MiR-29b-1-5p regulates the proliferation and differentiation of chicken primary myoblasts and analysis of its effective targets

Yuanfang Li,* Bin Zhai ,* Pengtao Yuan,* Shengxin Fan,* Wenjiao Jin,* Wenting Li,*† Guirong Sun,*† Yadong Tian,*† Xiaojun Liu,*† Xiangtao Kang,*†,1 and Guoxi Li,*†,1,2

*College of Animal Science and Technology, Henan Agricultural University, Zhengzhou 450046, China; and †Henan Key Laboratory for Innovation and Utilization of Chicken Germplasm Resources, Zhengzhou 450046, China

ABSTRACT Several recent studies investigated the role of the miR-29 family in muscle development. However, only a few studies focused on chicken skeletal muscle. In the present study, cell cycle, 5-ethynyl-2'-deoxyuridine (EdU), cell counting kit-8 (CCK-8), and other assays indicated that miR-29b-1-5p can inhibit the proliferation of chicken primary myoblasts (CPMs); the western blot assay and immunofluorescence detection of MYHC demonstrated that miR-29b-1-5p can promote the differentiation of myoblasts. The functional enrichment analysis revealed that the target genes of miR-29b-1-5p may be involved in muscle tissue development, muscle organ development, and striated muscle tissue development, which are biological processes related to muscle development. The correlation analysis showed that these 6 genes, that is, ankyrin repeat domain 9 (ANKRD9), lactate dehydrogenase A (LDHA), transcription factor 12 (TCF12), FAT atypical cadherin 1 (FAT1), lin-9 homolog (LIN9), and integrin beta 3 binding protein (ITGB3BP), can be used as effective candidate target genes of miR-29b-1-5p. Moreover, miR-29b-1-5p inhibits the expression of ANKRD9 by directly binding the 3'UTR of ANKRD9. Overall, these data indicate that miR-29b-1-5p inhibits the proliferation of primary chicken myoblasts, stimulates their differentiation, and is involved in the process of muscle development and that its effective target gene is ANKRD9. This study identified the molecular mechanism of miR-29b-1-5p in chicken muscle development.

Key words: miR-29b-1-5p, ANKRD9, chicken primary myoblast, muscle development

INTRODUCTION

In vertebrates, skeletal myogenesis is a highly coordinated process involving cell proliferation, differentiation, and fusion. Multistep myogenesis is regulated by a complex gene network (Yokoyama and Asahara, 2011; Imbriano and Molinari, 2018). For example, Sirt1 increases the proliferation of skeletal muscle precursor cells, which influence skeletal muscle growth (Rathbone et al., 2009). Myogenic differentiation is marked by the expression of the myogenic regulatory genes; Myf5 can be detected first and followed by MyoD, and myogenin. Subsequently, myoblasts expressing MYHC fuse into multinucleate myotubes and assemble to form the muscle fibers (Francis-West et al., 2003). In recent years, many miRNAs have been shown to target mRNAs and regulate gene expression, and miRNAs constitute an important part of the regulatory network of skeletal myogenesis (Williams et al., 2009; Kovanda et al., 2014).

Many miRNAs play important roles in muscle development and hypertrophy and early embryonic development in animals (Wang et al., 2013). MiRNAs are endogenous small RNA molecules involved in posttranscriptional gene regulation (Yekta et al., 2004). MiR-199a is induced in dystrophic muscles and influences myoblast proliferation and differentiation (Alexander et al., 2013). Four microRNAs specifically expressed in muscle tissue, i.e., miR-1, miR-133a, miR-133b, and miR-206, are expressed in human muscle cells and induced during differentiation, and their expression levels are increased depending on the developmental stage of the fetus (Koutsoulidou et al., 2011). The overexpression of these miRNAs in various differentiated tissues suggests that miRNAs are involved in the maintenance of the differentiated state of tissues.
Additional studies have shown that in duck myoblasts, miR-1 can target the histone deacetylase 4 (HDAC4) gene to promote myoblast differentiation, and miR-133 may influence the expression of serum response factor (SRF) and transforming growth factor β type I receptor (TGFBR1) to promote cell proliferation (Wu et al., 2019). Various miRNAs may play various important roles in the growth and development of skeletal muscle. Although many miRNA families have been identified, studies investigating their functions have not been very thorough.

The expression of the miR-29 family is increased in breast cancer (Gebeshuber et al., 2009) and acute myeloid leukemia (Han et al., 2010) but decreased in nonsmall cell lung cancer (Fabbri et al., 2007) and liver cancer (Xiong et al., 2010). Depending on the cell type, members of the miR-29 family may play various roles in cancer development, indicating that miR-29 has an important effect on cell proliferation. Recently, miR-29 was shown to influence muscle development (Qin et al., 2017). miR-29 can target Akt3 to reduce mouse myoblast proliferation and promote myotube formation (Wei et al., 2013). The electroporation of miR-29 into the muscles of young mice can inhibit proliferation and reproduce the aging-induced muscle response (Hu et al., 2017). miR-29 can target Akt3 to reduce mouse myoblast proliferation and promote myotube formation (Wei et al., 2013). The electroporation of miR-29 into the muscles of young mice can inhibit proliferation and reproduce the aging-induced muscle response (Hu et al., 2017). A single study investigating miR-29b-1-5p in chicken muscle development demonstrated that a novel circular RNA produced by the fibroblast growth factor receptor 2 (FGFR2) gene can promote the proliferation and differentiation of myoblasts by adsorbing miR-29b-1-5p (Chen et al., 2018). Currently, information regarding the role of miR-29b-1-5p in chicken muscle development is very limited.

Chicken has an important economic value. The local Gushi chicken, which is a meat- and egg-type breed in China, is famous for its delicious meat (Fu et al., 2018b). Our previous RNA-seq study demonstrated that ANKRD9 is differentially expressed in breast muscle tissue from wk 6 to wk 22 in Gushi chickens (Li et al., 2019b). ANKRD9 is an ANKRD protein involved in various cellular processes, including lipid metabolism (Wang et al., 2009) and cell proliferation (Lee et al., 2018a). A comparison of single nucleotide polymorphisms (SNPs) between gastric cancer patients and healthy controls indicated that ANKRD9 is related to cancer susceptibility (Lee et al., 2018b). Several studies have shown that the functions of the ANKRD family are regulated by miRNAs (John et al., 2019; Prabhakar et al., 2019). According to a target gene prediction, miR-29b-1-5p may target to the ANKRD9 gene. However, this mechanism has not been investigated in chicken muscle development. In the present study, the role of miR-29b-1-5p in the proliferation and differentiation of CPMs was investigated, and the relationship between miR-29b-1-5p and ANKRD9 in CPMs was assessed. Our study clarifies the molecular mechanism of miR-29b-1-5p in chicken muscle development.

### MATERIALS AND METHODS

#### Ethics Statement

Animal care in this study was performed in accordance with the Animal Experiment Management Regulations (Ministry of Science and Technology of China, 2004) approved by the Animal Care and Use Committee of Henan Agricultural University, China.

#### Sample Collection

In total, 60 Chinese native Gushi chicken 1-day-old embryos (E1) were obtained from the chicken farm of Henan Agricultural University (Zhengzhou, China) and incubated at 37.8°C at 60 ± 10% humidity. The breast muscles of 3 chicken embryos were collected at each stage of E10, E12, E14, E16, and E18.

#### Cell Culture

DF-1 cells (Himly et al., 1998) were cultured in DMEM culture medium (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (BI, Kibbutz Beit Haemek, Israel) and 0.1% penicillin/streptomycin (Solarbio, Beijing, China). The CPMs were isolated from the leg muscles of 11-day-old chicken embryos (O’Neill and Stockdale, 1972). After the removal of the skin and bones, the leg muscles were placed in a culture dish containing high glucose DMEM (HyClone, Logan, UT) supplemented with 10% FBS (BI, Kibbutz Beit Haemek, Israel) and 0.2% penicillin/streptomycin (Solarbio, Beijing, China). The muscles were cut into pieces and transferred to a 50 mL centrifuge tube; the suspension was vortexed for 1 min and filtered through a 70 μm sieve to obtain single cells. The appropriate culture medium was added, and the procedure was repeated approximately 5 times to obtain additional cells. The cells were collected by centrifugation at 1000 × g for 5 min at room temperature, and the supernatant was discarded. The cells were resuspended in complete high glucose DMEM and cultured in a cell culture flask. Finally, 3 successive differential attachments were performed to remove the fibroblasts, and the obtained CPMs were cultured in an incubator at 37°C at 5% CO₂ in a humidified environment. The cells were sampled daily to determine the degree of cell fusion (50% and 100%) and within the first 7 d of CPM differentiation (induce myoblast differentiation with 2% horse serum medium).

#### RNA Extraction, cDNA Synthesis and Quantitative Real-Time PCR (qRT-PCR)

The total RNA was extracted from the tissue and CPMs using TRIzol reagent (TaKaRa, Tokyo, Japan). The RNA concentration was quantified using a spectrophotometer (Thermo, Waltham, MA). cDNA was

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*Wienholds et al., 2005; Koutsoulidou et al., 2011.*
synthesized by reverse transcription using a PrimeScript RT reagent with gDNA eraser (TaKaRa, Japan). MiRNA was reverse transcribed using a ReverTra Ace qPCR RT kit (Toyobo, Tokyo, Japan). Then, qRT-PCR was performed using SYBR Green master mix (TaKaRa, Tokyo, Japan). The qRT-PCR reactions were performed using a LightCycler 96 qRT-PCR system (Roche, Basel, Switzerland). GAPDH was used as an internal reference gene for mRNA, and U6 was used as an internal reference for miRNA. All experiments were performed in triplicate. The relative quantification of genes was performed using the $2^{-\Delta\Delta C_t}$ method (Bubner and Baldwin, 2004). The B*LuLge-Loop miRNA qRT-PCR-specific primers, mimics, and inhibitors were designed by RiboBio (RiboBio, Guangzhou, China). The primer sequences are listed in Table S1.

**Plasmid Construction**

The 3′UTR fragment of ANKRD9 containing the binding sites was amplified by PCR from cDNA and then cloned into a psiCHECK-2 dual-luciferase reporter vector. To construct the mutant vectors, we designed a mutant primer sequence and changed the binding site (Table S1), 8 seed sequences were successfully mutated from AACCAGCA to TTTGTCGC to obtain the mutant primer sequence and changed the binding site vector. To construct the mutant vectors, we designed a binding site was amplified by PCR from cDNA and then cloned into a psiCHECK-2 dual-luciferase reporter vector. To construct the mutant vectors, we designed a mutant primer sequence and changed the binding site (Table S1), 8 seed sequences were successfully mutated from AACCAGCA to TTTGTCGC to obtain the mutant ANKRD9-3′UTR vector.

**Cell Transfection**

MiRNA was transfected with Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA), and siRNA was transfected with DharmaFECT transfection reagent (Dharmacon Inc., Lafayette, CO) as recommended by the manufacturer’s protocol.

**Luciferase Reporter Assays**

The miRNA target verification assay was performed using DF-1 cells. Wild-type or mutant ANKRD9-3′UTR dual-luciferase reporter vectors and miR-29b-1-5p mimics or NC duplexes were cotransfected into DF-1 cells in 24-well plates using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA). The activities of firefly and Renilla luciferase were measured 48 h after the transfection according to the instruction manual of the dual-luciferase reporter assay system (Promega, Madison, WI, USA).

**Cell Proliferation Assays**

Cell viability assays were performed using CCK-8 kit (Dojindo, Kumamoto, Japan). The cells were cultured in 96-well plates, and the cell viability was measured after the transfection. Two hours before the test, 10 μL of CCK-8 solution were added to each well according to the manufacturer’s instructions, and the plates were incubated in a 5% CO₂ incubator at 37°C. The cell viability was measured every 12 h using a fluorescence multimode microplate reader (BioTek, Winooski, VT.) at 450 nm. The cell proliferation analysis was performed by an EdU assay (Gauri, 1968). Cells seeded in 24-well plates were cultured, fixed 48 h after transfection, and incubated with a Cell-Light EdU Apollo 567 in vitro kit (RiboBio, Guangzhou, China) according to the manufacturer's instructions. A fluorescence microscope (Olympus, Tokyo, Japan) was used to capture the images. ImageJ software (NIH, Bethesda, MD) was used for the statistical measurement. All experiments were performed in triplicate.

**Cell Cycle Analysis**

The cell cycle analysis was performed by flow cytometry. Cells from the treatment and control groups were collected 48 h after the transfection, washed with PBS, and fixed in 70% ethanol at -20°C overnight. The DNA was incubated with propidium iodide (PI) (Solarbio, Beijing, China) staining solution at 4°C for 30 min. The cells were analyzed by a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA).

**Immunofluorescence**

The desmin immunofluorescence detection was performed using anti-desmin (Bioss, Beijing, China) and anti-rabbit IgG FITC-conjugated antibodies (Bioss, Beijing, China). The MYHC (DHSB, Iowa City, IA; B103) and anti-mouse IgG FITC-conjugated antibodies (Bioss, Beijing, China). The MyHC immunofluorescence detection was performed using anti-MYHC (DHSB, Iowa City, IA; B103) and anti-mouse IgG FITC-conjugated antibodies (Bioss, Beijing, China). DAPI (Bioss, Beijing, China) was used for the nuclear staining. The images were acquired under a fluorescence microscope (Olympus, Tokyo, Japan).

**Western Blot Assay**

The samples were incubated with anti-MYHC (DHSB, Iowa City, IA; B103) and anti-β-actin (Proteintech, Wuhan, China) antibodies at 4°C overnight, and the secondary antibody was conjugated to HRP (Proteintech, Wuhan, China) for 1 h at room temperature. The images were captured and analyzed by Odyssey FC (LI-COR, Lincoln, NE) and Image Studio V5.2 (LI-COR, Lincoln, NE). The expression of β-actin was used to normalize protein expression.

**miRNA Target Gene Analysis and Correlation Coefficient Analysis**

The miRNA target genes were analyzed through the miRDB database (http://www.mirdb.org) and microT-CDS database (http://www.microrna.gr/microT-CDS). The same breast muscle samples from Gushi chickens were used to construct miRNA and transcriptome profiles at 6 and 22 wk (Li et al., 2019b). Then, the miRNA-
mRNA interaction was calculated based on the miRNA and transcriptome expression profile data, a Pearson correlation analysis was used to determine the negatively related miRNA-mRNA pairs, and the network was constructed by Cytoscape (v3.6.1).

**Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Analyses**

The mRNAs and enriched genes among the miRNA-targeted mRNAs were annotated and classified by DAVID (https://david.ncifcrf.gov) and KOBAS (v3.0) (http://kobas.cbi.pku.edu.cn/kobas3), and Hiplot (https://hiplot.com.cn) was used to visualize the data. Only GO terms with P-values < 0.05 and KEGG pathways with corrected P-values < 0.05 were considered to indicate significant enrichment.

**Statistical Analysis**

All experiments were performed in triplicate, and the data are expressed as the mean ± S.E.M. A t-test was used to evaluate whether the values of 2 groups displayed statistically significant differences (*P < 0.05; **P < 0.01; ***P < 0.001). GraphPad Prism 7.0 software (GraphPad Software, Inc., San Diego, CA) was used for the statistical analysis of all data.

**RESULTS**

**Identification of Chicken Primary Myoblasts**

To determine the purity of the isolated primary chicken myoblasts, immunofluorescence detection of the cell-specific expression of desmin was performed; the results indicated that all isolated cells were myoblasts (Figure 1A). CPMs were collected at 50% and 100% confluence during the proliferation period, and CPMs from d 1 to d 7 of the differentiation period were used to investigate the effect of miR-29b-1-5p on CPM proliferation and differentiation (Figure 1B).

**miR-29b-1-5p Inhibits CPM Proliferation**

To determine the role of miR-29b-1-5p in the proliferation of primary chicken myoblasts, miR-29b-1-5p was overexpressed in cells; the overexpression efficiently increased the miR-29b-1-5p levels to 1043-fold (Figure 2A), indicating that the overexpression was very

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Identification of primary chicken myoblasts and model of proliferation and differentiation stages. (A) Identification of primary chicken myoblasts (CPMs). Desmin: staining of myosin-specific protein desmin; DAPI: staining of the nuclei; Merge: merged staining of desmin and DAPI. (B) Cell culture images of CPMs during the proliferation (50% and 100% confluence) and differentiation (d 1, d 3, d 5, and d 7) periods; DM indicates differentiation day.
successful. Then, the expression levels of the genes that can promote cell proliferation, such as G1/S-specific cyclin-D1 (CCND1), proliferating cell nuclear antigen (PCNA), and cyclin-dependent kinase 1 (CDK1) were measured, and the gene that can inhibits cell proliferation, p21 was assayed. CCND1, PCNA, and CDK1 were significantly decreased 48 h after miR-29b-1-5p overexpression, and p21 had an increasing trend, but the changes were not significant (Figure 2B). The changes in the expression of these genes indicate that miR-29b-1-5p may inhibit cell proliferation. The CCK-8 assay results indicated that cell activity was significantly decreased 12 h, 36 h, and 48 h after miR-29b-1-5p overexpression (Figure 2C). The results of the flow cytometry test indicated that the number of cells in the S phase was significantly decreased after miR-29b-1-5p overexpression (Figure 2D-F, Figure S1) (P < 0.05). The EdU experiments indicated that the overexpression of miR-29b-1-5p induced a significant decrease in cell proliferation (Figure 2G) (P < 0.05). These experiments indicate that miR-29b-1-5p can inhibit cell proliferation.

Additionally, miR-29b-1-5p inhibition experiments were performed. A significant 50-fold decrease in miR-29b-1-5p was detected (Figure 3A). Similarly, the assay of the expression levels of the p21, CCND1, PCNA, and CDK1 genes demonstrated that miR-29b-1-5p inhibition for 48 h induced a significant decrease in the expression of the p21 gene, and the expression levels of CCND1, PCNA, and CDK1 were increased, but the changes were not significant (Figure 3B). The inhibition of miR-29b-1-5p significantly increased cell viability at 24 h, 36 h, and 48 h (Figure 3C) according to the results of the CCK-8 assay. The flow cytometry results indicated that the inhibition of miR-29b-1-5p induced a significant increase in the number of cells in the S phase and a significant decrease in the number of cells in the G0/G1 phase (Figure 3D-F, Figure S2) (P < 0.05). The inhibition of the expression of miR-29b-1-5p significantly increased cell proliferation (Figure 3G) according to the results of the EdU assay (P < 0.05). These results indicate that the inhibition of miR-29b-1-5p can promote cell proliferation.

**miR-29b-1-5p Promotes CPM Differentiation**

To determine the role of miR-29b-1-5p in cell differentiation, CPMs at d 1 to 5 of differentiation were used to test the expression of miR-29b-1-5p and a myoblast differentiation marker, that is, MYOG; the results

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**Figure 2.** miR-29b-1-5p inhibits CPM proliferation. (A) Relative expression of miR-29b-1-5p in CPMs transfected with miR-29b-1-5p mimics. (B) Relative miRNA expression of p21, CCND1, PCNA, and CDK1 48 h after transfection with miR-29b-1-5p mimics in CPMs. (C) CCK-8 was used to detect cell growth after transfection with miR-29b-1-5p mimics in CPMs. (D-F) Cell cycle changes of CPMs after transfection with miR-29b-1-5p mimics as assayed by flow cytometry. (G) Proliferation of CPMs was assayed using EdU after transfection with miR-29b-1-5p mimics. All experiments were performed in triplicate, and the data are expressed as the mean ± S.E.M. (* P < 0.05; ** P < 0.01, *** P < 0.001).
indicated that in the first 3 d, the expression of the MYOG differentiation marker was gradually increased, and the levels of miR-29b-1-5p were also gradually increased. After the third day, the levels of MYOD and MYOG were significantly increased; however, the levels of MyHC did not significantly increase (Figure 4B) after miR-29b-1-5p overexpression. After the inhibition of miR-29b-1-5p, the MYOD and MyHC levels were significantly decreased, and the MYOG levels showed a downward trend, but the changes were not significant (Figure 4C). In the late stage of cell differentiation (after the third day), the results of the western blot assay and immunofluorescence staining of MYHC indicated that the overexpression of miR-29b-1-5p significantly promoted myoblast differentiation (Figure 4D and E), and the miR-29b-1-5p inhibitor inhibited myoblast differentiation (Figure 4F and G) during the cell differentiation stage. These results indicate that miR-29b-1-5p promotes CPM differentiation.

The Function Prediction of miR-29b-1-5p Target Genes and Screening of Candidate Target Genes

To further elucidate the potential mechanism of miR-29b-1-5p in chicken muscle development, we predicted and verified its target genes. We used the miRDB and microT-CDS databases to predict the target genes of miR-29b-1-5p. In total, 446 target genes were predicted in the miRDB database, and 101 target genes were predicted in the microT-CDS database. In total, 509 genes were predicted in the 2 databases, and 38 common genes were predicted (Figure 5A; Table S2). To further understand the functions of these target genes, we performed functional predictions of the 509 target genes of miR-29b-1-5p. The KEGG enrichment analysis revealed that there was only one significantly enriched pathway, that is, RNA degradation (Figure 5B). More interestingly, 3 GO terms related to muscle development were found in the GO enrichment analysis, namely, muscle tissue development, muscle organ development, and striated muscle tissue development (Figure 5C). This finding further illustrates that miR-29b-1-5p plays a role in muscle development. To narrow the scope and find the target genes that truly play a role in muscle development, we...
combined the 6-wk and 22-wk RNA-seq data of our previous Gushi chicken breast muscle tissue (Li et al., 2019b) and then analyzed the correlation coefficients of the expression profiles of 38 common target genes and miR-29b-1-5p in 6-wk and 22-wk RNA-seq data (Figure 5D; Table S3), which revealed that 26 genes were negatively correlated with miR-29b-1-5p. However, only 6 target genes (LDHA, ANKRD9, TCF12, FAT1, LIN9, and ITGB3BP) had correlation coefficients less than -0.8 and were significant ($P < 0.05$) (Figure 5E), and these genes can be used as candidate target genes for miR-29b-1-5p. To identify the most effective target gene of miR-29b-1-5p that affects muscle development, we also combined the differentially expressed genes between the 6-wk and 22-wk breast muscle tissue of Gushi chickens. The RNA-seq data revealed 547

Figure 4. miR-29b-1-5p promotes CPM differentiation. (A) Trend in miR-29b-1-5p and MYOG during 5 d of cell differentiation. (B) Changes in differentiation markers after transfection with miR-29b-1-5p mimics. (C) Changes in differentiation markers after transfection with a miR-29b-1-5p inhibitor. (D) Protein expression of MYHC after transfection with miR-29b-1-5p mimics. (E) MYHC staining of myoblasts after transfection with miR-29b-1-5p mimics at the cell differentiation stage. (F) Protein expression of MYHC after transfection with a miR-29b-1-5p inhibitor. (G) MYHC staining of myoblasts after transfection with a miR-29b-1-5p inhibitor during the cell differentiation stage.
differentially expressed genes between 6 wk and 22 wk (Table S2). Combined with the target genes predicted by the 2 databases, there were only 2 genes in common, namely, **ANKRD9** and **JMJD6** (Figure 5F). Based on the above results, we found that **ANKRD9** may be the most effective miR-29b-1-5p target gene that can affect muscle development.

**miR-29b-1-5p Directly Targets ANKRD9 and Influences the Proliferation and Differentiation of CPMs**

To confirm our prediction results, we conducted further studies of the candidate target gene of miR-29b-1-5p, that is, **ANKRD9**. Overall, we analyzed the changes in the expression of miR-29b-1-5p and **ANKRD9** from E10 to E18 (Figure 6A), and the results showed that the expression of miR-29b-1-5p increased from E10 to E16 and then showed a downward trend. **ANKRD9** showed a downward trend, with a slight increase at E16. This finding shows that miR-29b-1-5p and **ANKRD9** have temporal expression characteristics. These results also show that the expression trend of miR-29b-1-5p and **ANKRD9** from E10 to E18 showed a negative correlation, and the correlation coefficient was -0.57 (Table S4). In addition, we tested the expression changes of miR-29b-1-5p and **ANKRD9** during cell proliferation and differentiation (Figure 6B) and found that during cell proliferation, the expression of miR-29b-1-5p and **ANKRD9** showed the same upward trend. However, in contrast to **ANKRD9**, during cell differentiation, miR-29b-1-5p showed an upward trend at 1 to 3 d of differentiation. In addition, at 1 to 6 d of cell differentiation, the expression trend of the two was negatively correlated, and the correlation coefficient was -0.86 (Table S4). In addition, we constructed wild-type and mutant vectors of the 3’UTR of the **ANKRD9** gene (Figure 6C and D). The l reporter system showed that miR-29b-1-5p mimics were cotransfected with the wild-type 3’UTR of the **ANKRD9** gene, and the fluorescence activity was significantly reduced (Figure 6E). After the overexpression of miR-29b-1-5p, we found that **ANKRD9** was significantly reduced in CPMs (Figure 6F). These results strongly prove that there is a strong target relationship between miR-29b-1-5p and **ANKRD9** and that miR-29b-15p may affect the proliferation and differentiation of myoblasts by regulating **ANKRD9**.

**DISCUSSION**

Many miRNAs exhibit tissue- or time-specific expression patterns; and an increasing number of muscle-specific miRNAs and their functions have been identified.
In total, 25 miRNAs from various tissues of chicken embryos and adult chickens were identified for the first time, and 3 of these miRNAs were tissue-specific (Xu et al., 2006). Additional studies have shown that various miRNAs are expressed in the breast muscle tissue of chickens at various stages of postpartum development, indicating that these miRNAs may have spatiotemporal specificity (Fu et al., 2018a). The expression of many miRNAs is known to be spatiotemporal-specific or tissue-specific in animals (Mu et al., 2013; Wang et al., 2013). During the embryonic stage of chickens, myoblast proliferation reaches a peak at E12, and the cells begin to differentiate at E16 (Ouyang et al., 2017). Therefore, the changes in miR-29b-1-5p in breast muscle were assayed in E10-E18 embryos to assess the levels during peak proliferation and active differentiation of chicken embryonic myoblasts. The results of the present study indicate that miR-29b-1-5p has spatiotemporal specificity during the development of breast muscle in Gushi chickens; the levels gradually increase from E10 to E16, reach a peak at E16, and then decrease. The expression of miR-29b-1-5p was maximal at E16, indicating that miR-29b-1-5p likely plays an important role in the process of muscle differentiation.
The changes in miR-29b-1-5p expression during embryonic development suggest that miR-29b-1-5p is involved in skeletal muscle development.

The miR-29 family consists of miR-29a, miR-29b, and miR-29c, and the expression level of miR-29b is generally the highest (Yan et al., 2015). Most studies investigating the function of miR-29b-1-5p focused on human diseases. Recent studies have shown that miR-29b affects cancer progression by acting as a tumor suppressor (Zhu et al., 2016). However, miR-29b may play a role in the promotion of cancer (Xu et al., 2013). Some studies concerning muscle development have been reported. An increase in miR-29b and other myogenic miRNAs mediates the enhancement of muscle regeneration and resistance to muscle atrophy during hibernation (Luu et al., 2020). Only a few studies investigated miR-29b in chicken muscle development. The results of the present study confirm the role of miR-29b-1-5p in chicken skeletal muscle development. Numerous studies have reported that certain cell cycle-related genes are involved in the cell cycle (Li et al., 2019a). The regulatory cell cycle gene p21 is among the most important downstream genes of p53; p21 inhibits the activities of various cyclin-CDK complexes and PCNA, induces DNA damage and telomere shortening, and triggers cell cycle arrest and aging (Jiang et al., 2015). The detection of cell proliferation-related genes, such as CCND1, CDK1, PCNA, and p21, is usually used to detect the proliferation status of muscle cells (Cai et al., 2018; Luo et al., 2019). In CPMs, the overexpression of miR-29b-1-5p can significantly reduce the expression of proliferation-related genes, including CCND1, PCNA, and CDK1, and can increase the expression of p21. Therefore, it indicates that miR-29b-1-5p participates in and affects the activity of the proliferation state of myoblasts. The cell cycle analysis showed that the number of cells in the S phase was reduced after the overexpression of miR-29b-1-5p. Flow cytometry assays of the cell cycle indicated that the S and G2/M phases are usually used as important indicators to detect cell proliferation (Tirelli et al., 2002). Therefore, the cell flow cytometry results indicate that miR-29b-1-5p inhibits cell proliferation. Additionally, the CCK-8 and EdU assays confirmed that miR-29b-1-5p inhibits cell proliferation and that the inhibition of miR-29b-1-5p has the opposite effect. This result indicates that miR-29b-1-5p can inhibit the proliferation of CPMs.

Cell cycle arrest plays an important role in the initiation of muscle differentiation in myoblasts (Lee et al., 2002). Myoblasts permanently withdraw from the cell cycle during terminal differentiation (Shen et al., 2003). After myoblasts withdraw from the cell cycle, myogenic regulatory factors, such as MYOD and MYOG, promote muscle differentiation (Fernandez et al., 2002). Additionally, MyHC is essential for the molecular movement of muscles, the expression of various isoforms of MyHC is the main parameter associated with different types of muscle fibers (Tajsharghi and Oldfors, 2013). Whether miR-29b-1-5p plays a role in the differentiation of skeletal muscle cells is unclear. Studies have shown that miR-26a/b and miR-29b are upregulated during the osteogenic differentiation of unrestricted somatic stem cells (USSCs). These microRNAs may promote osteogenic differentiation by inhibiting the expression of osteogenic proteins, such as CDK6 and HDAC4 (Trompeter et al., 2013). Similar to previous studies, our results indicate that the expression trend of miR-29b-1-5p is consistent with the expression trend of MYOG during 5 d of myoblast differentiation; and miR-29b-1-5p and MYOG showed an upward trend in the first 3 d of differentiation. Myogenin accumulates just before myoblasts differentiate into myotubes (Buckingham, 1994). Therefore, in the first 3 d, it should be before myoblasts are converted into myotubes. At this time, the expression trends of miR-29b-1-5p and MYOG are consistent, indicating that miR-29b-1-5p is also involved in the differentiation of myoblasts into myotubes. Furthermore, miR-29b-1-5p can promote the expression of MYOD, MYOG, and MyHC in the early stage of cell differentiation, but the expression change of MyHC is not significant. MYOD is considered to be the pioneer transcription factor required to initiate muscle-specific gene expression, and subsequently allow the subsequent binding and transcriptional activity of MYOG (Loudhe and Davie, 2011). Then, myoblasts expressing MyHC fuse into multinucleated myotubes and assemble to form muscle fibers (Francis-West et al., 2003). Therefore, in the early stage of cell differentiation, MYOD and MYOG changed significantly after miR-29b-1-5p overexpression, while it had not yet formed muscle fibers, so MYHC expression was not significant. So we tested the expression of MYHC in the late stage of differentiation, the results of the MYHC protein expression and immunofluorescence assay indicated that miR-29b-1-5p promotes the expression of MYHC, confirming that miR-29b-1-5p plays a role in promoting the formation of muscle fibers.

MiRNAs often function by reducing the expression of target genes (He and Hamon, 2004; Li et al., 2017); hence, the interaction between miR-29b-1-5p and its target genes may be involved in the regulation of the development of chicken skeletal muscle. Thus, the miR-29b-1-5p target genes were identified based on the seed sequence of miR-29b-1-5p. We found that the target genes of miR-29b-1-5p are enriched in biological processes related to muscle development, further illustrating that miR-29b-1-5p may be involved in muscle development. In addition, our previous data indicated that the period between 14 and 22 wk is an important period for muscle development in Gushi chickens, and there were more differentially expressed genes between 6 wk and 22 wk. Based on the RNA-seq data of Gushi chicken breast muscle tissue at 6 wk and 22 wk, we found that the expression patterns of miR-29b-1-5p and 6 genes (LDHA, ANKR09, TCF12, FAT1, LIN9, and ITGB3BP) had a significantly negative relationship. These genes may be potential target genes of miR-29b-1-5p. This finding indicates that the function and structure of LDH are highly conserved in skeletal muscle and testis, but LDHA encodes skeletal muscle-specific lactate
dehydrogenase, which has unique characteristics in muscle tissue (Sugawara et al., 2016). In addition, TCF12 is a MYOD coactivator involved in muscle cell differentiation (Zhao and Hoffman, 2004). Studies have determined that FAT1 is a key determinant of muscle shape in mice (Caruso et al., 2013). Furthermore, the polymorphism of the ITGB3 coding gene plays an important role in the proliferation of smooth muscle cells, and ITGB3BP is the binding protein of ITGB3 (Li et al., 2016). Moreover, LIN9 is required for early embryonic development (Esterlechner et al., 2013). These results prove that these genes are related to muscle development. Subsequently, we identified the differentially expressed genes between 6 wk and 22 wk, and these differentially expressed genes are likely involved in the muscle development process. Subsequently, we combined the target genes of miR-29b-1-5p and found that there were only 2 genes, that is, ANKRD9 and JMJD6, in common. Furthermore, ANKRD9 is differentially expressed in Gushi chicken breast muscle tissue from wk 6 to wk 22 (Li et al., 2019b). Therefore, we hypothesized that ANKRD9 plays a role in muscle development. Members of the ANKRD protein family can regulate many cellular processes (Hui et al., 2019). ANKRD9 may be involved in the degradation of the nucleotide-processing enzyme inosine-5'-monophosphate dehydrogenase 2 (IMPDH2) and has antiproliferative activity. Additionally, the ANKRD9 protein may have variable localization and functions under various metabolic conditions (Hayward et al., 2019). Therefore, the expression of ANKRD9 in the breast muscle was assayed during the embryonic stage; the data indicate a downward trend from E10 to E18, except E16. These time-dependent changes in expression indicate that ANKRD9 may play a role in embryonic development. Additionally, the expression of miR-29b-1-5p and ANKRD exhibits a significant negative correlation trend during the embryonic and differentiation stages of CPMs. The results of the dual-luciferase reporter assay showed that miR-29b-1-5p can bind ANKRD9. Additionally, miR-29b-1-5p can significantly inhibit the expression of ANKRD9 in CPMs, indicating that ANKRD9 is a direct target of miR-29b-1-5p. Therefore, these results indicate that the expression of ANKRD9 is involved in posttranscriptional regulation. Thus, miR-29b-1-5p reduces the expression of ANKRD9 and influences the proliferation and differentiation of CPMs (Figure 7). However, there are certain additional considerations. For example, whether the effects of miR-29b-1-5p on the proliferation and differentiation of chicken myoblasts are mediated by ANKRD9 or the molecular mechanism of these effects remains unclear. miR-29b-1-5p may have many target genes in chicken myoblasts in addition to ANKRD9. These issues will be investigated in the future.

Thus, our data indicate that miR-29b-1-5p can inhibit the proliferation of chicken CPMs and promote their differentiation. miR-29b-1-5p partially reduces the expression of ANKRD9 and, thus, may influence muscle development. In addition, the target genes of miR-29b-1-5p may be involved in the development of muscle

**Figure 7.** A model of the regulatory network involved in the effects of miR-29b-1-5p on the proliferation and differentiation of chicken primary myoblasts. In brief, miR-29b-1-5 inhibits the expression of ANKRD9 mRNA by directly binding the 3′UTR of ANKRD9, participates in the inhibition of the proliferation of myoblasts and promotes the differentiation of myoblasts.
tissue, muscle organ development and striated muscle tissue development in biological processes related to muscle development. ANKRD9, LDHA, TCF12, FAT1, LIN9 and ITGB3/BP can also be used as miR-29b-1-5p efficient candidate target genes. These findings provide new insight into the molecular mechanism of chicken muscle development. The effect of some important target genes of miR-29b-1-5p, especially ANKRD9, on myoblasts will be investigated in our future studies.

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DISCLOSURES

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2021.101557.

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