

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

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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, and we studied its function to 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. Moreover, downregulation of bta-miR-365-3p increased expression of CCND1, CDK2 and PCNA but decreased 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 *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* to regulate its expression. Consistently, knock-down of *ACVR1* was associated with reduced CDK2, CCND1 and PCNA expression but increased MYOD1 expression.

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 differentiates 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 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 human 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 in all the studied tissues but with the highest expression level in muscle, which suggested a regulatory role in this tissue [13]. Furthermore, bta-miR-365-3p was differentially expressed between fast- and slow-type muscles (semitendinosus *versus* masseter) in Japanese Black steers[14]. Research has found a 2.6 fold higher expression of bta-miR-365-3p in adult stage of muscle tissues than in the fetal stage of Qinchuan cattle, the fold change was higher than a well-known muscle-specific miRNA, such as bta-miR-206 [15] (Fig S1A). Previous studies also revealed that the total number of sequence reads of bta-miR-365-3p in the proliferation stages of skeletal muscle-derived satellite cells from Chinese Simmental calves was almost 3.5 times higher than in the differentiation stages, which was similar to miRNAs such as bta-miR-378a-3p and bta-miR-23a [16–18]. 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 [19] but also to contribute to osteoarthritis pathogenesis in human [20]. 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: NWFAC1019). 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 [21]. 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). Differentiation was stimulated in DMEM containing 2% horse serum (HyClone) (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. 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. Cells were transfected with the vectors OPmiR-365-3p, 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% CO₂. 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 S2 .

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 were 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 phenylmethylsulfonyl 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™ 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 (Cambridge, MA, USA). Anti-β-actin were purchased from SunGenBio (Tianjin, China). HRP-conjugated Goat Anti-Rabbit IgG were 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).

2.5 EdU and flow cytometry assay

After 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, Guanzhou, 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 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 were 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).

2.7 Dual-Luciferase reporter assay

Dual-luciferase reporter assays were 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 [22,23]. VENNY (version 2.1) (<https://bioinfogp.cnb.csic.es/tools/venny/index.html>) was used to obtain the common targets from the two databases [24]. The R package *clusterProfiler* [25] 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 \pm 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 (Fig.1D), whereas the expression level of bta-miR-2333 and bta-miR-193a that map close to bta-miR-365-3p on bovine chromosome BTA19 were unchanged (Fig.1E), which indicated that the expression vector was constructed successfully (Fig.1D, 1E and Fig.S2 a,b).

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 [26–28]. 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 PBM cells with OPmiR-365-3p that overexpresses bta-miR-365-3p (Fig. 2A). Flow cytometer assays showed that PBMs numbers were reduced in the G₂-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 G₀/G₁-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 G₀/G₁-phase, when we knock downed bta-miR-365-3p in PBMs (Fig. 3B, 3C and 3D). Also, the number of EdU-positive cells were increased 15.4% in the bta-miR-365-3p inhibitor group (Fig. 3E and 3F). Based our results, we conclude 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 in PBM differentiation, we first monitored its expression levels following induction of differentiation and overexpression by means of transfection with OPmiR-365-3p, and as expected, quantitative PCR showed an increased expression level of bta-miR-365-3p (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), but reduced by the inhibitor of bta-miR-365-3p (Fig. 4E). Moreover, immunofluorescence staining showed that bta-miR-365-3p overexpression gave a higher amount of MyHC-positive myotubes than in the control group (Fig. 4F), while the opposite result was found by treatment with the bta-miR-365-3p inhibitor (Fig. 4G). 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. Intersection of the predicted targets gave 101 genes, which were used as input 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-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 of ~0.1. 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 site especially in *ACVR1* (Fig 5C). Consistently, overexpression of bta-miR-365-3p negatively regulated the expression level of *ACVR1* at both mRNA and protein levels in PBMs (Fig 5D). 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.5E).

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 and Fig S1C). Furthermore, 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 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 [29]. Understanding the molecular genetics of bovine skeletal muscle development will therefore provide important information for use 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 [6,13–15,18,30]. 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 [31]. Six miRNAs regulated proliferation, apoptosis and differentiation of PBMs through targeting of various functional genes [32–37] (Table 1). Thus, miR-744 was abundantly expressed in the fetal stage of Qinchuan cattle and positively regulated skeletal muscle satellite cell myogenic proliferation, while bta-miR-365-3p showed higher expression in muscle tissues of adult stage than in fetal stages and with expression levels similar to bta-miR-1 [15,34] (Fig S1A). Moreover, bta-miR-356-3p showed the same tendency towards increased expression as bta-miR-125b, bta-miR-378, bta-miR-27b in the cell differentiation stage (Fig. S1B) [16,18], but showed an opposite tendency of bta-miR-885, which have been shown to promote proliferation and inhibit differentiation of myoblast [37].

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

Here, we demonstrated that bta-miR-365-3p was developmentally regulated and highly expressed in heart and skeletal muscle tissues in Qinchuan cattle (Fig 1A), which is consistent with previous transcriptome data in various tissues of Angus crossbreed cattle[13]. Other studies have shown that expression levels of bta-miR-365-3p are regulated according to fast- and slow-type skeletal muscles, stage of myoblast differentiation and development stages in cattle [13–15,18]. Also, bta-miR-365-3p expression was increased during PBMs differentiation, which consistent with the study of Zhang et al (Fig. 1C and Fig. S1B) [18]. Furthermore, miR-365-3p has been reported to serve as a therapeutic biomarker for various cancers and tumors such as lung cancer [38,39], colon cancer [30], pancreatic cancer [40], breast cancer [41] and gastric tumorigenesis [42]. Moreover, miR-365-3p inhibited vascular smooth muscle cell proliferation through targeting of *CCND1*[43]. This agrees well with the present study, showing that bta-miR-365-3p acted as a negative regulator of PBM proliferation based on cell cycle analysis, which demonstrated that 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 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 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 [20].

4.3 Gene targets of miR-365-3p

Several targets of miR-365-3p has been validated such as cyclinD1 (*CCND1*) [43,44], histone deacetylase 4 (*HDAC4*) [19,20], nuclear factor I/B (*NFIB*) [45], Pax 6 [46] thyroid transcription factor 1 (*TTF1*) [38], src

homology domain containing 1 (SHC1) and Bax [40] (Table2). Here, we observed that *ACVR1* is a direct target of bta-miR-365-3p. Importantly, knock-down of *ACVR1* significantly inhibited myoblast proliferation and promoted myoblast differentiation similarly to overexpression of bta-miR-365-3p. Interestingly, mesenchymal stem cells can give rise either to myoblast or osteoblast lineages, and it has shown that a constitutively activating mutation of *ACVR1* induces expression of *Tmem176b* expression in C2C12 cells, resulting promotion of differentiation of myoblast into osteoblasts [47]. As an essential member of TGF- β family, *ACVR1* have functions in mouse embryonic development [48], lens formation [49], chondrogenesis [50], osteogenesis [51], and cardiac hypertrophy [52]. Additionally, recurrent heterozygous mutations of *ACVR1* have been associated with diseases 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* located were associated with meat weight, eye muscle area, silverside weight in cattle [56].

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*.

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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: Phenylmethylsulfonyl fluoride

RIPA: Radioimmunoprecipitation assay buffer

SHC1: src homology domain containing 1

TTF1: thyroid transcription

Tables

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

get gene abbreviation	Target ID	Associated phenotype	References PMID
AC4	517559	Skeletal muscle satellite cell myogenic differentiation	26424132
AC4	517559	Skeletal muscle satellite cell myogenic differentiation	26424132
536229	536229	Skeletal muscle satellite cell myogenic differentiation	26424132
536229	536229	Skeletal muscle satellite cell myogenic differentiation	26424132
F423	508025	Adipogeneses in skeletal muscle	28255176
TN	281187	Skeletal muscle hypertrophy	23510267
KN1A	513497	Promoted skeletal muscle cell proliferation	30317561
t5a	530005	Promoted skeletal muscle cell proliferation while inhibited the apoptosis and differentiation	31051333
AKII6	109560236	Promoted skeletal muscle cell proliferation while inhibited the apoptosis and differentiation	31051333
76	505884	Inhibited muscle cell proliferation but promoted apoptosis	30793769
AC4	517559	Promoted myoblast differentiation and inhibited proliferation	27661135
2	281240	Sponged by lncMD to promote myoblast differentiation	27589905
t3a	522467	Suppress cell differentiation and did not	29858062

		affect cell proliferation				
oD1	281938	Promote	proliferation	but	inhibit	331985035
		differentiation				

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

Gene name	Functions	References PMID
Histone deacetylase 4	Stimulate chondrocyte differentiation in chicken or mouse/osteoarthritis development in human	21856783
Histone deacetylase 4	Osteoarthritis development in human	27023516
Thyroid transcription factor1	Regulate lung cancer	22185756 and 26337230
Cyclin D1/Bcl2 apoptosis regulator	Regulate colon cancer	22072615
Src homology domain containing 1	Gemcitabine Regulate pancreatic cancer	24216611
Nuclear factor I/B	Promote cutaneous squamous cell carcinoma	24949940
Cyclin D1	Contribute to gastric tumorigenesis	24149576
Cyclin D1	Inhibit vascular smooth muscle cell proliferation	24819721 and 24936138
Paired box 6	Regulate human retinoblastoma cells	23660406
	Transport-related stress in turkeys	26760121
Potassium voltage-gated channel	Regulate nociceptive behaviors	26937014

subfamily	H			
member 2				
Interleukin-6	Host defense			21518763
Bcl regulator 2	apoptosis stimulation	Response	low-density lipoprotein	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 ten previously identified miRNAs. (A) in muscle tissues of adult and fetal stage Qinchuan cattle base on the Sun et al's study (Sun et al., 2013). (B) in primary muscle cell proliferation (P), differentiation for 1 day (D1) and differentiation for 3 days (D3) based on Zhang et al's study (Zhang et al., 2016).

Figure S2. (A) The expression level of bta-miR-2333 after transfected with OPmiR-365-3p. (B) The expression level of bta-miR-193a after transfected with OPmiR-365-3p. (C) The expression level of *ACVR1* after transfected with siACVR1s.

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.

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Authors' contributions

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

Figures

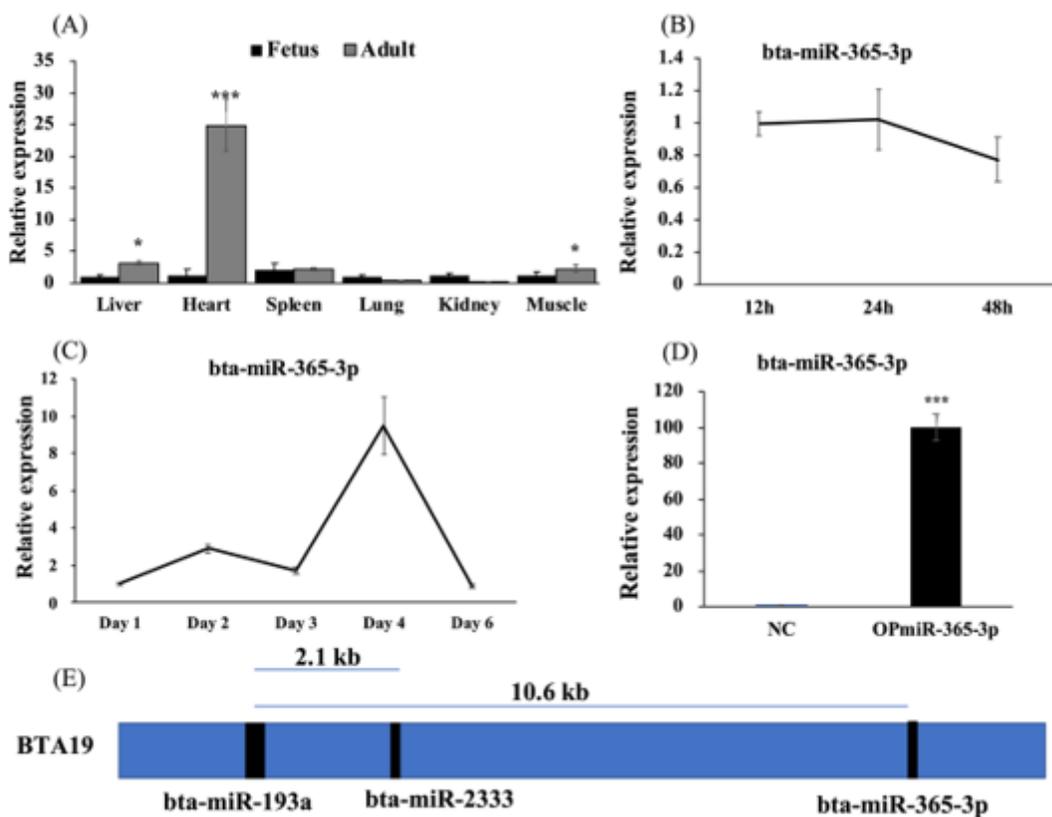


Figure 1

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-365-3p in

PBMs, when transfected with OPmiR-365-3p. NC, negative control of transfected empty plasmid pcDNA 3.1. (E) The location of bta-miR-365-3p and its neighbor miRNAs in the bovine chromosome BTA19. All data are shown as mean \pm 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. * $P < 0.05$, *** $P < 0.001$.

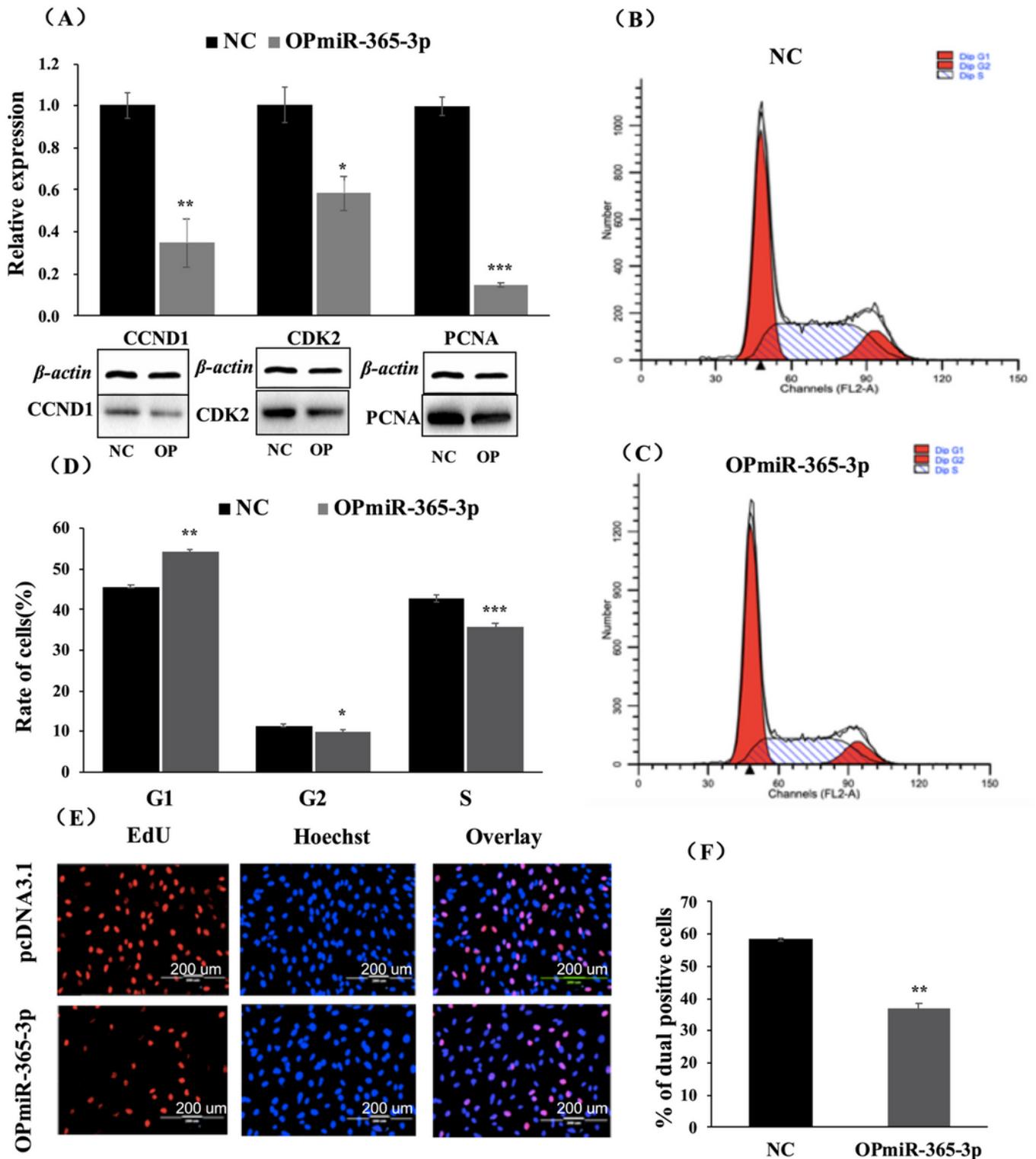


Figure 2

Transfection with OPmiR-365-3p and pcDNA3.1 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 μ m. (F) Statistics of the percentage of dual positive cells. Error bars indicates the SD among three repeats. Values are mean \pm 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.

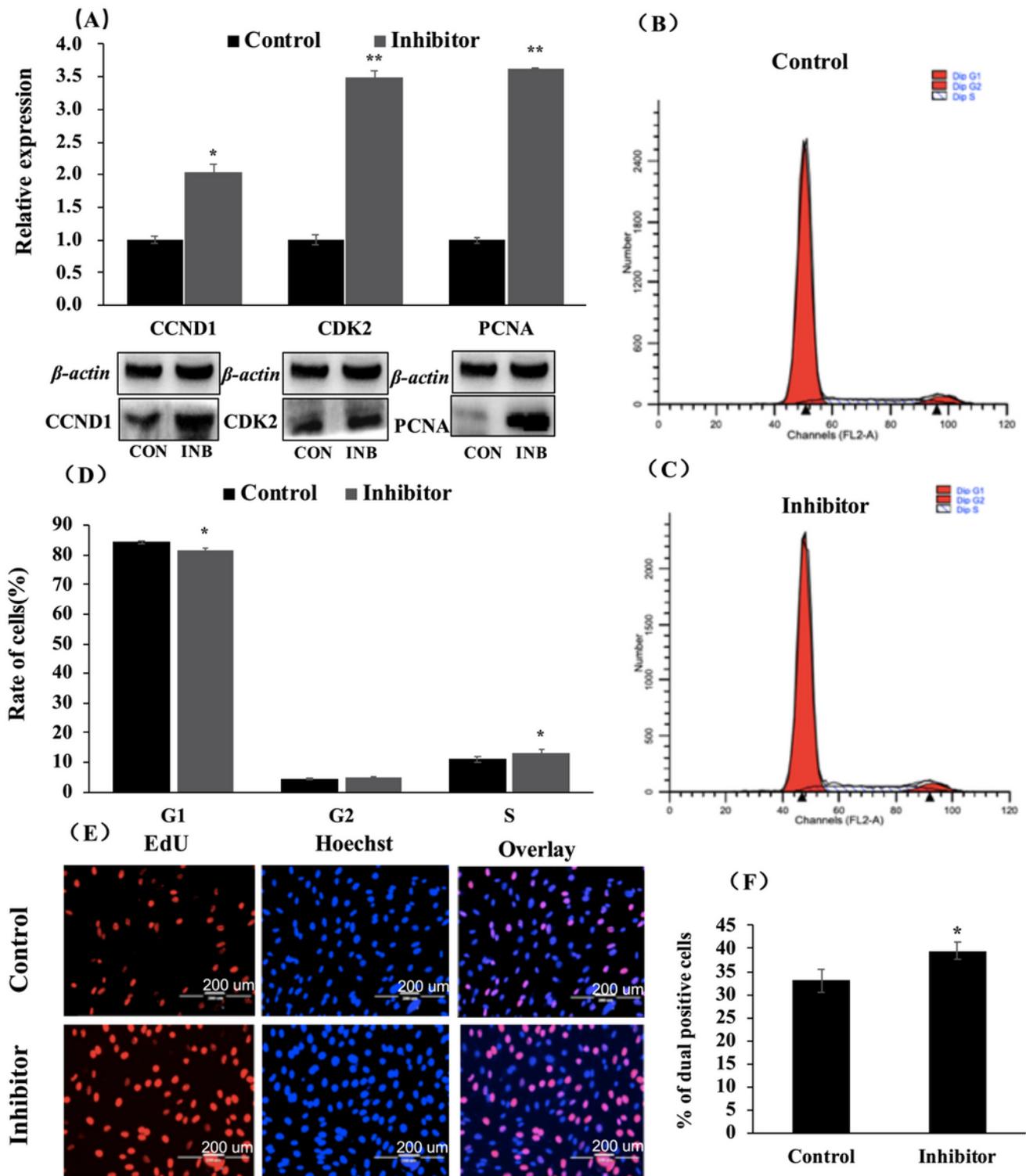


Figure 3

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's 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 μ m. (F) Statistics of the percentage of dual positive cells. Values are mean \pm 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.

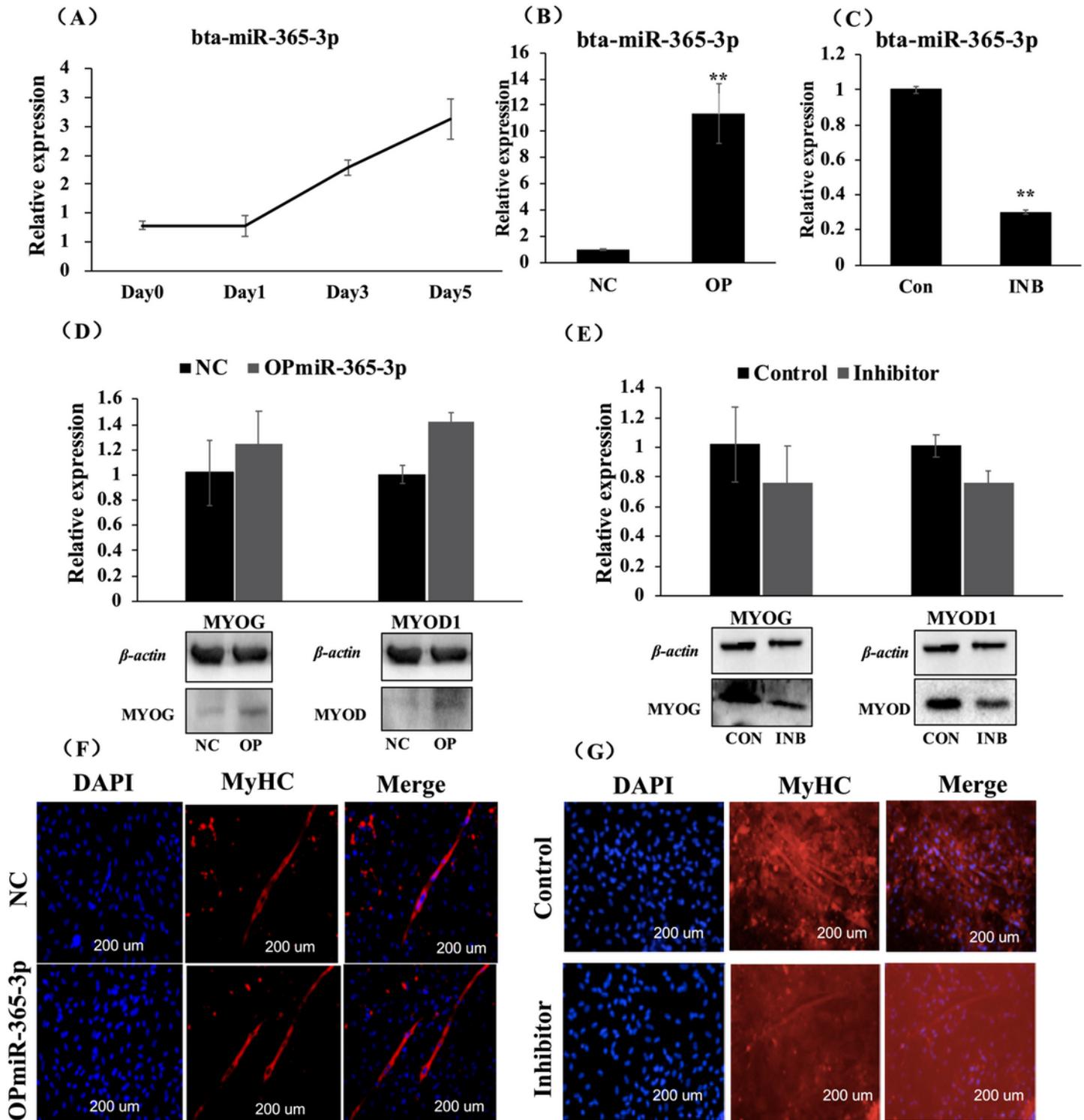


Figure 4

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. (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 and protein expression of MYOD1 and MYOG in PBMs after treatment with OPmiR-365-3p for four days. (E) The mRNA and protein expression of MYOD1 and MYOG in PBMs after treatment with the inhibitor of bta-miR-365-3p for four days. (F) MyHC (red)-positive myotubes were detected by immunofluorescence after transfection with OPmiR-365-3p, scale bars: 2000 μm . (G) MyHC (red)-positive myotubes were detected by immunofluorescence after transfection with the inhibitor of bta-miR-365-3p. Scale bars: 2000 μm . Values are mean \pm SD for three biological replicates. Note: MYOD1: Myogenic differentiation 1; MYOG: Myogenin. SD: standard deviation *P < 0.05, ** P < 0.01, *** P < 0.001.

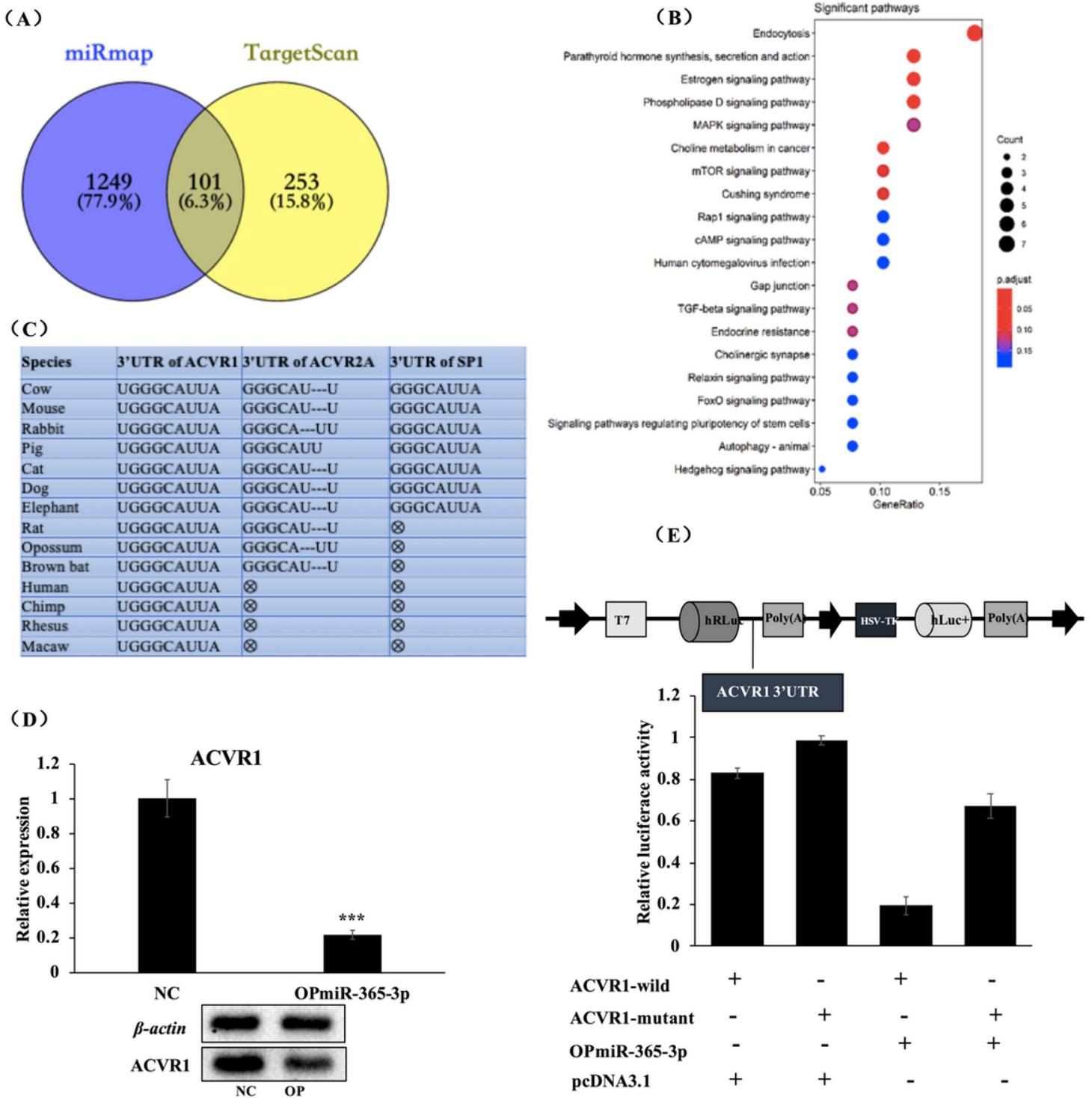


Figure 5

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 mRNA and protein expression of ACVR1 when transfected with the

interference siACVR1-1. (E) 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

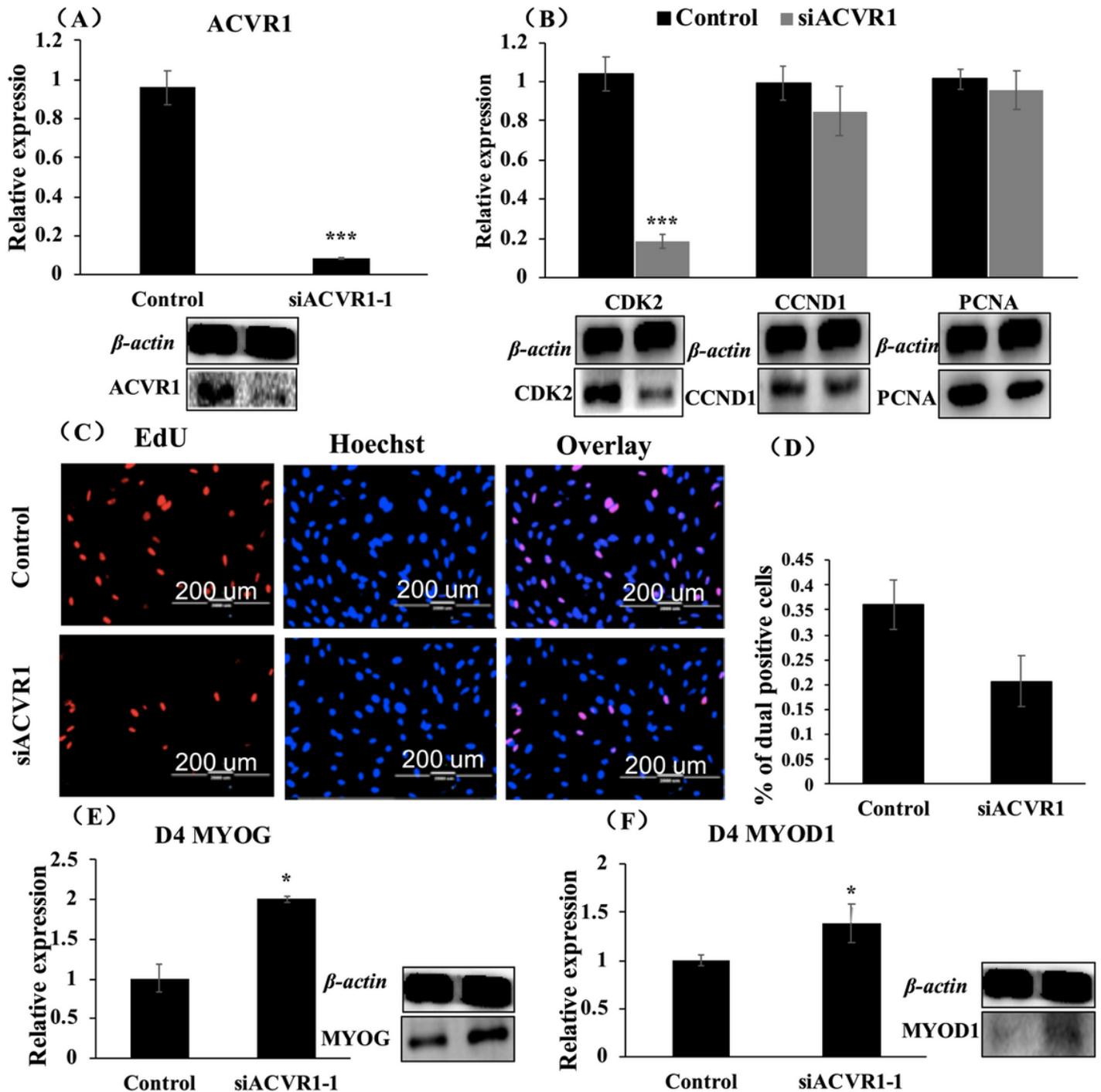


Figure 6

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 μ m. (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 \pm 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

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