

circFGFR4 Promotes Differentiation of Myoblasts via Binding miR-107 to Relieve Its Inhibition of Wnt3a

Hui Li,^{1,5} Xuefeng Wei,^{1,5} Jiameng Yang,^{1,5} Dong Dong,¹ Dan Hao,¹ Yongzhen Huang,¹ Xianyong Lan,¹ Martin Plath,¹ Chuzhao Lei,¹ Yun Ma,² Fengpeng Lin,³ Yueyu Bai,⁴ and Hong Chen¹

¹Shaanxi Key Laboratory of Molecular Biology for Agriculture, College of Animal Science and Technology, Northwest A&F University, Yangling, 712100 Shaanxi, China;

²College of Life Sciences, Xinyang Normal University, Institute for Conservation and Utilization of Agro-Bioresources in Dabie Mountains, Xinyang, Henan 464000, PRC;

³Bureau of Animal Husbandry of Biyang County, Biyang, Henan 463700, China; ⁴Animal Health Supervision in Henan Province, Zhengzhou, Henan 450008, China

Muscle development is regulated under a series of complicate processes, and non-coding RNAs, such as microRNAs (miRNAs) and circular RNAs (circRNAs), have been reported to play important roles in regulating myoblast proliferation and differentiation. We found that miR-107 expression was high in skeletal muscle of Qinchuan cattle. Overexpression of miR-107 inhibited bovine myoblasts differentiation and protected cells from apoptosis. Wnt3a was identified as a target of miR-107 by luciferase activity, real-time qPCR, and western blotting assays. Knockdown of Wnt3a inhibited bovine myoblasts differentiation and apoptosis, and this effect was similar to miR-107 overexpression. We also found circFGFR4 to promote myoblasts differentiation and to induce cell apoptosis. Via luciferase screening and RNA pull-down assays, circFGFR4 was observed to sponge miR-107. Overexpression of circFGFR4 increased the expression of Wnt3a, whereas this effect was abolished by miR-107. These results demonstrated that circFGFR4 binding miR-107 promotes cell differentiation via targeting Wnt3a in bovine primary myoblasts.

INTRODUCTION

MicroRNAs (miRNAs) are a class of small non-coding RNAs about 18~24 nucleotides length that bind to mRNAs of coding genes to repress their protein production.¹ miRNAs are involved in diverse biological processes, including cell proliferation,^{2,3} cell apoptosis,^{2,4} and myoblast differentiation.⁵ Circular RNAs (circRNAs), with covalently closed loop structure, are generated from back-spliced exons and intron-derived RNAs⁶ and were first found in human cells in 1991⁷ but hardly recognized until 2013.^{8,9} Although there is limited knowledge related to the function of circRNAs,^{10,11} the role of two circRNAs, *ciRS-7* and *Sry*, is known well. Both *ciRS-7* and *Sry* inhibit miRNA function by sponging miR-7 and miR-138,^{9,12} respectively, hence revealing that circRNAs may play important roles in post-transcriptional regulation. Recently, a novel subclass of circRNAs, named exon-intron circRNAs (EiRNAs), interacted with RNA polymerase II and U1 small nuclear ribonucleoproteins (snRNPs) and functioned as *cis* inducers of host-gene transcription.¹³ The development of skeletal muscle and its physio-

logical and biochemical characteristics directly affect the meat quantity and quality. Skeletal muscle is the most important component of animal body (constitutes 40% of the animal body) and provides structural support and energy storage. Recent researches demonstrate that the physiology and pathology of skeletal muscle are profoundly influenced by miRNAs, such as miR-133,¹⁴ miR-29,⁵ miR-1/206,^{15,16} and miR-378a.^{2,17,18} In our previous miRNA sequencing results, the expression of miR-107 was high in muscle tissue.¹⁹ miR-107 has been reported to promote cancer cell proliferation and metastasis;^{20–22} however, the role of miR-107 in the development of bovine primary myoblast has not been established.

It is reported that Wnt3a promotes cell proliferation in NIH 3T3 fibroblast cells and stimulates the G1 to S phase cell cycle progression through activation of the Wnt/ β -catenin and ERK pathways.²³ Wnt3a/ β -catenin signaling plays a critical role in cell differentiation and induces expression of muscle-specific genes, such as myogenin.²⁴ Wnt3a promotes neurogenesis and improves neurocognitive function through inducing differentiation of neural stem cells.²⁵ Wnt3a is expressed by developing skeletal muscles and mediates its dispersal activity by downregulating rapsyn expression via a β -catenin-dependent but T cell factor (TCF)-independent pathway.²⁶

In this study, we transfected miR-107 mimic and inhibitor into bovine primary myoblasts to explore the role of miR-107 in myoblast differentiation and proliferation. Moreover, we found circFGFR4 regulates myoblast differentiation via sponging miR-107 to increase the expression of Wnt3a. These evidences shed light on the current understanding of circRNA and miRNA function during muscle progression.

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⁵These authors contributed equally to this work.

Correspondence: Hong Chen, College of Animal Science and Technology, Northwest A&F University, Yangling, 712100 Shaanxi, China.

E-mail: chenhong1212@263.net



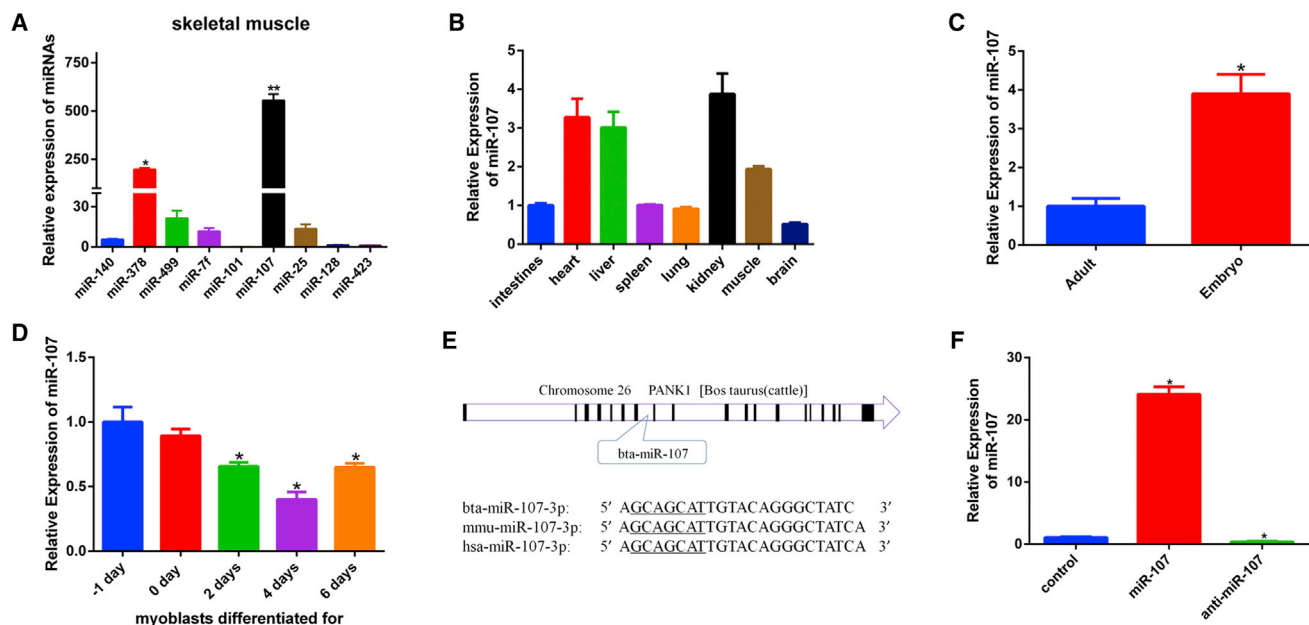


Figure 1. miR-107 Identification in Bovine Skeletal Muscle

(A) The expression of nine miRNAs in skeletal muscle of Qinchuan cattle at embryonic stage is shown. (B) The expression of miR-107 in different tissues of Qinchuan cattle at embryonic stage is shown. (C) The expression of miR-107 in skeletal muscle of Qinchuan cattle at the embryonic stage compared with the adult stage is shown. (D) The expression of miR-107 in myoblasts differentiated for -1, 0, 2, 4, and 6 days is shown. (E) The location of miR-107 in genomic and conservative property analysis in different species is shown. (F) The expression levels of miR-107 in bovine primary myoblasts transfected with miR-107 mimic and inhibitor are shown. Values are means \pm SEM for three individuals. * $p < 0.05$.

RESULTS

miR-107-Specific Expression in Muscle Tissue

In our previous study, we obtained 510 mature miRNAs in muscle tissue of Qinchuan bovine through Solexa SBS technology sequencing.¹⁹ In order to explore the function of miRNAs in myoblasts, we selected nine miRNAs, including miR-107 as the candidates. We found that miR-107 had the highest expression level in these miRNAs, and its expression was high in muscle tissue (Figures 1A and 1B). We found the expression of miR-107 to be much higher (3.9-fold) at the embryonic stage compared with the adult stage (Figure 1C). The expression of miR-107 was higher at proliferation period compared with differentiation period and was down-regulated during myoblasts differentiation (Figure 1D). These results suggest that miR-107 may be as a negative regulator of muscle development. Sequence analysis revealed that miR-107 derives from the seventh intron of pantothenate kinase 1 in chromosome 26, and the sequence of miR-107 was highly conserved between different species (Figure 1E). The level of miR-107 in cells transfected with miR-107 mimic was over 20 times higher than the negative control (Figure 1F). For the inhibition experiment, miR-107 inhibitor resulted in an about 60% decrease in miR-107 (Figure 1F).

miR-107 Suppressed Cell Differentiation

To assess the effect of miR-107 on myoblast differentiation, the expression of established myogenic markers, MyoD, myogenin (MyoG), and myosin heavy chains (MyHCs), was detected in primary

bovine myoblasts treated with miR-107 mimic or inhibitor differentiated for 4 days. From immunofluorescence assay in Figure 2A, we found that miR-107 suppressed MyHC expression and myotube forming and anti-miR-107 promoted myotube forming. The mRNA expression of *MyHC* was also inhibited by miR-107 (Figure 2B). Similarly, miR-107 decreased expression of established myogenic markers, MyoD and MyoG, at both mRNA and protein levels (Figures 2B and 2C). These results suggest that miR-107 inhibits myoblast differentiation.

miR-107 Did Not Affect Cell Proliferation

To establish the role of miR-107 in myoblast proliferation, cell phase, cell counting kit-8 (CCK-8), 5-ethynyl-2'-deoxyuridine (EdU), real-time qPCR, and western blotting assays were conducted. Cell cycle analysis revealed that miR-107 decreased the number of myoblasts in S phases and increased the proportion of cells in G2 phase but had no effect on the proportion of cells in the S+G2 phases, suggesting that miR-107 may have no effect on cell proliferation (Figures 3A and 3B). CCK-8 assay revealed that miR-107 could inhibit cell viability lightly, but not significantly ($p > 0.05$; Figure 3C). EdU assay had similar results (Figures 3D and 3E). Then, we also detected the effect of miR-107 on the expression of cell-proliferation-related genes proliferating cell nuclear antigen (PCNA), cyclin D1, and cyclin-dependent-kinase 2 (CDK2) and found that miR-107 did not significantly increase or decrease the expression of these genes at mRNA and protein levels

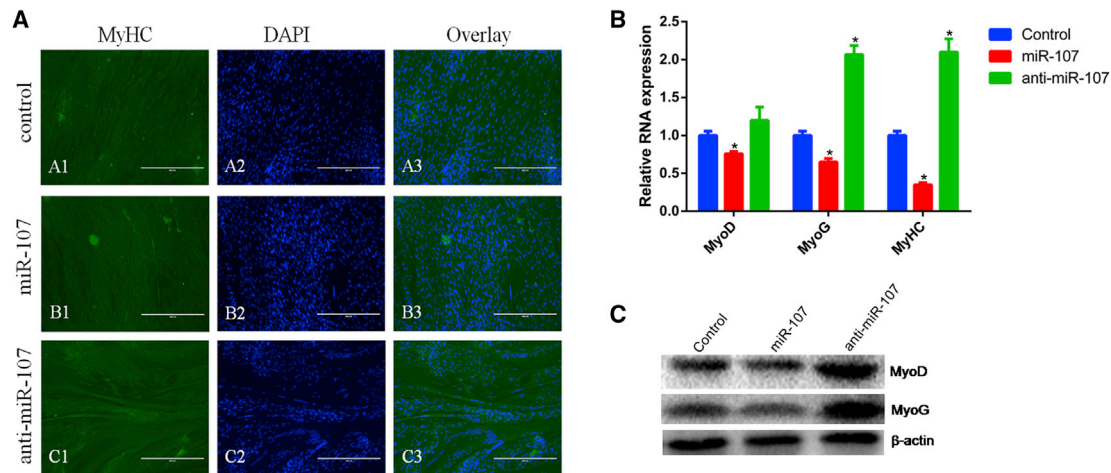


Figure 2. miR-107 Suppresses Differentiation of Bovine Primary Myoblasts

(A) Bovine primary myoblasts were transfected with miR-107 mimic and inhibitor, and cell differentiation was detected by immunofluorescence. (B) The mRNA expression of cell differentiation markers *MyHC*, *MyoD*, and *MyoG* was detected by real-time qPCR. (C) The protein expression of *MyoD* and *MyoG* was analyzed by western blotting. Values are means \pm SEM for three individuals. * $p < 0.05$.

(Figures 3F–3H). These results reveal that miR-107 does not affect cell proliferation.

miR-107 Suppressed Cell Apoptosis

To explore whether miR-107 regulates myoblast apoptosis, we performed the annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining, qPCR, and western blotting assays. Results showed miR-107 overexpression protected cells from apoptosis, whereas anti-miR-107 induced myoblasts apoptosis (Figures 4A and 4B). Bcl-2 has been described as an anti-apoptotic protein and plays important roles in regulating proliferation and apoptosis in myoblasts.^{27,28} We asked whether Bcl-2 is involved in the anti-apoptotic effect of miR-107 in myoblast and thus quantified the expression of Bcl-2 pretreated with miR-107 mimic or inhibitor using qPCR or western blotting. We found miR-107 to promote the expression of Bcl-2 in primary bovine myoblasts (Figures 4C and 4F). Moreover, we found miR-107 to inhibit the expression of P53 and caspase-9 (Figures 4D–4F). Thus, our results confirm that miR-107 has an anti-apoptotic effect in primary bovine myoblasts.

miR-107 Targeted Wnt3a

In order to explore the regulatory mechanism of miR-107, which inhibits myoblasts differentiation, we speculated and screened *Wnt3a* as a potential target gene of miR-107 by bioinformatics software TargetScan 7.1 and DAVID. *Wnt3a* has a highly conserved binding site in the mRNA 3' UTR, which is complementary to the seed sequence of miR-107. Through Dual luciferase activity assay, we found that miR-107 could significantly decrease Renilla luciferase activity in co-transfect with miR-107 mimic and psi-Wnt3a-3' UTR-W. Conversely, the luciferase activity did not reduce in psi-Wnt3a-3' UTR-Mut treatment group, which had three mutated

nucleotides at seed binding site (Figures 5A and 5B). Similarly, we found that miR-107 markedly suppressed the expression of *Wnt3a* at both mRNA and protein levels (Figures 5C and 5D).

Effect of Wnt3a on Primary Myoblasts Differentiation, Proliferation, and Apoptosis

To determine the function of *Wnt3a* in myoblasts development, we knock down *Wnt3a* using small interfering RNA (siRNA) and found its mRNA and protein levels were both significantly decreased (Figure 6A). Meanwhile, we detected the effects of si-Wnt3a on myogenic differentiation and found that si-Wnt3a inhibited myotube forming and the expression of *MyHC*, *MyoD*, and *MyoG* all were decreased (Figures 6B–6D). In addition, we also detected the expression of *CyclinD1*, *PCNA*, and *CDK2* and found si-Wnt3a to decrease the expression of these genes lightly (Figure 6E). The EdU results revealed si-Wnt3a did not affect cell proliferation (Figures 6F and 6G). For cell apoptosis, marker genes caspase-9, caspase-3, and *p53* were detected using qPCR and western blotting assays. Results showed that si-Wnt3a suppressed their expression and revealed an anti-apoptosis effect (Figures 6H–6J).

circFGFR4 Acts as a Competing Endogenous RNA for miR-107

To identify circRNAs in cattle skeletal muscle, three longissimus muscle samples were obtained from Qinchuan cattle at embryonic stage (90 days) and adult stage (24 months old), respectively. We found that circFGFR4 was highly expressed in muscle (Figure 7A), suggesting a potential role in muscle. The full length of circFGFR4 was 963 nucleotides and named based on its host gene *FGFR4*, which is located on chromosome 7 (Text S1). We verified the amplified PCR product with specific circRNA junctions by Sanger sequencing (Figure S1). In order to understand the role of circFGFR4 in muscle development, we

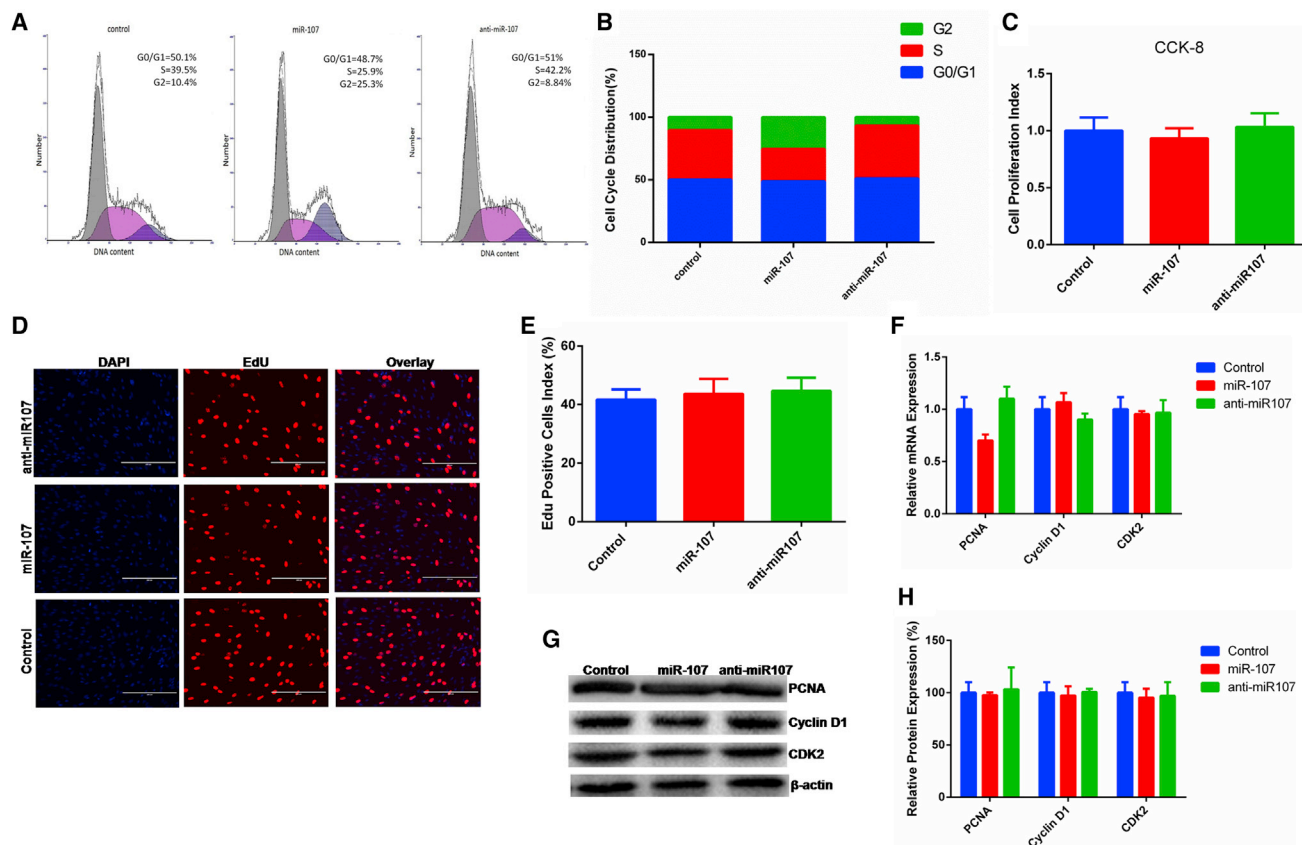


Figure 3. miR-107 Does Not Affect Proliferation of Bovine Primary Myoblasts

(A and B) Bovine primary myoblasts were transfected with miR-107 mimic and inhibitor and cell phase analyzed (A) and counted (B) by flow cytometry. (C) Cell proliferation index was detected by cell counting kit-8 (CCK-8) assay. (D) Cell proliferation was detected with 5-ethynyl-2'-deoxyuridine (EdU); the scale bar represents 200 μ m. (E) EdU-positive cell index statistics are shown. (F–H) The expression of proliferation marker gene PCNA, cyclin D1, and CDK2 was detected by real-time qPCR (F) and western blotting (G), and protein band density was also analyzed (H). Values are means \pm SEM for three individuals. * $p < 0.05$.

transfected pcDNA-circFGFR4 into bovine primary myoblasts and found that circFGFR4 overexpression led to about 6-fold induction of *circFGFR4* RNA (Figure 7B). Softwares RNAhybrid and TargetScan used for miRNA recognition sequences on bovine circFGFR4 revealed the presence of eighteen putative miR-107 binding sites. Luciferase assay revealed that miR-107 significantly inhibited Rluc expression of pCK2-circFGFR4^W (Figure 7C). We have already shown that miR-107 directly targets 3' UTR of Wnt3a to inhibit its expression at mRNA and protein levels (Figure 5). To confirm the effects of circFGFR4 on muscle development were due to miR-107-mediated regulation of Wnt3a, we transfected pcDNA-circFGFR4 and (or) miR-107 mimic into bovine primary myoblasts. In order to confirm that circFGFR4 could bind directly to miR-107, using a biotin-coupled miR-107 mimic, we observed a more than 8-fold enrichment of circFGFR4 in the miR-107-captured fraction compared with the negative control (Figure 7D). Furthermore, biotinylated circFGFR4 pull-down was also performed to provide further evidence for circFGFR4 as a candidate competitive endogenous RNA (ceRNA), and we observed a more than 4-fold enrichment of miR-107 in the

circFGFR4-captured fraction compared with the negative control (Figure 7E). As expected, circFGFR4 increased the mRNA expression of Wnt3a, but this effect was inhibited by miR-107 overexpression (Figure 7F). Together, these findings indicate that circFGFR4, by binding miR-107, acts as a decoy to relieve miRNA inhibiting effect on Wnt3a.

Effects of circFGFR4 on the Cell Differentiation, Proliferation, and Apoptosis

To establish the involvement of circFGFR4 in myoblast differentiation, the expression of MyoG and MyHC was detected in primary bovine myoblasts differentiated for 4 days. We found circFGFR4 significantly increased expression of MyoG at mRNA and protein levels, but when co-transfected with miR-107, the expression of MyoG decreased significantly (Figures 7G and 7H). From immunofluorescence assay in Figure 7I, we found that circFGFR4 promoted MyHC expression and myotube forming, and this effect could be abolished by miR-107 overexpression (Figure 7I). These results suggest that circFGFR4 promotes myoblasts differentiation via regulating level of miR-107.

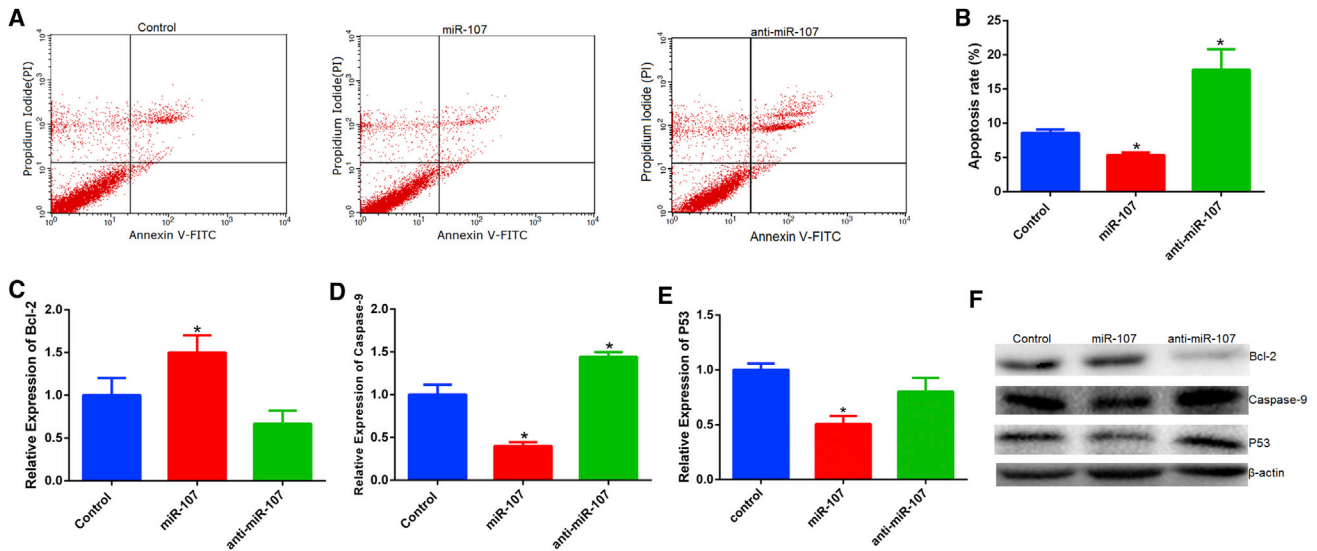


Figure 4. miR-107 Inhibits Apoptosis of Bovine Primary Myoblasts

(A and B) Bovine primary myoblasts were transfected with miR-107 mimic and inhibitor, and cell apoptosis was determined (A) and counted (B) by annexin V-FITC/PI binding followed by flow cytometry. (C–E) The mRNA expression of apoptosis marker genes *Caspase9* (C), *Caspase3* (D), and *P53* (E) was detected by real-time qPCR. (F) The protein expression of apoptosis marker genes was detected by western blotting. Values are means \pm SEM for three individuals. * $p < 0.05$.

To assess the effect of circFGFR4 on cell proliferation, we pretreated primary bovine myoblasts with pcDNA-circFGFR4 and (or) miR-107 mimic using EdU, CCK-8, qPCR, and western blotting assays. We found circFGFR4 did not affect cell proliferation (Figure 8). It has been shown that miR-107 inhibited myoblasts apoptosis; thus, we wanted to know whether circFGFR4 could regulate myoblast apoptosis by sponging miR-107. The annexin V-FITC/PI staining assay showed circFGFR4 relieved the protection effect of primary bovine myoblasts from apoptosis induced by miR-107 overexpression (Figures 9A–9E). Consistently, circFGFR4 significantly increased expression of *p53* (Figure 9F). These results demonstrate that circFGFR4 promotes myoblast differentiation and apoptosis via binding miR-107 (Figure 10).

DISCUSSION

Most studies on muscle development and growth focus on protein-coding genes, miRNAs,^{14,29} and long noncoding RNAs (lncRNAs).^{30–32} However, the occurrence of circRNAs in muscle remains largely unknown. This study is designed to explore circFGFR4 function acting as a sponge for miR-107 in primary bovine myoblasts. We found circFGFR4 binding miR-107 promotes bovine myoblast differentiation via targeting *Wnt3a*.

miR-107 Suppressed Bovine Myoblast Differentiation

miRNA functions mainly focus on tumors and cancers,^{33–35} and there is limited knowledge about muscle development, especially in cattle muscle. In this study, we chose miR-107, whose expression level was higher than the other miRNAs in muscle tissue via the high-throughput RNA-sequence analysis and qPCR. Hence, we hypothesize that miR-107 plays an important role in muscle

development. Ruan et al.³⁶ reported that miR-107 promotes chronic myeloid leukemia cell differentiation. We found that miR-107 significantly inhibited myotube forming and decreased the expression of *MyoD*, *MyoG*, and *MyHC* at mRNA and protein levels, hence revealing its function as a negative regulatory factor on myoblast differentiation. Previous study has shown that miR-107 participates in cell proliferation by negatively regulating *Axin2* in hepatocellular carcinoma.²⁰ However, Feng et al.³⁷ indicate that miR-107 inhibits gastric cancer cell proliferation via targeting cyclin-dependent kinase 6 expression. Our study indicated that miR-107 could inhibit cell proliferation lightly, but not significantly ($p > 0.05$). These results provide new evidence for the function of miR-107 regulating cell proliferation. Apoptosis plays essential roles in maintaining tissue homeostasis and remodeling, especially in the immune system. Many miRNAs have been involved in regulating cell apoptosis, such as miR-146a,³⁸ miR-14,³⁹ and miR-34a.^{40,41} Our study indicated that miR-107 inhibited the apoptosis of myoblasts intensely, suggesting that miR-107 has an anti-apoptotic effect in primary bovine myoblasts.

circFGFR4 Directly Bound miR-107

circRNAs have been demonstrated to sponge miRNAs; however, only several circRNAs have been reported to have functions. For example, a circRNA from the testes-specific *Sry* gene acts as a sponge for miR-138, and circRNAs *CDR1* acts as a sponge for miR-7,^{9,12} suggesting that miRNA sponge effect achieved by circRNAs is a vital mechanism and even very few miRNA binding sites can be functionally important. In this study, we expanded the repertoire of endogenous miRNA sponges. We showed that

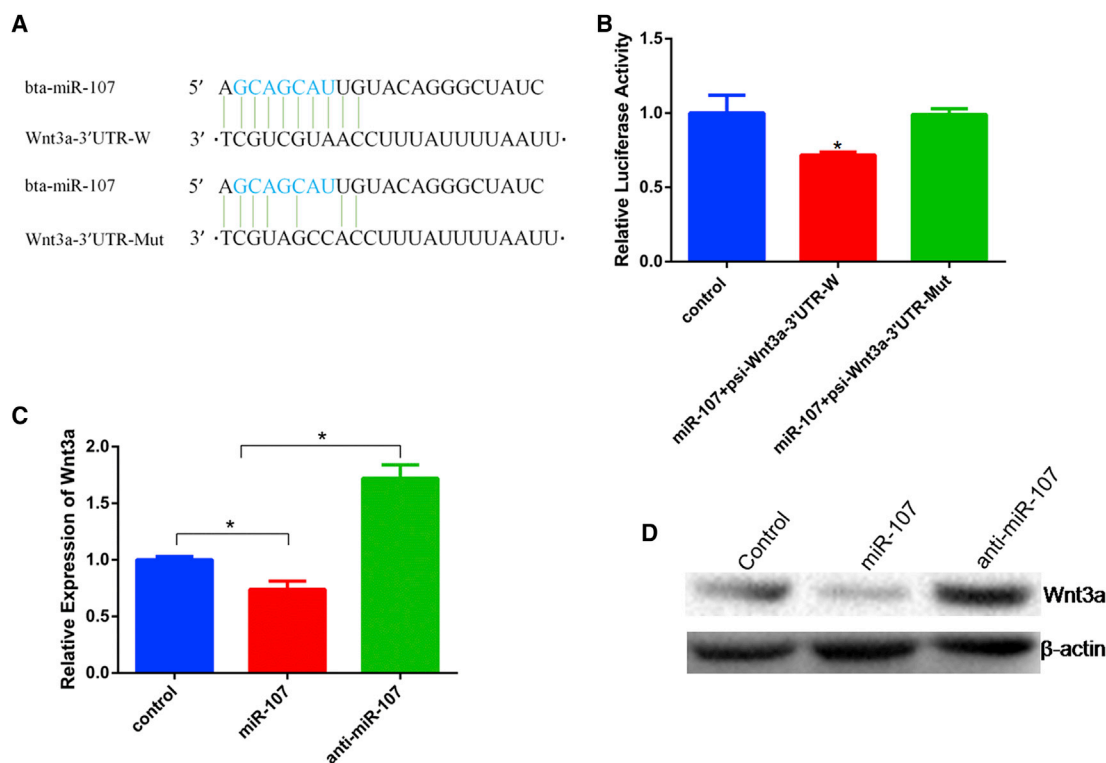


Figure 5. miR-107 Directly Targets Wnt3a Gene

(A) The binding sites analysis in Wnt3a 3' UTR. (B) Cells were co-transfected with miR-107 mimic and psi-Wnt3a-3' UTR-W/Mut, and Renilla luciferase activity was normalized to the firefly luciferase activity. (C) The mRNA expression *Wnt3a* was detected by real-time qPCR. (D) The protein expression of Wnt3a was detected by western blotting. Values are means \pm SEM for three individuals. * $p < 0.05$.

circFGFR4 is a bovine-muscle-development-related circRNA. Luciferase screening and RNA pull-down assays demonstrated that circFGFR4 is a direct target of miR-107 in bovine primary myoblasts. We found circFGFR4 or miR-107 in myoblasts produced an opposite effect in the myoblast differentiation. Consistently, miR-107 protected myoblasts from apoptosis, and this effect could be abolished by circFGFR4 overexpression, suggesting that miRNA sponge effect achieved by circRNAs is a vital mechanism. These results further demonstrated that circFGFR4 could serve as a modulator of cell differentiation and cell survival by sponging miR-107.

circFGFR4 Promotes Myoblasts Differentiation via Binding miR-107 to Relieve Its Inhibition of Wnt3a

Wnt3a plays an important role in Wnt/ β -catenin pathway, and activated Wnt3a induces β -catenin translocating to nuclear and finally activates gene expression, such as cyclin D1.²³ Wnt3a was identified as a target of miR-107 by luciferase activity, real-time qPCR, and western blotting assays in bovine primary myoblasts. We speculate the effects of circFGFR4 on myogenesis were due to miR-107-mediated regulation of Wnt3a. circFGFR4 modulates Wnt3a expression by competing for miR-107 as a ceRNA to regulate myogenic differentiation. The ceRNA regulatory network,

circFGFR4/miR-107/Wnt3a, sheds light on understanding the mechanisms of non-coding RNA regulating muscle development. Thus, modulation of circFGFR4 expression in muscle tissue may emerge as a potent tool to regulate muscle development in the cattle.

MATERIALS AND METHODS

Tissue Sample Preparation

Animal samples used in this study were approved by the Animal Care and Use Committee of Northwest A&F University. All samples from Qinchuan cattle at embryonic stage (90 days) and adult stage (24 months old) were collected at a local slaughterhouse in Xi'an, Shaanxi province. Samples, including muscle, liver, heart, lung, spleen, kidney, brain, and adipose tissues, were collected and immediately frozen in liquid nitrogen. The samples were kept at -80°C until RNA isolation.

RNA Isolation, cDNA Synthesis, and Real-Time qPCR

Total RNA extraction, cDNA synthesis, and real-time qPCR were performed as previously described.^{42,43} For RNase R treatment, 1 μg of total RNA was incubated for 15 min at 37°C with 2 units μg^{-1} of RNase R (Epicenter Technologies, Madison, WI). miRNA-specific stem-loop primers (Table S1) were used for

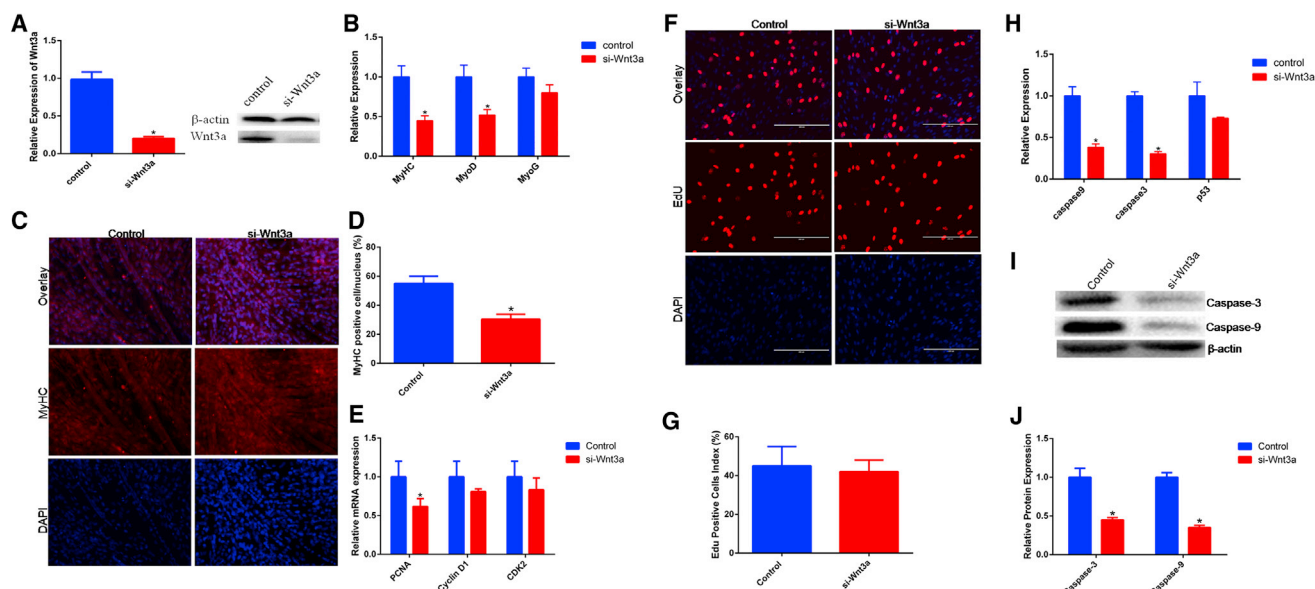


Figure 6. si-Wnt3a Inhibits Differentiation and Apoptosis of Bovine Primary Myoblasts

(A) Bovine primary myoblasts were transfected by si-Wnt3a, and the mRNA and protein expression of Wnt3a were detected by real-time qPCR and western blotting. (B) The mRNA expression of differentiation marker genes was detected by qPCR. (C and D) Bovine primary myocytes were transfected with si-Wnt3a, and cell differentiation was detected by immunofluorescence (MyHC) (C) and observed under a fluorescence microscope (D). (E) The mRNA expression of proliferation marker genes was detected by qPCR. (F and G) Cell proliferation was detected (F) and counted (G) with EdU. (H–J) The expression of apoptosis marker genes caspase-9, caspase-3, and *P53* was detected by qPCR (H) and western blotting (I), and protein band density was also analyzed (J). Values are means \pm SEM for three individuals. * $p < 0.05$.

reverse-transcribed cDNA of miRNAs. The expression levels of miRNAs and the transcript mRNAs were calculated by the $2^{-\Delta\Delta CT}$ method. *U6* (for miRNA) and β -actin were used as the internal control for normalization of the data. All spanning the distal ends of circRNAs with sequence specificity checked using BLAST, qPCR primers were listed in Table S2. For each time point, qPCR was done on three biological replicates.

Vector Construction

The whole length of circFGFR4 was cloned into overexpression vector of pCDNA-3.1(+) using PrimerSTAR Max DNA Polymerase Mix (Takara, Dalian, China). The fragment of the Wnt3a 3' UTR, including binding site of miR-107, was amplified and inserted into psiCHECK-2 vector (Promega, Madison, WI, USA) at the 3' end of Renilla gene using restriction enzymes *Xho* I and *Not* I (TaKaRa, Dalian, China) and T4 DNA ligase (psi-Wnt3a-3' UTR-W). The mutant psiCHECK2-Wnt3a-3' UTR-Mut (psi-Wnt3a-3' UTR-Mut) was generated by mutating complementary to the seed region of the miR-107 using mutagenic primer. Similarly, the vectors of psi-CHECK-circFGFR4-W/Mut (pCK-circFGFR4-W/Mut) were obtained using the same method. Primer sequences are shown in Table S3. All constructs were verified by sequencing.

Cell Culture and Treatment

Primary bovine myoblasts were isolated and cultured from bovine longissimus muscle as previously described.⁴⁴ HEK293T cells

(ATCC, USA) and myoblasts were cultured in high-glucose DMEM supplemented with fetal bovine serum (Hyclone, USA; 10% and 20%, respectively) and double antibiotics (1% penicillin and streptomycin; growth medium [GM]) at 5% CO₂, 37°C. To induce myoblasts differentiation, cells were switched to differential medium (DMEM; 2% horse serum; differentiation medium [DM]) in nearly 90% confluence. Myoblasts were transfected with miR-107 mimic, inhibitor, siWnt3a, or pcDNA-circFGFR4 using Lipofectamine 2000 (Invitrogen, USA) when cell confluence reached approximately 80%. The siWnt3a sequence is 5'-GGGUCUCA UACCUAAGGAC-3'.

Cell Proliferation Assay

To gain insights into the effect of circFGFR4 and miR-107 on myoblasts proliferation, CCK-8 (Multisciences, Hangzhou, China) and EdU incorporation assays (Ribobio, Guangzhou, China) were performed. For the CCK-8 assay, cells were plated into 96-well culture plates at a density of 1×10^4 cells/well in 100 μ L culture medium, and each treatment group had eight independent replicates. After 24 hr of culture at 37°C, 10 μ L of CCK-8 reagent was added to each well and incubation was continued for 4 hr. The absorbance value of all samples was detected using an automatic microplate reader (Molecular Devices, Sunnyvale, USA) at 450-nm wavelength. We also assessed cell proliferation using the Cell-Light EdU DNA cell proliferation kit according to the manufacturer's instructions, and each treatment group had three independent replicates.

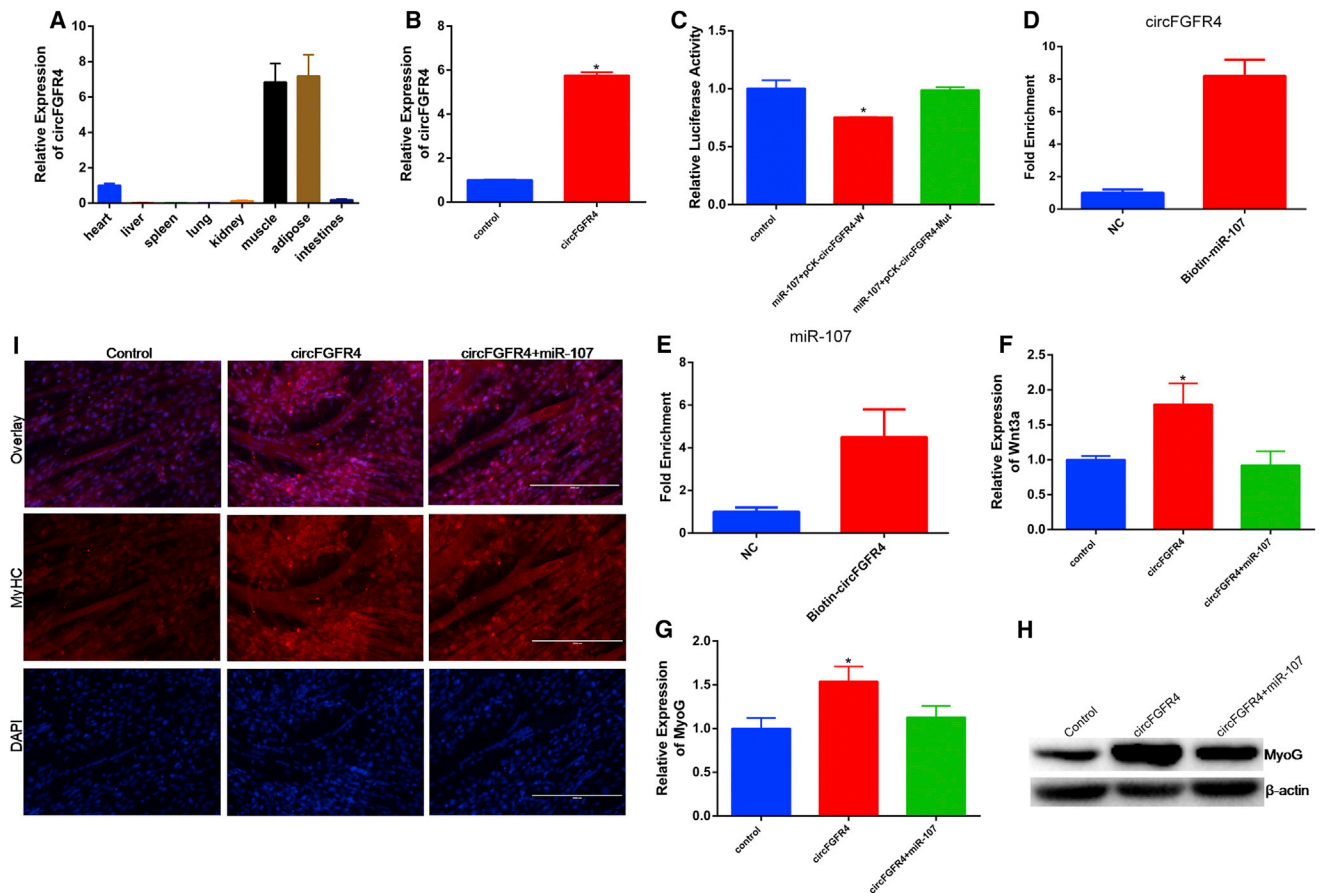


Figure 7. circFGFR4 Binding miR-107 to Promote Cell Differentiation

(A) circFGFR4 expression in different tissues from embryonic Qinchuan cattle detected by real-time qPCR. (B) The expression efficiency of pcDNA-circFGFR4 is shown. (C) Bovine primary myoblasts were co-transfected with miR-107 mimic and pCK-circFGFR4-W or pCK-circFGFR4-Mut. Renilla luciferase activity was normalized to the Firefly luciferase activity. (D) qPCR analysis of circFGFR4 level in the streptavidin captured fractions from the bovine primary myoblasts lysates after transfection with 3' end biotinylated miR-107 or control RNA (NC). (E) Biotin-labeled circRNA was purified and subjected to RNA pull-down assays by incubation with bovine primary myoblasts lysates, followed by qPCR analysis of miR-107 level. (F) The mRNA expression of *Wnt3a* in primary bovine myoblasts transfected with miR-107 mimic and (or) circFGFR4 for 24 hr was detected by qPCR. (G and H) The expression of MyoG in primary bovine myoblasts was detected by qPCR (G) and western blotting (H). (I) Bovine primary myocytes were transfected with pcDNA-circFGFR4 and (or) miR-107 mimic, and cell differentiation was detected by immunofluorescence (MyHC) and observed under a fluorescence microscope. Values are means \pm SEM for three individuals. * $p < 0.05$.

Flow Cytometry for the Cell Cycle and Apoptosis Assays

We analyzed the cell cycle of different treatment groups using the cell cycle testing kit (Multisciences, Hangzhou, China). Myoblasts were grown in six-well plates (1×10^6 cells/well) with 2 mL culture medium. After treatment for 24 hr, we harvested cells and washed them in PBS buffer. Then, 1 mL of DNA stain solution and 10 μ L of permeabilization solution were added to the resuspended cells. After incubating for 30 min in the dark at room temperature, the cell suspension was used for flow cytometry (FACS Canto II, BD BioSciences, USA), and each treatment group had three independent replicates.

Cell apoptosis was measured by annexin V-FITC/PI staining assay. After incubation, cells with different treatment were washed three times with PBS buffer and then resuspended in 500 μ L $1 \times$ binding

buffer. Then, cells were incubated for 10 min in the dark at room temperature in the presence of annexin V-FITC (5 μ L) and PI (10 μ L; Multisciences, Hangzhou, China). Afterward, cells were analyzed using flow cytometry, and each treatment group had three independent replicates. Cells stained with only annexin V were evaluated as being in early apoptosis; cells stained with both annexin V and PI were evaluated as being in late apoptosis stage.

Luciferase Activity Assay

When the cell confluence reached about 80%, the miR-107 mimic and psi-Wnt3a-3' UTR-W or psi-Wnt3a-3' UTR-Mut were co-transfected into HEK293T cells using Lipofectamine 2000. Similarly, miR-107 and pck-circFGFR4-W or pck-circFGFR4-Mut were co-transfected into cells. After incubation for 24 hr, the cells were

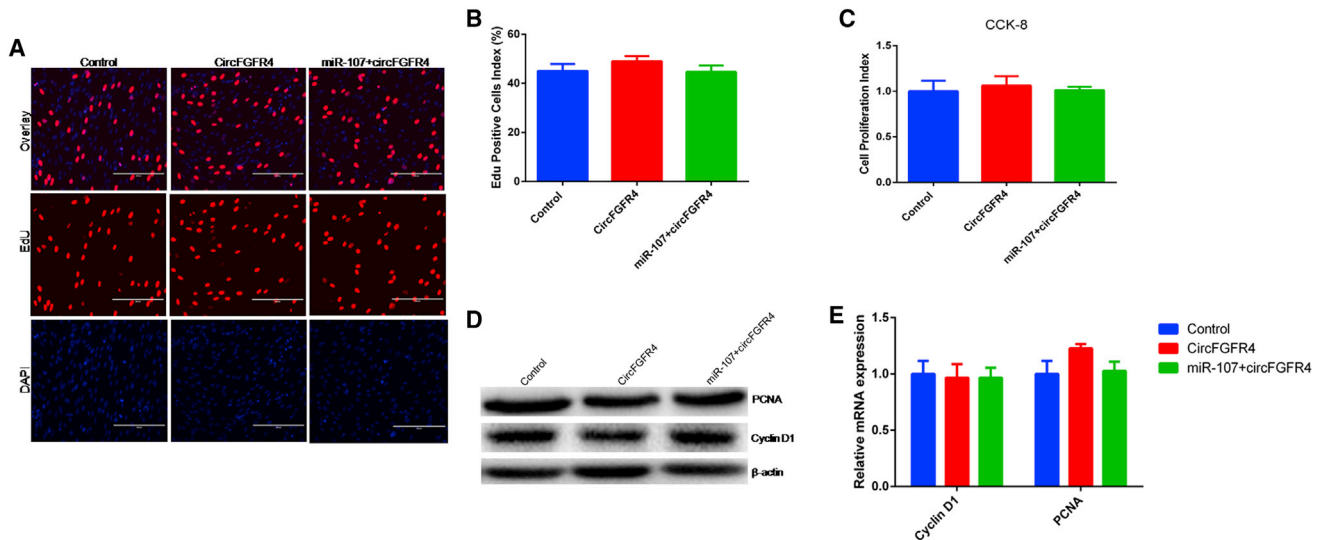


Figure 8. circFGFR4 Did Not Affect Proliferation of Bovine Primary Myoblasts

(A) Cell proliferation index of primary bovine myoblasts was detected with EdU assay; the scale bar represents 200 μ m. (B) The statistics of EdU-positive cell index are shown. (C) Cell proliferation index was detected by CCK-8 assay. (D and E) The expression of PCNA and cyclin D1 was detected by real-time qPCR (D) and western blotting (E). Values are means \pm SEM for three individuals. * $p < 0.05$.

washed with PBS and harvested using 200 μ L passive lysis buffer (PLB). Dual-luciferase activity was measured using an automatic microplate reader (Molecular Devices, Sunnyvale, USA), and the Renilla luciferase activity was normalized against firefly luciferase activity.

Western Blotting

The total proteins were extracted from cells using protein lysis buffer radioimmunoprecipitation assay (RIPA) containing 1 mM PMSF (Solarbio; Beijing, China). The extracts were boiled with 4 \times SDS loading buffer (150 mM Tris-HCL [pH 6.8], 12% SDS, 30% glycerol, 0.02% bromophenol blue, and 6% 2-mercaptoethanol) at 98 $^{\circ}$ C for 10 min, and then 20 μ g total proteins were loaded and separated on 10% SDS-PAGE gels. After electrophoresis, the proteins were transferred to a 0.2 μ m polyvinylidene fluoride (PVDF) membrane that was soaked in formaldehyde and then blocked with 5% skim milk in Tris saline with Tween (TBST) buffer for about 2 hr at room temperature. The membrane was then incubated overnight with primary antibodies specific for anti-Wnt3a, anti-MyoD, anti-MyHC, anti-MyoG, anti-PCNA, anti-cyclin D1, anti-CDK2 (Abcam, Cambridge, England), anti-Bcl-2, anti-caspase-3, anti-caspase-9, anti-P53 (Wanleibio, Haerbin, China), and anti- β -actin (Sungene Biotech, Tianjin, China) at 4 $^{\circ}$ C. The PVDF membrane was washed three times with TBST buffer and then incubated with secondary antibody for 2 hr at room temperature. β -actin was used as the internal control with a secondary antibody that was horseradish peroxidase (HRP)-labeled anti-mouse immunoglobulin G (IgG) (Sungene Biotech, China). Finally, antibody reacting bands were detected using enhanced chemiluminescence (ECL) luminous fluid (Solarbio, China).

Immunofluorescence and Microscopy

Primary bovine myoblasts at the stage of 95% confluence were washed three times with PBS (pH 7.4) and permeabilized for 15 min in PBS plus 0.5% Triton X-100 before fixation in 4% paraformaldehyde in PBS for 30 min. Immunostaining was carried out as follows: the cells were incubated at 4 $^{\circ}$ C overnight with primary antibody-MyHC, diluted in 1% BSA. The cells were washed and then incubated at room temperature for two hours with the corresponding secondary antibody goat anti-rabbit IgG heavy and light chain (H&L; 1:1,000; Abcam, Cambridge, MA) diluted in 1% BSA in PBS. DNA was visualized using 5 mg/mL DAPI. Finally, the cells were washed three times with PBS and observed with fluorescence microscope (DM5000B; Leica Microsystems, Germany).

Biotin-Coupled miRNA and circRNA Capture

The biotin-coupled miRNA and circRNA pull-down assays were performed as described.^{45,46} Briefly, the 3' end biotinylated miR-107 or circFGFR4 (Genesee, Guangzhou, China) were transfected into bovine primary myoblasts at a final concentration of 20 nM for 1 day. The biotin-coupled RNA complex was pulled down by incubating the cell lysates with streptavidin-coated magnetic beads (Life Technologies). The abundance of circFGFR4 or miR-107 in bound fractions was evaluated by qPCR analysis.

Statistical Analysis

The quantitative results are presented as mean \pm SEM based on at least three independent experiments. All data in this study were analyzed by ANOVA for p value calculations using SPSS v17.0

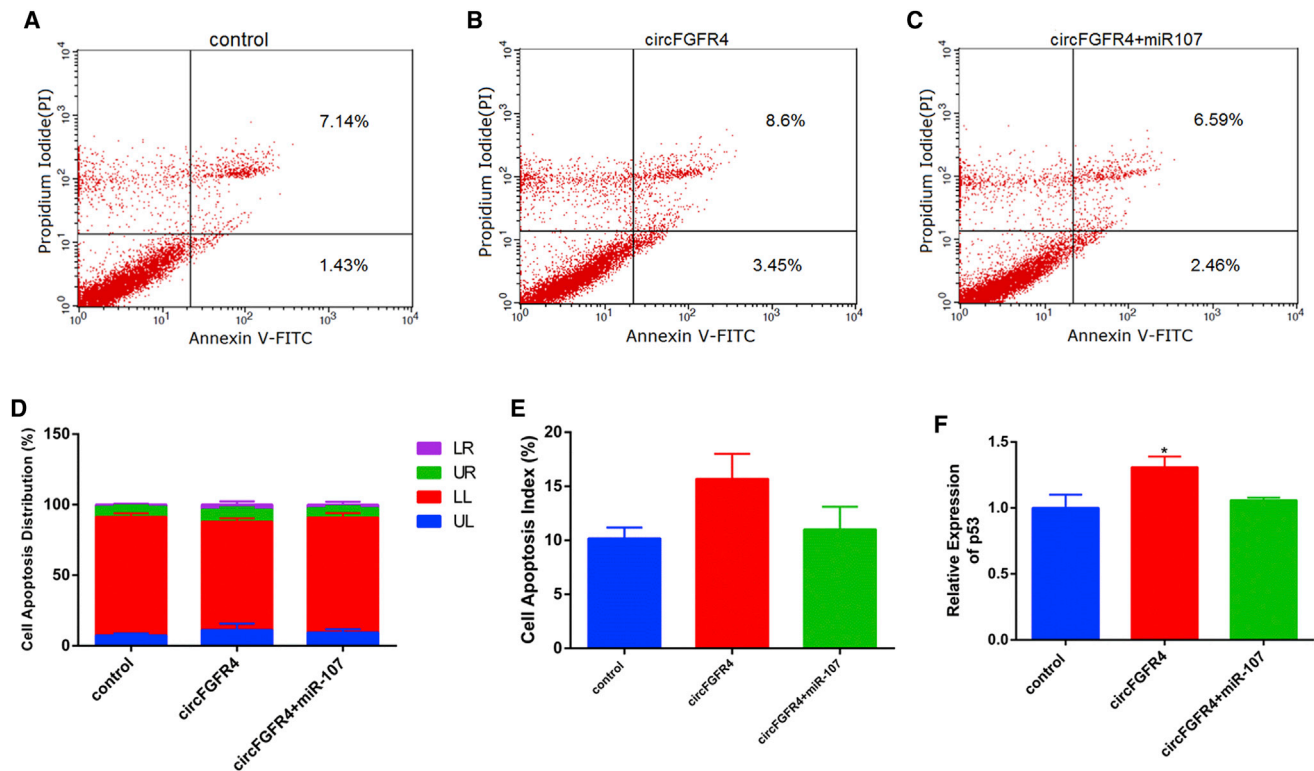


Figure 9. circFGFR4 Promotes Apoptosis of Bovine Primary Myoblasts

(A–E) Bovine primary myoblasts were transfected with miR-107 mimic and (or) circFGFR4, and cell apoptosis was determined (A–C) and counted (D and E) by annexin V-FITC/PI binding followed by flow cytometry. (F) The mRNA expression of *P53* detected by real-time qPCR is shown. Values are means ± SEM for three individuals. **p* < 0.05.

software. *p* < 0.05 was considered statistically significant differences among means. The software ImageJ was utilized for gels image gray value analysis.

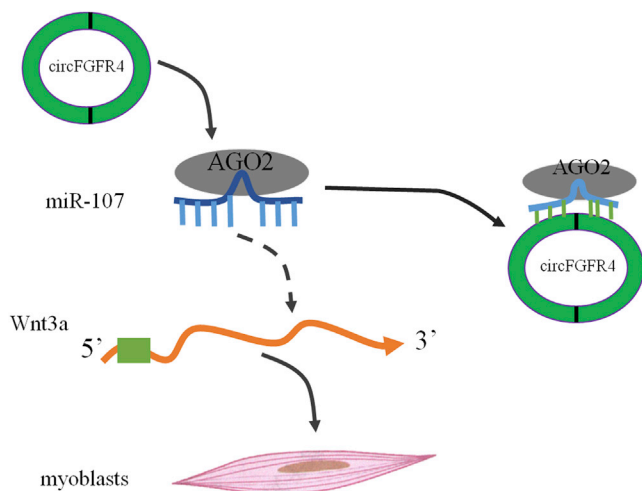


Figure 10. Schematic Diagram of circFGFR4 Competitively Binding miR-107 in Bovine Primary Myoblasts

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure, three tables, and text and can be found with this article online at <https://doi.org/10.1016/j.omtn.2018.02.012>.

AUTHOR CONTRIBUTIONS

H.C., H.L., and X.W. designed the study. H.L., X.W., and J.Y. performed the experiments, drafted the manuscript, and contributed equally to this work. D.D., J.Y., D.H., Y.H., X.L., and C.L. helped perform the experiments and analyzed the data. Y.M., F.L., and Y.B. helped collect tissue samples. M.P. helped modify the language of this manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Supplemental Information

circFGFR4 Promotes Differentiation of Myoblasts via Binding miR-107 to Relieve Its Inhibition of Wnt3a

Hui Li, Xuefeng Wei, Jiameng Yang, Dong Dong, Dan Hao, Yongzhen Huang, Xianyong Lan, Martin Plath, Chuzhao Lei, Yun Ma, Fengpeng Lin, Yueyu Bai, and Hong Chen

Supplementary information

Table S1 Stem-loop primers for reverse-transcribed miRNAs

miRNA ID	stem-loop primer
bta-miR-378	gtcgtatccagtcaggggtccgaggtattcgactggatacgacGCCTTC
bta-miR-107	gtcgtatccagtcaggggtccgaggtattcgactggatacgacGATAGC
bta-miR-25	gtcgtatccagtcaggggtccgaggtattcgactggatacgacTCAGAC
bta-miR-101	gtcgtatccagtcaggggtccgaggtattcgactggatacgacTTCAGT
bta-miR-128	gtcgtatccagtcaggggtccgaggtattcgactggatacgacAAAGAG
bta-miR-140	gtcgtatccagtcaggggtccgaggtattcgactggatacgacTCCGTG
bta-miR-7f	gtcgtatccagtcaggggtccgaggtattcgactggatacgacAACTAT
bta-miR-499	gtcgtatccagtcaggggtccgaggtattcgactggatacgacAAACAT
bta-miR-423-5p	gtcgtatccagtcaggggtccgaggtattcgactggatacgacAAAGTC

Table S2 Primers for qPCR

miRNA ID	Forward primer 5'→3'	Reverse primer 5'→3'
Bta-miR-107	ggAGCAGCATTGTACAGGGCTATC	GCAGGGTCCGAGGTATTC
bta-miR-378	gcgcACTGGACTTGGAGTC	GCAGGGTCCGAGGTATTC
bta-miR-25	gcgcgcCATTGCACTTGTCTC	GCAGGGTCCGAGGTATTC
bta-miR-101	gcgcgcgcTACAGTACTGTGATA	GCAGGGTCCGAGGTATTC
bta-miR-128	gcgcTCACAGTGAACCGGT	GCAGGGTCCGAGGTATTC
bta-miR-140	gcgcgcTACCACAGGGTAGAA	GCAGGGTCCGAGGTATTC
bta-miR-7f	gcgcgcacaTGAGGTAGTAGATTG	GCAGGGTCCGAGGTATTC
bta-miR-499	gcgcgcTTAAGACTTGCAGTG	GCAGGGTCCGAGGTATTC
bta-miR-423-5p	attTGAGGGGCAGAGAGC	GCAGGGTCCGAGGTATTC
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT
Wnt3a	GCAGAGGGCATCAAATC	CTGTTGTTGATGGTGGTG
β-actin	CATCCTGACCCTCAAGTA	CTCGTTGTAGAAGGTGTG
MyHC	TGCTCATCTACCAAGTTCC	CACTCTTCACTCTCATGGACC
MyoG	CAAATCCACTCCCTGAAA	GCATAGGAAGAGATGAACA
MyoD	ACGGCATGATGGACTACAGC	AGGCAGTCGAGGCTCGACA
BCL-2	ATGACCGAGTACCTGAAC	CATACAGCTCCACAAAGG
Caspase9	TGGTGGTCATCCTGTCTC	CATCCATCTGTGCCATAAAC
Caspase3	AGAACTGGACTGTGGTATT	TCTGCCTCAACTGGTATT
P53	CACAGCCAAAGAAGAAAC	CTCTCGGAACATCTCATAG

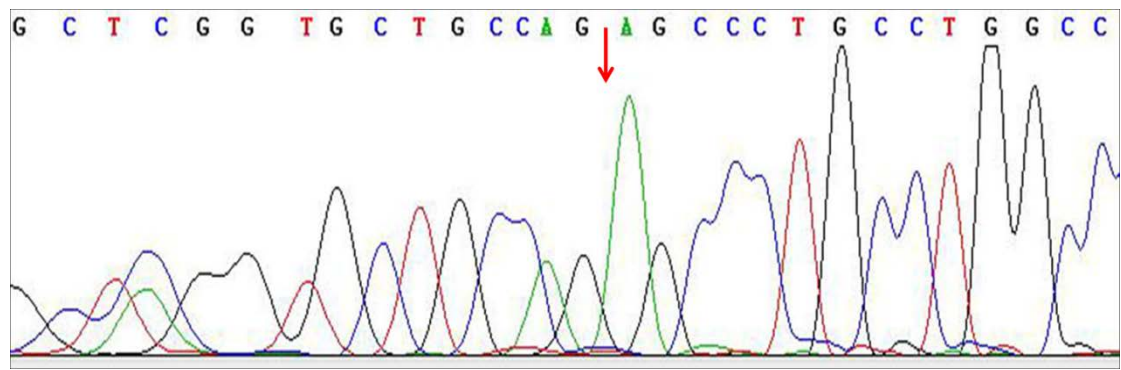
PCNA	TCCAGAACAAGAGTATAGC	TACAACAGCATCTCCAAT
CyclinD1	CCGTCCATGCGGAAGATC	CAGGAAGCGGTCCAGGTAG
CircFGFR4	GGCAACTCCATCGGCCTTTC	CCGCAGCATAACCGCACCA

Table S3 Primers for vector construction

Name	Primer sequence 5'→3'
pcDNA-circFGFR4-F	CCCA <u>AAGCTTTT</u> TACCCCCAGCAAGCACCT
pcDNA-circFGFR4-R	GGGGTACCACGTGCCCATTC AAGGGGC
Psi-CHECK-circFGFR4-W-F	CCGCTCGAGACGACTTCTTTGTCAGAAAGAC
Psi-CHECK-circFGFR4-W-R	ATAAGAATGCGGCCGCCATTCTCTAAGTCAGGATCCA
Psi-CHECK-circFGFR4-Mut-F	GCCTGTGCGGTTATCATACGGGCGGGCTGAGC
Psi-CHECK-circFGFR4-Mut-R	GCTCAGCCCGCCCGTATGATAACCGCACAGGC
Psi-CHECK-Wnt3a-3'UTR-W-F	CCGCTCGAGGGCTTACCTGGTGTGTTTCG
Psi-CHECK-Wnt3a-3'UTR-W-R	ATAAGAATGCGGCCGCTCTGTTTGTCTAGCTCCTGTG
Psi-CHECK-Wnt3a-3'UTR-Mut-F	ATTTTATTTCCACCGATGCTATATCCACCAT
Psi-CHECK-Wnt3a-3'UTR-Mut-R	ATGGTGGATATAGCATCGGTGGAAATAAAAT

Notes: The nucleotides with underline is the restriction enzyme cutting site.

Figure S1



Text S1: CircFGFR4 sequence

circFGFR4>AGCCCTGCCTGGCCCCAGCCCGGAGCAGCAAGAGCAGGAGT
TGACGGTGGCCCTTGGGCAGCCTGTGCGGTTATGCTGCGGGCGGGCTGAG
CGCAGTGGCCACTGGTACAAGGAGGGCAGTCGCCTGACACCTGCTGGCCG
GGTACGAGGCTGGAGAGGCCGCTTGGAGATTGCCAGCTTCCTACCCGAGG
ATGCTGGCCAGTACCTCTGCCTATCACGAGGCTCCTTGCTTCTGCACAACG
TCACCTTGGTTGTGGACGACTCCATGACCTCCAGCAATGGCGACGAGGAC
CCCAAGATCCACAGGGGCCCCTTGAATGGGCACGTTTACCCCCAGCAAGC
ACCCTACTGGACGCACCCCCAGCGCATGGAGAAGAACTGCATGCTGTGC
CTGCCGGGAACACCGTCAAGTTCCGCTGTCCAGCTGCAGGCAACCCCATG
CCCACCATCCGCTGGCTCAAGGATGGACAGGACTTCCACGGGGAGCATCG
CATTGGAGGCATTCGGCTGCGCCACCAGCACTGGAGCCTGGTGATGGAAA
GCGTGGTGCCCTCTGACCGTGGCACTTACACCTGCCTCGTGGAGAATTCTT
TGGGCAGCATTGCTATAGCTACCTGCTGGACGTGCTGGAGCGGTCCCCGC
ACCGGCCCATCCTTCAGGCAGGGCTCCCAGCCAACACCACGGCTGTGGTG
GGCAGTGACGTGGAAGTCTGCTCTGCAAGGTGTACAGCGACGCCAGCCCCA
CATCCAGTGGCTGAAGCACATCGTCATCAACGGCAGCAGCTTCGGTGCCG
ACGGCTTCCCCTATGTGCAAGTCTTAAAGACAGCGGACATCAATAGCTCAG
AGGTGGAGGTCTTGTACCTTCGGAATGTATCTGCTGAGGATGCAGGCGAGT
ACACCTGCCTGGCGGGCAACTCCATCGGCCTTTCCTACCAGTCGGCCTGGC
TCACGGTGCTGCCAG