CircRIMKLB Promotes Myoblasts Proliferation and Inhibits Differentiation via Sponging miR-29c to Release KCNJ12

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Research

Keywords: non-coding RNA, myoblast, protein Immunoprecipitation, vivo

Posted Date: July 23rd, 2020

DOI: https://doi.org/10.21203/rs.3.rs-43665/v1

Abstract

Background

Muscle development is a complex progress that regulated by many factors, and non-coding RNA (ncRNA) is found to act as a vital part of performing normal function of muscle cells. Circular RNA(circRNA), a kind ncRNA with closed RNA, is reported to affect life process. However, there are limited studies about how circRNAs affect muscle development and this study is aimed to find how circ RNA Ribosomal modification protein rimK like family member B (circRIMKLB) affects muscle development.

Results

We found circular circRIMKLB expressed differentially at different stages of muscle development. The results revealed that circRIMKLB could promote myoblasts proliferation and inhibits differentiation. MicroRNA-29c (miR-29c) was identified as a downstream of circRIMKLB by dual-luciferase reporter assay and RNA binding proteinImmunoprecipitation (RIP) assay. Besides, Channel subfamily J member 12 (KCNJ12) was proved to be a novel target of miR-29c via dual-luciferase reporter assay, quantitative real time polymerase chain reaction(qRT-PCR) and western blot. We also explored the effect of circRIMKLB and KCNJ12 on muscle regeneration after injury *in vivo* and found that circRIMKLB and KCNJ12 could participate in regulating cell cycle.

Conclusions

In conclusion, we proved circRIMKLB could sponge miR-29c to affect the expression level of KCNJ12 and eventually affect myoblast proliferation and differentiation and participate in cell cycle regulation in muscle regeneration after injury *in vivo*.

Background

As one of the most abundant tissue in the body, skeletal muscle, consisting of muscle fibers, neurons, vasculature, connective tissue and so on, performs multiple functions, such as generating movements, producing heat, storing protein reserves and so on[1-3]. Skeletal muscle originally originated from the progenitor cells of the somatic mesoderm except esophagus and facial muscles during the period of embryonic development[4]. Progenitor cells continue to proliferate meanwhile expressing *MyoG*enic determinants and differentiating into mononuclear myoblasts via mesenchymal stem cells. Mononuclear myoblasts will continue to fuse into myotubes and eventually differentiate into various muscle fibers. Adult mammalian skeletal muscle is stable under normal conditions, with only sporadic fusion of satellite cells to compensate for muscle turnover caused by daily wear and tear[5]. However, when injury occurs, satellite cells activate, proliferate and differentiate to give rise to myoblasts. Myoblasts then fuse with each other to form multinucleated myotubes, thereby producing adult muscle fibers, which is very similar to the myogenesis during embryonic period, suggesting that studying muscle development is also helpful in understanding muscle damage repair[6]. Muscle growth, development, and regeneration are important

components of human and animal health. The function loss or reduction of skeletal muscle often leads directly or indirectly to increased morbidity and mortality by developing secondary diseases such as diabetes, obesity, cardiovascular and respiratory diseases[7]. Thus, to understand the mechanisms that influence the process of regulating skeletal muscle function and development is one of key priorities.

It has been reported that non-coding RNAs (ncRNAs) regulate the myogenesis and muscle regeneration. Circular RNAs (CircRNAs), terminated with 5'caps and 3'tails, are a category of endogenous non-coding RNAs with the character of covalently closed loop structures, which makes circRNA more stable and more tolerable to RNase R than other ncRNAs[8-10]. Compared with other kind of non-coding RNAs such as miRNAs and long non-coding RNAs, knowledge about circRNA is in the rising stage but still with relatively few studies. Increasing studies reveal the multiple manners of circRNAs in regulating gene expression, for example, circRNAs could sponge miRNAs in a ceRNA manner or bind proteins as a scaffolding and part of circRNAs were found to have the ability of being translated into peptide[11-13]. According to their sources, circular RNAs can be divided into three types, exonic circRNAs, intronic circRNAs and exonic-intronic circRNAs[14, 15]. In general, most exonic circRNAs are localized in the cytoplasm and could function as a sponge for miRNAs to affect post-transcriptional regulation, which means that circRNAs could be a member of competing endogenous RNAs (ceRNAs)[16]. For instance, *ciRS-7/CDR1* is regarded as the inhibitor of miR-7 to regulate hepatocellular carcinoma[17]. Some intronic circRNAs and exonic-intronic circRNAs regulated the transcription in the nucleus, for instance, ci-ankrd52 is concentrated in the transcription site of its host gene, and acts with RNA polymerase to promote the transcription of its own coding gene in the manner of cis-regulator[18]. Besides, *EIciRNA*s interact with *U1* snRNP and enhance transcription of their parental genes[19]. CircRNAs are widely involved in a variety of life activities, such as cell proliferation, differentiation, tissue development, cancer occurrence, immune response and so on[20-22]. It has been found that CircRNAs are highly enriched in striated muscle, indicating that circRNAs service as a new regulatory network that contributes to skeletal muscle development, myogenesis and regeneration, etc.[16]. However, the existing research on how circular RNA affecting muscle development is relatively rare, so the study on circular RNA can help to gain a deeper understanding of the regulatory network for muscle development.

Here, we focused on the regulatory function(s) of the circular RNA Ribosomal modification protein rimK like family member B (circRIMKLB) during myogenesis. In this study, circRIMKLB*,* which is abundant in skeletal musclewas identified as a novel circRNA. Our work showed that circRIMKLB promoted myoblasts proliferation and inhibited myoblasts differentiation via sponging *miR-29cin vitro*, and participated in cell cycle regelation in muscle regeneration after injury *in vivo*. Besides, potassium inwardly rectifying channel subfamily J member 12 (KCNJ12) was conformed as a novel target of *miR-29c*. In a word, circRIMKLB releases its function as a molecular sponge to absorbing *miR-29c* and enhancing the expression ofKCNJ12 in muscle cells.

Materials And Methods

Animal experiment

Adult C57BL/6 mice of ~5 weeks old were purchased from Air Force Medical University (Xi'an, China). All experiments were performed according to the protocols approved by the Animal Care Committee of Northwest A&F University. Mice tibialis anterior of the left leg were injected with 10 μM cardiotoxin (CTX; Whiga) to induce muscle injury model. In order to make sure that the muscle injury was induced successfully, Paraffin sections of tibialis anterior was used for hematoxylin–eosin staining 24 h after the injection. Plasmid at the mass of 6.25 μg of PCD2.1-circ RIMKLB or PCD2.1 plasmid as well as pcDNA3.1-*KCNJ12* or pcDNA3.1(+) were injected with EntransterTM-in vivo transfection reagent (Engreen Biosystem Co., Ltd.) following manufacturer's instructions at 12 and 48h after cardiotoxin treatment. Mice tibialis anterior of the left leg were harvested at 72h for HE staining, RNA and protein extraction.

Vectors construction

The full length of circRIMKLB and the coding sequences (CDS) of KCNJ12 was cloned into PCD2.1 plasmid and pcDNA3.1 plasmid, respectively. Besides, part of pri-*miR-29c* containing pre-*miR-29c* was inserted into pcDNA3.1(+). The 3'-UTR fragment of KCNJ12（Wt-*KCNJ12*） and circRIMKLB (Wt-circRIMKLB) containing binding site of *miR-29c* was amplified and inserted into psiCHECK-2 vector (Promega, Madison, WI, USA). The mutant psiCHECK2-KCNJ12-3'UTR-Mut (Mut-KCNJ12) and psiCHECK2-circRIMKLB-Mut (Mut-circRIMKLB) was generated by mutating complementary to the seed region of the miR-29c using mutagenic primer. Primers used above are listed in Supplementary Table 1.

Cell culture and transfections

Bovine primary myoblast cells were isolated and cultured from bovine longissimus muscle as previously described[23]. HEK293T cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) with 10% FBS (20% FBS for Bovine primary myoblasts) and 1% of antibiotics (100 U/ml penicillin, 0.1 mg/L streptomycin) 37°C, 5% CO_2 environment. KCNJ12 expression plasmid (pcDNA3.1) was constructed by GENEWIZ (Nanjing, China) using HindIII and EcoRI. *KCNJ12*-siRNA (5′-ACATCGAGTTCGCCAACAT-3′) and circRIMKLB-siRNA (5′-GCTGAACCTCTACAACACT-3′) was purchased from Ribobio (Guang zhou, China). Plasmids and siRNAs were transfected into bovine primary myoblasts by using XfectTM Transfection Reagent or XfectTM RNA Transfection Reagent (Takara, Dalian, China) following manufacturer's instructions, respectively.

Cell proliferation assay

In order to investigate the effects of *KCNJ12* and circRIMKLB on bovine primary myoblasts, Cell Counting Kit-8(CCK-8) assay and 5-Ethynyl-20-deoxyuridine (EdU) proliferation assay were performed. Cells at the density of 1×10^4 in 96-well plates were added 10μL of CCK-8 reagent 20h after transfection. After 4h incubation, the absorbance value of all samples was detected using an automatic microplate reader (Molecular Devices, Sunnyvale, USA) at 450-nm wavelength. Cell proliferation was also detected by using the Cell-Light EdU DNA cell proliferation kit (RiboBio, Guangzhou, China) in 96-well plates at a density of 5×10^3 cells/well as the manufacturer's instructions listed.

Flow cytometry for the Cell Cycle and Apoptosis Assays

Bovine primary myoblasts were grown in 60mm plates with the number of cells is about 2× 10^6 per well. While transfected 24 hr later, myoblasts were collected and washed in PBS buffer. Then, 1mL PI/RNase Staining Buffer (BD Pharmingen™, USA) were added and 15min later, cell suspension was subjected to flow cytometry (BD FACSAria, BD BioSciences, USA).

Cell apoptosis was detected by annexin V-FITC/PI staining assay. Cell were treated based on the protocol of Cell Cycle Testing Kit (BestBio, Shanghai, China) according to the manufacture and the cell suspension was analyzed by flow cytometry (FACS Canto TM II, BD BioSciences, USA).

Quantitative real-time polymerase chain reaction(qRT-PCR)

Total RNA in bovine primary myoblasts and mice tibialis anterior were extracted via using RNAiso Plus (TaKaRa, Dalian, China). Total RNA was reverse-transcribed to cDNA with Prime Script RT reagent Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The SYBR Green Master Mix (genstar, Beijing, China) was used for qRT-PCR applications. GAPDH was used to normalize data and the relative expression level of genes was calculated by the $2^{-\Delta\Delta Ct}$ method. All primers used were listed in table 1.

Western blot

The total proteins from bovine primary myoblasts were extracted using protein lysis buffer radioimmunoprecipitation assay (RIPA) containing 1 mM PMSF (Solarbio, Beijing, China). Besides, total proteins from mice tibialis anterior were extracted by using RNAiso Plus regent (Takara, China). A BCA Kit (Takara, Dalian, China) was used to determine protein concentration. Then protein samples were boiled with 5×SDS loading buffer(Heart, Xian, China) for 10 min at 98 ℃ and 20 μg total proteins were separated via using 10% SDS-PAGE gels, and then transferred the separated protein samples onto a 0.2 μM polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After sealed with 5% skim milk in Tris saline with Tween (TBST) buffer for about 2 hr at room temperature, membrane was incubated with primary antibodies specific for anti-p27, anti-CDK2, anti-*MyoD*, anti-*MyoG*, anti-β-actin at 4 ℃ overnight. The PVDF membrane was incubated with secondary antibody for 1.5h after washed with TBST for 3 times. The membranes were exposed with ECL Plus (Sangon, Shanghai, china) and quantified with the ChemiDoc XRS+ system (Bio-Rad Laboratories, Shanghai, China). All of the antibodies used are listed in Supplementary Table 2.

Fluorescence in situ hybridization (FISH)

RNA Fish assay was used to find the location of circRIMKLB using Dig-labeled probe(5'-ATATAGACTCCTCCCAGGTTTCCTTGTTCA-3') as well as bta-*miR-29c* by using a Cy3-labeled probe (5'-TAACCGATTTCAGATGGTGCTA-3'). Cells were first treated with 4% paraformaldehyde for 20min and digested with proteinase K(20ug/ml) for 8 minutes. After washed with PBS for 3 times, cells on a glass

coverslip was hybridized with circRIMKLB probe at 37℃ overnight. Then cells were washed with 2×SCC, 1×SCC, 0.5×SCC for 10min, respectively. Dropped with blocking serum of BSA for 30min, cells were added with anti-DIG-HRP and then FITC-TSA. As last, bta-*miR-29c* probe and DAPI, respectively. cells were observed with an inverted fluorescence microscope (ECLIPSE TI-SR, NIKON, Japan).

Immunocytochemical analysis

Muscle primary myoblasts were induced to differentiation for 4 days and then added with 4% paraformaldehyde for 20 min after washed by PBS. Then 0.5% Triton X-100 dissolved in PBS was used to incubated myoblasts for 20 min. After incubated with 5% BSA for 30 min, myoblasts were added with anti-MyHC antibody overnight. Next, myoblasts were incubated with Alexa Fluor 594-conjugated goat anti-mouse IgG for 2 h and then DAPI for 15 min, followed by acquiring image.

Dual-Luciferase Activity Assay

For dual-luciferase activity assay, pcDNA3.1-*miR-29c* or pcDNA3.1(+) and Wt- circ*RIMKLB*/Mut-circ*RIMKLB* were co-transfected into HEK293T cells using X-FECT (Takara, Dalian, China) when the cell confluence reached approximately 80% in 96-well dish. Besides, pcDNA3.1-*miR-29c* or pcDNA3.1(+) and Wt-*KCNJ12* /Mut-*KCNJ12* were co-transfected into HEK293T cells using the same method. After incubation for 24 hrs, the cells were harvested and luciferase activities were measured by using the Dual-Luciferase Reporter Assay System (Promega, Madison, AL) on an MPPC luminescence analyzer (HAMAMATSU, Beijing, China).

RNA-binding protein immunoprecipitation (RIP)

In order to explore whether circRIMKLB function as a molecular sponge through Ago-2 protein, RNA-binding protein immunoprecipitation assay of AGO protein was performed with an RNA-Binding Protein Immunoprecipitation Kit (17–701, Merck, Millipore) as the manufacturer said. Then, qRT-PCR was performed on the Ago-2 associated RNA mixture absorbed by the magnetic beads.

Statistics

All experiments were performed in triplicate, and data are presented as the mean value ±standard deviation (SD). The statistical significance between groups was determined using the Student's t-test using SPSS statistical software. $p < 0.05$ was considered to be statistically significant.

Results

Identification of the circular structure and expression file of circRIMKLB

CircRIMKLB, arising from the *RIMKLB* gene, is located at chromosome 5 and consists of the head-to-tail splicing of exon 2 (Fig. 1A). In order to find whether circRIMKLB was a real circular RNA, the Sanger sequencing was performed to find the head-to-tail splicing site with a pair of back-to-back primers (Fig.

1A). Then a PCR assay was performed with divergent and convergent primers to detect the tolerance of RNase R and the results showed that circRIMKLB is more stable than liner *RIMKLB* while treated with RNase R (Fig. 1B). Additionally, a qRT-PCR was performed to detect the stability of circRIMKLB and liner RIMKLB under actinomycin D treatment and the results suggested that circRIMKLB has better survivability (Fig. 1C). The secondary structure of circRIMKLB was analyzed (Fig. 1D). All results above conformed that circRIMKLB is a real circular RNA. The expression files of circRIMKLB indicated that it expressed in heart, liver, spleen, lung, kidney, and skeletal muscle of fetal stage and highly expressed in lung, kidney and skeletal muscle relatively (Fig. 1E). Though circcircRIMKLB also expressed in heart, liver, spleen, lung, kidney, and skeletal muscle of adult stage, it has a low expression in skeletal muscle relatively (Fig. 1F).

CircRIMKLB promotes proliferation and inhibited differentiation of bovine primary myoblasts.

To clarify the function of circRIMKLB on the development of skeletal muscle, CCK-8 assay, EdU assay, cell phase assay, qRT-PCR and western blot were performed while circRIMKLB was overexpressed or inhibited 24h later. CCK-8 assay revealed that circRIMKLB could enhance the cell viability (Fig. 2A). EdU assay showed that circRIMKLB increased the percentage of proliferative cells (Fig. 2B). Cell cycle analysis revealed that circRIMKLB increased the proportion of myoblasts in S phases and decreased the percentage of cells in G1/G0 phase but had no effect on the proportion of cells in the G2 phases, suggesting that circRIMKLB may have a crucial effect on cell proliferation (Fig. 2C). Besides, qRT-PCR and western blot shown that circRIMKLB promoted the expression of cell-proliferation-related genes such as Cyclin Dependent Kinase Inhibitor 1B (CDKN1B/p27), Cyclin D1(CCND1) and Cyclin-dependent-kinase 2(CDK2) in mRNA and protein levels (Fig. 2D and E). Next, we also explored the effect of circ*RIMKLB* on myoblasts differentiation and the results of MyHC immunofluorescence staining of myotubes showed that the number of myotubes was reduced and increased when circRIMKLB was overexpressed and inhibited (Fig. 2F). qRT-PCR and western blot also indicated that circRIMKLB could inhibit the expression of the differentiation related gene MyoD and MyoG, suggesting that circRIMKLB inhibit the differentiation of bovine primary myoblasts (Fig. 2G ang H). In these cases, we can conclude that circRIMKLB participated in regulating cell cycle thus to promote the proliferation, inhibiting the differentiation of bovine primary myoblasts as well.

CircRIMKLB acts as a competing endogenous RNA for miR-29c.

Since previous studies implicate the roles of exonic circRNAs mostly located in cytoplasm and mainly function as miRNA sponges[24, 25], fluorescence in situ hybridization（FISH）assay was used to figure out the localization of circRIMKLB and the result showed that circRIMKLB mainly exited in cytoplasm(Fig. 3A). Further experiments of RNA Binding Protein Immunoprecipitation Assay indicated the endogenous binding of Ago-2 to circRIMKLB (Fig. 3B). All above results suggesting that circRIMKLB might function in binding miRNAs. In order to find the target miRNAs, RNAhybrid was used to choose a potential target miRNA and circRIMKLB was predicted to contain a miR-29c bind site with a minimum free energy of −23.0kcal/mol (Fig. 3C). Dual-luciferase assay shown that miR-29c markedly suppressed the Renilla

luciferase reporter activity of the wild-type circRIMKLB (Fig. 3D). To verify the direct binding between circRIMKLB and miR-29c, we first constructed a miR-29c sensor by inserting two copies of perfectly matched miR-29c fragments into the 3'UTR of a Renilla luciferase (Rluc) gene of the psiCHECK^{TM}-2 vector (Fig. 3E). Then, the miR-29c sensor was transfected into HEK293T cells, along with pcDNA3.1(NC), miR-29c, or increasing amounts of PCD2.1-circRIMKLB (circRIMKLB). The decreased Rluc activity induced by miR-29c was recovered in response to PCD2.1-circRIMKLB (Fig.3F), indicating that circRIMKLB specifically sponged miR-29c, thereby preventing it from inhibiting Rluc expression. It is also observed that the location of circRIMKLB and miR-29c overlapped through FISH assay, suggesting that circRIMKLB could bind miR-29c in cytoplasm (Fig.3G).

KCNJ12 is a novel target of miR-29c.

previous evidence shows that miRNAs positively regulate gene transcription by targeting the 3’-untranslated region(3’-UTR) of mRNA[25] and in this case, KCNJ12 was predicted as a potential target of miR-29c by using targetscan (Fig. 4A). Besides, the conservation of miR-29c in cattle, human, mouse and rat was analyzed and it’s highly conserved (Fig. 4B). In order to detect the interaction of miR-29c and 3’-UTR of KCNJ12, dul-Luciferase assays were performed and the results indicated that miR-29c markedly repressed the activity of Renilla luciferase reporter with the wild-type KCNJ12(WT-KCNJ12) 3'-UTR but not affect the activity of Renilla luciferase reporter of mutant-type of KCNJ12 (MUT-KCNJ12) 3'-UTR (Fig. 4C and D). Next, qRT-PCR and western blot shown that miR-29c significant suppressed the expression of KCNJ12 in mRNA and protein level (Fig. 4E and F). In a word, KCNJ12 was proved as a target of miR-29c.

KCNJ12 promotes the proliferation and inhibits the differentiation of bovine primary myoblasts.

In order to find the effects of KCNJ12 on muscle development, KCNJ12 was overexpressed and inhibited in bovine primary myoblasts. Results of CCK-8 assay shown that the overexpression of KCNJ12 enhanced the cell verbality of myoblasts and inhibition of KCNJ12 decreased the cell viability (Fig.5A). EdU assay indicated that KCNJ12 increased the percentage of EdU positive cells and inhibited the expression of KCNJ12 decreased the percentage of EdU positive cells (Fig. 5B and C). Cell cycle assay showed that KCNJ12 increase the percentage of S phase cells and decreased the percentage of G1 phase cells (Fig.5D and E). Besides, overexpressed KCNJ12 also promoted the protein levels of CDK2 as well as CCND1 and inhibited the expression of p27, and inhibited the expression of KCNJ12 showed the opposite results (Fig. 5F). All results above indicated that KCNJ12 could affect the proliferation of bovine primary myoblasts. Differentiation is one of the most important progress to muscle development. So as to find the effects of KCNJ12 on myoblasts differentiation, KCNJ12 was overexpressed or inhibited. The result of qRT-PCR showed that the mRNA level of MyoD and MyoG were down-regulated while KCNJ12 was overexpressed and inhibited the expression showed the opposite results (Fig. 5G and H). These results indicated that KCNJ12 inhibited the differentiation.

CircRIMKLB and KCNJ12 participates in cell cycle regulation in muscle regeneration after injury i*n vivo*.

Since circRIMKLB could influence the proliferation of bovine primary myoblasts, it is possible that circRIMKLB has an impact on progress of muscle cell regeneration. Then mice were injected with Cardiotoxin, and Hematoxylin-eosin staining showed that the skeletal muscle injury model was constructed successfully (Fig. 6A). Hematoxylin-eosin staining also shown that muscle injury repair was enhanced while overexpressing circRIMKLB (Fig. 6B). Additionally, qRT-PCR and western blot also showed that the expression efficiency of circRIMKLB and levels of CKD2 and CCND1 were upregulated and p27 decreased when circRIMKLB was overexpressed (Fig. 6D and E). Together, these results suggested that circRIMKLB participated in muscle cell regeneration and play a role in cell cycle regulation*in vivo*. As a target gene of miR-29c, KCNJ12 is likely to have an effect on muscle injury repair. Here, we first analyzed the conservation of KCNJ12 among different species and found that its amino acid sequence is highly conserved (Fig. 6F). Ordered and disordered regions in KCNJ12 of human, mice, rat and cattle are highly conserved (Fig. 6G). Hematoxylin–eosin staining showed that the repair of injury treated with KCNJ12 was promoted (Fig 6H). After detecting the expression efficiency of KCNJ12, The next test proved that after overexpressing KCNJ12, the mRNA and protein level of CDK2 and CCND1 raised while p27 decreased (Fig. 7I, J and K). All in all, we can conclude that KCNJ12 participate in cell cycle regulation and affect the progress of muscle repair after injury.

Discussion

Skeletal muscle development is a process orchestrated by a complex network involving transcription factors, epigenetic regulators and so on[26]. Among them, there are fewer studies about how circRNAs affect muscle development due to previous limited knowledge of circRNAs. To date, the specific functions of ncRNAs are largely unknow. In this study, circRIMKLB was proved to be a circular RNA and function as a promoter of myoblasts proliferation, an inhibitor of myoblasts differentiation, and a regulator of cell cycle as well as muscle regeneration via sponging miR-29c to release KCNJ12.

CircRNAs were regard as an endogenous RNA splicing by-products or intermediates escaped from intron lariat debranching with unless function[27-30]. However, emerging studies found circRNAs exert an enormous function on regulating life activities in not only cells, but also in extracellular fluid such as exosomes[31-33]. In this case, circRIMKLB was proved to be a circular RNA with closed loop at first. Then, the function of circRIMKLB on bivine primary myoblasts was studied by using CCK8, EDU, flow cytometry, qRT-PCR, and WB. According to previous researches about circRNAs, circRIMKLB, an exonic circRNA, probably acts as a sponge of miRNAs. To explore the underlying mechanism of circR-RIMKIB action, RNAhybrid were used to predicted the target. Dul-luciferase assay and qRT-PCR revealed that miR-29c is a target of circRIMKLB. MiR-29c is a highly expressed miRNA in muscle tissue that is important for muscle development. we also found KCNJ12 a novel target gene of miR-29c. According to the high conservation of circRIMKLB between mouse and cattle, circRIMKLB was used to study the influence on mouse muscle injury. It is found that cicrRIMKLB was involved in cell cycle regulation *in vivo* and affected the muscle regeneration after injury.

miR-29 family contains miR-29a, b, and c, which are global expressed miRNAs and influence many physiological and pathological processes[34]. For instance, miR-29 participates in regulating TCL1 oncogene in disease of B-cell chronic lymphocytic leukemia and directly targets DNMT 3a and DNMT 3 b in lung cancer and acute myeloid leukemia[35-37]. Besides, all three members of the miR-29 family were significantly down-regulated in livers of hepatic fibrogenesis mice[38]. Moreover, emerging evidence indicated that miR-29 attach importance to muscle function. In human muscle, miR-29c is downregulated in quiescent satellite cells compared with proliferating satellite cells. MiR-29 family affects the expression of many muscle-important factor, such as HDAC4, YY1, Ring1, Rybp, Akt3, Col, and Lims1, which means miR-29 family is critical to muscle development[39-43]. In some muscle-related diseases, miR-29 family also plays an important regulatory role. For example, in the muscle of mice with chronic kidney disease(CKD), 12 miRNAs such as miR-29a and miR-29b and miR-23a are significantly reduced[44]. In primary muscular dystrophy, 185 miRNAs, including miR-29c, were found to be upregulated or downregulated in 10 major muscular disorders in humans[45]. All of the above studies have shown that miR-29 family is closely related to muscle development. Factors involved in the regulation of miR-29 expression and genes regulated by miR-29 family are concern about muscle function. Our study found that circRIMKLB regulated the expression of miR-29c. We have also identified a new target gene for miR-29c in muscle tissue.

KCNJ12 (also called Kir2.2), a member of Kir2 subfamily, encodes an inwardly rectifying potassium channel protein which plays an important physiological role in maintaining normal excitability of cells and maintaining blood potassium balance[46, 47]. The blocking of normal cellular trafficking of KCNJ12 and KCNJ2 to the cell surface has connection with hyperthyroidism in thyrotoxic periodic paralysis[48]. Besides, Recent studies showed that KCNJ12 is involved in the contraction process of muscle[49]. Our study demonstrated that KCNJ12 promoted the proliferation and inhibited differentiation of bovine primary myoblasts. Besides, we explored the influence of KCNJ12 on muscle regeneration and our experiments in mice suggested that KCNJ12 is able to influence the cell cycle *in vivo* in the process of muscle regeneration after injury. We speculated that this may be achieved by KCNJ12 by affecting the viability of mitochondria. Previous studies have found that KCNJ12 can affect the aging of tumor cells by affecting the vitality of mitochondria. Muscle tissue is one of the tissues which contain abundant mitochondria in the body, and the repair process of muscle after injury requires attendance of numerous mitochondria. Similarly, the functioning of normal muscle function also requires a large number of mitochondria. Therefore, KCNJ12 is likely (at least partly) to participate in the regulation of muscle function by affecting mitochondria activity in muscle cells. As for the mechanism of how KCNJ12 affects mitochondrial viability still requires more research.

Because of the important role of miR-29 in muscle diseases, we studied the effects of circRIMKLB on the repair process of muscle damage. It was found that circRIMKLB was able to participate in the cycle regulation of myoblasts in muscle regeneration caused by damage, resulting in down-regulation of p27 and up-regulation of CDK2 and CCND1 expression. It is predicted that circRIMKLB might affect the expression of miR-29 in some muscle diseases, and might be involved in the development of some muscle diseases. Therefore, circRIMKLB might be a potential marker for muscle diseases. Because of the

high conservation of circRIMKLB and KCNJ12 in mice and cattle, we were able to study in mice. But due to differences in species, whether there are other factors affecting the results of the trial still require further research.

In summary, for the first time, we identified a novel circRIMKLB, researched its function, and explored its mechanism. CircRIMKLB acts as an endogenous miR-29c sponge to release KCNJ12, resulting in promoting bovine myoblast proliferation, inhibiting differentiation, and affecting cell phase in muscle regeneration, which may be used as a potential candidate to regulating muscle function.

Conclusion

Our findings showed that, circ*RIMKLB* promotes myoblasts proliferation and inhibits myoblasts differentiation by sponging miR-29 to release *KCNJ12*. Besides, circ*RIMKLB* and *KCNJ12* affect the cell cycle regulation of muscles and participate in muscle regeneration after injury.

Abbreviations

ncRNA: non-coding RNA

circRIMKLB: circular RNA Rribosomal modification protein rimK like family member B

KCNJ12: potassium inwardly rectifying channel subfamily J member 12

qRT-PCR: quantitative real time polymerase chain reaction

miR-29c: microRNA 29c

ceRNAs: competing endogenous RNAs

CCK-8: Cell Counting Kit-8

EdU: 5-Ethynyl-20-deoxyuridine

p27:cyclin dependent kinase inhibitor 1B

CCND1: cyclin D1

CDK2: cyclin-dependent-kinase 2

MyHC: myosin: heavy chain

MyoD: myogenic differentiation 1

MyoG: myogenin

FISH: fluorescence in situ hybridization

Rluc: renilla luciferase

3’-UTR: 3’-untranslated region

HDAC4: histone deacetylase 4

YY1: YY1 transcription factor

Ring1: ring finger protein 1

Rybp: ring1 and YY1 binding protein

Akt3: AKT serine/threonine kinase 3

Col: collagen type XVII alpha 1 chain

Lims1: LIM zinc finger domain containing 1.

Declarations

Ethics approval and consent to participate

All animal experiments and study protocols were approved by the Animal Care Commission of the College of Veterinary Medicine, Northwest A&F University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Founding

This study was supported by the National Natural Science Foundation of China (31601926 and 31772574), Program of National Beef Cattle and Yak Industrial Technology System (CARS-37).

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Author's contributions

Jian Wang performed most experiments, summarized most part of data, and drafted the manuscript. Yifan Wen and Jiawei Xu performed qRT-PCR. Binglin Yue performed animal experiments. Li zheng and Jialin Zhong summarized part of data and did informatic analysis. Hong chen and Yongzhen huang contributed to the experimental design and approved the final version.

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Acknowledgements

We appreciate help of all members of laboratories. We thank Dr. Chengchuang Song and Dr. Xiukai Cao for advice to this research. We are grateful to Mr. Jintao Hu for his suggestions about flow cytometry assay.

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Figures

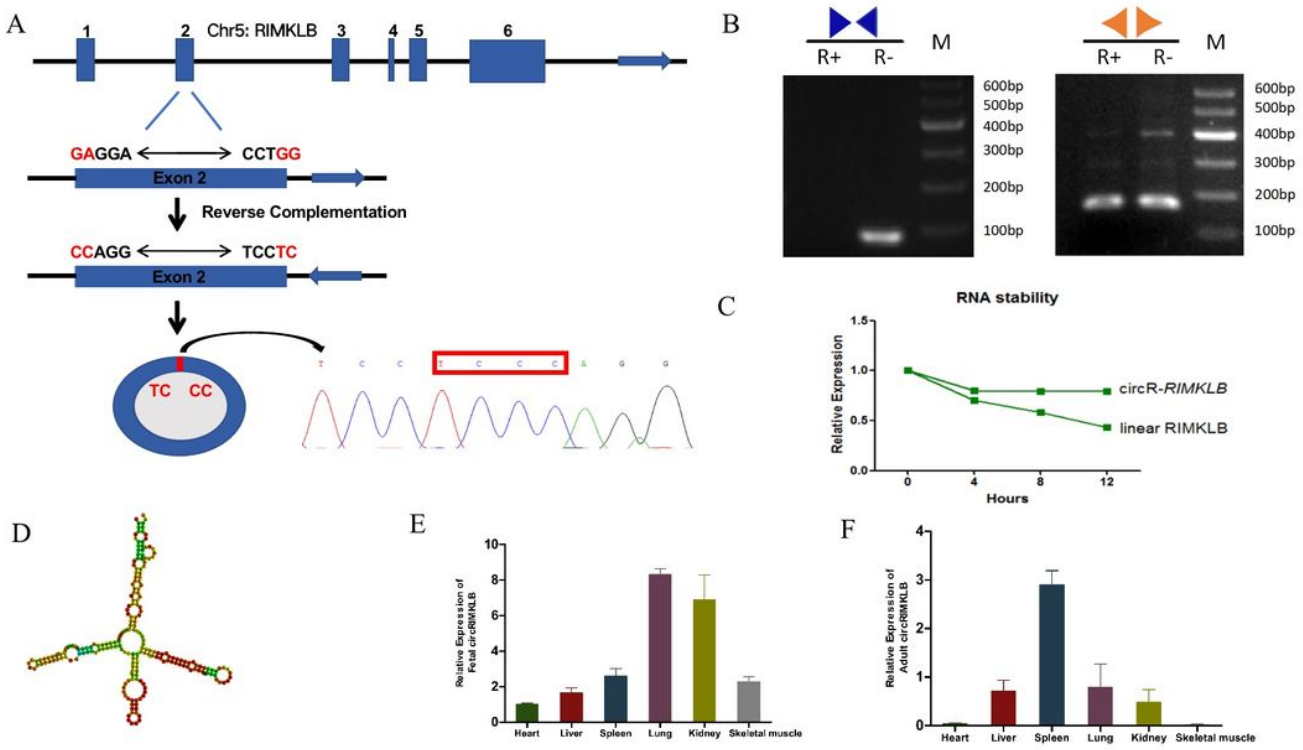

Figure 1

CircRIMKLB is a real circular RNA. (A) The head-to-tail splicing site of circRIMKLB was found by Sanger sequencing. (B) PCR product of circRIMKLB was detected by using divergent and convergent primers while RNA was treated RNase R or not. (C) circRIMKLB and liner RIMKLB were detected by qRT-PCR after cells were treated with actinomycin D (a transcription inhibitor). (D) The secondary structure of circRIMKLB was analyzed via using RNAfold (http://rna.tbi.univie.ac.at/). (E) The expression of circRIMKLB in fetal stage was analyzed by using qRT-PCR. (F) The expression of circRIMKLB in adult stage was analyzed by using qRT-PCR. *p ≤ 0.05; **p ≤ 0.01.

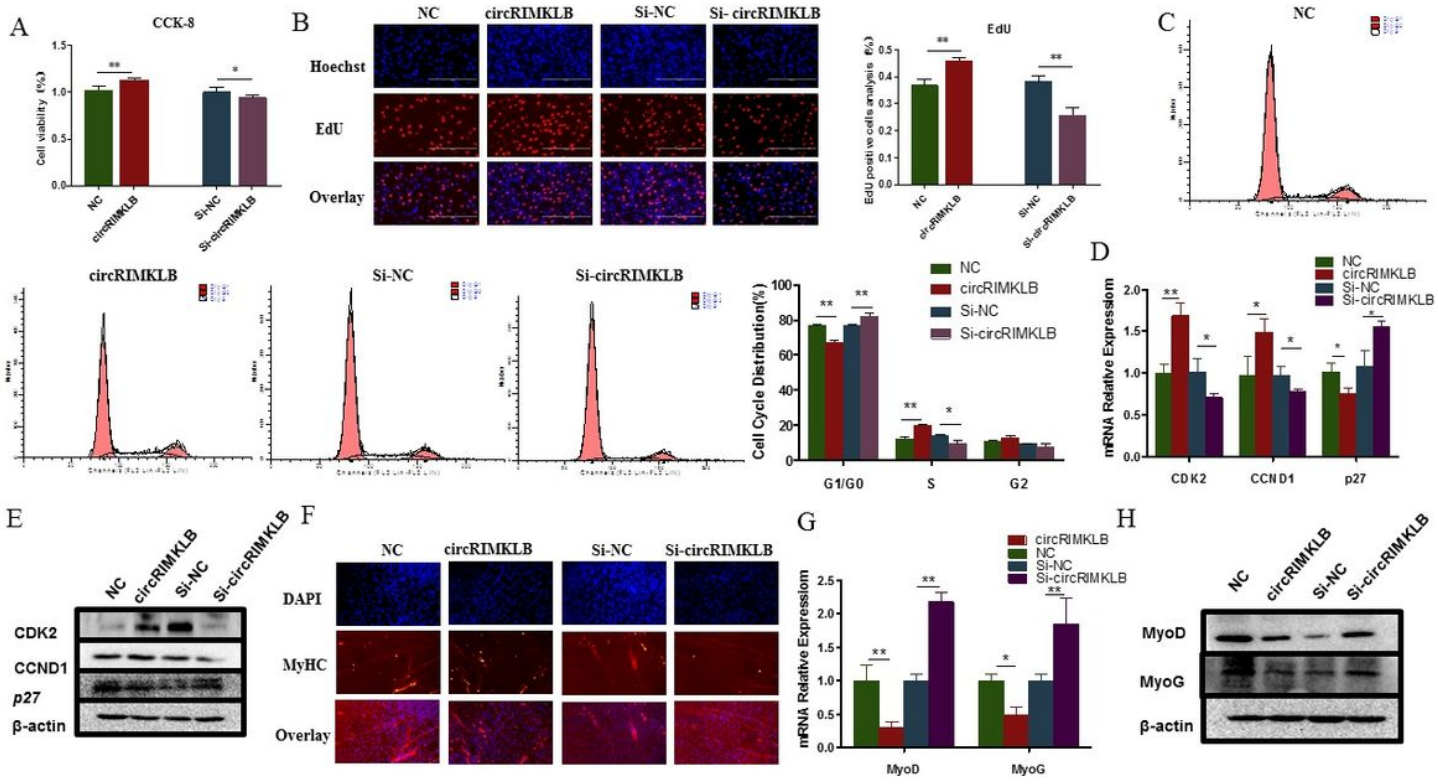

Figure 2

circRIMKLB promotes proliferation and inhibits differentiation of bovine primary myoblasts. (A) Cell viability was detected by CCK-8 assay while circRIMKLB was overexpressed and inhibited 24h later. (B) EdU assay was used to detect the cell proliferation index and the percentage of EdU positive cells were counted. (C) Cell cycle phase index were detected by flow cytometry and the percentage of different phases were analyzed. (D) The mRNA level of CDK2, CCND1 and p27 in bovine primary myoblasts were detected by qRT-PCR at 24 h post-transfection. (E) The protein level of CDK2, CCND1 and p27 in bovine primary myoblasts were detected by western blot at 24 h post-transfection. (F) Cell differentiation was measured by MyHC immunofluorescence assay, ×400. (G) The mRNA level of MyoD and MyoG were detected by qRT-PCR after cattle primary myoblasts were induced to differentiate for 4 days. (H) The protein level of MyoD and MyoG were detected by western blot after cattle primary myoblasts were induced to differentiate for 4 days. *p ≤ 0.05; **p ≤ 0.01.

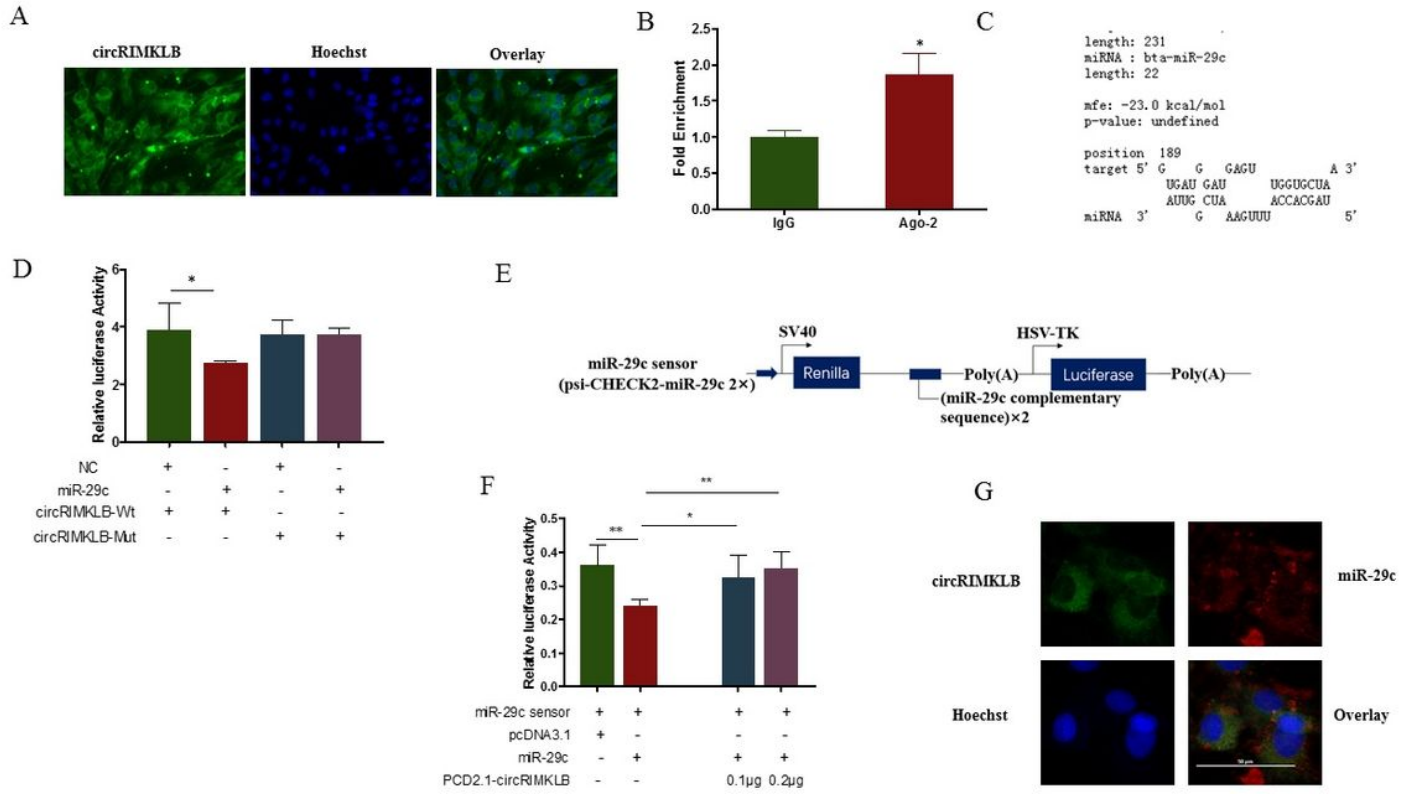

Figure 3

CircRIMKLB functions as a sponge of miR-29c. (A) The location of circRIMKLB was detected by FISH assay, ×400. (B) RIP assay of Ago-2 was used to find whether circRIMKLB could function a miRNA sponge. (C) MiR-29c and circRIMKLB folding energy was predicted by RNAhybrid. (D) miR-29c with psi-CHECK2 plasmid of circRIMKLB-Wt or circRIMKLB-Mut were transfected into HEK293T cells, and Renilla luciferase activity was normalized to the firefly luciferase (hLuc) activity. (E) miR-29c sensor was constructed. (F) The miR-29c sensor was transfected into HEK293T cells together with miR-29c and increasing amounts of PCD2.1-circRIMKLB. Luciferase activities were measured 24 h after transfection. (G) The co-location of miR-29c and circRIMKLB were detected by FISH assay. *p ≤ 0.05; **p ≤ 0.01.

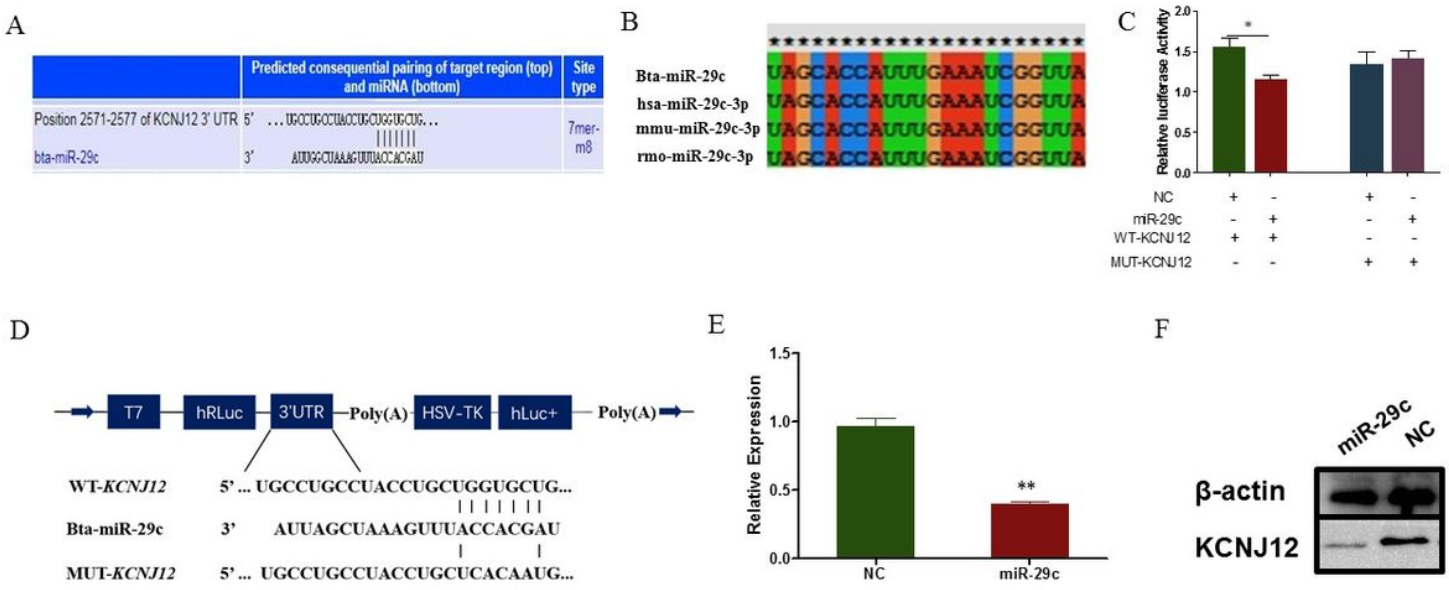

Figure 4

KCNJ12 is a novel target of miR-29c. (A) The binding sites of miR-29c and KCNJ12 were predicted by Targetscan(http://www.targetscan.org/vert_72/). (B) The conservation of miR-29c in human, mice, rat and cattle were analyzed via ClustalX2. (C) miR-29c and psi-CHECK2 plasmid inserted with 3'UTR of WT-KCNJ12 or MUT-KCNJ12 were transfected into HEK293T cells, and Renilla luciferase activity was normalized to the firefly luciferase (hLuc) activity. (D) Sequence of predicted sites of KCNJ12 binding with miR-29c their locations in the psi-CHECK2 plasmid. (E) KCNJ12 mRNA expression in cattle primary cells was detected by qRT-PCR at 24 h posttransfection with miR-29c. (g) Protein expression of KCNJ12 was detected by western blot. *$p \leq 0.05$; **$p \leq 0.01$.

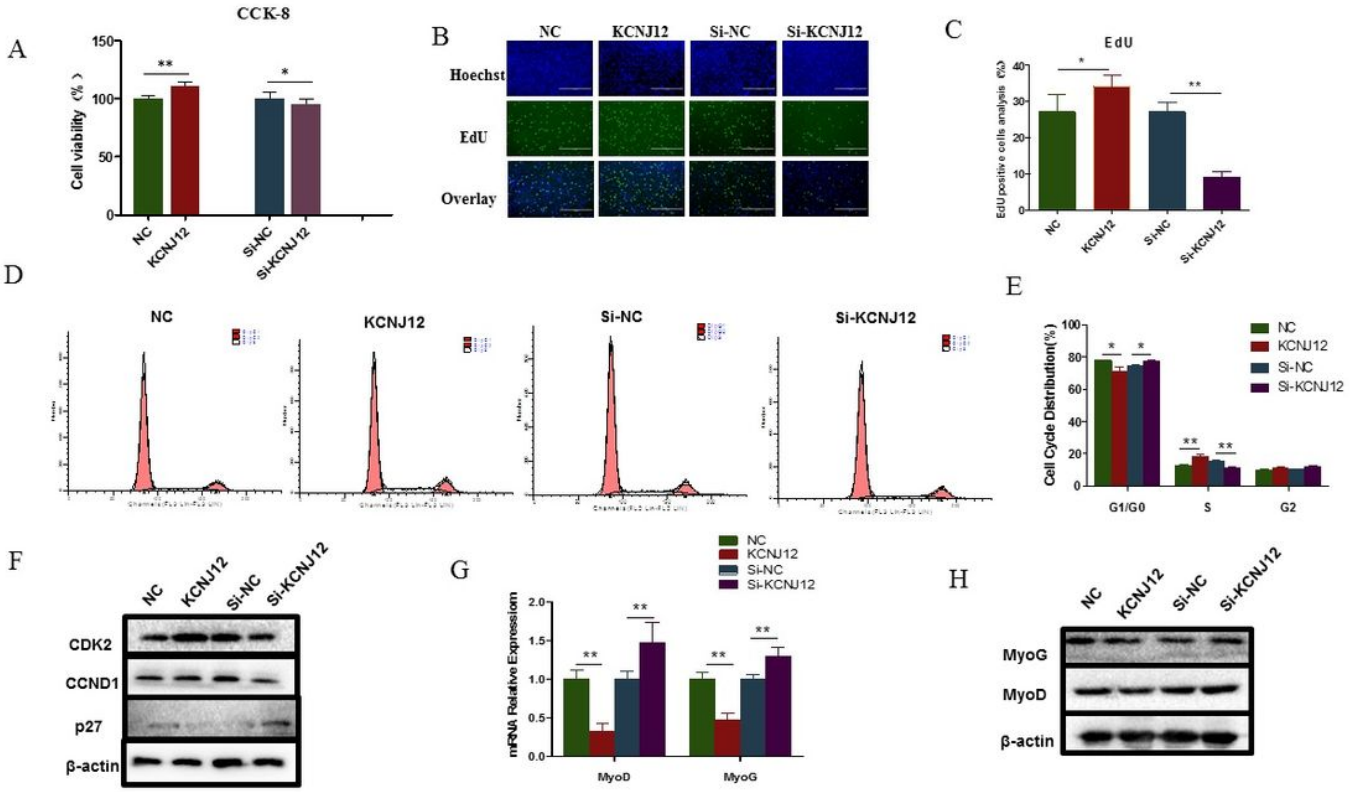

Figure 5

KCNJ12 promotes the proliferation and inhibits the differentiation of bovine primary myoblasts. (A) The cells were treated with pcDNA3.1(NC), pcDNA3.1-KCNJ12(KCNJ12), Si-RNA negative control (Si-NC), and Si-RNA for KCNJ12(Si-KCNJ12) and cell verbality was detected via CCK-8 assay 24h after transection. (B) (C) EdU assay detected the cell proliferation index and analysis results of EdU positive cells. (E) Cell cycle phase indexes were analyzed by flow cytometry and the distribution of cell phase was analyzed. (F) The protein levels of CDK2, CCND1 and p27 were detected by Western Blot. (G)(H) The mRNA and protein levels of MyoD and MyoG were detected by qRT-PCR and Western Blot, respectively. *p<0.05; **p<0.01.

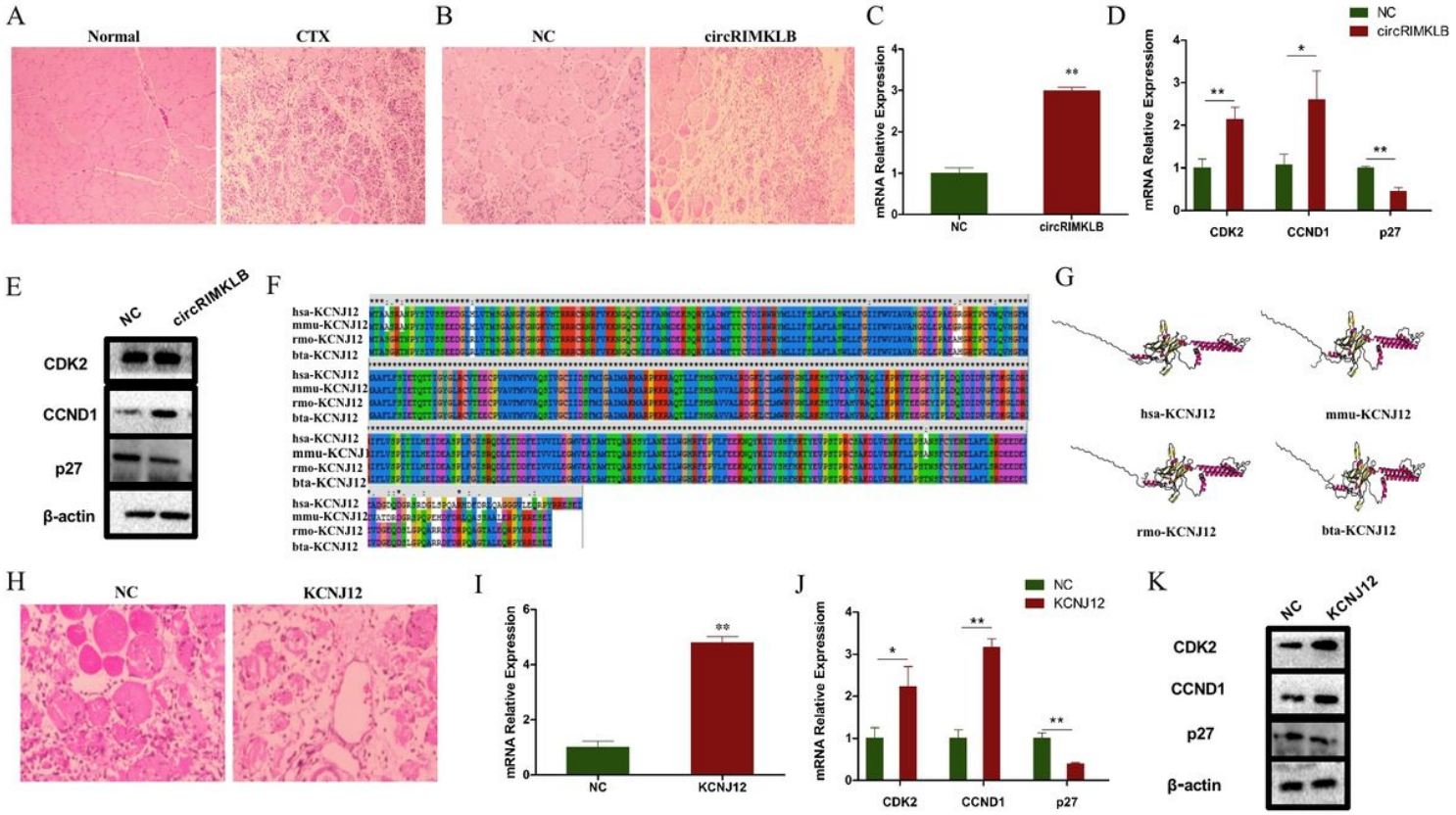

Figure 6

CircRIMKLB and KCNJ12 participates in cell cycle regulation in muscle regeneration after injury in vivo. (A) Normal and injured muscle at the tibialis anterior of the left leg of mice was checked by hematoxylin–eosin staining, ×100. (B) Injured muscle injected with NC or circRIMKLB 2 times was checked by hematoxylin–eosin staining 3 days later, ×200. (C) The expression efficiency of circRIMKLB was checked by qRT-PCR. (D) CDK2, CCND1 and p27 mRNA expressions of mouse tibialis anterior were detected by qRT-PCR at 3 days post-transfection. (E) CDK2, CCND1 and p27 protein levels of mouse tibialis anterior were detected by western blot at 3 days after transfection. (F) The conservation of KCNJ12 Amino acid of human, mice, rat and cattle were analyzed via ClustalX2. (G) Ordered and disordered regions in the KCNJ12 protein of human, mice, rat and cattle were predicted by RaptorX Structure Prediction (raptorx.uchicago.edu). (H) Injured muscle injected with NC or KCNJ12 2 times was checked by hematoxylin–eosin staining 3 days later, ×200. (I) The expression efficiency of KCNJ12 was checked by qRT-PCR. (J) CDK2, CCND1 and p27 mRNA expressions of mouse tibialis anterior were detected by qRT-PCR at 3 days post-transfection. (K) CDK2, CCND1 and p27 protein levels of mouse tibialis anterior were detected by western blot at 3 days after transfection. *p ≤ 0.05; **p ≤ 0.01.

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