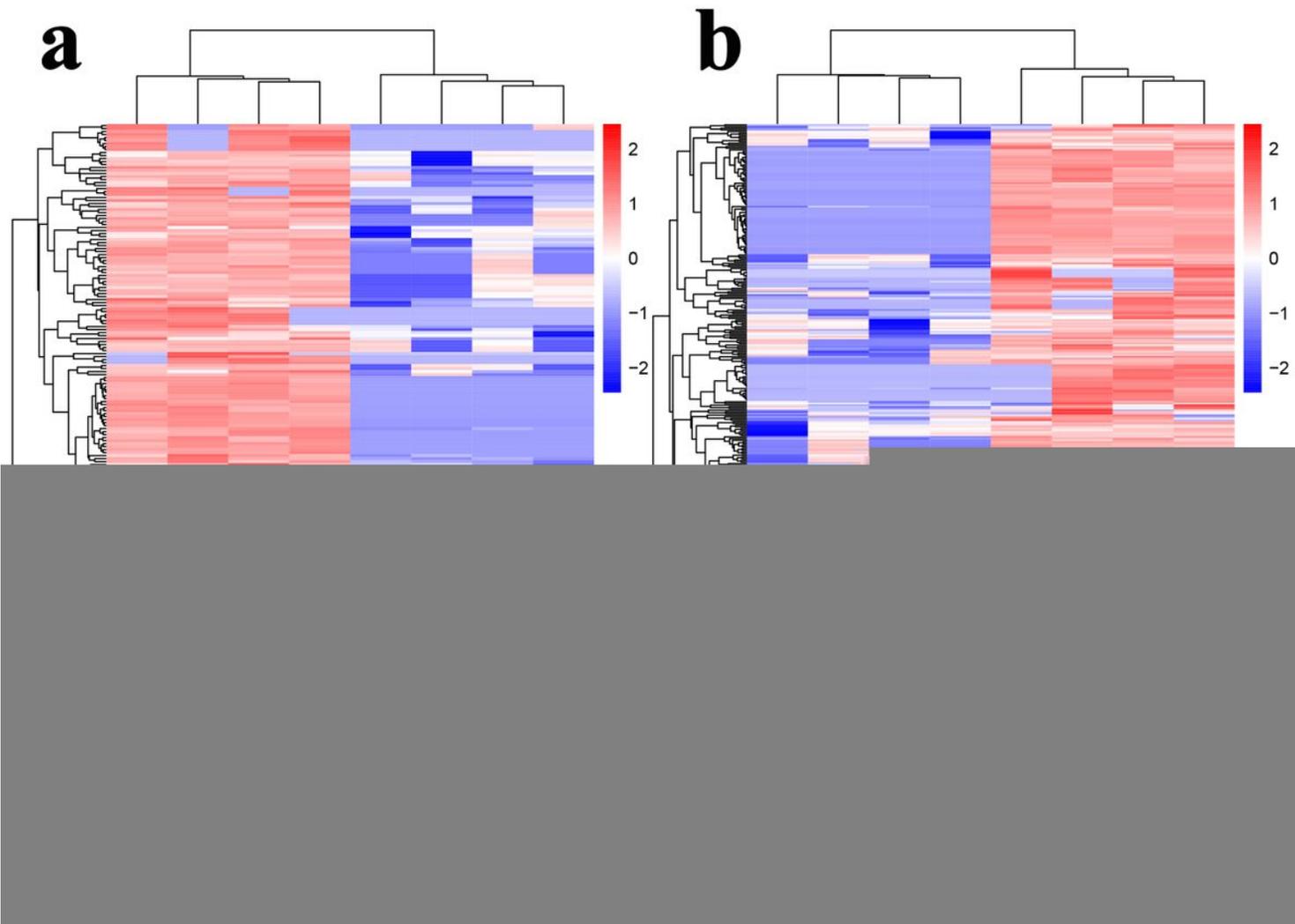


Figure 2

Results of hierarchical clustering analysis. **(a)** Hierarchical clustering results for differentially expressed (DE) circRNAs in F14vsF20; **(b)** Hierarchical clustering results for DE circRNAs in S14vsS20.

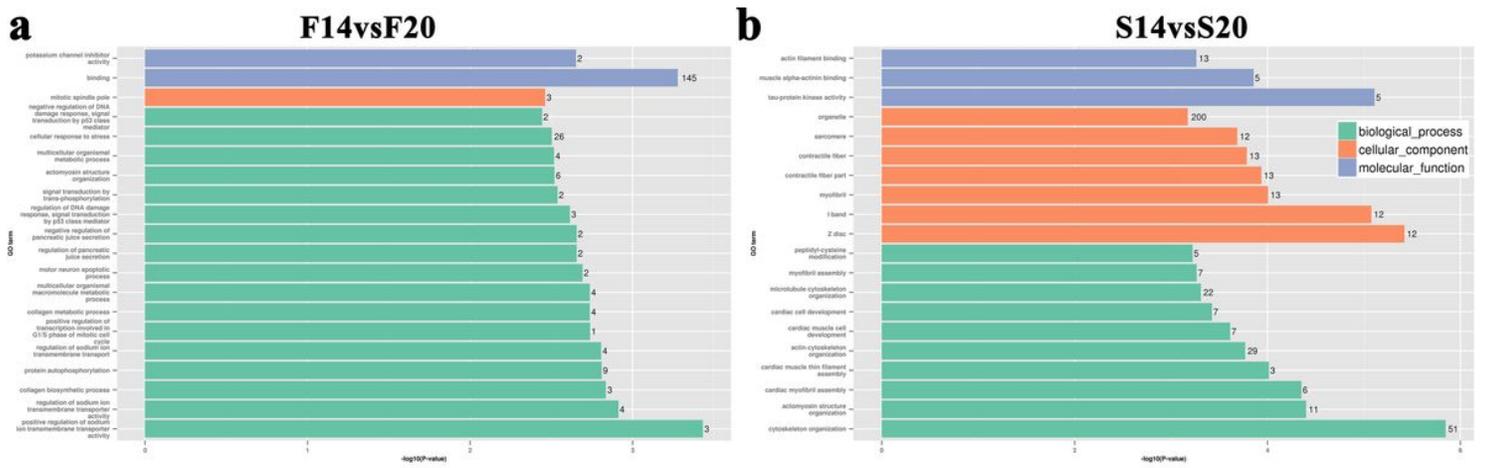


Figure 3

Functional enrichment analysis for host genes of the differentially expressed (DE) circRNA. **(a)** GO enrichment analysis of F14vsF20; **(b)** GO enrichment analysis of S14vsS20; **(c)** KEGG pathway enrichment of F14vsF20; **(d)** KEGG pathway enrichment of S14vsS20.

Figure 4

Visualization for the significant enrichment pathways and enriched genes of F14vsF20 and S14vsS20. **NOTE:** The innermost square with blue represents the seven significantly enriched pathways; The middle layer represents enriched host genes: red nodes were simultaneously enriched in the significant pathways of the two comparison groups, pink nodes were only significantly enriched pathways of F14vsF20 group and green nodes were significantly enriched in S14vsS20; The outermost gray circle represents circRNA.

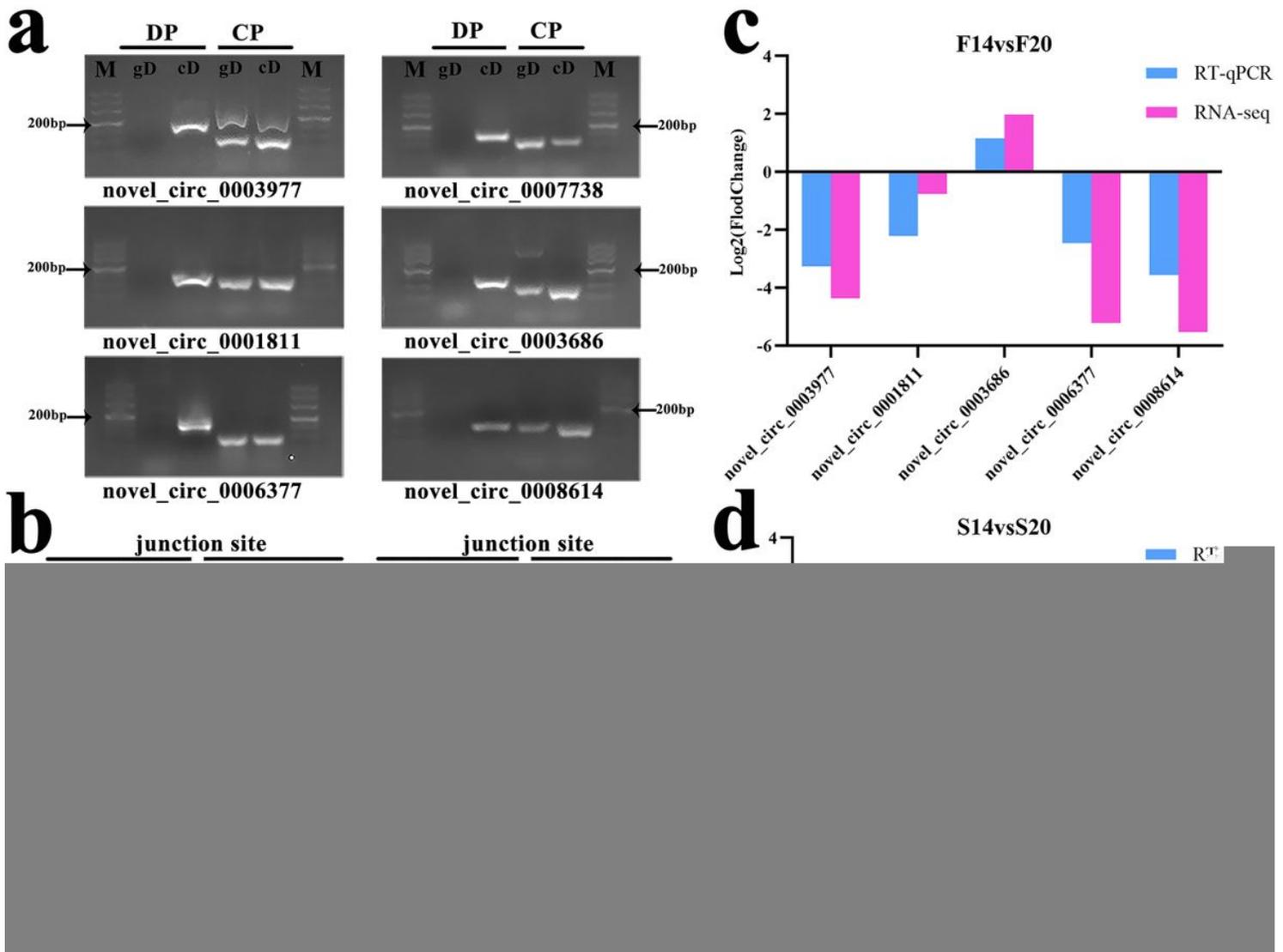


Figure 5

Validation of differentially expressed circRNAs. **(a)** agarose gel electrophoresis test for PCR products of divergent primers (DP) and convergent primers (CP) using cDNA (cD) and gDNA (gD). **(b)** Sanger sequencing confirmed the back-splicing junction of circRNAs; **(c)** RT-qPCR validation of five differentially expressed circRNAs in F14vsF20 and S14vsS20 respectively. **NOTE:** Original gels are presented in Supplementary Figure S1; M: maker; DP: divergent primers; CP: convergent primers.

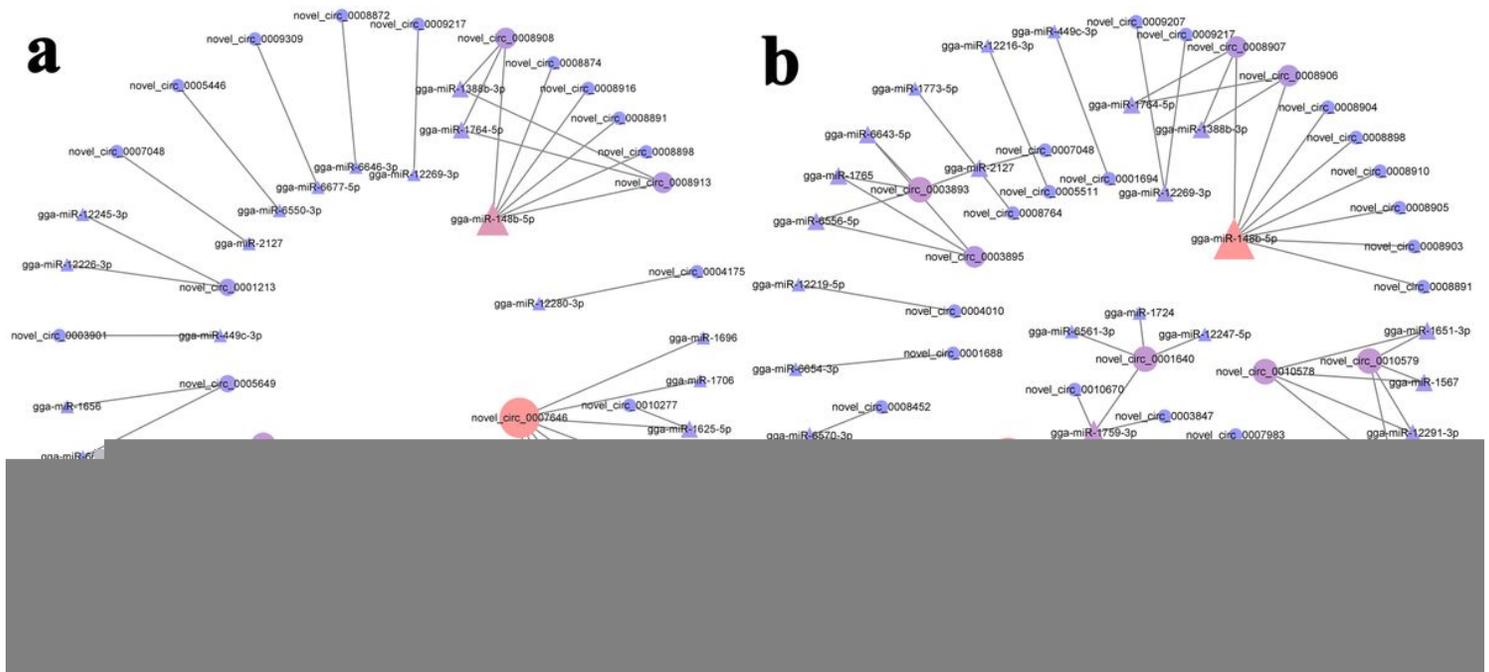


Figure 6

circRNA-miRNA interaction network. **(a)** circRNA-miRNA pairs of F14vsF20. **(b)** circRNA-miRNA pairs of S14vsS20. **NOTE:** Circles represent circRNAs and triangles represent miRNAs.

Supplementary Files

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