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

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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

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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

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(Wienholds et al., 2005; Koutsoulidou et al., 2011). 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 non-small 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., 2014). 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% CO_2 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

synthesized by reverse transcription using a PrimeScript RT reagent kit 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 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 $2^{-\Delta\Delta Ct}$ method (Bubner and Baldwin, 2004). The BμLge-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 *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% CO_2 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

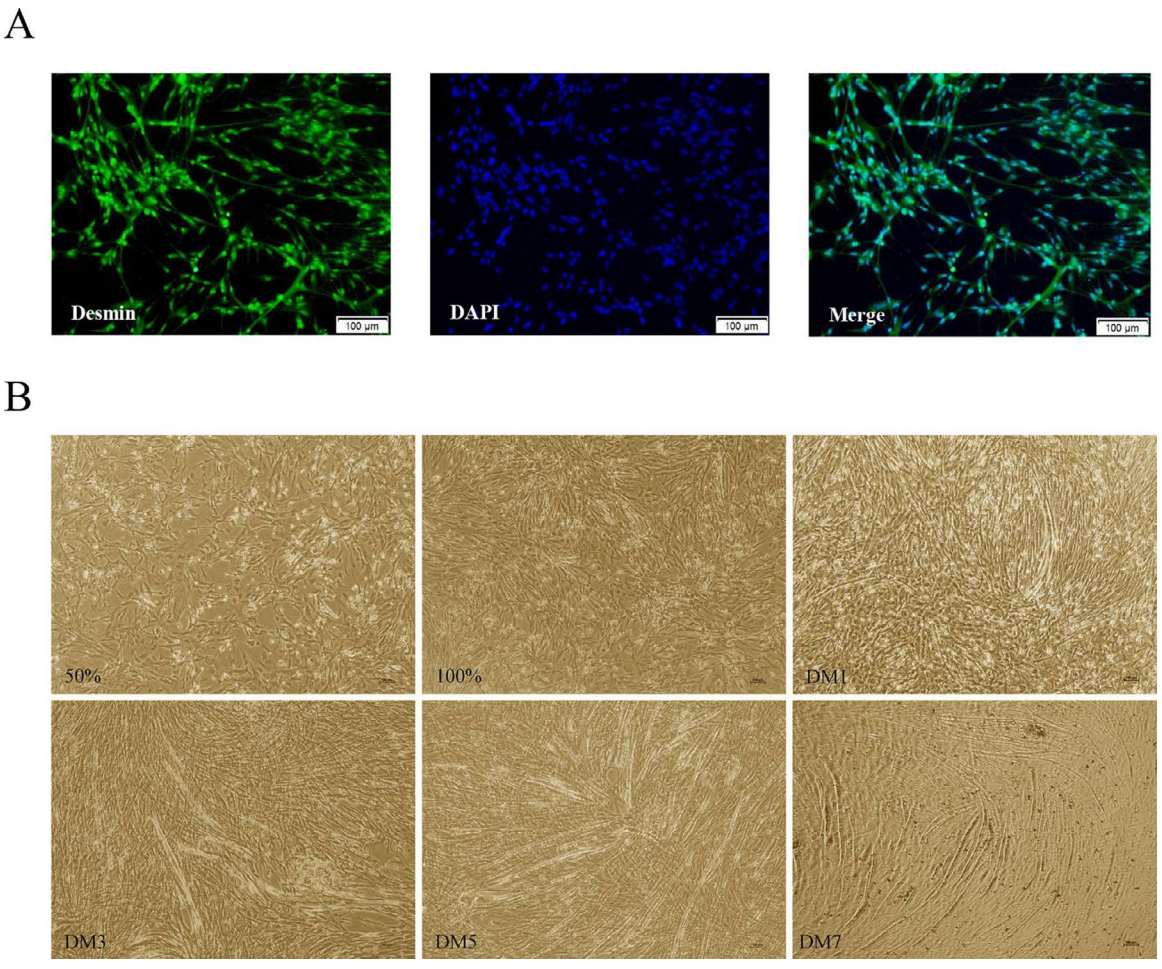

Figure 1. Identification of primary chicken myoblasts and model of proliferation and differentiation stages. (A) Identification of primary chicken myoblasts (CPMs). Desmin: staining of myosin-specific protein desmin; DAPI: staining of the nuclei; Merge: merged staining of desmin and DAPI. (B) Cell culture 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.

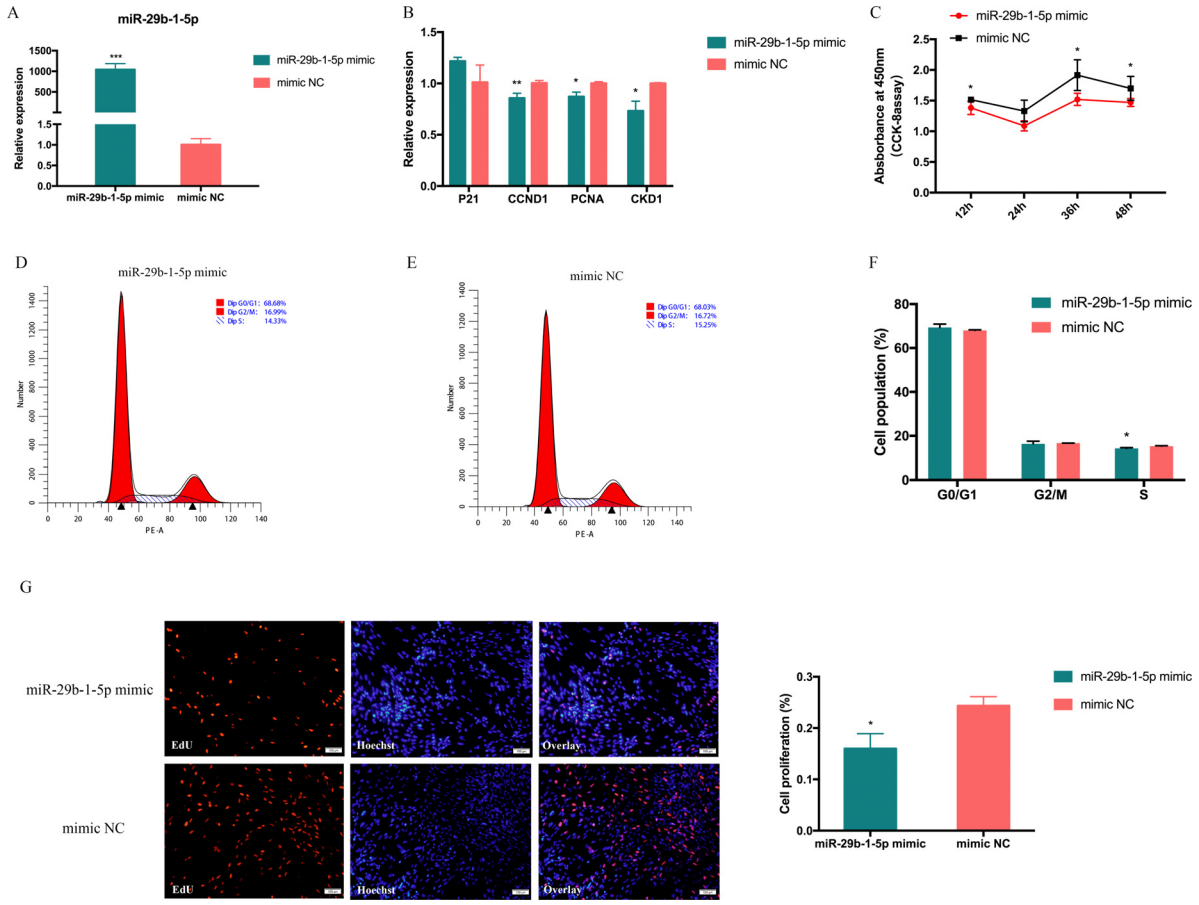

Figure 2. miR-29b-1-5p inhibits CPM proliferation. (A) Relative expression of miR-29b-1-5p in CPMs transfected with miR-29b-1-5p mimics. (B) Relative mRNA expression of *p21, CCND1, PCNA*, and *CDK1* 48 h after transfection with miR-29b-1-5p mimics in CPMs. (C) CCK-8 was used to detect cell growth after 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. (* $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$).

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 the proliferation of CPMs.

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

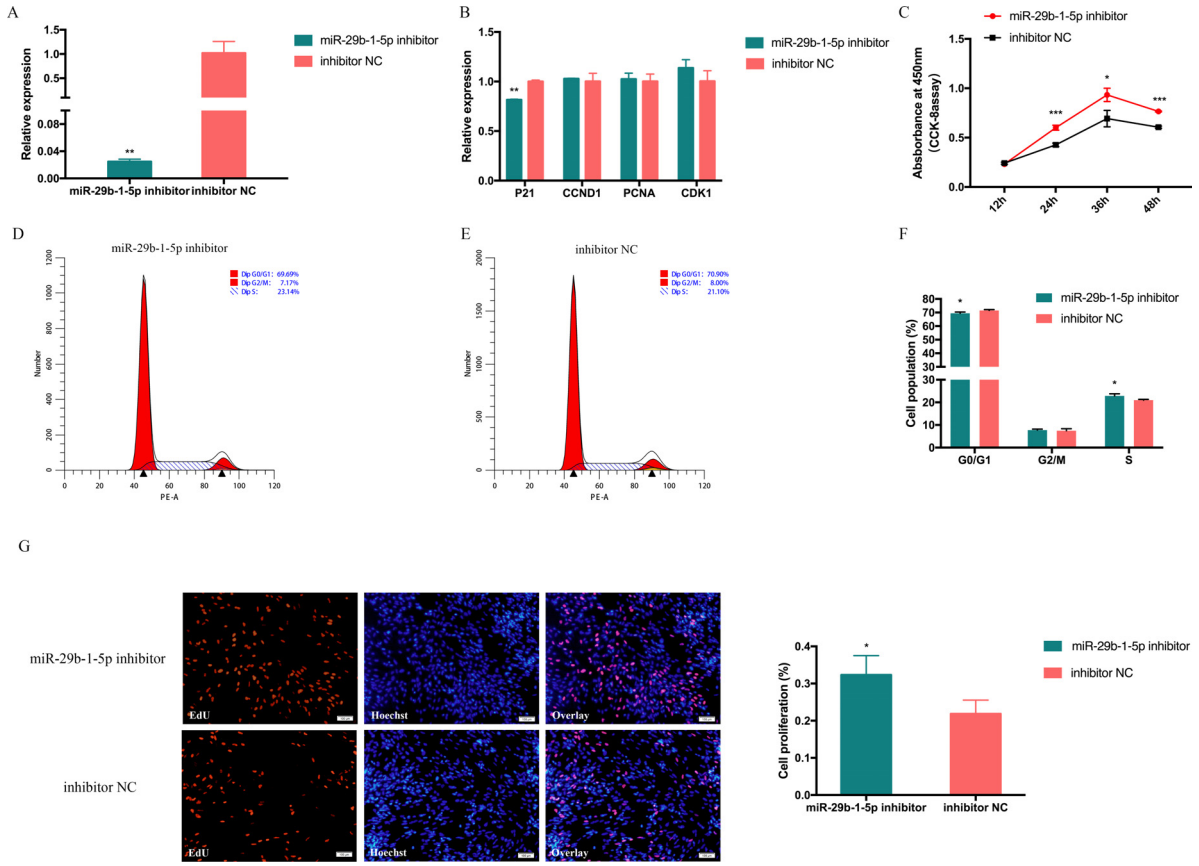

Figure 3. miR-29b-1-5p inhibitor promotes CPM proliferation. (A) Relative expression of miR-29b-1-5p in CPMs after transfection with a miR-29b-1-5p inhibitor. (B) Relative mRNA expression of *p21, CCND1, PCNA*, and *CDK1* 48 h after transfection with a miR-29b-1-5p inhibitor in CPMs. (C) CCK-8 was used to detect cell growth after transfection with a miR-29b-1-5p inhibitor in CPMs. (D–F) Cell cycle analysis of CPMs after transfection with a miR-29b-1-5p inhibitor as assayed by flow cytometry. (G) EdU was used to detect the proliferation of CPMs after transfection with a miR-29b-1-5p inhibitor. 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 *MYOG* and miR-29b-1-5p showed a downward trend (Figure 4A). After the transfection with the miR-29b-1-5p mimics, the myoblast differentiation markers *MYOD, MYOG*, and myosin heavy chain (*MyHC)* were assayed in the early stage of cell differentiation (before 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 significant enrichment 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

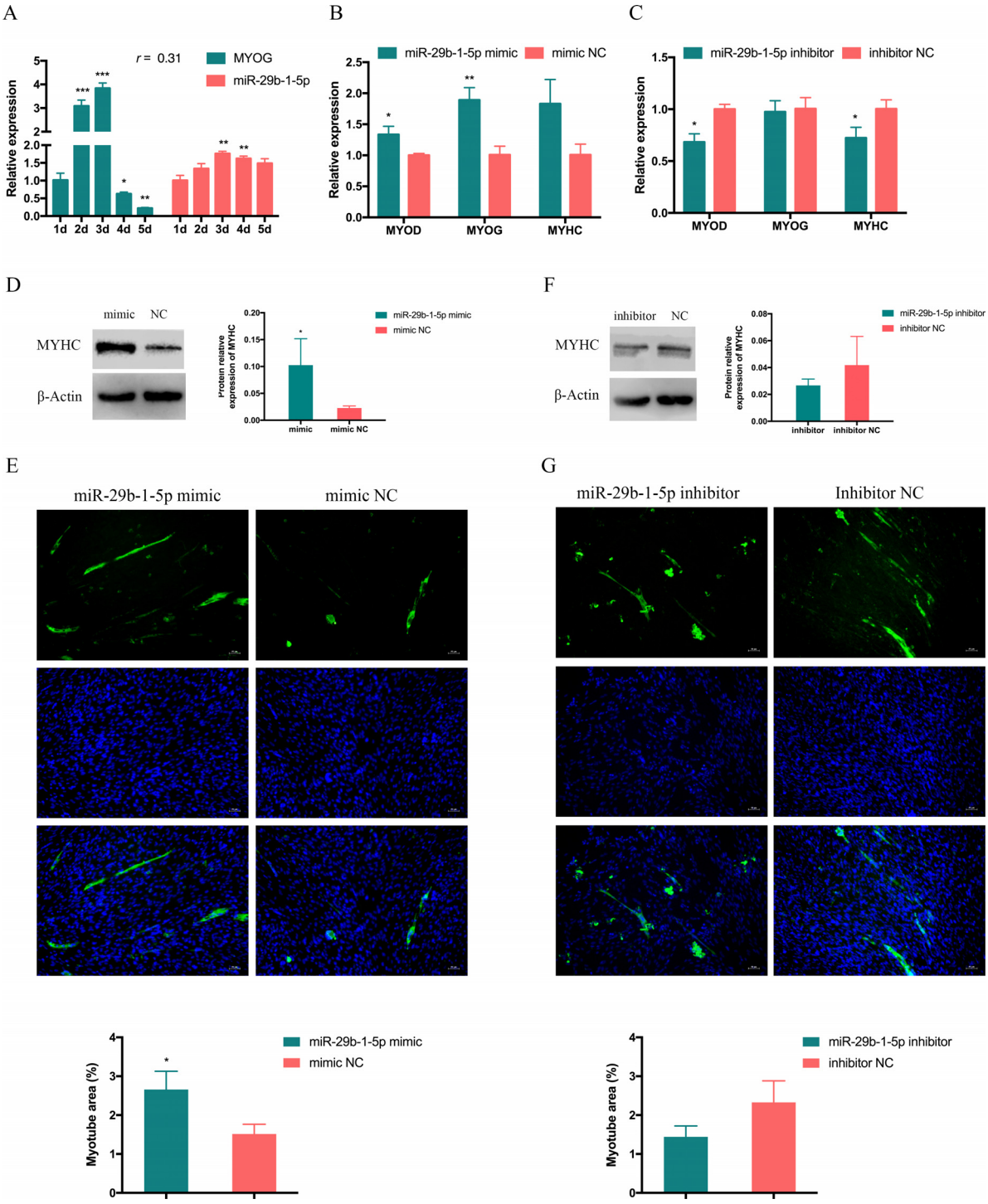

Figure 4. miR-29b-1-5p promotes CPM differentiation. (A) Trend in miR-29b-1-5p and *MYOG* during 5 d of cell differentiation. (B) Changes in differentiation markers after transfection with miR-29b-1-5p mimics. (C) Changes in differentiation markers after transfection with a miR-29b-1-5p inhibitor. (D) 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.

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

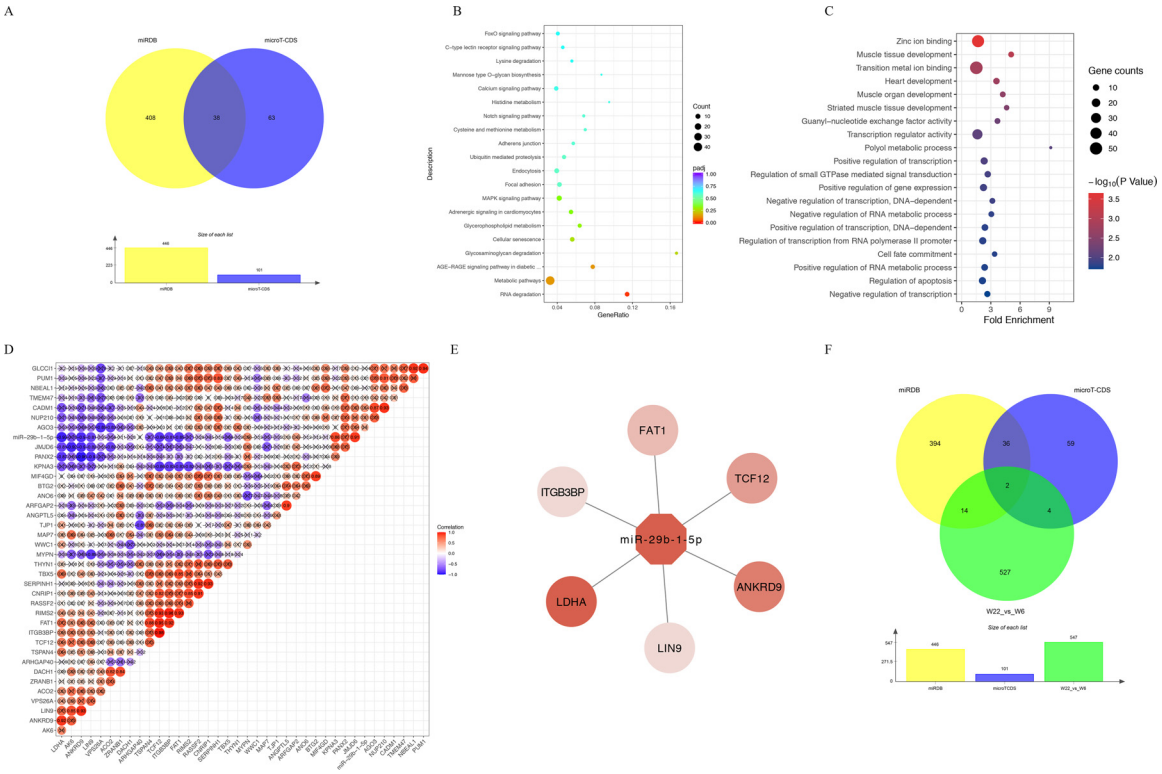

Figure 5. The function prediction of miR-29b-1-5p target genes and screening of candidate target genes. (A) Venn diagram of miR-29b-1-5p target genes predicted by the miRDB database and microT-CDS database. (B) KEGG enrichment analysis of all target genes of miR-29b-1-5p in both the miRDB database and microT-CDS database. (C) GO enrichment analysis of all target genes of miR-29b-1-5p in the miRDB database and microT-CDS database. (D) Correlation analysis of the expression of miR-29b-1-5p and its 38 common target genes in Gushi chicken breast muscle at 22 wk and 6 wk. (E) The 6 candidate target genes most negatively related to miR-29b-1-5p. (F) Venn diagram of the differentially expressed genes between 22 and 6 wk in breast muscle of Gushi chickens, the miR-29b-1-5p target genes predicted by the miRDB database and the target genes predicted by the microT-CDS database.

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.

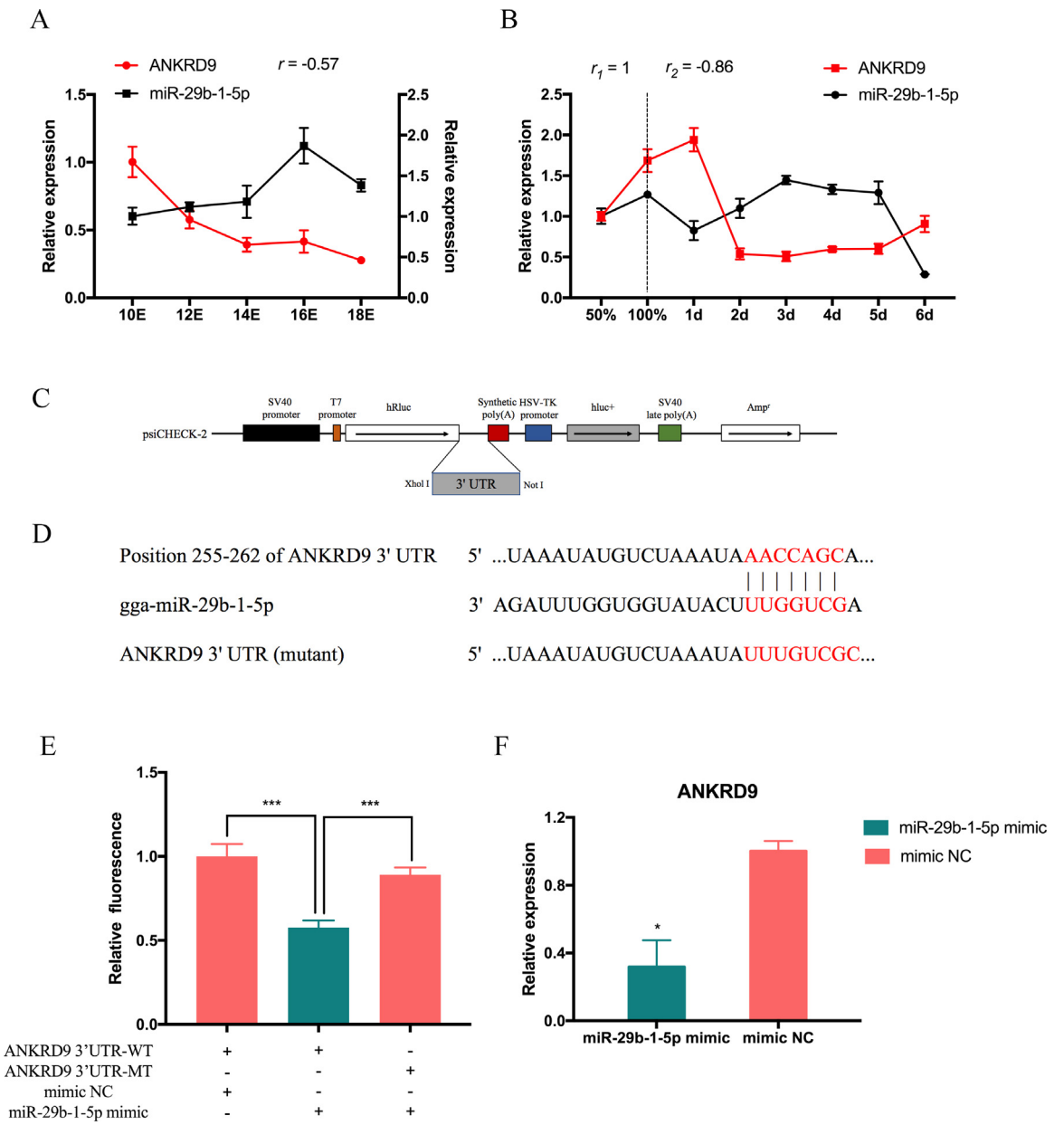

Figure 6. miR-29b-1-5p can directly target *ANKRD9*. (A) Expression of miR-29b-1-5p and *ANKRD9* in the breast muscle of Gushi chicken embryos from E10 to E18. *r* is the coefficient of the correlation between the expression of miR-29b-1-5p and *ANKRD9*. (B) Expression trends of miR-29b-1-5p and *ANKRD9* at 50% and 100% cell fusion and on d 1 to 6 of differentiation. r_1 is the coefficient of the correlation between the expression of miR-29b-1-5p and *ANKRD9* during the proliferation period, and r_2 is the coefficient of the correlation between the expression of miR-29b-1-5p and *ANKRD9* during the differentiation period. (C) Constructed *ANKRD9* 3′UTR dual luciferase reporter vector. (D) Potential binding site of miR-29b-1-5p in the 3′UTR of *ANKRD9* mRNA. Sequences of the wild-type and mutant vectors of the miR-29b-1-5p binding site are highlighted in red. (E) A dual luciferase reporter system was used to detect the fluorescence activity of DF-1 cells by cotransfecting wild-type or mutant *ANKRD9* 3′UTR with miR-29b-1-5p mimics or mimics NC. F. Changes in ANKRD9 48 h after transfection with miR-29b-1-5p mimics.

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 isotypes 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* (Londhe 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 Hannon, 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, ANKRD9, 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

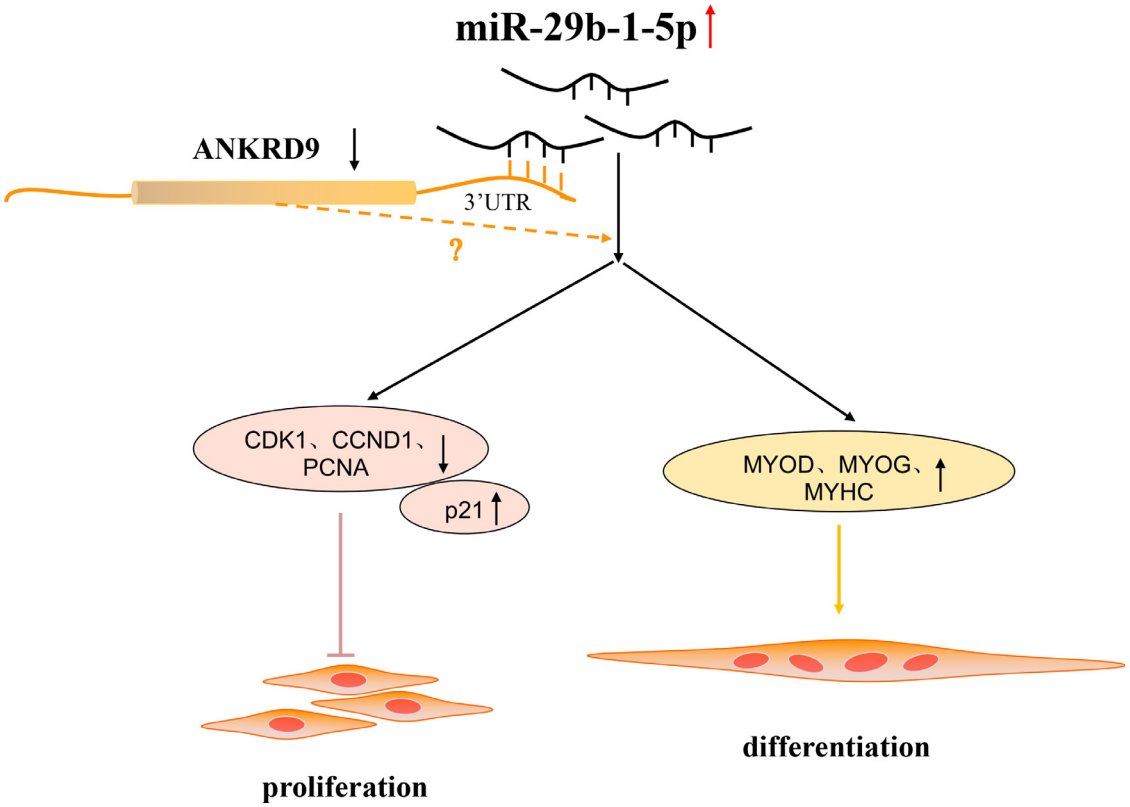

Figure 7. A model of the regulatory network involved in the effects of miR-29b-1-5p on the proliferation and differentiation of chicken primary myoblasts. In brief, miR-29b-1-5 inhibits the expression of *ANKRD9* mRNA by directly binding the 3′UTR of *ANKRD9*, participates in the inhibition of the proliferation of myoblasts and promotes the differentiation of myoblasts.

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

tissue, muscle organ development and striated muscle tissue development in biological processes related to muscle development. *ANKRD9, LDHA, TCF12, FAT1, LIN9* and *ITGB3BP* 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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