MicroRNA bta-miR-365-3p inhibits proliferation but promotes differentiation of primary bovine myoblasts by targeting the activin A receptor type I

Dan Hao
NWSUAF https://orcid.org/0000-0002-9023-0488
Xiaogang Wang
Northwest Agriculture and Forestry University
Xiao Wang
Danmarks Tekniske Universitet
Bo Thomsen
Aarhus Universitet
Yu Yang
Northwest Agriculture and Forestry University
Xianyong Lan
Northwest Agriculture and Forestry University
Yongzhen Huang
Northwest Agriculture and Forestry University
Hong Chen (chenhong1212@263.net)
Northwest A&F University

Research

Keywords: ACVR1, Bta-miR-365-3p, Cattle, Primary bovine myoblast

Posted Date: August 26th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-27805/v2

Version of Record: A version of this preprint was published on January 12th, 2021. See the published version at https://doi.org/10.1186/s40104-020-00528-0.

Abstract

Background: MicroRNAs act as post-transcriptional regulators that repress translation or degrades mRNA transcripts. Each microRNA has many mRNA targets and each mRNA may be targeted by several microRNAs. Skeletal muscles express a plethora of microRNA genes that regulate muscle development and function by controlling the expression of protein-coding target genes. To expand our understanding of the role of microRNA, specifically bta-miR-365-3p, in muscle biology, we studied its function in regulating primary bovine myoblast proliferation and differentiation.

Results: We first show that bta-miR-365-3p is predominantly expressed in skeletal muscle and heart tissue in Chinese Qinchuan beef cattle. Quantitative PCR and western blotting showed that overexpression of bta-miR-365-3p significantly reduced the levels of cyclinD1 (*CCND1*), cyclin dependent kinase 2 (*CDK2*) and proliferating cell nuclear antigen (*PCNA*) but stimulated the expression of muscle differentiation markers *MYOD1*, *MYOG*, both at mRNA and protein level. Moreover, downregulation of bta-miR-365-3p increased the expression of *CCND1*, *CDK2* and *PCNA* but decreased the expression of *MYOD1* and *MYOG* at both mRNA and protein levels. Furthermore, flow cytometry, EdU proliferation assays and immunostaining showed that increased levels of bta-miR-365-3p suppressed cell proliferation but promoted myotube formation, whereas a decreased level of bta-miR-365-3p had opposite consequences. Finally, we determined that activin A receptor type I (*ACVR1*) is a direct target of bta-miR-365-3p. Thus, dual luciferase gene reporter assays demonstrated that bta-miR-365-3p can bind to the 3'UTR of *ACVR1* gene to regulate its expression. Consistently, knock-down of *ACVR1* was associated with reduced *CDK2*, *CCND1* and *PCNA* expression but increased *MYOG* and *MYOD1* expression both at mRNA and protein level.

Conclusion: Collectively these data suggest that bta-miR-365-3p represses proliferation but promotes differentiation of bovine myoblasts through a mechanism involving downregulation of *ACVR1*.

Background

Skeletal muscles originate from embryonic structures called the somites in which mononuclear myoblasts proliferate, differentiate and fuse to produce multinucleated myotubes that subsequently differentiate into myofibers [1]. Myofibers vary with respect to their myosin heavy chain isoforms (fast *versus* slow) and types of energy metabolism (oxidative *versus* glycolytic). The number of myofibers is constant, but myofibers can increase in size by fusion with muscle stem cells, the satellite cells [2]. Furthermore, adult skeletal muscle has a remarkable ability to repair after injury, leading to new myofiber formation in a process that involves satellite cells [3]. Skeletal muscle mass and muscle fiber characteristics play key roles in the determination of meat yield and quality in cattle. Therefore, understanding the molecular processes and genetic networks underlying myogenesis and muscle development will provide fundamental information for cattle breeding programs.

The mature microRNAs (miRNAs) are small RNA molecules (~ 22 nucleotides), which have been widely identified in humans and animals since they were first discovered in the nematode *Caenorhabditis elegans* [4]. MicroRNAs act as post-transcriptional regulators that repress translation or degrades mRNA transcripts through either complete (canonical sites) or incomplete (non-canonical sites) complementarity with the 3'UTR of target mRNAs. Nowadays, the sequencing technologies are accelerating the discovery of microRNAs, which are deposited in the searchable miRNAs database. Also, effective microRNA target sites have been accurately predicted using various computational approaches [5]. The latest database of miRbase (Release 22.1) contains 48,860 distinct mature miRNA (miR) sequences from 271 organisms, including 1143 miRNAs from cattle [6]. Previous studies have shown that tissue-specific and developmental stage-specific miRNAs play critical functional roles in diverse cellular, physiological and developmental processes [7–9]. For example, muscle-specific miRNAs such as miR-1, miR-133 and miR-206 participates in ontogenesis and skeletal myogenesis through modulation of muscle differentiation genes [10–12]. Profiling of miRNA expression patterns among eleven different tissues from beef cattle revealed that bta-miR-365-3p was ubiquitously expressed but with the highest expression level in muscle, which suggested its regulatory role in muscle tissue [13]. Furthermore, bta-miR-365-3p was differentially expressed between fast- and slow-type muscles (semitendinosus *versus* masseter) in Japanese Black steers [14]. Researchers also has found a 2.6 fold higher expression of bta-miR-365-3p in the adult stage of muscle tissues than in the fetal stage of Qinchuan cattle, which showed the same tendency with bta-miR-1, and showed an opposite tendency with well-known muscle-specific miRNA, such as bta-miR-206 [15] (Fig. S1A). Previous studies also revealed that the total sequence reads of bta-miR-365-3p in the proliferation stages of skeletal muscle-derived satellite cells from Chinese Simmental calves were almost 3.5 times higher than in the differentiation stages, which was similar to bta-miR-378a-3p and bta-miR-23a [16]. Thus, we speculate that bta-miR-365-3p plays an important role in muscle tissue development. The target genes and the regulatory networks of bta-miR-365-3p in muscle cells are essentially uncharacterized. However, in other tissues, it has been shown that miR-365-3p negatively regulates histone deacetylase 4 (*HDAC4*) not only to stimulate primary chondrocyte proliferation and differentiation in mouse and chicken [17] but also to contribute to osteoarthritis pathogenesis in humans [18]. The objective of our study is to assess the expression level of bta-miR-365-3p in various bovine tissues and to investigate how it influences primary myoblast proliferation and differentiation.

Materials And Methods

2.1 Animal and cell culture

All animal experiments were approved by the Animal Care Commission of the College of Veterinary Medicine Northwest A&F University (Permit Number: NWAFAC1019). Six tissue samples, i.e., heart, liver, spleen, lung, kidney, *longissimus dorsi* muscle were collected from sixty-day-old fetuses (n = 3) and two-year-old adults (n = 3) of the Chinese Qinchuan (QC) beef cattle breed. All the samples were provided by Shanxi Kingbull Livestock Co., Ltd., Baoji city, China. Primary bovine myoblasts (PBMs) were isolated from fetal *longissimus dorsi* muscle following established protocols [19]. Myoblasts were cultured in growth medium (GM) consisting of high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco)

with 1% penicillin-streptomycin (HyClone) and 20% fetal bovine serum (TransGen, Beijing, China). Myoblast differentiation was stimulated in DMEM containing 2% horse serum (HyClone) and 1% penicillin-streptomycin (differentiation medium, DM). Cells were incubated at 37 °C with 5% CO2.

2.2 Plasmid construction and transfection

A DNA fragment containing the precursor sequence of bta-miR-365-3p was obtained from QC cattle genomic DNA by PCR and inserted into the pcDNA-3.1(+) vector using T4 DNA ligase (Takara, Dalian, China). The resulting plasmid was named OPmiR-365-3p and used for overexpression of bta-miR-365-3p in PBMs.

Next, we used the inhibitor of bta-miR-365-3p and negative control (NC) as treatment and control groups, respectively. The sequence of bta-miR-365-3p inhibitor is AUAAGGAUUUUUAGGGGCAUUA. With a 21-23 nt 2'-methoxy modified RNA oligonucleotide design, bta-miR-365-3p inhibitor is a purified molecules that inhibit endogenous mature bta-miR-365-3p's activities specifically and effectively. The sequence of the inhibitor's negative control is CAGUACUUUUGUGUAGUACAA, which acted as the control group for the bta-miR-365-3p inhibitor treatment group (Table S1).

A DNA fragment containing the target site of bta-miR-365-3p of the 3'UTR of bovine *AVCR1* was amplified by PCR and cloned into the *XhoI* and *NotI* sites of the psiCHECK-2 dual-luciferase reporter vector (Promega, Madison, WI, USA) and named ACVR1-wild. Mutagenic primers were used to mutagenize the bta-miR-365-3p target site, which was then cloned into psiCHECK-2 to create ACVR1-mutant. Three siRNAs of *ACVR1* were used to inhibit the expression of *ACVR1* in PBMs, including siACVR1-1, siACVR1-2 and siACVR1-3, the sequence of the siACVR1s were shown in Table S1.

Cells were transfected with OPmiR-365-3p, the inhibitor of bta-miR-365-3p, inhibitor N.C, ACVR1-wild, ACVR1-mutant and siACVR1s using Lipofectamine 2000 (Invitrogen, Grand Island, NY) and incubated at 37 °C with 5% CO_2. The inhibitors and siRNAs were purchased from GenePharma (Shanghai, China). All experiments were done in triplicate. All the primers, inhibitor and the siRNAs sequences were listed in Table S1.

2.3 RNA extraction and qRT-PCR

Total RNA was extracted from six different tissues and from PBMs using TRIzol reagent (Takara, Japan). After assessing RNA purity and concentration by spectrophotometry using a NanoDrop 2000 (Wilmington, USA) and 0.8% agarose gel electrophoresis, 1000 ng RNAs were transcribed into complementary DNA (cDNA) with PrimeScript RT reagent kit for use in qRT-PCR with SYBR Green Master Mix Reagen kit (GenStar, Beijing). The specific stem-loop of bta-miR-365-3p was used for synthesizing the first cDNA. All the primers were listed in Table S1. The method of $2^{-\Delta\Delta Ct}$ was used to calculate the relative expression levels.

2.4 Western blot analysis

All proteins were extracted from PBMs at 4 °C using the radioimmunoprecipitation assay lysis buffer (RIPA buffer) and phenylmethylsulfnoyl fluoride (PMSF) (Solarbio, Beijing, China). Proteins were measured and adjusted by using the BCA protein assay kit (MULTI SCIENCE, China) and denatured with 5 × SDS loading buffer (Beyotime) at 98 °C for 10 min. The prepared proteins were separated by SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes. After being blocked with 5% skim milk solution, we incubated membranes with the specific primary antibodies and the secondary antibody. We visualized the membranes using ChemiDoc^{TM} XRS+ system (Bio-Rad Laboratories) and ECL Plus reagents (Solarbio, Beijing, China). The primary antibodies including anti-CDK2 and anti-PCNA were obtained from Sangon Biotech (Shanghai, China), anti-ACVR1, anti-cyclinD1, anti-MyoD and anti-MyoG were purchased from Abcam (Cambrige, MA, USA). Anti-β-actin were purchased from SungenBio (Tianjin, China). HRP-conjugated Goat Anti-Rabbit IgG was obtained from BBI Life Science (Shanghai, China). All the primary antibodies were diluted with primary antibody dilution buffer that was obtained from Beyotime (Haimen, China). Image Lab ^{TM} Software 6.0.1 was used to calculate the grayscale value of the proteins.

2.5 EdU and flow cytometry assay

After the transfection of PBMs with the expression vectors, inhibitor and siRNAs, we employed the EdU proliferation assay to measure their influences on DNA synthesis using the Cell Light EdU DNA cell proliferation kit according to the instruction (RiboBio, Guangzhou, China). The stained cells were detected and calculated by fluorescence microscopy (DM5000B, Leica Microsystems). Cell cycle phases were assessed by a cell cycle testing kit (Multisciences, Hangzhou, China) on a flow cytometry instrument (FACS Canto II, BD Biosciences, USA). Briefly, the cells were seeded in 6-well plates and transfected for 24 h after the cells reached 60% confluence. Cold 70 % ethanol was used to fix the harvested cells. After staining with 100 μg/ml of the PI master mix at 37°C for 30 minutes, the cell suspension was subjected to flow cytometry.

2.6 Immunofluorescence Staining

After inducing PBMs differentiation for 4 days, 4% paraformaldehyde in PBS was used to fix differentiated myoblast in a plate for 20 min. 0.5% of Triton-X-100 was added to permeabilize the fixed myoblast for 10 min and the cells were blocked with 5% bovine serum albumin solution (BSA) at 4 °C for 2 h. Subsequently, we incubated primary antibody (anti-MyHC diluted 1:250; Abcam, Cambridge, MA) at 4 °C overnight and incubated the corresponding fluorescent secondary antibody at 4°C for 2.5 h. Finally, the cell nuclei were stained with DAPI and images were captured by fluorescence microscope (DM5000B, Leica Microsystems, Germany). The degree of differentiation was measured by the fusion index which was calculated as the number of nuclei in the myotube as a percentage of the total nuclei.

2.7 Dual-luciferase reporter assay

Dual-luciferase reporter assay was performed to test the interaction of bta-miR-365-3p with its predicted targets. HEK293T cells were co-transfected with OPmiR-365-3p vector (or the empty vector) and vectors

containing ACVR1-wild or ACVR1-mutant. The dual-luciferase activity was analyzed on an MPPC luminescence analyzer (HAMAMATSU, Beijing, China) using the luciferase reporter assay kit (Promega, Madison, WI) according to the manufacturer's instructions. The results were calculated as the ratio of firefly to Renilla luciferase activities in three independent replicates.

2.8 Bioinformatics analysis

The online databases TargetScan (http://www.targetscan.org/vert_72/) and miRmap (https://mirmap.ezlab.org/) were used to search for targets for bta-miR-365-3p [20,21]. VENNY (version 2.1) (https://bioinfogp.cnb.csic.es/tools/venny/index.html) was used to obtain the common targets from the two databases [22]. The R package *clusterProfiler* [23] was used to cluster the Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) for the common genes.

2.9 Statistical analysis

All the quantitative data are presented as the mean ± SD. Each group has three independent experiments. Student' *t*-test procedure was used to analyze the statistical significance between groups were analyzed by SPSS v19.0. An asterisk indicated $P < 0.05$, two asterisks indicated $P < 0.01$ and three asterisks indicated $P < 0.001$ extremely significant between groups.

Results

3.1 bta-miR-365-3p expression in cattle tissue and PBMs

In order to investigate the functional roles of bta-miR-365-3p, we first detected the expression levels of bta-miR-365-3p in six different tissues from two developmental stages of QC cattle using quantitative PCR. The results showed that muscle had the highest expression level in the fetus, while adult stage expression of bta-miR-365-3p was highest in heart tissue (Fig. 1A). Furthermore, expression levels of bta-miR-365-3p were significantly different between adult and fetal stages in the liver, heart and muscle tissues (Fig. 1A). Also, we found that the expression levels of bta-miR-365-3p exhibited a slightly decreased trend during PBM proliferation (Fig. 1B), but a dynamic expression profile that peaked on day four after which expression was downregulated again on day six (Fig. 1C). In cultured myoblast cells transfected with the expression vector OPmiR-365-3p, quantitative PCR showed that bta-miR-365-3p was significantly overexpressed, whereas the expression level of bta-miR-2333 and bta-miR-193a that map close to bta-miR-365-3p on BTA19 were not significantly overexpressed (Fig.1D and Fig.1E), which indicated that the expression vector was constructed successfully.

3.2 Bta-miR-365-3p suppresses PBM proliferation

The proteins encoded by *CDK2* (cyclin-dependent kinase 2*)*, *PCNA* (proliferating cell nuclear antigen) and *CCND1* (cyclinD1) have been identified to perform critical functions in G1, S and G2 phases during cell cycle progression [24–26]. The results of qRT-PCR and western blotting showed that expression of *CCND1*, *CDK2* and *PCNA* were significantly reduced both at the mRNA and protein levels after transfecting

PBMs with OPmiR-365-3p that overexpressed bta-miR-365-3p (Fig. 2A). Flow cytometer assays showed that PBM numbers were reduced in the G_2-phase (12.78%) and in the S-phase (16.91%) ($P < 0.05$ and $P = 0.08$, respectively), whereas the proportion of PBMs was increased in the G_0/G_1-phase, when bta-miR-365-3p was overexpressed (Fig. 2B, 2C and 2D). Moreover, the results of EdU proliferation assays revealed a 36.86% reduction in mitotic activity of PMBs transfected with OPmiR-365-3p ($P < 0.01$) (Fig. 2E and 2F). However, inhibition of bta-miR-365-3p significantly increased the expression of proliferation marker genes *CCND1*, *CDK2* and *PCNA* at mRNA and protein levels (Fig. 3A). And the proportion of PBMs was increased 20.06% in S-phase ($P < 0.05$), and decreased 3.1% in G_0/G1-phase, when we knock downed bta-miR-365-3p in PBMs (Fig. 3B, 3C and 3D). Also, the number of EdU-positive cells was increased 15.4% in the bta-miR-365-3p inhibitor group (Fig. 3E and 3F). Based on our results, we concluded that overexpression of bta-miR-365-3p suppressed PBM proliferation, whereas knockdown of bta-miR-365-3p promoted PBM proliferation.

3.3 Bta-miR-365-3p promotes PBMs differentiation.

To understand the function of bta-miR-365-3p for PBMs differentiation, we first monitored its expression levels following induction of differentiation and overexpression by means of continuously transfected with OPmiR-365-3p. As expected, quantitative PCR showed a peak expression level of bta-miR-365-3p in the differentiation for four days too (Fig. 4A). Subsequently, we assessed the effect of bta-miR-365-3p on PBM differentiation by overexpressing bta-miR-365-3p approximately 10 fold by transfection with OPmiR-365-3p (Fig. 4B), or alternatively by reducing the expression level about 5 times using an inhibitor of bta-miR-365-3p on differentiation day four (Fig. 4C). The data revealed that the mRNA and protein expression levels of two different muscle differentiation marker genes, *MYOD1* and *MYOG*, were both increased by OPmiR-365-3p (Fig. 4D and Fig. 4E), but reduced by the inhibitor of bta-miR-365-3p (Fig. 4F and Fig. 4G). Moreover, immunofluorescence staining showed that bta-miR-365-3p overexpression gave a higher amount of MyHC-positive myotubes than in the control group (Fig. 4H and Fig. 4J), while the opposite result was found by treatment with the bta-miR-365-3p inhibitor (Fig. 4I and Fig. 4J). Taken together, these results revealed that bta-miR-365-3p promoted PBM differentiation.

3.4 *ACVR1* is a target gene of the bta-miR-365-3p

In silico prediction using TargetScan and miRmap revealed 1354 and 354 putative target genes of bta-miR-365-3p, respectively. The intersection of the predicted targets gave 101 genes, which were used and inputted in a KEGG pathway analysis (Fig. 5A). Five significant signaling pathways (adjusted *P*-value <0.05) were observed comprising Parathyroid hormone synthesis, secretion and action (bta04928), Endocytosis (bta04144), Estrogen signaling pathway (bta04915), Phospholipase D signaling pathway (bta04072), Choline metabolism in cancer (bta05231) (Fig 5B). Interestingly, three putative target genes of bta-miR-365-3p, i.e., activin A receptor type 2A (*ACVR2A*), Sp1 transcription factor (*SP1*) and activin A receptor type 1 (*ACVR1*) are associated with the TGF-beta signaling pathway (bta04350), which is significant at an adjusted *P*-value about 0.1 (Table S2). Moreover, analysis for the presence of target sites in the 3' UTR of these three genes in fourteen different animal species showed strong conservation of the

bta-miR-365-3p target sites, especially in *ACVR1* (Fig. 5C). The *ACVR1* was conversely expressed with the bta-miR-365-3p during the PBM differentiation both under the OPmiR-365-3p treated or without treated (Fig. 5D and Fig. 5E). Consistently, overexpression of bta-miR-365-3p negatively regulated the expression level of *ACVR1* at both mRNA and protein levels in PBMs (Fig. 5F). To test directly whether bta-miR-365-3p interacts with the 3'UTR of *ACVR1* we conducted a dual-luciferase activity experiment. The data showed that the luciferase activity was significantly decreased when co-transfecting the OPmiR-365-3p and ACVR-wild vectors in PBMs. In contrast, the luciferase activity was unaffected in co-transfections of OPmiR-365-3p and ACVR1-mutant. Collectively, these data suggested that *ACVR1* is a direct target gene for bta-miR-365-3p (Fig. 5G).

3.5 siACVR1 inhibited PBM proliferation but promoted PBM differentiation

Next, we employed siRNA technology to address the role of *ACVR1* in PBM. Three different siRNAs were designed to target the bovine *ACVR1* and transfected in PBMs; the data showed that siACVR1-1 efficiently downregulated the expression of *ACVR1* at both the mRNA and protein level in PBMs (Fig. 6A). Furthermore, the results of q-RT-PCR and western blotting showed that siACVR1-1 also significantly decreased *CDK2* expression at both mRNA and protein levels. *CCND1* and *PCNA* were only slightly decreased at mRNA level but significantly reduced at the protein level (Fig. 6B). In the EdU proliferation assay, the percentage of dual positive PBMs was significantly reduced in cells with knock-down of *ACVR1* compared to non-treated cells (Fig. 6C and Fig. 6D). Conversely, the muscle differentiation markers *MYOD1* and *MYOG* were increased at both mRNA and protein levels when *ACVR1* expression was knocked down in PBMs (Fig. 6E and Fig. 6F). Thus, overexpression of bta-miR-365-3p and knock-down of *ACVR1* expression have similar molecular phenotypes.

Discussion

4.1 miRNAs in bovine skeletal muscle

Skeletal muscle mass and muscle fiber characteristics are highly associated with economically important traits such as meat quality and yield in beef cattle [27]. Understanding the molecular genetics of bovine skeletal muscle development will, therefore, provide important information for using in cattle breeding programs. Recently, researches have revealed several miRNAs associated with bovine skeletal muscle development and differentiation of satellite cells supported by advanced sequencing and bioinformatics technologies as well as annotated databases such as miRbase [6,13–16,28]. Moreover, miRNAs regulate protein synthesis by targeting mRNAs; so far four microRNAs modulating bovine skeletal muscle development and function have been deposited in miRTarBase, which provides information about experimentally validated miRNA-target interactions [29]. Six miRNAs regulated proliferation, apoptosis and differentiation of PBMs through targeting of various functional genes have been confirmed by various experimental methods [30–35] (Table 1). Such as, miR-744 was abundantly expressed in the fetal stage of Qinchuan cattle and have been confimed to positively regulate skeletal muscle satellite cell myogenic proliferation [15,31].

4.2 The expression profile and functional roles of bta-miR-365-3p

Previous studies have shown that expression levels of bta-miR-365-3p are significant differently expressed in fast- and slow-type skeletal muscles, stage of myoblast differentiation and development stages in cattle [13–16], which underlying its functional roles for skeletal muscle development. Skeletal muscle development can be divided into the prenatal stage which decided the muscle fibers numbers and the postnatal stage which mainly generated the muscle fiber size [36]. Firstly, we demonstrated that bta-miR-365-3p was highly expressed in heart and skeletal muscle tissues in Qinchuan cattle (Fig. 1A), which were consistent with previous transcriptome data in various tissues of Angus crossbred cattle [13]. Moreover, bta-miR-365-3p was highly expressed in adult muscle tissues than in fetal stages which was similar to bta-miR-1 on Sun et al's study [15] (Fig. S1A). The results may indicate that it played more important roles for the skeletal muscle postnatal stage development (to influence the fiber size) than the prenatal stage development (to determine the muscle fibers numbers).

The dynamic process of myoblast developed to myofiber involved proliferation, determination, differentiation and maturation phases [37,38]. Endogenous bta-miR-365-3p showed high expression in the maturation stage (72h-96 h, myotubes to form myofiber) than the early differentiation stage (24 h-72 h, mononucleated fuse to multinucleated myotubes) in our study, and same expression tendency was found in the specific-related skeletal muscle development miRNAs, such as bta-miR-1 and bta-miR-23a, but not bta-miR-125b in previous study (Fig. S1B) [16]. Previous research has found that the expression of bta-miR-1 higher in the early myoblast differentiation process than bta-miR-365-3p, indicated that bta-miR-1 play important roles for the early differentiation stage, which consistent with our study that the endogenous bta-miR-365-3p plays an important role in myoblast differentiation process of maturation stage, while lower expressed after 4 days differentiation, indicated that once the myofiber was fused, the function of bta-miR-365-3p may be reduced.

Furthermore, miR-365-3p has been reported to serve as a therapeutic biomarker for various cancers and tumors such as lung cancer [39,40], colon cancer [28], pancreatic cancer [41], breast cancer [42] and gastric tumorigenesis [43]. Moreover, miR-365-3p inhibited vascular smooth muscle cell proliferation through targeting of *CCND1* [44]. This agrees well with the present study, demonstrated that bta-miR-365-3p acted as a negative regulator of PBM proliferation based on cell cycle analysis with high levels of bta-miR-365-3p increased the percentage of cells in the G0/G1-phase and reduced the number of cells in S-phase. In agreement with this observation, downregulated bta-miR-365-3p with its inhibitor showed opposite effects. Consistently, *CDK2*, *CCND1* and *PCNA* were all shown to be downregulated when overexpressing bta-miR-365-3p, while the marker genes were upregulated when the expression of bta-miR-365-3p was inhibited in PBMs. In contrast, overexpression of bta-miR-365-3p increased the expression of muscle differentiation markers *MYOD1*, *MYOG* and promoted myoblast differentiation and myotube formation, while inhibition of bta-miR-365-3p showed the reverse effects. These observations are similar to previous results, showing that miR-365-3p promoted chondrocyte differentiation [18].

4.3 Gene targets of miR-365-3p

Several targets of miR-365-3p has been validated such as cyclinD1 (CCND1) [44,45], histone deacetylase 4 (HDAC4) [17,18], nuclear factor I/B (NFIB) [46], Pax 6 [47] thyroid transcription factor 1 (TTF1) [39], src homology domain containing 1 (SHC1) and Bax [41] (Table 2). After bioinformatics analysis, we filtered that *ACVR1*, also known as ALK2, a member of bone morphogenetic protein receptors type I, was another target of bta-miR-365-3p. As an essential member of TGF-β family, *ACVR1* has been reported to play functional roles in early embryonic development [48], lens formation [49], chondrogenesis, osteogenesis [50,51] and cardiac hypertrophy [52]. Additionally, recurrent heterozygous mutations of *ACVR1* have been associated with diseases in human such as fibro dysplasia ossificans progress (FOP) [53], diffuse intrinsic pontine gliomas (DIPGs) [54] and pediatric midline high-grade astrocytoma (mHGAs) [55]. The mutations of *ACVR1* also associated with meat weight, eye muscle area, silverside weight, and growth traits in cattle [56,57]. Moreover, a constitutively activating mutation of *ACVR1* induces the expression of Tmem176b in C2C12 cells, promoted myoblast differentiation into osteoblasts [58]. While, in our study, we found that *ACVR1* is a direct target of bta-miR-365-3p, and the decreased expression of *ACVR1* significantly inhibited myoblast proliferation but promoted myoblast differentiation. Our study was consistent to Shi et. al's research, who designed the antisense oligonucleotides (AONs) to knockdown *ACVR1* expression in mouse and resulted in inducing muscle differentiation and repressing osteoblast differentiation [59].

Conclusions

In conclusion, we have observed that bta-miR-365-3p was predominantly expressed in muscle tissues from adult and fetal stages, and moreover that bta-miR-365-3p can repress proliferation and promote the differentiation of PBMs through downregulation of *ACVR1* in cattle.

Abbreviations

ACVR1: Activin A receptor type I

CDK2: Cyclin dependent kinase 2

DIPGs: Diffuse intrinsic pontine gliomas

DM: Differentiation medium

FOP: Sporadic fibro dysplasia ossificans progressive

GM: Proliferation medium

GO: Gene Ontology

HDAC4: Histone deacetylase 4

KEGG: Kyoto Encyclopaedia of Genes and Genomes

MyoD1: Myogenic differentiation 1

MyoG: Myogenin

myomiR: Muscle-specific miRNAs

midline high-grade astrocytoma: mHGAs

NFIB: nuclear factor I/B

PCNA: Proliferating cell nuclear antigen

PBM: Primary bovine myoblast

PMSF: Phenylmethylsulfnoyl fluoride

RIPA: Radioimmunoprecipitation assay buffer

SHC1: src homology domain containing 1

TTF1: thyroid transcription

Declarations

Ethics approval and consent to participate

All the animal procedures were carried out according to the protocols approved by the College of Animal Science and Technology, Northwest A&F University, China. All the experimental animals were approved by the Institutional Animal Care and Use Committee in the College of Animal Science and Technology, Northwest A&F University, China.

Consent for publication

Not applicable

Availability of data and material

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

Funding

This work was supported by the National Natural Science Foundation of China (No.31772574) the Program of National Beef Cattle and Yak Industrial Technology System (CARS-37). Dan Hao and Xiao Wang appreciated the scholarship from the China Scholarship Council (CSC), China.

Authors' contributions

DH performed major parts of the experiments. XGW and YY performed rest parts of the experiments. DH analyzed the data and drafted the manuscript. XW and BT revised the manuscript. XYL and YZH collected the animal samples. HC conceived and designed the experiments.

Reference

1. Chal J, Pourquié O. Making muscle: Skeletal myogenesis in vivo and in vitro. Development (Cambridge). 2017;144:2104–22.
2. Yin H, Price F, Rudnicki MA. Satellite cells and the muscle stem cell niche. Physiological Reviews. 2013;93:23–67.
3. Collins CA, Olsen I, Zammit PS, Heslop L, Petrie A, Partridge TA, et al. Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Cell. 2005;122:289–301.
4. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 1993;75:843–54.
5. Li L, Xu J, Yang D, Tan X, Wang H. Computational approaches for microRNA studies: A review. Mammalian Genome. 2010;21:1–12.
6. Kozomara A, Birgaoanu M, Griffiths-Jones S. MiRBase: From microRNA sequences to function. Nucleic Acids Research. 2019;47:D155–62.
7. Braicu C, Gulei D, de Melo Maia B, Berindan-Neagoe I, Calin GA. miRNA Expression Assays. Netto GJ, Kaul KL, editors. Genomic Applications in Pathology. Cham: Springer International Publishing; 2019;51–71.
8. Huang Y, Shen XJ, Zou Q, Wang SP, Tang SM, Zhang GZ. Biological functions of microRNAs: A review. Journal of Physiology and Biochemistry. 2011;67:129–39.
9. Plasterk RHA. Micro RNAs in animal development. Cell. 2006;124:877–81.
10. Ge Y, Chen J. MicroRNAs in skeletal myogenesis. Cell Cycle. 2011;10:441–8.
11. Güller I, Russell AP. MicroRNAs in skeletal muscle: Their role and regulation in development, disease and function. Journal of Physiology. 2010;588:4075–87.
12. McCarthy JJ. The myomiR network in skeletal muscle plasticity. Exercise and Sport Sciences Reviews. 2011;39:150–4.
13. Jin W, Grant JR, Stothard P, Moore SS, Guan LL. Characterization of bovine miRNAs by sequencing and bioinformatics analysis. BMC Molecular Biology. 2009;10:90.

14. Muroya S, Taniguchi M, Shibata M, Oe M, Ojima K, Nakajima I, et al. Profiling of differentially expressed microRNA and the bioinformatic target gene analyses in bovine fast- and slow-type muscles by massively parallel sequencing. Journal of Animal Science. 2013;91:90–103.
15. Sun J, Li M, Li Z, Xue J, Lan X, Zhang C, et al. Identification and profiling of conserved and novel microRNAs from Chinese Qinchuan bovine longissimus thoracis. BMC Genomics. 2013;14:42.
16. Zhang WW, Sun XF, Tong HL, Wang YH, Li SF, Yan YQ, et al. Effect of differentiation on microRNA expression in bovine skeletal muscle satellite cells by deep sequencing. Cellular and Molecular Biology Letters. 2016;21:1–18.
17. Guan YJ, Yang X, Wei L, Chen Q. MiR-365: A mechanosensitive microRNA stimulates chondrocyte differentiation through targeting histone deacetylase 4. FASEB Journal. 2011;25:4457–66.
18. Yang X, Guan Y, Tian S, Wang Y, Sun K, Chen Q. Mechanical and IL-1β responsive miR-365 contributes to osteoarthritis development by targeting histone deacetylase 4. International Journal of Molecular Sciences. 2016;17.
19. Miyake M, Takahashi H, Kitagawa E. AMPK activation by AICAR inhibits myogenic differentiation and myostatin expression in Cattle. Cell and Tissue Research. 2012;349:615–23.
20. Agarwal V, Bell GW, Nam JW, Bartel DP. Predicting effective microRNA target sites in mammalian mRNAs. eLife. 2015;4:e05005.
21. Vejnar CE, Zdobnov EM. MiRmap: Comprehensive prediction of microRNA target repression strength. Nucleic Acids Research. 2012;40:11673–83.
22. Oliveros JC. VENNY. An interactive tool for comparing lists with Venn Diagrams (2007-2015). 2018.
23. Yu G, Wang LG, Han Y, He QY. ClusterProfiler: An R package for comparing biological themes among gene clusters. OMICS A Journal of Integrative Biology. 2012;16:284–7.
24. Ekholm S V., Reed SI. Regulation of G1 cyclin-dependent kinases in the mammalian cell cycle. Current Opinion in Cell Biology. 2000;12:676–84.
25. Naryzhny SN. Proliferating cell nuclear antigen: A proteomics view. Cellular and Molecular Life Sciences. 2008;65:3789–808.
26. Stacey DW. Cyclin D1 serves as a cell cycle regulatory switch in actively proliferating cells. Current Opinion in Cell Biology. 2003;15:158–63.
27. Lee SH, Joo ST, Ryu YC. Skeletal muscle fiber type and myofibrillar proteins in relation to meat quality. Meat Science. 2010;86:166–70.
28. Nie J, Liu L, Zheng W, Chen L, Wu X, Xu Y, et al. MicroRNA-365, down-regulated in colon cancer, inhibits cell Cycle progression and promotes apoptosis of colon cancer cells by probably targeting Cyclin D1 and Bcl-2. Carcinogenesis. 2012;33:220–5.
29. Chou CH, Shrestha S, Yang CD, Chang NW, Lin YL, Liao KW, et al. MiRTarBase update 2018: A resource for experimentally validated microRNA-target interactions. Nucleic Acids Research. 2018;46:D296–302.

30. Wang J, Song C, Cao X, Li H, Cai H, Ma Y, et al. MiR-208b regulates cell cycle and promotes skeletal muscle cell proliferation by targeting CDKN1A. Journal of Cellular Physiology. 2019;234:3720–9.
31. Peng S, Song C, Li H, Cao X, Ma Y, Wang X, et al. Circular RNA SNX29 Sponges miR-744 to Regulate Proliferation and Differentiation of Myoblasts by Activating the Wnt5a/Ca2+ Signaling Pathway. Molecular Therapy - Nucleic Acids. Elsevier Ltd.; 2019;16:481–93.
32. Song C, Yang J, Jiang R, Yang Z, Li H, Huang Y, et al. miR-148a-3p regulates proliferation and apoptosis of bovine muscle cells by targeting KLF6. Journal of Cellular Physiology. 2019;234:15742–50.
33. Sun X, Li M, Sun Y, Cai H, Lan X, Huang Y, et al. The developmental transcriptome sequencing of bovine skeletal muscle reveals a long noncoding RNA, lncMD, promotes muscle differentiation by sponging miR-125b. Biochimica et Biophysica Acta - Molecular Cell Research. 2016;1863:2835–45.
34. Li H, Wei X, Yang J, Dong D, Hao D, Huang Y, et al. circFGFR4 Promotes Differentiation of Myoblasts via Binding miR-107 to Relieve Its Inhibition of Wnt3a. Molecular Therapy - Nucleic Acids. 2018;11:272–83.
35. Elsaeid Elnour I, Dong D, Wang X, Zhansaya T, Khan R, Jian W, et al. Bta-miR-885 promotes proliferation and inhibits differentiation of myoblasts by targeting MyoD1. Journal of Cellular Physiology. 2020;1–12.
36. Hocquette JF. Endocrine and metabolic regulation of muscle growth and body composition in cattle. Animal. 2010;4:1797–809.
37. Perry RL, Rudnick MA. Molecular mechanisms regulating myogenic determination and differentiation. Frontiers in bioscience : a journal and virtual library. 2000;5:d750-767.
38. Montesano A, Luzi L, Senesi P, Mazzocchi N, Terruzzi I. Resveratrol promotes myogenesis and hypertrophy in murine myoblasts. Journal of Translational Medicine. 2013;11, 310.
39. Qi J, Rice SJ, Salzberg AC, Runkle EA, Liao J, Zander DS, et al. MiR-365 regulates lung cancer and developmental gene thyroid transcription factor 1. Cell Cycle. 2012;11:177–86.
40. Liu Y, Zhang G, Li H, Han L, Fu A, Zhang N, et al. Serum microRNA-365 in combination with its target gene TTF-1 as a non-invasive prognostic marker for non-small cell lung cancer. Biomedicine and Pharmacotherapy. 2015;75:185–90.
41. Hamada S, Masamune A, Miura S, Satoh K, Shimosegawa T. MiR-365 induces gemcitabine resistance in pancreatic cancer cells by targeting the adaptor protein SHC1 and pro-apoptotic regulator BAX. Cellular Signalling. 2014;26:179–85.
42. Han JG, Jiang YD, Zhang CH, Yang YM, Pang D, Song YN, et al. A novel panel of serum miR-21/miR-155/miR-365 as a potential diagnostic biomarker for breast cancer. Annals of Surgical Treatment and Research. 2017;92:55–66.
43. Guo SL, Ye H, Teng Y, Wang YL, Yang G, Li X Bin, et al. Akt-p53-miR-365-cyclin D1/cdc25A axis contributes to gastric tumorigenesis induced by PTEN deficiency. Nature Communications. Nature Publishing Group; 2013;4:1–11.

44. Zhang P, Zheng C, Ye H, Teng Y, Zheng B, Yang X, et al. MicroRNA-365 inhibits vascular smooth muscle cell proliferation through targeting cyclin D1. International Journal of Medical Sciences. 2014;11:765–70.
45. Kim MH, Ham O, Lee SY, Choi E, Lee CY, Park JH, et al. MicroRNA-365 inhibits the proliferation of vascular smooth muscle cells by targeting cyclin D1. Journal of Cellular Biochemistry. 2014;115:1752–61.
46. Zhou M, Zhou L, Zheng L, Guo L, Wang Y, Liu H, et al. miR-365 promotes Cutaneous Squamous Cell Carcinoma (CSCC) through targeting Nuclear Factor I/B (NFIB). PLoS ONE. 2014;9.
47. Wang J, Wang X, Wu G, Hou D, Hu Q. MiR-365b-3p, down-regulated in retinoblastoma, regulates cell cycle progression and apoptosis of human retinoblastoma cells by targeting PAX6. FEBS Letters. 2013;587:1779–86.
48. Gu Z, Reynolds EM, Song J, Lei H, Feijen A, Yu L, et al. The type I serine/threonine kinase receptor ActRIA (ALK2) is required for gastrulation of the mouse embryo. Development (Cambridge, England). 1999;126:2551–61.
49. Rajagopal R, Huang J, Dattilo LK, Kaartinen V, Mishina Y, Deng CX, et al. The type I BMP receptors, Bmpr1a and Acvr1, activate multiple signaling pathways to regulate lens formation. Developmental Biology. Elsevier Inc.; 2009;335:305–16.
50. Lin S, Svoboda KKH, Feng JQ, Jiang X. The biological function of type i receptors of bone morphogenetic protein in bone. Bone Research. 2016;4:16005.
51. Zhang D, Schwarz EM, Rosier RN, Zuscik MJ, Puzas JE, O'Keefe RJ. ALK2 functions as a BMP type I receptor and induces Indian hedgehog in chondrocytes during skeletal development. Journal of Bone and Mineral Research. 2003;18:1593–604.
52. Shahid M, Spagnolli E, Ernande L, Thoonen R, Kolodziej SA, Leyton PA, et al. BMP type I receptor ALK2 is required for angiotensin II-induced cardiac hypertrophy. American Journal of Physiology - Heart and Circulatory Physiology. 2016;310:H984–94.
53. Shore EM, Xu M, Feldman GJ, Fenstermacher DA, Brown MA, Kaplan FS. A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. Nature Genetics. 2006;38:525–7.
54. Taylor KR, Mackay A, Truffaux N, Butterfield YS, Morozova O, Philippe C, et al. Recurrent activating ACVR1 mutations in diffuse intrinsic pontine glioma. Nature Genetics. 2014;46:457–61.
55. Fontebasso AM, Papillon-Cavanagh S, Schwartzentruber J, Nikbakht H, Gerges N, Fiset PO, et al. Recurrent somatic mutations in ACVR1 in pediatric midline high-grade astrocytoma. Nature Genetics. 2014;46:462–6.
56. Cheng J, Cao X, Hao D, Ma Y, Qi X, Chaogetu B, et al. The ACVR1 gene is significantly associated with growth traits in Chinese beef cattle. Livestock Science. 2019;229:210–5.
57. Irida Novianti. Molecular genetics of cattle muscularity. 2011.
58. Yano M, Kawao N, Tamura Y, Okada K, Kaji H. A novel factor, Tmem176b, induced by activin-like kinase 2 signal promotes the differentiation of myoblasts into osteoblasts. Experimental and Clinical

Endocrinology and Diabetes. 2014;122:7–14.

59. Shi ST, Cai J, de Gorter DJJ, Sanchez-Duffhues G, Kemaladewi DU, Hoogaars WMH, et al. Antisense-Oligonucleotide Mediated Exon Skipping in Activin-Receptor-Like Kinase 2: Inhibiting the Receptor That Is Overactive in Fibrodysplasia Ossificans Progressiva. PLoS ONE. 2013;8:e69096.

Tables

Table 1 The published miRNAs to skeletal muscle myoblast development in cattle

miRNAs Name	Target gene Abbreviation	Target ID	Associated phenotype	References PMID
bta-miR-1	HDAC4	517559	Skeletal muscle satellite cell myogenic differentiation	26424132
bta-miR-206	HDAC4	517559	Skeletal muscle satellite cell myogenic differentiation	26424132
bta-miR-1	LOC536229	536229	Skeletal muscle satellite cell myogenic differentiation	26424132
bta-miR-206	LOC536229	536229	Skeletal muscle satellite cell myogenic differentiation	26424132
bta-miR-23a	ZNF423	508025	Adipogeneses in skeletal muscle	28255176
bta-miR-27b	MSTN	281187	Skeletal muscle hypertrophy	23510267
bta-miR-208b	CDKN1A	513497	Promoted skeletal muscle cell proliferation	30317561
bta-miR-744	Wnt5a	530005	Promoted skeletal muscle cell proliferation while inhibited the apoptosis and differentiation	31051333
bta-miR-744	CaMKIIδ	109560236	Promoted skeletal muscle cell proliferation while inhibited the apoptosis and differentiation	31051333
bta-miR-148a-3p	KLF6	505884	Inhibited muscle cell proliferation but promoted apoptosis	30793769
bta-miR-378a-3p	HDAC4	517559	Promoted myoblast differentiation and inhibited proliferation	27661135
bta-miR-125b	IGF2	281240	Sponged by lncMD to promote myoblast differentiation	27589905
bta-miR-107	Wnt3a	522467	Suppress cell differentiation and did not affect cell proliferation	29858062
bta-miR-885	MyoD1	281938	Promote proliferation but inhibit differentiation	331985035

Table 2. The published functions of miR-365-3p and its validated targets

Target Gene	Gene name	Functions	References PMID
HDAC4	Histone deacetylase 4	Stimulate chondrocyte differentiation in chicken or mouse/osteoarthritis development in human	21856783
HDAC4	Histone deacetylase 4	Osteoarthiritis development in human	27023516
TTF1	Thyroid transcription factor1	Regulate lung cancer	22185756 and 26337230
CycD1/Bcl2	Cyclin D1/Bcl apoptosis regulator 2	Regulate colon cancer	22072615
SHC1	Src homology domain containing 1	Gemcitabine Regulate pancreatic cancer	24216611
NFIB	Nuclear factor I/B	Promote cutaneous squamous cell carcinoma	24949940
CycD1/cdc25A	Cyclin D1	Contribute to gastric tumorigenesis	24149576
CycD1	Cyclin D1	Inhibit vascular smooth muscle cell proliferation	24819721and 24936138
Pax6	Paired box 6	Regulate human retinoblastoma cells	23660406
Not clearly		Transport-related stress in turkeys	26760121
Kcnh2	Potassium voltage-gated channel subfamily H member 2	Regulate nociceptive behaviors	26937014
IL-6	Interleukin-6	Host defense	21518763
Bcl-2	Bcl apoptosis regulator 2	Response low-density lipoprotein stimulation	21640710

Supporting Information

Table S1. The primers

Table S2. The pathway of the common target genes of bta-miR-365-3p from two database

Figure S1. The expression level of previously identified miRNAs. Figure S1. The expression level of previously identified miRNAs. (A) The fold change (FC) values between adult stage of muscle tissues and fetal stage of muscle tissues in Qinchuan cattle based on the Sun et al's study (Sun et al., 2013). (B) The FC values among primary muscle cell proliferation stage (P), primary muscle cell differentiation stage for 1 day (D1) and primary muscle cell differentiation stage for 3 days (D3) based on Zhang et al's study

(Zhang et al., 2016). D1/P indicated the FC values between D1 and P. D3/P indicated the FC values between D3 and P. D3/D1 indicated the FC values between D3 and D1. All the FC calculation is based on A reads - B reads/min (A reads, B reads).

Figure S2. The expression level of *ACVR1* after transfected with siACVR1s.

Figures

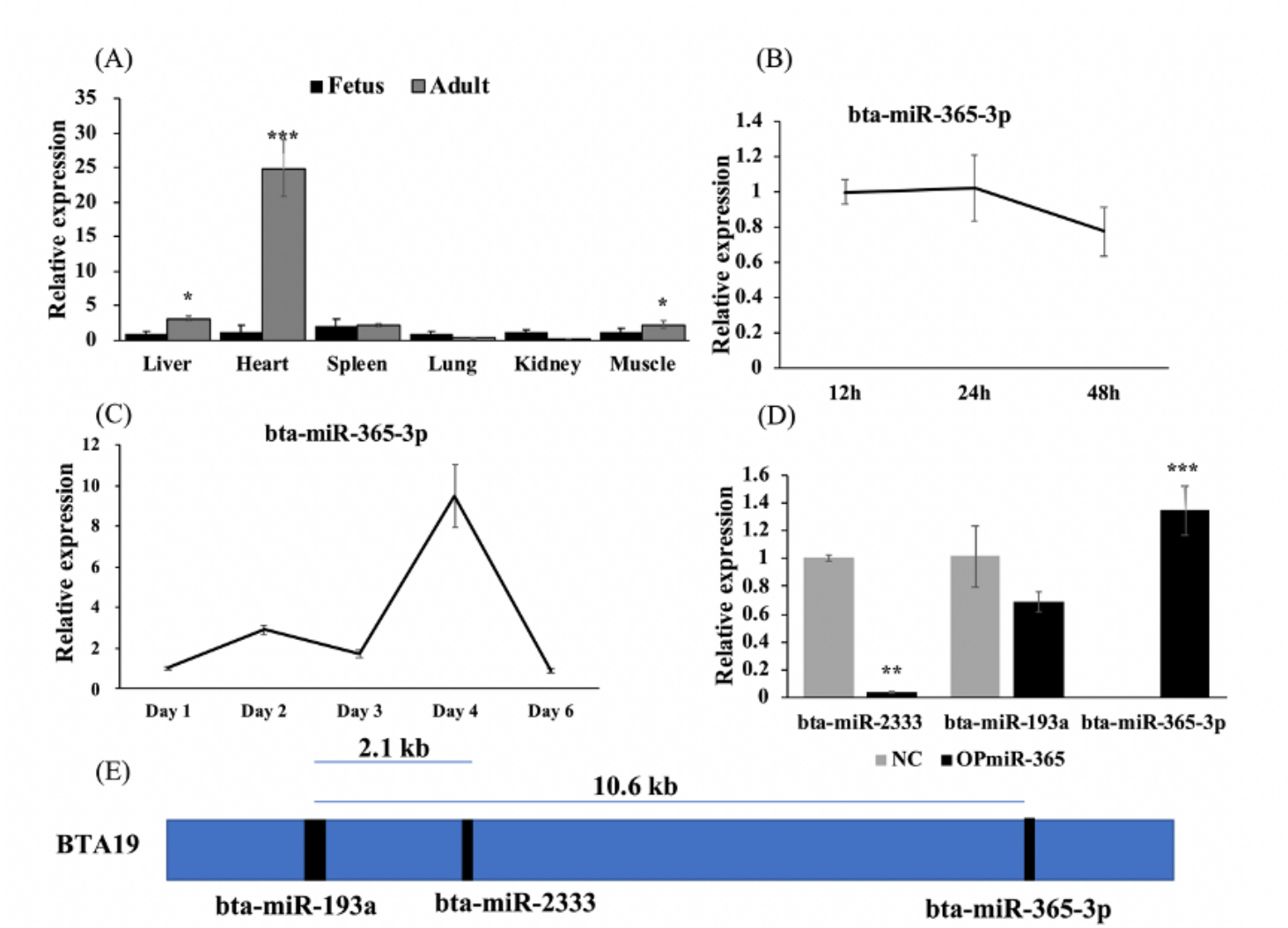


Figure 1

The expression level of bta-miR-365-3p at different development stages of cattle tissues and PBMs. (A) Differential expression level of bta-miR-365-3p in different tissues between fetal stage and adult stage of QC cattle. (B) bta-miR-365-3p expression levels during PBMs proliferation. (C) bta-miR-365-3p expression levels during PBMs differentiation with no treatment. (D) The expression levels of bta-miR-2333, bta-miR-193a and bta-miR-365-3p in PBMs, when transfected with OPmiR-365-3p in PBMs for 24 h in proliferation stage. NC, negative control with transfecting empty plasmid pcDNA 3.1. Note: The expression level of bta-

miR-365-3p was showed in the histogram that is 100 times smaller. (E) The location of bta-miR-365-3p and its neighbor miRNAs in the bovine chromosome BTA19. All data are shown as mean ± SD for three biological replicates, the error bars indicated the SD among three repeats. Note：PBMs: primary bovine myoblasts; OPmiR-365-3p: the constructed pcDNA3.1 vector overexpressing bta-miR-365-3p; SD: standard deviation.*P < 0.05, *** P < 0.001.

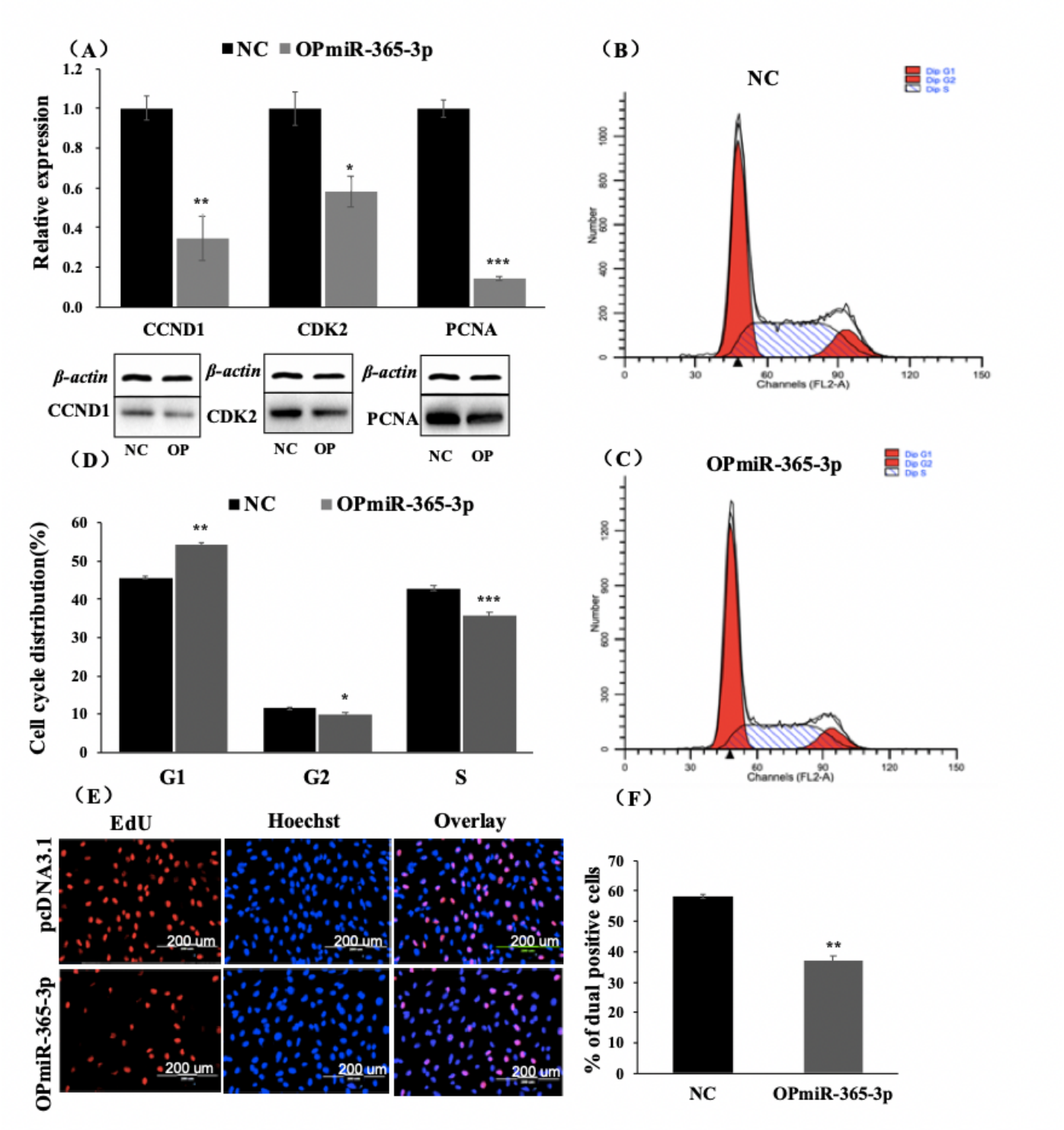


Figure 2

Transfection with OPmiR-365-3p and pcDNA.3.1 for 24 hr in PBMs for detecting effects on cell proliferation. (A) The expression of CDK2, PCNA and CCND1 at the mRNA level and protein level by qRT-PCR and western blotting, respectively. The OP group denotes the group treated with the vector OPmiR-365-3p, which overexpresses bta-miR-365-3p. (B) The PBMs transfected with empty plasmid pcDNA3.1 by flow cytometer assay. (C) The PBMs transfected with OPmiR-365-3p by flow cytometer assay. (D) Statistics of the counts of G1, G2 and S stage cells of flow cytometer assay. Error bars indicates the SD among three repeats. (E) EdU cell proliferation index, EdU (red), DAPI (blue), scale bars 200 μm. (F) Statistics of the percentage of dual positive cells. Error bars indicates the SD among three repeats. Values are mean ± SD for three biological replicates. Note CDK2: cyclin-dependent kinase 2; PCNA: Proliferating cell nuclear antigen; CCND1: CyclinD1; NC: Negative control transfected with the empty plasmid pcDNA3.1; OP: OPmiR_365-3p, the vector overexpressing bta-miR-365-3p; qRT-PCR: quantitative real-time polymerase chain reaction; SD: standard deviation. *P < 0.05, ** P < 0.01, *** P < 0.001.

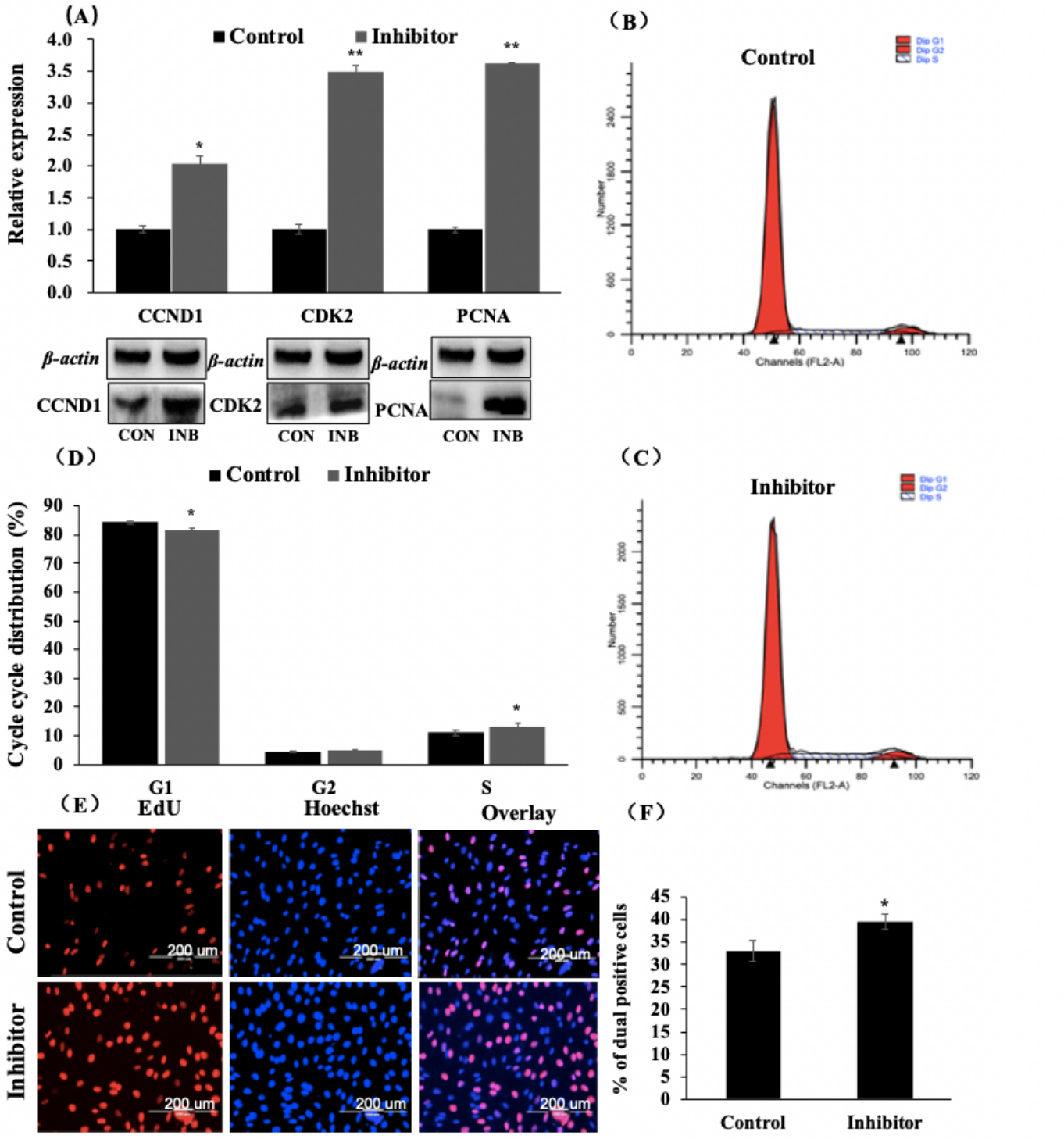


Figure 3

Transfection with the inhibitor of bta-miR-365-3p for 24 hr in PBMs for detecting effects on cell proliferation. (A) The expression of CDK2, PCNA and CCND1 at the mRNA level and protein level by qRT-PCR and western blotting, respectively. The CON group indicates the group treated with NC, and the INB group indicates the group treated with the inhibitor of bta-mR-365-3p. (B) The PBMs transfected with NC by flow cytometer assay. (C) The PBMs transfected with the inhibitor of bta-miR-365-3p by flow cytometer

assay (D) Statistics of the counts of G1, G2 and S stage cells of flow cytometer assay. (E) EdU cell proliferation index. EdU (red), DAPI (blue), scale bars 200 μm. (F) Statistics of the percentage of dual positive cells. Values are mean ± SD for three biological replicates. Note：CDK2: cyclin-dependent kinase 2; PCNA: Proliferating cell nuclear antigen; CCND1: CyclinD1; NC: Negative control. qRT-PCR: quantitative real-time polymerase chain reaction; SD: standard deviation. *P < 0.05, ** P < 0.01, *** P < 0.001.

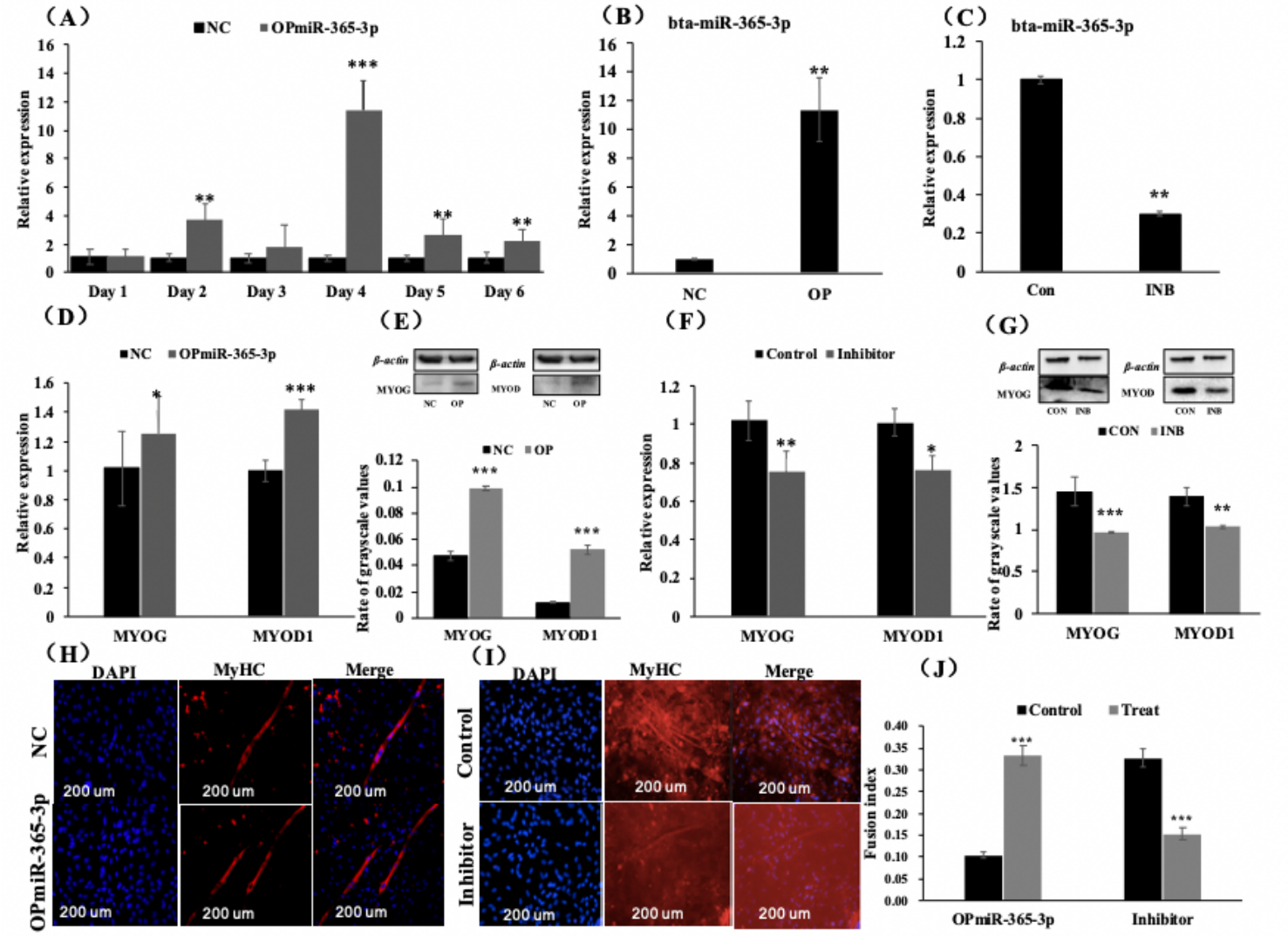
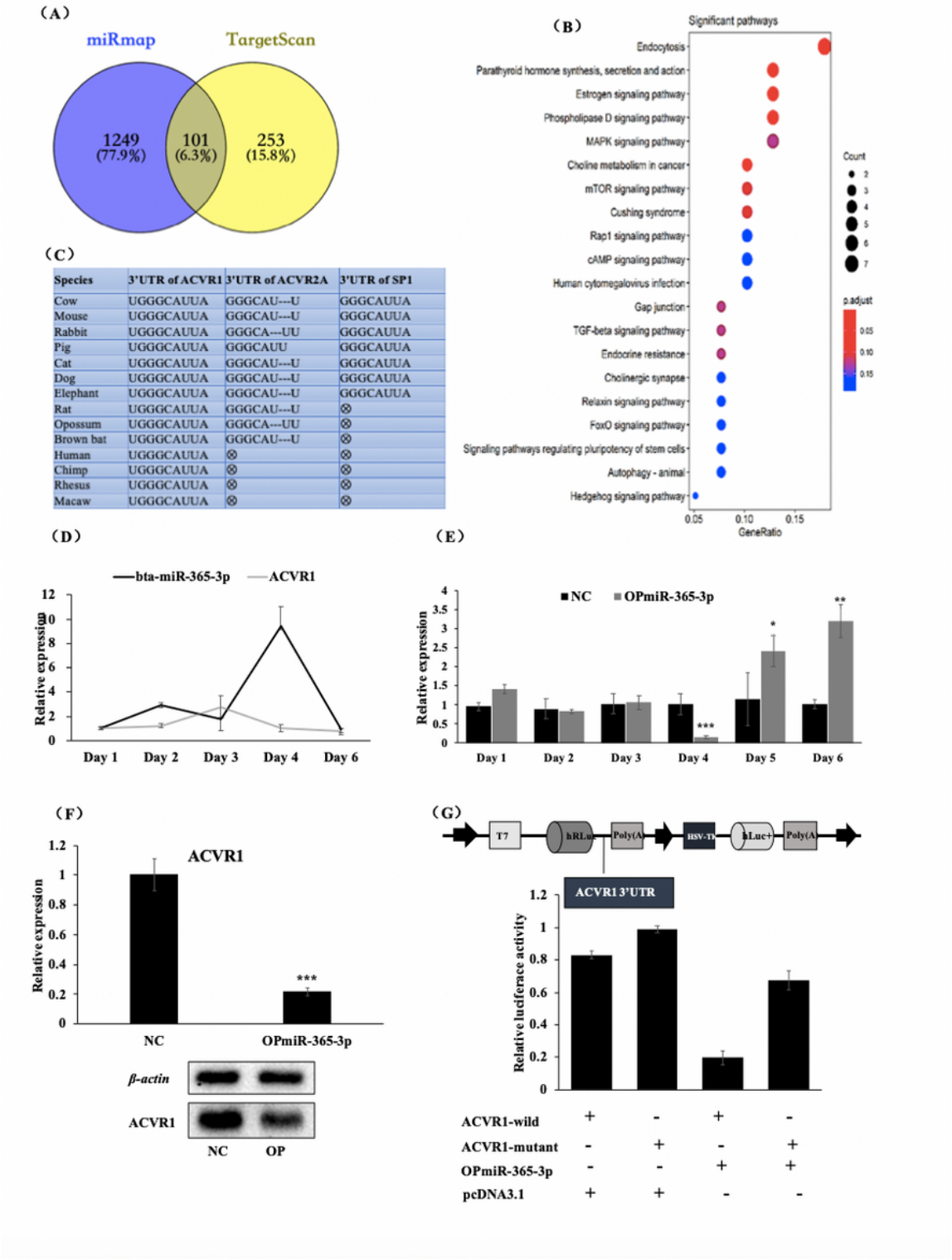


Figure 4

Overexpression of bta-miR-365-3p promoted primary cattle myoblast differentiation. (A) The expression level of miR-365-3p during PBMs differentiation after treatment with OPmiR-365-3p for differentiation six days. (B) The expression level of bta-miR-365-3p after treatment with OPmiR-365-3p in PBMs at four days. (C) The expression level of bta-miR-365-3p after treatment with its inhibitor for four days (D) The mRNA expression of MYOD1 and MYOG in PBMs after treatment with OPmiR-365-3p for four days. (E) The protein expression of MYOG and MYOD1 under the treatment of OPmiR-365-3p, and the statistic relatively rate grayscale values of MYOG or MYOD1 with β-actin with Image Lab software. (G) The mRNA expression of MYOD1 and MYOG in PBMs after treatment with the inhibitor of bta-miR-365-3p for four

days. (G) The protein expression of MYOG and MYOD1 under the treatment of bta-miR-365-3p inhibitor, and the statistic the relatively rate grayscale values of MYOG and MYOD1 (F) MyHC (red)-positive myotubes were detected by immunofluorescence after transfection with OPmiR-365-3p, scale bars: 200 μm. (G) MyHC (red)-positive myotubes were detected by immunofluorescence after transfection with the inhibitor of bta-miR-365-3p. Scale bars: 200 μm. (F) The fusion indexes calculation, the number of nuclei in the myotube as a percentage of the total nucleus. The 'Treat' indicated that the groups were treated with OPmiR-365-3p or the inhibitor. The 'Control' indicated the groups of their negative control. Values are mean ± SD for three biological replicates. Note：MYOD1: Myogenic differentiation 1; MYOG: Myogenin. SD: standard deviation *P < 0.05, ** P < 0.01, *** P < 0.001.



Species	3'UTR of ACVR1	3'UTR of ACVR2A	3'UTR of SP1
Cow	UGGGCAUUA	GGGCAU---U	GGGCAUUA
Mouse	UGGGCAUUA	GGGCAU---U	GGGCAUUA
Rabbit	UGGGCAUUA	GGGCA---UU	GGGCAUUA
Pig	UGGGCAUUA	GGGCAUU	GGGCAUUA
Cat	UGGGCAUUA	GGGCAU---U	GGGCAUUA
Dog	UGGGCAUUA	GGGCAU---U	GGGCAUUA
Elephant	UGGGCAUUA	GGGCAU---U	GGGCAUUA
Rat	UGGGCAUUA	GGGCAU---U	⊗
Opossum	UGGGCAUUA	GGGCA---UU	⊗
Brown bat	UGGGCAUUA	GGGCAU---U	⊗
Human	UGGGCAUUA	⊗	⊗
Chimp	UGGGCAUUA	⊗	⊗
Rhesus	UGGGCAUUA	⊗	⊗
Macaw	UGGGCAUUA	⊗	⊗

Figure 5

Bovine ACVR1 is a target gene for miR-365-3p. (A) The common target genes of bta-miR-365-3p from two databases, miRmap and TargetScan. (B) The KEGG pathways associated with the common target genes of bta-miR-365-3p. The red to blue color indicated that p.adjust values was increasing, and the red circle means the genes were enriched in the significant pathway; the size of the circle indicates the gene counts in the pathway. (C) Conservation among various species of the bta-miR-365-3p target sequences in the 3'

UTR of ACVR1, ACVR2A, SP1. (D) The expression level of ACVR1 during PBMs differentiation (E) The expression level of ACVR1 after transfected with OPmiR-365-3p for continuously inducing differentiation 6 days (F) The mRNA and protein expression of ACVR1 when transfected with the interference siACVR1-1 cultured with growth medium (GM) for proliferating 24 h . (G) The structure of the psiCHECK-2 dual-luciferase reporter vector, and the luciferase activity test when transfected with ACVR1- wild, ACVR1-mutant, OPmiR-365-3p and pcDNA3.1(NC). Note: ACVR1: activin A receptor type I; ACVR2A: activin A receptor type 2A; Sp1: transcription factor; ACVR1- wild: psiCHECK-2 dual-luciferase reporter vector of the ACVR1 3'UTR region. ACVR1-mutant: psiCHECK-2 dual-luciferase reporter vector of the ACVR1 3'UTR region with mutation; SD: standard deviation. *** P < 0.001

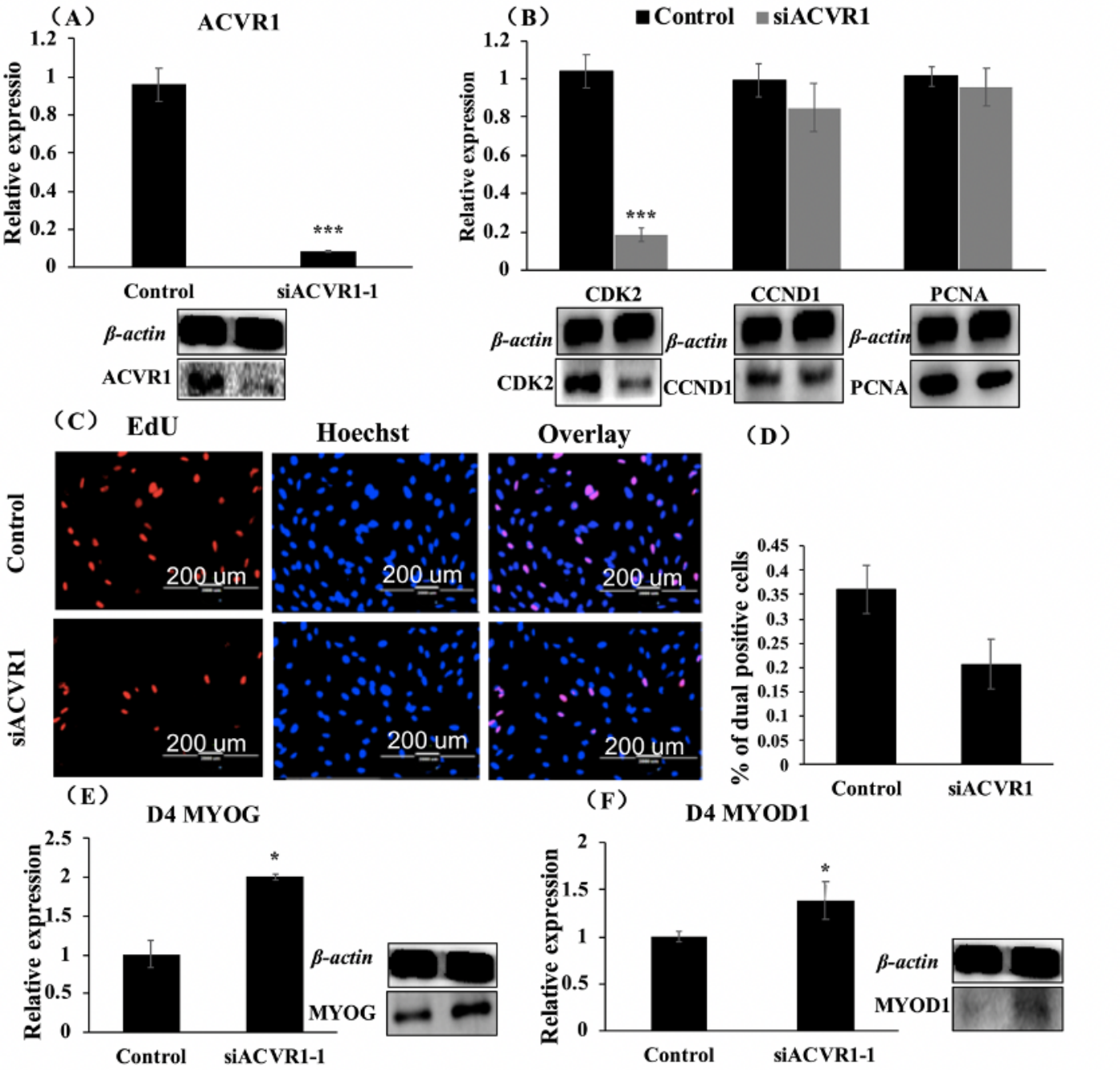


Figure 6

Knock-down of ACVR1 inhibited primary myoblast proliferation but promoted PBMs differentiation. (A) RNA interference using siACVR1-1 decreases expression of ACVR1 at mRNA and protein levels compared with the control group by qRT-PCR and western blotting in PBMs, respectively. (B) The mRNA and protein expression levels of CDK2, CCND1, and PCNA at 24 hr. post transfection with siACVR1-1 in PBMs. (C) EdU proliferation assay detected the cell proliferation index. EdU (red), Hoechst (blue), scale bars 2000 μm. (D) Statistics of the percentage of dual positive cells. Error bars indicates the SD among three repeats. (E) The mRNA and protein expression of MYOG in PBMs after treated with siACVR1-1 and induced differentiation for four days. (F) The mRNA and protein expression of MYOD1 in PBMs after treated with siACVR1-1 induced for four days. Values are mean ± SD. Note: CDK2: cyclin-dependent kinase 2; PCNA: Proliferating cell nuclear antigen; MYOD1: Myogenic differentiation 1; MYOG: Myogenin; siACVR1: The interference RNA of ACVR1 in cattle; SD: standard deviation. *P < 0.05; *** P < 0.001.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplemental.docx