Circular RNAs are abundant and dynamically expressed during embryonic muscle development in chickens

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Abstract

The growth and development of skeletal muscle is regulated by proteins as well as non-coding RNAs. Circular RNAs (circRNAs) are universally expressed in various tissues and cell types, and regulate gene expression in eukaryotes. To identify the circRNAs during chicken embryonic skeletal muscle development, leg muscles of female Xinghua (XH) chicken at three developmental time points 11 embryo age (E11), 16 embryo age (E16) and 1 day post hatch (P1) were performed RNA sequencing. We identified 13,377 circRNAs with 3,036 abundantly expressed and most were derived from coding exons. A total of 462 differentially expressed circRNAs were identified (fold change >2; q-value < 0.05). Parental genes of differentially expressed circRNAs were related to muscle biological processes. There were 946 exonic circRNAs have been found that harbored one or more miRNA-binding site for 150 known miRNAs. We validated that circRBFOX2s promoted cell proliferation through interacted with miR-206. These data collectively indicate that circRNAs are abundant and dynamically expressed during embryonic muscle development and could play key roles through sequestering miRNAs as well as other functions.

Key words: circular RNA, chicken, skeletal muscle, embryonic development, cell proliferation

1. Introduction

Non-coding RNA such as microRNAs (miRNA) and long non-coding RNAs exist in many cells and regulate gene expression and possibly perform other biological functions. Circular RNAs (circRNAs) are a novel non-coding RNA that forms a covalently closed continuous loop and have been observed for decades in plant viroids and in a few mammalian genes. CircRNAs were generally considered to be splicing artifacts or by-products until recent advances in RNA-sequencing (RNA-seq) technology. CircRNAs are served as a new class of RNAs that have been identified widespread in eukaryotic tissues and cells from humans, mice, nematodes, fruit flies and plants.

CircRNAs can arise from exons, introns, untranslated regions, non-coding RNA loci as well as intergenic and antisense transcripts.
They are abundantly and widely expressed in eukaryotes, and often show tissue and developmental stage-specific expression patterns. In some genes, circular RNA isoforms are even more abundant than their linear counterparts. CircRNAs are also expressed across eukaryotes, and in humans, hundreds of exonic circRNAs are found to be with circular orthologues in murine. The biological functions of circRNA are still largely unknown but they are implicated in the regulation of gene expression at multiple levels. Some exonic circRNAs (ecircRNA) can function as miRNA sponges, which compete with miRNAs for miRNA binding and thus up-regulate the expression of miRNAs target gene. One representative example is the cerebellar degeneration-related 1 antisense transcript (CDR1as) that contains over 60 conserved miR-7 target sites. A CDR1as knockdown leads to reduced expression of miRNAs containing miR-7-binding sites. Overexpression of CDR1as has an effect similar to a miR-7 knockdown and this impaired midbrain development in zebrafish. Circular RNAs can regulate transcription via RNA Pol II interactions and have been demonstrated for the ciRNA (intronic circular RNA) ci-ankrd52 and ci-sirt7. EclircRNAs (exon-intron circular RNA) regulate their parental genes through interaction with the spliceosome component U1 snRNP (small nuclear ribonucleoprotein). CircRNA biogenesis may also compete with pre-mRNA splicing and thus regulate linear mRNA levels. In addition, some circRNAs have been found to serve as templates for coding peptides or proteins, especially in that modified by N6-methyladenosine or with the presence of internal ribosomal entry sites (IRES).

Skeletal muscle is the most important component of food animals and directly correlates with meat quantity and quality. The growth and development of skeletal muscle involves a series of very complex biological processes, regulated by many signaling pathways, genes, transcription factors and ncRNAs. CircRNAs can play important roles in biologic processes as well as human disease. CircRNAs also have been identified abundantly expressed in monkey skeletal muscle and may play key roles in growth and development. In this study, we identified circRNAs during chicken embryonic skeletal muscle development by RNA sequencing to explore the functions of circRNAs in chicken skeletal muscle.

2. Materials and methods

2.1. Ethics standards

Animal experiments were handled in compliance and all efforts were made to minimize suffering. It was approved by the Animal Care Committee of South China Agricultural University (Guangzhou, China) with approval number SCAU#0014.

2.2. Samples for circular RNA sequencing

A total of 240 XH chickens of E10 embryonic age were obtained from the Chicken Breeding Farm of South China Agricultural University (Guangzhou, China), and incubated in Automatic Incubator (Oscilla, Shandong, China) at 37.8 °C, with 60 ± 10% humidity. During E10 to 1 day post hatch (P1), leg muscles of 20 XH chickens were collected daily. The sex of chicken embryo was identified by PCR of the CHD1 gene. Leg muscles of female XH chickens at E11, E16 and P1 were used for circular RNA sequencing.

2.3. Circular RNA library construction and Illumina sequencing

Total RNAs of six female chickens (E11, E16, and P1; each stage two individuals), were isolated by using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and then treated with DNase (Promega, Madison, WI, USA) following the manufacturer’s instructions. The quantity and quality of RNAs were evaluated by NanoDrop2000 (Thermo, Waltham, MA, USA) and gel electrophoresis. Total RNA samples (5 μg) were treated with the Ribo-Zero-magnetic-kit (Epicenter, Madison, Wisconsin, USA) to remove RNA, and then digested with 20 U of RNase R (RNR07250, Epicenter). RNA-Seq libraries were prepared using the Tru Seq RNA LT Sample Prep Kit v2 (Illumina, San Diego, CA, USA) following the manufacturer’s instructions. The libraries were sequenced using an Illumina HiSeq 3000 instrument with a Paired-End module (at a depth of 50 million reads) at Genery Biotechnology Co., Ltd. (Shanghai, China).

2.4. Annotation of chicken circRNAs

All sequencing data are available in the Gene Expression Omnibus (GEO) with accession number (GSE89355). For all raw sequencing data of each sample, adapter reads and low-quality reads were removed using Trim Galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/ (16 September 2017, date last accessed)). The filtered data were mapped to the chicken genome (Gallus_gallus-4.0/jgiGal4) using TopHat software. The mapping reads was performed transcript assembly using Cufflinks software. Identification of circRNAs was performed using CIRI.

2.5. Differential expression analysis

Expression levels of circRNAs were quantified using the number of reads spanning back-spliced junctions (circular reads). The relative expression of circRNAs was denoted as BSRP (back-spliced reads per million mapped reads), using circular reads normalized to per million mapped reads. Differentially expressed circRNAs among three groups (E11, E16 and P1) were identified using the DESEQ2 software package (http://www.bioconductor.org/packages/release/bioc/html/DESeq.html (16 September 2017, date last accessed)) with a t test q-value < 0.05 and fold change > 2. The top 200 expressed circRNAs were log2 transformed, gene mean centered and visualized as heatmaps using the Multi Experiment Viewer (http://www.tm4.org/ (16 September 2017, date last accessed)).

2.6. Target miRNA prediction, pathway and network analysis

All exonic circRNAs were used to predict miRNAs potential binding sites using miRanda (http://www.microrna.org/microrna/home.do (16 September 2017, date last accessed)) with threshold parameters as follows: single-residue-pair match scores > 140, AG < −10 kcal/mol and demand strict 5’ seed pairing. All parental genes of differentially expressed circRNAs were subjected to Gene Ontology (GO) (http://www.geneontology.org/ (16 September 2017, date last accessed)) and KEGG (http://www.genome.jp/kegg/ (16 September 2017, date last accessed)) pathway enrichment analysis using DAVID 6.7 Functional Annotation Tool (http://david.abcc.ncifcrf.gov/ (16 September 2017, date last accessed)). All parental genes of circRNAs were set as the background gene list. Gene network analysis was performed by Ingenuity Pathways analysis (IPA; Ingenuity Systems, http://www.ingenuity.com (16 September 2017, date last accessed)).

2.7. Validation of circRNAs by Sanger sequencing

The circRNAs were validated using PCR with divergent and convergent primers as previously described. Divergent primers were designed in regions about 100 bp from a junction, and convergent
primers were designed in regions of one exon (Details of primers are summarized in Supplementary Table S1). To confirm the junction sequence of circRNAs, PCR products of divergent primers were gel purified and submitted for Sanger sequencing at Sangon Biotech Co. Ltd (Shanghai, China).

2.8. cDNA synthesis and quantitative real-time PCR (qRT-PCR)
Total RNA was used for reverse transcription using RevertAid First Strand cDNA Synthesis Kit (Fermentas, Waltham, MA, USA) with either random hexamers or specific primers for miRNAs as indicated. The relative expression levels of circRNAs or miRNAs were determined by qRT-PCR using SsoFast Eva Green Supermix (Bio-Rad, Hercules, CA, USA) in a final volume of 20 μl. To check the sensitivity of circRNA to RNaseR, qRT-PCR was also performed using RNA samples with and without RNaseR treatment. Primers used for circRNAs were designed as divergent primers to detect backsplicing junctions, and bulge-loop primers were synthesized by Ribobio (Guangzhou, China) for miRNAs (Details of primers are summarized in Supplementary Table S1). The β-actin gene was used as reference genes for circRNAs, and U6 snRNA was used as reference gene for miRNAs. The qRT-PCR program was performed in a BIO-RAD CFX96 system as follows: 95°C for 3 min; 40 cycles of 95°C for 10 s, annealing temperature (58–62°C) for 30 s, and 72°C for 30 s; and 72°C for 1 min. The relative expression level of miRNA was calculated using the comparative 2^−ΔΔCt (ΔΔCt = Ct(target gene) − Ct(reference gene)), in which ΔΔCt = ΔCt (target sample) − ΔCt (control sample). All reactions were run in triplicate and presented as means ± S.E.M. The Student’s t-test was used to compare expression levels among different groups.

2.9. Vector construction and RNA oligonucleotides
The perfect match sequence of gga-miR-1a-3p or gga-miR-206 was synthesized and cloned into the psiCHECK-2 vector (Promega) using the NotI and XhoI restriction sites. The circRNA overexpression vectors were constructed using the linear sequences of circRBFOX2.2-3 and circRBFOX2.2-4 amplified from chicken leg muscle cDNA using PCR. They were then cloned into pCD-ciR2.1 vector (Supplementary Fig. S1, Genesee Biotech, Guangzhou, China) according to the manufacturer’s protocol using the KpnI and BamHI restriction sites. The wild-type and mutated 3'UTRs sequences of CCND1, CCND2, PAX7 and HDAC4 were synthesized and cloned into the pmirGLO dual-luciferase reporter vector (Promega) using the Nhel and XhoI restriction sites. The exon2-3 or exon2-4 of RBFOX2 were synthesized and cloned into the pcDNA3.1+ vector (Invitrogen) using the Nhel and SalI restriction sites. Small interfering RNA (siRNA) of circRNAs (sequences are summarized in Supplementary Table S1), miRNA mimics and inhibitors were synthesized by Ribobio.

2.10. Cell culture and transfection
Chicken primary myoblasts were isolated from the leg muscles of E11 chickens. Leg muscle (1 g) was minced into sections of approximately 1 mm with scissors and digested with 0.25% trypsin (Gibco, Grand Island, NY, USA) at 37°C in a shaking water bath (90 oscillations/min). Digestions were terminated by adding foetal bovine serum (Gibco) after 30 min. The mixture was filtered through a nylon mesh with 70 μm pores (BD Falcon). The filtered cells were centrifuged at 350g for 5 min, and maintained in Dulbecco’s modified Eagle medium (DMEM) (Gibco), supplemented with 20% foetal bovine serum, and 0.2% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO2 humidified atmosphere. Serial plating was performed to enrich myoblasts and remove fibroblasts. DF-1 cells were cultured in DMEM with 10% foetal bovine serum and 0.2% penicillin/streptomycin. QM-7 cells were cultured in high-glucose M199 medium (Gibco) with 10% foetal bovine serum, 10% tryptose phosphate broth solution (Sigma, Louis, MO, USA) and 0.2% penicillin/streptomycin. Cells were transfected with 50 nM of miRNA mimics, 100 nM of miRNA inhibitors, 100 nM of siRNA or plasmid (1 μg/ml) using Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer’s instructions.

2.11. Luciferase reporter assay and Northern blot analysis
DF-1 cells were seeded in 96-well plates and co-transfected with the dual-luciferase reporter (pSCEHECK-2) and miRNA mimics or NC (negative control) and with circRNA overexpression vector, circRNA mutated vector or empty vector (EV) using Lipofectamine 3000 reagent. After transfection for 48 h, the luminescent signals of firefly and Renilla luciferase were detected using Dual-GLO Luciferase Assay System Kit (Promega) with a Fluorescence/Multi-Detection Microplate Reader (Biotek, Winooski, VT, USA). Northern blotting for miR-206 and U6 were performed with miRNA Northern Blot Assay Kit (Signosis, Santa Clara, CA, USA) following the manufacturer’s instructions.

2.12. Flow cytometry analysis of the cell cycle
After transfection 48 h, myoblasts were collected and fixed in 70% ethanol overnight at −20°C. The cells were incubated with 50 μg/ml propidium iodide (Sigma), 10 μg/ml RNaseA (Takara) and 0.2% (v/v) Triton X-100 (Sigma) for 30 min at 4°C. Analyses were performed using a BD AccuriC6 flow cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo (v7.6) software (Treestar Incorporated, Ashland, OR, USA).

2.13. EdU assay
QM-7 cells were seeded in 12-well plates, and at 80% confluence, cell were transfected with overexpression vector or siRNA of circRNAs, miRNA mimic, miRNA inhibitor or negative control. After transfection for 36 h, cell proliferation was tested using a Cell-Light EdU Apollo 567 in Vitro Flow Cytometry Kit (Ribobio). The cells were first incubated with 50 μM EdU for 2 h at 37°C, and then fixed in 4% paraformaldehyde for 30 min and permeabilized with 0.5% Triton X-100. The proliferating cells were stained with Apollo Dye Solution and Hoechst 33342 (used as control). The EdU-stained cells were visualized by fluorescence microscopy (Nikon, Tokyo, Japan). The cell proliferation rate was calculated using images of randomly selected fields obtained from the fluorescence microscope. We performed four repeats for each group, and three images were used to calculate the cell proliferation rate in each repeat.

2.14. Histology
The leg muscle samples were fixed in 4% paraformaldehyde for a minimum of 24 h and then embedded in paraffin and 10-mm thick serial sections were made. The sections were then subjected to H-E staining following standard protocols. Images were taken using a Moticam 2306 CCD imaging system (Motic Instruments, CA, USA).
2.15. RNA immunoprecipitation and biotin-coupled miRNA pull down

RIP experiments were performed by using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA) following the manufacturer’s instructions. Immunoprecipitation of AGO2 was performed in QM7 cells co-transfected with circRBFOX2s expression vector and miR-1a-3p or miR-206 and miR-203 control. The mRNA levels of circRBFOX2s were quantified by qRT–PCR and were normalized to GAPDH gene. The relative immunoprecipitate/input ratios are plotted.

The 3' end biotinylated miR-1a-3p, miR-206 or miR-203 mimic (Ribobio) were transfected into QM7 cells along with circRBFOX2s expression vector. The biotin-coupled RNA complex was pull-down by Dynabeads MyOne Streptavidin C1 kit (Invitrogen) following the manufacturer’s instructions. RNA bound to the beads (pull-down RNA) was isolated using Trizol LS reagent (Invitrogen). The mRNA levels of circRBFOX2s in the streptavidin captured fractions were quantified by qRT–PCR and the enrichment ratios of the miR-1a-3p or miR-206 to the miR-203 control were plotted.

3. Results and discussion

3.1. Overview of circular RNA deep sequencing data

In recent years, a number of reports demonstrated that circRNAs were widespread and abundant existed in various eukaryotes, especially in mammals. However, little information is known regarding circRNAs in domestic animals with the exception of brain cortex of pigs and mammary gland tissues of cattle. Here, we performed circRNAs in domestic animals with the exception of brain cortex of pigs and mammary gland tissues of cattle. 3.1. Overview of circular RNA deep sequencing data

3.2. Identification of differentially expressed circRNAs

To address the potential functions of circRNA, differentially-expressed circRNAs (DEcircRNAs) were identified by DESeq analysis (fold change >2, q-value <0.05) and clustered based on their expression profiles (Fig. 2A and Supplementary Fig. S4). A total of 462 DEcircRNAs were detected in the three developmental groups, 236, 285 and 89 circRNAs in E11_VS_E16, E11_VS_P1, and E16_VS_P1 comparison groups, respectively. These circRNAs were more abundant in E16 and P1 than in E11, whereas a small fraction of group E11 was quantitatively higher (Fig. 2B, Supplementary Tables S4–S6). We found that 223, 526 and 255 circRNAs were specifically expressed in E11, E16 and P1, respectively (Fig. 2C). A larger number of circRNAs are differentially expressed in chicken leg muscle development. We performed RNA sequencing using two individuals in each stage but not mix RNA pool. The variation of circRNAs abundance was also existed between the two duplicates used for sequencing. The numbers of circRNAs were larger in E16 or P1 than that in E11 of leg muscle, consistent with that circRNAs were found to accumulate during aging in Drosophila heads. 3.2. Identification of differentially expressed circRNAs

3.3. Putative functions of chicken circRNAs as miRNA sponges

The biogenesis of circRNA can compete with pre-mRNA splicing, and intron or exon-intron circRNAs can regulate the transcription of their parental gene. In this study, we performed GO Enrichment Analysis for the parental genes of differentially-expressed circRNAs (fold change >2, P-value <0.05) in E11_VS_E16, E11_VS_P1 and E16_VS_P1 comparison groups (Supplementary Tables S7–S9). The parental gene functions were involved in muscle-related biological processes, including muscle structure development, muscle tissue development, muscle system processes and muscle cell differentiation (Fig. 2D–F). Using this data, we organized a functional network using Ingenuity pathway analysis (Supplementary Table S10). The most prevalent diseases and function interaction network was related to skeletal and muscular disorders and this group involved 29 parental genes (Supplementary Fig. S5).
Figure 1. Annotation of chicken embryonic muscle circRNA. (A) Distribution of sequencing reads on chicken chromosome. (B) Venn diagrams of circRNAs in embryonic muscle with three different development stages. E11, 11 embryo age; E16, 16 embryo age; P1, 1 day post hatch. (C) Genomic origin of chicken circRNA. (D) Distribution of circRNAs among genes; 11,064 circRNAs in 3,589 genes. (E, F) The number of circRNAs expressed at various cutoff expression levels. (G) Venn diagrams of highly expressed circRNAs ($n = 3,036$, BSRP > 1). (H) Numbers and percentages of different types of highly expressed circRNAs. The Y axis (left) represents the numbers of highly expressed circRNAs, and the Y axis (right) represents the percentage is the number of abundant circRNAs of type divided by the total number of circRNAs specific type. ecRNA, exonic circRNA; EicRNA, exon-intron circRNA; ciRNA, intronic circRNA.
Table 1. The top 10 circRNAs expressed in chicken embryonic muscle

| circRNA_ID | circRNA_type | Parental gene | Junction | Linear distance of junction (nt) | Length (nt) | BSRP |
|------------|--------------|---------------|----------|---------------------------------|-------------|------|
| 1:106020993| eciRNA       | MORC3         | Exon 5-7 | 3,464                           | 428         | 328.06|
| 5:24409706 | eciRNA       | MAPKBP1       | Exon 25-27 | 1,751                           | 396         | 279.82|
| 2:14642365 | EicRNA       | SVIL          | Exon 6-14 | 6,296                           | 6296        | 271.62|
| 2:14634516 | eciRNA       | SVIL          | Exon 6-10 | 3,412                           | 504         | 179.63|
| 7:14755660 | eciRNA       | MYPN          | Exon 100-104 | 2,179                          | 501         | 154.00|
| 1:51782860 | eciRNA       | RBFOX2        | Exon 2   | 2,25                            | 225         | 134.95|
| 4:1285476 | eciRNA       | FND3C3A       | Exon 5-9 | 4,123                           | 695         | 126.41|
| 26:5104947 | eciRNA       | TAF8          | Exon 2-5 | 2,185                           | 444         | 120.12|
| 1:51819796 | eciRNA       | RBFOX2        | Exon 9-11 | 13,323                          | 262         | 106.49|

The expressed abundances of circRNAs were normalized as number of back-spliced reads per million mapped reads (BSRP). This column represented the sum of the BSRP values for all samples. eciRNA, exonic circRNA; EicRNA, exon-intron circRNA.

Figure 2. Differentially expressed circRNAs in three different development stages of embryonic muscle. (A) Heatmap of top 100 differentially expressed circRNAs in E11, E16 and P1 of leg muscle. (B) Venn diagrams of differentially expressed circRNAs (n = 462; q value < 0.05, fold change > 2). (C) The circRNA specific expression in three different development stages of embryonic muscle. The top 10 GO enrichment term for the parental genes of differentially expressed circRNAs in E11_VS_E16 (D), E11_VS_P1 (E) and E16_VS_P1 (F). The Y-axis represents GO terms and the X-axis represents –Log p-value.
Divergent primers and convergent primers were designed to amplify six candidate circRNAs in cDNA and genomic DNA samples. Divergent PCR products of divergent primers were further detected by Sanger sequencing to confirm the back-splicing junctions (Fig. 5B and Supplementary Fig. S6). To detect the resistance of circRNA to the digestion by RNaseR, we quantified the six candidate circRNAs with RNaseR treatment compared to control by qRT-PCR. All tested circRNAs showed much more resistant than the linear mRNA control (Fig. 5C). In addition, the expression of six differentially expressed circRNAs was also validated by qRT-PCR in E11_VS_E16, E11_VS_P1, and E16_VS_P1 comparison groups. Except the expression of circRBFOX2s and circHIPK3, the deep sequencing results were reliable (Fig. 5D).

### 3.5. CircRBFOX2 interacts with mir-206 and mir-1a

Two circular isoforms of RBFOX2 which we designated as circRBFOX2.2-3 and circRBFOX2.2-4 were derived from exon2-3 and exon 2-4 of RBFOX2, respectively. Both these molecules possessed miR-1a-3p and miR-206 binding sites (Fig. 6A and B). The prediction of a miRNA target site does not necessarily mean that a miRNA binds to that site. To further validate that these two circRBFOX2s can be interacted with miR-1a-3p and miR-206, we constructed two dual-luciferase reporters by inserting perfect miR-1a-3p or miR-206 target sites into the 3' end of Renilla luciferase. The knockdown potential of the miRNA was assessed by the presence or absence of circRBFOX2s. After co-transferring the dual-luciferase reporter with their corresponding miRNAs, the relative luminescence was significantly decreased compared with miR-NC. However, the relative luminescence returned when co-transfected with either circRBFOX2.2-3 or circRBFOX2.2-4 expression vectors, but not the mutated vectors of circRBFOX2s (Fig. 6C and D). These results demonstrated that the knockdown potential of miR-1a-3p or miR-206 was significantly diminished in the presence of circRBFOX2.2-3 and circRBFOX2.2-4. Immunoprecipitation of AGO2 from myoblasts co-transfected with miR-1a-3p, miR-206 or miR-203 and circRBFOX2 overexpression vector was performed and the circRNA levels were quantified by qRT–PCR and normalized to GAPDH.

### 3.4. Experimental validation of chicken circRNAs

The expression and back-splicing sites of several circRNAs were validated by divergent reverse-transcription PCR, RNaseR digestion and qRT-PCR, according to previously described methodologies.1,13 Divergent primers and convergent primers were designed to amplify six candidate circRNAs in cDNA and genomic DNA samples. Divergent primers from each circRNA produced a single distinct band of the expected product size only in cDNA samples suggesting the presence of back-splicing junctions but not genomic rearrangements (Fig. 5A). PCR products of divergent primers were further detected by Sanger sequencing to confirm the back-splicing junctions (Fig. 5B).

### Table 2. The top 10 differentially expressed eciRNAs with miRNA binding sites

| circRNA_ID | E11_base Mean | E16_base Mean | P1_base Mean | Parental gene | Junction | Length (nt) | Target miRNAs |
|------------|---------------|---------------|--------------|---------------|----------|------------|---------------|
| 2:14633486/14648660 | 313.35 | 5345.71 | 7148.93 | SVIL | Exon6-14 | 1594 | miR-31-5p (2), miR-103-3p (2), miR-135a-5p (2), miR-203a, miR-1720-3p, miR-1737 |
| 7:14755660/14757838 | 14.79 | 2547.25 | 5155.27 | TNN | Exon100-104 | 501 | miR-31-5p, miR-1a-2-5p, miR-22-5, miR-451 |
| 26:5104947/5107131 | 561.47 | 2995.87 | 2333.66 | TAF8 | Exon2-5 | 444 | miR-34a-5p |
| 1:51819796/51833118 | 626.95 | 2756.90 | 1891.00 | RBFOX2 | Exon9-11 | 262 | miR-18b-3p |
| 2:14643486/14644033 | 354.24 | 1777.35 | 1973.62 | SVIL | Exon11-14 | 1090 | miR-31-5p, miR-103-3p (2), miR-135a-5p (2), miR-1720-3p |
| 5:13397775/13397627 | 0.00 | 3666.12 | 0.00 | TNNT3 | Exon15-17 | 297 | miR-6631-5p |
| 1:84430731/84432412 | 1992.44 | 171.80 | 690.86 | ABIBP | Exon1-13-4 | 273 | miR-499-3p (2) |
| 6:30457170/30457149 | 2015.87 | 101.28 | 47.48 | FGFR2 | Exon3-6 | 636 | miR-15a, miR-200a-3p |
| 1:51782680/51793309 | 165.19 | 775.20 | 1025.64 | RBFOX2 | Exon2-3 | 372 | miR-1a-3p, miR-206 |
| 7:35565240/35577784 | 246.91 | 912.57 | 451.10 | GPD2 | Exon1-5 | 667 | miR-103-3p (2), miR-133a-3p, miR-133b, miR-133c-3p, miR-30a-5p |

Read counts of each sample were normalized by DESEQ software. Base Mean is the average normalized value of two samples at same time point. The numbers in brackets are target sites for each miRNA.
miR-203 control, circRBFOX2.2-4 was specifically captured by both 3-biotinylated miR-1a-3p and miR-206, and circRBFOX2.2-3 was also captured by 3-biotinylated miR-206. Together, these results suggested that circRBFOX2s are able to interact with miR-1a-3p and miR-206.

3.6. Effects of circRBFOX2 on expression of miRNAs and their target genes

We over expressed or knocked down circRBFOX2.2-3 and circRBFOX2.2-4 in chicken primary myoblast, and then measured miR-1a-3p and miR-206 expression levels by qRT-PCR. We constructed the pcDNA3.1 vector with linear exon2-3 or exon2-4 of RBFOX2 as control, which give rise to linear RBFOX2 products. We transfected these linear control vector in myoblast and then the miR-206/miR-1a-3p level were quantified by qRT-PCR. The results showed that linear RBFOX2 did not affect the expression of miR-206 and miR-1a-3p (Fig. 7A).

Compared to empty vector control, overexpressed circRBFOX2.2-3 or circRBFOX2.2-4 inhibited both miR-1a-3p and miR-206, while the mutation of circRBFOX2 vector did not have significant change (Fig. 7B). Furthermore, the expression level of miR-206/miR-1a-3p tend to be lower when the level of circRBFOX2s higher (Fig. 7C). We knock down circRBFOX2.2-3...
and circRBFOX2.2-4 using siRNAs, and the results showed that the siRNAs could knockdown the circRBFOX2s but not linear RBFOX2. Knockdown of either circRBFOX2.2-3 or circRBFOX2.2-4 increased both the expression of miR-1a-3p and miR-206 (Fig. 7D). Northern blot analysis for miR-206 was also confirmed that the miR-206 expression level was increased by knockdown of circRBFOX2s (Fig. 7H). The expression of miR-203 used as control did not affect by neither overexpressed nor knockdown of circRBFOX2s. Together, our results found that circRBFOX2s downregulated the expression of miR-1a-3p and miR-206. This finding was not consisted with the standard model of circRNA miRNA sponge. The circRNAs that act as miRNA sponge can sequesters the miRNA away from other targets, but does not necessarily alter the expression of the miRNA. Recently, there are some circRNAs, such as CircRan1, circ-ABCB10 and hsa-circ-0016347 were also reported to downregulated the expression of their target miRNA.55–57 However, the exact mechanism for this association is unknown, probably upregulation of circRNAs might degrade miRNA expression.

We further validated whether circRBFOX2.2-3 and circRBFOX2.2-4 regulated the target gene of miR-206. According to previous studies and predicted by Targetscan (http://www.targetscan.org/vert_71/ (16 September 2017, date last accessed)), we chose the CCND1, CCND2, PAX3 and HDAC4 as candidate target gene for miR-206, and then verified their relations by luciferase assay.58–60 The CCND2 and HDAC4 were confirmed as the target gene of miR-206 (Fig. 7A). Thus, we further determine their expression level after overexpression/knockdown of circRBFOX2s in myoblast. The results showed that both circRBFOX2.2-3 and circRBFOX2.2-4 upregulated the mRNA level of CCND2 but not HDAC4 (Fig. 7F and G).

### 3.7. Effects of circRBFOX2 and miR-206 on myoblast proliferation

To observe the functions of circRBFOX2.2-3 or circRBFOX2.2-4 on myoblast, we over expressed and knocked down the circRBFOX2.2-3 or circRBFOX2.2-4 by transfected with overexpression vector or siRNAs. Overexpression of either circRBFOX2.2-3 or circRBFOX2.2-
resulted in increased levels of circRBFOX2.2-3 or circRBFOX2.2-4 above that of the empty vector control (Fig. 8A and B). Similarly, when we transfected circRBFOX2.2-3 or circRBFOX2.2-4 siRNAs, the transcript levels of both these RNAs decreased (Fig. 8C and D). These results indicated that our expression system worked in myoblasts. We used flow cytometry for cell cycle analysis of transfected myoblasts. Overexpression of circRBFOX2.2-3 resulted in a greater number of S-phase cells than controls, and fewer G0/G1 cells (Fig. 8E and F, Supplementary Figs S7 and S8). Both the mutated vector of circRBFOX2.2-3 and circRBFOX2.2-4 did not affect the cell cycle of myoblast. Knockdown of circRBFOX2.2-3 resulted in a greater number of G0/G1 cells and fewer S and G2/M phase cells than controls. However, the circRBFOX2.2-4 knockdown and overexpression groups showed significant differences only in S-phase (Fig. 8E and F, Supplementary Figs S7 and S8). The 5'-Ethynyl-2'-deoxyuridine (EdU) incorporation assays were also performed after over expressed and knocked down the circRBFOX2.2-3 or circRBFOX2.2-4 in myoblast. The number of EdU-stained cells increased in both the circRBFOX2.2-3 and circRBFOX2.2-4 overexpression groups. In contrast, proliferation decreased when circRBFOX2.2-3 and circRBFOX2.2-4 were knocked down (Fig. 8G and H).

Muscle-specific miR-206 has been reported to inhibit cell proliferation in human and mouse. 58–59 The effects of miR-206 in myoblast proliferation were also examined using cell cycle and EdU incorporation analysis. We overexpressed and knocked down miR-206 by transfecting myoblasts with a miR-206 mimic or anti-miR-206 (Fig. 9A and B). Myoblasts transfected with the miR-206 mimic decreased the number of S-phase cells compared with the scrambled negative control group (Fig. 9C). Conversely, S-phase cells in the anti-miR-206 group were significantly increased (Fig. 9D). Consistent with the result

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**Figure 6.** CircRBFOX2s interacted with miR-206 and miR-1a-3p. (A) The predicted binding site of miR-1a-3p and miR-206 in the exon3 of RBFOX2. Mut indicates the mutation sequences of binding sites. (B) miR-1a-3p and miR-206 targeting site in circRBFOX2s analysed by RNAhybrid software. Luciferase reporter assays for miR-1a-3p (C) and miR-206 (D). Luminescence was measured 48h after transfected with the luciferase reporter and miRNA mimics or NC (negative control) with circRNAs overexpression vector, circRNA mutated vector or empty vector. The relative levels of Renilla luminescence normalized to firefly luminescence are plotted. Error bars represent S.D. (n = 6). OV1, overexpression vector of circRBFOX2.2-3; OV2, overexpression vector of circRBFOX2.2-4; Mut1, mutated vector of circRBFOX2.2-3; Mut2, mutated vector of circRBFOX2.2-4; EV, empty vector. (E) Immunoprecipitation of AGO2 from myoblasts co-transfected with miR-1a-3p, miR-206 or miR-203 and circRBFOX2 overexpression vector. The circRNA levels were quantified by qRT–PCR and normalized to the GAPDH, the fold change of immunoprecipitate/input are plotted. Error bars represent S.D. (n = 3). (F) qRT–PCR analysis of circRBFOX2s level in the streptavidin captured fractions from the myoblast lysates after transfection with 3-end biotinylated miR-1a-3p, miR-206 or miR-203 control. Error bars represent S.D. (n = 3). Student’s t test (two-tailed) was performed for data analysis. *P < 0.05; **P < 0.01.
Figure 7. Effects of circRBFOX2 on expression of miRNAs and their target gene. (A) Linear RBFOX2 did not affect the expression of miR-206 and miR-1a-3p. The miR-206 and miR-1a-3p level were quantified by qRT-PCR after transfected with linear RBFOX2 overexpression vector or pcDNA3.1 empty vector. L1 represents linear exon2-3 of RBFOX2 overexpression vector; L2 represents linear exon2-4 of RBFOX2 overexpression vector; NC control represents pcDNA3.1 empty vector. (B) Overexpression of circRBFOX2.2-3 or circRBFOX2.2-4 inhibits miR-1a-3p and miR-206 expression. The expression level of miR-1a-3p, miR-206 and miR-203 control was detected after transfected with circRNAs overexpression vector, circRNA mutated vector or empty vector. Bars represent S.D. (n = 4). (C) The effects different fold change of circRBFOX2.2-3 or circRBFOX2.2-4 on miR-1a-3p and miR-206 expression. (D) Knockdown of circRBFOX2.2-3 or circRBFOX2.2-4 increases miR-1a-3p and miR-206 abundance. The expression level of miR-1a-3p, miR-206 and miR-203 control was detected after transfected with siRNA of circRBFOX2 or si-NC. Bars represent s.d. (n = 4). (E) Luciferase assay for miR-206 and their target genes. The relative luminescence was measured 48h after co-transfected with miR-206 mimic and luciferase reporters of no-insert control, wide type target sequence of target genes (WT) or mutated target sequence of target genes (MUT) in DF-1 cell. Error bars represent s.d. (n = 4). (F) The relative levels of CCND2 and HDAC4 were quantified by qRT–PCR after overexpressed (F) or knockdown (G) of circRBFOX2.2-3 or circRBFOX2.2-4 in chicken myoblast. Error bars represent s.d. (n = 4). (H) Knockdown of circRBFOX2.2-3 or circRBFOX2.2-4 increases the expression of miR-206. The expression level of miR-206 was detected after transfected with siRNA of circRBFOX2 or si-NC by Northern blotting. OV1, overexpression vector of circRBFOX2.2-3; OV2, overexpression vector of circRBFOX2.2-4; Mut1, mutated vector of circRBFOX2.2-3; Mut2, mutated vector of circRBFOX2.2-4; EV, empty vector; Si-1, siRNA of circRBFOX2.2-3; Si-2, siRNA of circRBFOX2.2-4; Si-NC, negative control. Student's t-test (two-tailed) was performed for data analysis. *P < 0.05; **P < 0.01.
of cell cycle analysis, EdU incorporation indicated that myoblast proliferation was suppressed by miR-206 overexpression and increased by miR-206 knockdown (Fig. 9A and F). Interestingly, the effects of miR-206 on myoblast proliferation was impaired when either circRBFOX2.2-3 or circRBFOX2.2-4 vectors were co-transfected (Fig. 9E and F). Together, these data implied that miR-206 could suppress myoblast proliferation, whereas circRBFOX2.2-3 and circRBFOX2.2-4 can promote myoblast proliferation, and antagonize the functions of miR-206 in myoblasts. Our results support previous findings that circRNAs sequester miRNAs to suppress their functions in chickens. Nevertheless, only a few circRNAs that function in this manner have been described. Some reports argue that most circRNAs...
do not function as miRNA sponges in human and mouse. Whether this mechanism is a common theme in circRNA biology awaits future research.

In summary, we performed genome-wide identification of chicken circRNAs by RNA sequencing, and found they are abundant and differentially expressed during chicken embryonic development. Most of the exonic circRNAs harbored miRNA binding sites and could play key roles in embryonic muscle development through sequestering miRNAs as well as other functions.

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Accession numbers
RNA-seq data are available in the Gene Expression Omnibus (GEO) with accession number (GSE89355).

Supplementary data
Supplementary data are available at DNARES online.

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Conflict of interest
None declared.

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