

# miR-144 regulates bovine skeletal muscle satellite cell proliferation and differentiation by targeting the NACC1 gene

**yanling Ding**

Ningxia University

**Yanfeng Zhang**

Ningxia University

**Xiaonan Zhou**

Ningxia University

**Chenglong Li**

Ningxia University

**Zonghua Su**

Ningxia University

**Junjie Xu**

Ningxia University

**Yufgang Shi**

Ningxia University

**congjun li**

USDA

**xiaolong kang**

kangx1@nxu.edu.cn

Ningxia University

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## Research Article

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# Abstract

miRNAs are encoded by eukaryotic genomes and are characterized by tissue-specific and temporal expression, suggesting that miRNAs play multiple roles in different tissues and developmental periods of a species through a variety of regulatory pathways. miR-144 regulates cell development in other species, but its regulatory mechanism in bovine skeletal muscle satellite cells (BSMSCs) is unknown. So, this experiment was designed to elucidate the function of miR-144 in BSMSCs development. It was found that miR-144 promoted the proliferation of BSMSCs, but it plays an inhibitory role in the differentiation process. After transfection of the miR-144 mimic, 476 differentially expressed genes (DEGs) were detected by RNA-seq, and these DEGs mainly regulate adrenergic, MAPK, and PI3K-AKT signaling pathways. Further studies revealed that bta-miR-144 targets binding to the *NACC1* gene; whereas *NACC1* regulates BSMSCs in a manner opposite to bta-miR-144. These findings suggest that miR-144 negatively regulates BSMSCs development by targeting the *NACC1* gene.

## 1. Introduction

Skeletal muscles are mainly distributed in the trunk and limbs of livestock and poultry, which are the major components of body weight. Skeletal muscle growth and development directly affect meat production and the economic benefits of the farm. Apart from age, breed, and feeding method, genetics is also important in influencing skeletal muscle development. Skeletal muscle formation is driven by a variety of transcription factors that play essential roles at different stages of development, such as Pax3 is an upstream regulator of skeletal muscle development, which is necessary for myogenic progenitor cells to differentiate and migrate to other sites of muscle formation during early embryonic development[1]. As cells develop, Pax7 expression is activated and Pax3 expression is down-regulated, and thus the function of Pax3 is replaced by Pax7. At later stages of cell development, MYOD expression is up-regulated, while myogenic regulators such as MYF5, MYOG, and MRF4/MYF6 are activated during myofibroblast differentiation, these myogenic regulators interact with E proteins and finally bind to DNA to regulate skeletal muscle development[2]. The study found that satellite cell numbers were higher in mice with deletion of the *MYF5* gene during myogenesis[3]. In myofibers, the expression level of the *MYF4* gene was higher, whereas low expression of the *MYOD* gene was associated with fast muscle fiber types, knockdown of the *MYOD* gene altered the composition of muscle fiber types, in that fast muscle fiber was converted to slow muscle fiber [4]. Except for the transcription factors, signaling pathways such as Wnt, Foxo, and AKT [5, 6], and non-coding RNA also regulated skeletal muscle development [7, 8]. It has been found that circUBE3C down-regulated the *p27* gene expression through sponge adsorption of miR-191, which ultimately modulates the development of bovine primary myoblasts[9]. The Wnt signaling pathway regulates transcription factor LRP6 expression levels and ADAMTSL2 enhances the rate of Wnt signaling by binding to the LRP6, thereby promoting muscle differentiation[10].

miRNAs are widely expressed in eukaryotes and are characterized by tissue-specific and time-specific expression, thus determining the functional properties of tissues and cells. miRNAs are essential

regulators in energy metabolism[11], adipogenesis[12] and cancer[13]. In addition, miRNAs also play critical roles in skeletal muscle development[14, 15]. Many miRNAs are specifically expressed in skeletal muscle cells, such as miR-1, miR-133, and miR-206[16]. miR-133 enhances myofibroblast proliferation, whereas miR-1 and miR-206 stimulates myofibroblast differentiation[17]. miR-1 was significantly highly expressed in goat muscle tissues and promoted muscle development by targeting the *HDAC4* gene[18]. In addition to the specifically expressed miRNAs, a large number of non-specific miRNAs also regulate muscle development. miR-204-5p[19] and miR-664-5p[20] were associated with myofibroblast development. miR-9-5p regulated osteogenic differentiation and estrogen signaling by inhibiting the target mRNA expression[21]. miR-29b was a molecular marker for muscle atrophy, and it diminished muscle atrophy, increased myotube diameter, and reduced apoptosis and autophagy in mice[22]. Research on miR-144 has mainly focused on diseases, such as miR-144-3p inhibited colorectal cell proliferation by regulating the Wnt/β signaling pathway[23]. The miR-144 expression level was dose-dependently increased by the addition of Berberine, which inhibited lung cancer cell proliferation and promoted autophagy[24]. In muscles, miR-144 expression level was upregulated and miR-144 inhibited insulin signaling by targeting *IRS1* gene, further regulating the pathogenesis of type 2 diabetes mellitus[25]. RNA-seq analysis revealed that miR-144 was differentially up-regulated in rhesus monkeys' skeletal muscles at different ages[26]. However, there were few studies on the mechanism of miR-144 in BSMSCs development.

NACC1 is located on bovine chromosome 7 and consists of 6 exons. The biological function of NACC1 is mainly mediated through interaction with BTB-ZF proteins[27]. The regulatory mechanisms of NACC1 have been extensively studied in tumors. For example, as a transcription factor, NACC1 mediates the NF-κB signaling pathway and inhibits the growth of melanomas[28], and exon 2 of NACC1 affects neuroglial tumors through fusion with exon 3 of SREBF1[29]. In addition, NACC1 induces gene mutations during embryonic development, which in turn leads to neurological disorders such as epilepsy and psychiatric disorders[30].NACC1 was involved in osteoclastogenesis, and overexpression of NACC1 increased osteoblast proliferation but diminished differentiation [31]. Thus, NACC1 was an important factor affecting skeletal development and muscle homeostasis.

Muscle is one of the important tissues in energy metabolism and mitochondrial respiration in the organism[32]. In the previous studies, we found that miR-144 was one of the key miRNAs related to bovine RFI [33]. We hypothesized that miR-144 and its target genes might regulate energy metabolism by affecting feed digestion and absorption, which in turn regulates muscle growth and development. Based on the above findings, this experiment takes miR-144 as the research objective, analyzing its expression patterns in different tissues and muscle types, and exploring the miR-144 regulatory mechanism in BSMSCs development. This study aims to provide molecular markers for muscle development of beef cattle.

## 2. Materials and Methods

### 2.1 Ethical statement

Animal experiments were conducted following the guidelines established by the China Animal Care Committee, and the experimental procedures followed the standards set by the Association for the Protection of Laboratory Animals of Ningxia University (Permit No. NXUC20190904).

## **2.2 Tissue collection and cell culture**

Heart, longissimus dorsi muscle, and subcutaneous fat tissue were obtained from 36-month-old adult castrated cattle. The newborn male calf at 1 week old was slaughtered in a humanitarian manner, collecting tissues from different parts and storing them in a -80°C refrigerator. The primary BSMSCs used in this study were preserved in our laboratory[34].

## **2.3 Cell transfection**

The pcDNA3.1-NACC1 vector, si-NACC1, and the miR-144 mimic/inhibitor were synthesized from Tsingke Bio-technology Co., Ltd. (Beijing, China). The BSMSCs were transfected and RNAs were collected after 48h, or induced to differentiate. Table S1 lists all RNA sequences and primer information.

## **2.4 RNAs isolation, cDNA library, and RT-qPCR**

Total RNA was extracted, assayed for concentration, and reverse transcribed to cDNA for RT-qPCR reaction templates. The endogenous control for mRNA was GAPDH, and 18S snRNA was used as an exogenous control for miRNA. The RT-qPCR reaction procedure was consistent with the previous method in our laboratory[35].

## **2.5 Western blotting analysis**

Proteins were extracted from cells of different treatment groups and protein concentrations were detected using the BCA method. 15 $\mu$ g protein samples were added to 12% SDS-polyacrylamide gel, and then the gel was correctly trimmed and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). Then, washing membranes with 3% BSA for 1h, and overnight incubation of primary antibodies. The antibodies including *CDK2*(1:800; ProteinTech, Wuhan, China), *CDK4* (1:800; ProteinTech, Wuhan, China), *PCNA* (1:1000; ProteinTech, Wuhan, China), *MYOD* (1:1000, Abways, Shanghai, China), *MYOG1* (1:1000; Abways, Shanghai, China), *MYF6* (1:1000; Abways, Shanghai, China), and *GAPDH* (1:2000; Abways, Shanghai, China). After the primary antibodies were incubated, washing membranes with TBST solution and incubated with the corresponding secondary antibodies (1:2000; Always, Shanghai, China) for 1h.

## **2.6 Dual-luciferase reporter assay**

The psiCHECK2-NACC1-3'UTR-Wt and psiCHECK2-NACC1-3'UTR-Mut were constructed by Sangon Biotech (Shanghai, China). Co-transfecting 500ng of psiCHECK2-NACC1-3'UTR-Wt or psiCHECK2-NACC1-3'UTR-Mut with either 50nM of miR-144 mimic or mimic NC using Lip3000 (Invitrogen, Carlsbad, CA, USA) in 293T cells, and then the relative fluorescence activity was measured.

## **2.7 RNA-seq analysis**

Total cellular RNAs were collected from miR-144 mimic (n=8) or mimic NC (n=8) after transfection 48h, then synthesizing cDNA libraries and assessing libraries quality. Generating raw data using the Illumina Hiseq sequencing platform (150bp paired-end reads) (BioMarker Technology; Qingdao, Shandong, China). After sequencing, the raw reads were filtered by FastQc v0.1, and mapping clean reads to the bovine reference genome (*Bos\_taurus.AR5\_UCD1.2.new.genome.fa*) using Hisat2 v2.2.1 [36]. StringTie v2.1.2 [37] was used to assemble the mapped results.

## 2.8 DEGs analysis

Comparing the gene expression levels of miR-144 mimic and mimic NC groups using DESeq2 v1.20 [38], with  $|Fold Change| \geq 1.5$  and  $FDR < 0.05$  as the threshold of significant difference between the groups. The functional and pathway enrichment of DEGs were analyzed using clusterProfiler v4.0.0 [39], and  $P < 0.05$  was used as the criterion for significant enrichment.

## 2.9 Statistical analysis

The  $2^{-\Delta\Delta Ct}$  was used to calculate the relative expression of genes and data were analyzed by one-way ANOVA using SAS 9.4 software. Each sample contained three technical replicates, and all data were represented as mean  $\pm$  standard error of the mean (SEM). \*\* $P < 0.01$ , \*  $P < 0.05$ , and  $^{ns} P > 0.05$ .

# 3. Results

## 3.1. Identification of primary BSMSCs

Culturing primary BSMSCs in complete medium to 80% confluence, then replacing the complete medium with 2% horse serum differentiation medium. Microscopic observation of morphological changes of myotubes differentiation at 2d, 4d, and 6d, the mRNA expression levels in differentiation at 2d, 4d, 6d, and 8d was detected by RT-qPCR. The results demonstrated that the cells were spindle-shaped during the proliferation period(Fig 1a). As cells differentiated, the number of myotubes increased gradually (Fig 1b). In addition, the mRNAs of *MYOD1*, *MYOG*, and *MYH3* were differentially expressed at differentiation periods, which were closely related to myocyte differentiation. Compared to 2d, the *MYOD1*, *MYOG*, and *MYH3* expression levels were higher in 6d and 8d (Fig 1c). The above results suggest that the differentiation model of primary BSMSCs has been constructed successfully in vitro.

## 3.2. Expression pattern analysis of bta-miR-144

To explore the influence of bta-miR-144 on skeletal muscle, this study compared the conservation and sequence features of miR-144 among different species using miRbase software (<https://www.mirbase.org/>). The results indicated that the mature sequence of miR-144 was not different among humans, bovines, mice, pigs, and chickens, indicating that the miR-144 mature sequence was strongly conserved (Fig 2a). In different tissues between newborn calf and adult cattle, the miR-144 expression pattern was investigated by RT-qPCR. The results found that miR-144 was expressed

highly in the newborn calf ' liver, heart, and leg muscles, with the lowest expression in the rumen and subcutaneous fat (Fig 2b). In addition, compared to the newborn calf, miR-144 was higher in heart and longissimus dorsi muscle tissues in adult cattle ( $P<0.05$ ), while significantly lower in subcutaneous fat ( $P<0.01$ ) (Fig 2c). Total cellular RNAs were extracted when primary BSMSCs reached 30%, 50%, 70%, and 100 % confluence, and differentiation 0d (70%), 2d, 4d, 6d, and 8d, respectively, and then detecting miR-144 expression levels by RT-qPCR. It was demonstrated that miR-144 expression levels decreased significantly with increasing cell confluence(Fig 2d), but with the differentiation, which was increased and higher at 6d (Fig 2e). The above studies preliminarily revealed that bta-miR-144 expression levels in muscle tissues were higher than those in other tissues.

### **3.3. Effect of miR-144 on BSMSCs proliferation and differentiation**

To detect the function of bta-miR-144 in the proliferation and differentiation, transfecting miR-144 mimic/inhibitor into BSMSCs. As shown in Fig 3a, the miR-144 expression levels in the mimic group were significantly increased than mimic NC, however, the results were reversed after inhibition of miR-144, indicating a better transfection efficiency. CCK-8 assay confirmed that miR-144 mimic decreased cell viability (Fig 3b), instead miR-144 inhibitor increased cell viability(Fig 3c). Moreover, compared to the mimic NC, the *CyclinD1*, *PCNA*, *CDK2*, and *CDK4* were decreased in the miR-144 mimic (Fig 3d). In contrast, *CyclinD1*, *PCNA*, *CDK2*, and *CDK4* were notably higher in the miR-144 inhibitor (Fig 3e). In the mimic or inhibitor group, PCNA, CDK2, and CDK4 proteins were consistent with the mRNA (Fig 3f). Furthermore, over-expression of miR-144 significantly increased MYOD1, MYOG, and MYF6 expression levels in mRNA and protein, but the opposite result was obtained after interfering with miR-144 (Fig 3g-i). These results suggest that miR-144 inhibits BSMSCs proliferation and promotes differentiation.

### **3.4. RNA-Seq analysis of overexpression bta-miR-144 in BSMSCs**

To further clarify the miR-144 regulatory role on BSMSCs development, RNA-seq was performed after overexpression of miR-144. Table S2 lists the specific information of the RNA-seq data. Furthermore, compared to the mimic NC, 310 DEGs were increased and 166 DEGs were decreased in the miR-144 mimic( $P< 0.05$ ) (Fig 4a, Table S3). The top 20 up-regulated DEGs with the highest fold change mainly included *Fbp1*, *Egr3*, and *Fgf18* (Table 1, Table S4), these genes were associated with muscle development and skeletal muscle cell differentiation [40, 41]. Go enrichment analysis showed that muscle tissue development (GO:0060537,  $P=0.00004$ ), neuromuscular process (GO:0050905,  $P=0.00014$ ), and heart development (GO:0007507,  $P=0.00046$ ) were the main enriched GO terms (Fig 4b, Table S5). Meanwhile, the DEGs were mainly enriched in the adrenergic signaling pathway in myocardial cells, MAPK, and PI3K-AKT signaling pathway (Fig 4c, Table S6). To verify the RNA-seq results accuracy, 12 DEGs were validated by RT-qPCR. Based on the results, the expression levels of 12 DEGs were consistent with those in the RNA-seq results (Fig 4d).

Table 1. Top 20 differentially expressed up-regulated genes

Gene name	M FPKM Mean	NC FPKM Mean	FDR	$\log_2 FC$
<i>F13A1</i>	7.035039	1.318023	0.00000	1.870328
<i>ZAN</i>	0.597852	0.091796	0.00000	1.718992
<i>SCN7A</i>	0.451005	0.098903	0.00000	1.584998
<i>FBP1</i>	5.458683	1.044844	0.00000	1.416713
<i>EGR3</i>	5.947703	1.995266	0.00000	1.314022
<i>SESN1</i>	15.40213	5.032959	0.00000	1.308761
<i>TRIM29</i>	1.604893	0.393962	0.00000	1.241698
<i>GFRA3</i>	10.83929	2.573364	0.00000	1.189316
<i>EGR2</i>	2.194426	0.657466	0.00000	1.139428
<i>SUSD4</i>	4.134281	1.345084	0.00000	1.13047
<i>FGF18</i>	3.708817	1.289289	0.00000	1.129788
<i>EPHA1</i>	1.09562	0.291105	0.00001	1.127428
<i>GRHL3</i>	5.50091	1.458888	0.00001	1.126464
<i>GREB1</i>	0.322969	0.091872	0.00001	1.11084
<i>INA</i>	1.502615	0.499747	0.00000	1.10667
<i>NEXMIF</i>	0.562676	0.183181	0.00000	1.10643
<i>SERPINB10</i>	2.14725	0.794903	0.00000	1.101352
<i>CEND1</i>	5.229122	1.590594	0.00002	1.081006
<i>LY6G6C</i>	3.854651	0.977842	0.00005	1.079324
<i>ALS2CL</i>	0.503287	0.166944	0.00000	1.075241

M: miR-144 mimic; NC: mimic NC

### 3.5. The target gene of bta-miR-144 was *NACC1*

To further investigate the downstream targets, bta-miR-144 potential target genes were predicted, and then 29 gene sets were screened by TargetScan, miRDB, and RNA-seq (Table 2, Fig 5a). Sequence comparison analysis showed that miR-144 was highly conserved in different species (Fig 5b). To ascertain the interaction between the bta-miR-144 and the *NACC1* gene, we introduced mutations at the miR-144 binding sites within the *NACC1* 3'-UTR (Fig 5c). Dual-luciferase reporter assays showed that miR-144 mimic significantly inhibits the *NACC1*-3'-UTR-Wt luciferase activity, while there was no remarkable difference with *NACC1*-3'-UTR-Mut in the 293T cells (Fig 5d). Furthermore, *NACC1* gene

expression levels were significantly down-regulated after over-expression of miR-144 ( $P<0.05$ ), while transfection of miR-144 inhibitor significantly increased *NACC1* gene expression levels ( $P<0.01$ ) (Fig 5e). These results suggest that the target of bta-miR-144 was the *NACC1* gene.

Table 2. The target gene statistics of bta-miR-144

Source	List of primary target genes
TargetScan	<i>NACC1</i> <i>MAFK</i> <i>TFAP4</i> <i>SNTB2</i> <i>PHACTR2</i> <i>CAV3</i> <i>PIM1</i> <i>ATP1B1</i>
miRDB	<i>NACC1</i> <i>MAFK</i> <i>TFAP4</i> <i>SNTB2</i> <i>NACC2</i> <i>ATP1B1</i> <i>MAFK</i> <i>MDM4</i>
RNA-seq	<i>NACC1</i> <i>MAFK</i> <i>TFAP4</i> <i>SNTB2</i> <i>AFF4</i> <i>MDM4</i> <i>ADAMTS15</i> <i>NACC2</i> <i>ATP1B1</i>
TagetScan∩miRDB∩RNA-seq	<i>NACC1</i> <i>UBE2D1</i> <i>ADAMTS15</i> <i>AFF4</i> <i>ATP1B1</i> <i>CALCR</i> <i>CAV3</i> <i>EIF5A2</i> <i>HTRA3</i> <i>ITPR1</i> <i>JAKMIP2</i> <i>LIFR</i> <i>MAFK</i> <i>MDM4</i> <i>MED12L</i> <i>MMP15</i> <i>NACC2</i> <i>PHACTR2</i> <i>PHTF2</i> <i>PIM1</i> <i>PLXNC1</i> <i>RASGRF1</i> <i>RGMA</i> <i>RNF125</i> <i>ROBO2</i> <i>SLC20A2</i> <i>SNTB2</i> <i>TFAP4</i> <i>TMED5</i>

### 3.6 Effect of *NACC1* on BSMSCs proliferation and differentiation

To explore the influence of the *NACC1* gene on the BSMSCs development, the *NACC1* was examined at different periods. The results revealed that the expression levels of the *NACC1* gene gradually increased and were higher at 70% and 100% confluence ( $P<0.01$ ) (Fig 6a). During the differentiation period, the *NACC1* gene expression levels gradually increased from 0d to 4d and then decreased to 4d ( $P<0.01$ ) (Fig 6b), which was the opposite of the expression pattern of miR-144. Furthermore, we designed pcDNA3.1-*NACC1* and si-*NACC1* vectors and then transfected them into BSMSCs. The results found that the transfection efficiency satisfied the experimental requirements (Fig 6c-d). In addition, pcDNA3.1-*NACC1* significantly increased the PCNA, CDK2, and CDK4 expression levels; In contrast, knockdown *NACC1* significantly decreased the PCNA, CDK2, and CDK4 expression levels (Fig 6e-g). Furthermore, overexpression of *NACC1* inhibits the MYOD1, MYOG, and MYF6 expression levels, but the MYOD1, MYOG, and MYF6 expression levels were significantly increased when transfecting si-*NACCA* (Fig 6h-j). In conclusion, these results suggest that miR-144 regulates BSMSCs mainly by targeting the *NACC1* gene.

## 4. Discussion

Research on miR-144 has been focused on cancer. For example, miR-144 was decreased in thyroid cancer tissues ( $P<0.05$ ), and miR-144 inhibited tumorigenesis in breast cancer by targeting the *ZEB1* gene [42]. miR-144 inhibited breast cancer cell proliferation and migration by down-regulating the *CEP55* gene [43]. Furthermore, miR-144 regulated muscle development and adipogenesis, as such miR-144 was highly associated with the differentiation of fibroblasts [44], cardiomyocytes [45], and smooth muscle cells [46]. miR-144 inhibited mouse cardiomyocyte proliferation and promoted apoptosis [47]. *EZH2* gene expression was diminished after transfecting of the miR-144-3p mimic, thereby reducing HASMC proliferation [46]. Fatty liver is an essential factor for obesity. miR-144 regulated the tricarboxylic

acid cycle and energy metabolism pathway in the liver, and silencing miR-144 reduced the production of fumarate, an activator of NRF2, and then inhibited the expression of NRF2 protein activity, which ultimately alleviated the development of obesity[48]. Studies on miR-144 have focused on model animals such as humans and mice, but fewer studies have been conducted on livestock. In this study, the transfecting of miR-144 mimic/inhibitor and negative control into BSMSCs, and the miR-144 expression pattern and its function were initially investigated in BSMSCs. The results showed that miR-144 was decreased during proliferation and up-regulated during differentiation. These results tentatively suggest that miR-144 diminishes BSMSCs proliferation and promotes differentiation.

To further elucidate the miR-144 regulatory functions on BSMSCs, the target genes of bta-miR-144 were predicted and validated by RNA-seq and molecular experiments. Furthermore, we found that *NACC1* was one of the target genes of bta-miR-144. *NACC1* plays a crucial function in cancers, such as *NACC1* was up-regulated in nasopharyngeal carcinoma cells, and AKT and mTOR phosphorylation levels were reduced after silencing the *NACC1* gene, which suggests that *NACC1* inhibits nasopharyngeal carcinoma cells proliferation and invasion[49]. miR-218-5p inhibits human retinoblastoma cell proliferation by targeting the *NACC1* gene[50]. *NACC1* was a transcriptional regulator of the nervous systems and urinary systems, and it was highly expressed in the brain and bladder, such as *NACC1* increased proliferation, migration, and invasion of uroepithelial cells by targeting adsorption of miR-331-3p, and hence could be a potential target molecule for uroepithelial cancer therapy[51]. In muscle, *NACC1* is an actin-binding protein, and actin is a component of the cytoskeleton[52]. During mouse development, deletion of *NACC1* leads to proliferation, and the viability of chondrocytes is reduced, rendering the spine underdeveloped, which consequently reduces the survival rate of mouse offspring[53]. Mutations in the c.892C>T locus of the *NACC1* gene cause diseases such as myoclonus and dystonia in children, which may be attributed to the fact that mutations in the *NACC1* gene affect mitochondrial function and metabolism in fibroblasts[54]. The present study similarly showed that miR-144 regulates bovine skeletal muscle development, which further suggests that *NACC1* is a key factor in myogenesis. Moreover, functional enrichment showed in this study that miR-144 target genes were significantly enriched in PI3K-AKT and MAPK signaling pathways. The MAPK signaling pathway was involved in muscle development and energy metabolism [55], which was a major intracellular signaling pathway for muscle development and adipogenesis [56]. The c-Jun, and p38-MAPK could be activated by the *FABP4* gene, and the MAPK signaling pathway increased *CyclinD1*, *MMP2*, and *CCL2* gene expression, which was involved in cell proliferation and migration [57]. The PPAR $\gamma$  protected muscle fibers by inhibiting ERK and p38-MAPK signaling pathways and NF- $\kappa$ B translocation from cytoplasm to the nucleus in differentiated C2C12 cells [58]. PI3K-AKT was a key pathway for muscle production and glucose metabolism, and differentiation of myoblasts was blocked after inhibition of the PI3K-AKT pathway[59]. Furthermore, in mouse skeletal and cardiac muscle, ApoA-IV inhibited lipid droplet production and accumulation in myofibroblasts by mediating the PI3K-AKT pathway[60]. Therefore, the present study preliminarily hypothesized that miR-144 regulates bovine muscle development by targeting the *NACC1* gene and by mediating the MAPK or PI3K-AKT signaling pathway, but the mechanism remains to be further investigated.

## 5. Conclusions

In conclusion, our study demonstrated that miR-144 regulates BSMSCs development by negatively modulating the *NACC1* gene (Fig 7). miR-144 participates in several life activity processes, and it is significant to understand its molecular regulatory mechanism for skeletal muscle development and muscular disease prevention. This study intensification the understanding of the regulatory mechanisms of small RNAs in bovine muscle development.

## Abbreviations

BSMSCs, bovine skeletal muscle satellite cells; DEGs, differentially expressed genes; SEM, standard error of the mean.

## Declarations

**Credit authorship contribution statement:** X.K. and Y.S. designed the experiments; Y.D., X.Z., and Z.S. finished the experiments; J.X. and C.Q. drew the pictures; Y.Z., C.L., and X.W. completed data analysis; C.-J.L. and X.K. revised the manuscript; Y.D. drafted the manuscript.

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**Declaration of competing interest:** The authors declare no conflict of interest.

## References

1. Lagha M, Rocancourt D, Relaix F: Pax3/Pax7-dependent population of skeletal muscle progenitor cells. *M S-Med Sci* 2005, 21(10):801-803. <https://doi.org/10.1051/medsci/20052110801>
2. Wang C, Wang M, Arrington J, Shan TZ, Yue F, Nie YH, Tao WGA, Kuang SH: Ascl2 inhibits myogenesis by antagonizing the transcriptional activity of myogenic regulatory factors. *Development* 2017, 144(2):235-247. <https://doi.org/10.1242/dev.138099>
3. Wright WE, Sasoon DA, Lin VKJC: Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. 1989, 56(4):607-617. [https://doi.org/10.1016/0092-8674\(89\)90583-7](https://doi.org/10.1016/0092-8674(89)90583-7)
4. Davie JK, Cho JH, Meadows E, Flynn JM, Knapp JR, Klein WH: Target gene selectivity of the myogenic basic helix-loop-helix transcription factor myogenin in embryonic muscle. *Dev Biol* 2007,

- 311(2):650-664. <https://doi.org/10.1016/j.ydbio.2007.08.014>
5. Segales J, Perdiguero E, Munoz-Canoves P: Regulation of Muscle Stem Cell Functions: A Focus on the p38 MAPK Signaling Pathway. *Front Cell Dev Biol* 2016, 4:91.  
<https://doi.org/10.3389/fcell.2016.00091>
6. Lei Y, Jin X, Sun M, Ji Z: RNF7 Induces Skeletal Muscle Cell Apoptosis and Arrests Cell Autophagy via Upregulation of THBS1 and Inactivation of the PI3K/Akt Signaling Pathway in a Rat Sepsis Model. *Infect Immun* 2023, 91(4):e0053522. <https://doi.org/10.1128/iai.00535-22>
7. Huang Y, Chen H, Gao X, Ren H, Gao S: Identification and functional analysis of miRNAs in skeletal muscle of juvenile and adult largemouth bass, Micropterus salmoides. *Comp Biochem Physiol Part D Genomics Proteomics* 2022, 42:100985. <https://doi.org/10.1016/j.cbd.2022.100985>
8. Shen J, Luo Y, Wang J, Hu J, Liu X, Li S, Hao Z, Li M, Zhao Z, Zhang Y *et al*: Integrated transcriptome analysis reveals roles of long non-coding RNAs (lncRNAs) in caprine skeletal muscle mass and meat quality. *Funct Integr Genomics* 2023, 23(1):63. <https://doi.org/10.1007/s10142-023-00987-4>
9. Yang H, Yue B, Yang S, Qi A, Yang Y, Tang J, Ren G, Jiang X, Lan X, Pan CJJoCP: circUBE3C modulates myoblast development by binding to miR-191 and upregulating the expression of p27. 2024, 239(2):e31159. <https://doi.org/10.1002/jcp.31159>
10. Taye N, Singh M, Baldock C, Hubmacher DJMB: Secreted ADAMTS-like 2 promotes myoblast differentiation by potentiating WNT signaling. 2023, 120:24-42.  
<https://doi.org/10.1016/j.matbio.2023.05.003>
11. Li J, Yang ZR, Yan J, Zhang K, Ning XH, Wang T, Ji J, Zhang GS, Yin SW, Zhao C: Multi-omics analysis revealed the brain dysfunction induced by energy metabolism in pelteobagrus rachelle under hypoxia stress. *Ecotox Environ Safe* 2023, 254. <https://doi.org/10.1016/j.ecoenv.2023.114749>
12. Afonso J, Lima AO, Sousa MAPD, de Athayde FRF, Fortes MRS: Transcription factors and miRNA act as contrary regulators of gene expression in the testis and epididymis of gene expression in the testis and epididymis of Bos indicus animals. *Gene* 2024, 899.  
<https://doi.org/10.1016/j.gene.2024.148133>
13. Wu SY, Chu CA, Lan SH, Liu HS: Degradative autophagy regulates the homeostasis of miRNAs to control cancer development. *Autophagy* 2024. <https://doi.org/10.1080/15548627.2024.2312035>
14. Cao XN, Tang SY, Du F, Li H, Shen XX, Li DY, Wang Y, Zhang ZC, Xia L, Zhu Q *et al*: miR-99a-5p Regulates the Proliferation and Differentiation of Skeletal Muscle Satellite Cells by Targeting MTMR3 in Chicken. *Genes-Basel* 2020, 11(4). <https://doi.org/10.3390/genes11040369>
15. Elsaeid Elnour I, Dong D, Wang X, Zhansaya T, Khan R, Jian W, Jie C, Chen H: Bta-miR-885 promotes proliferation and inhibits differentiation of myoblasts by targeting MyoD1. *J Cell Physiol* 2020, 235(10):6625-6636. <https://doi.org/10.1002/jcp.29559>
16. Van Rooij E, Sutherland LB, Qi XX, Richardson JA, Hill J, Olson EN: Control of stress-dependent cardiac growth and gene expression by a microRNA. *Science* 2007, 316(5824):575-579.  
<https://doi.org/10.1126/science.1139089>

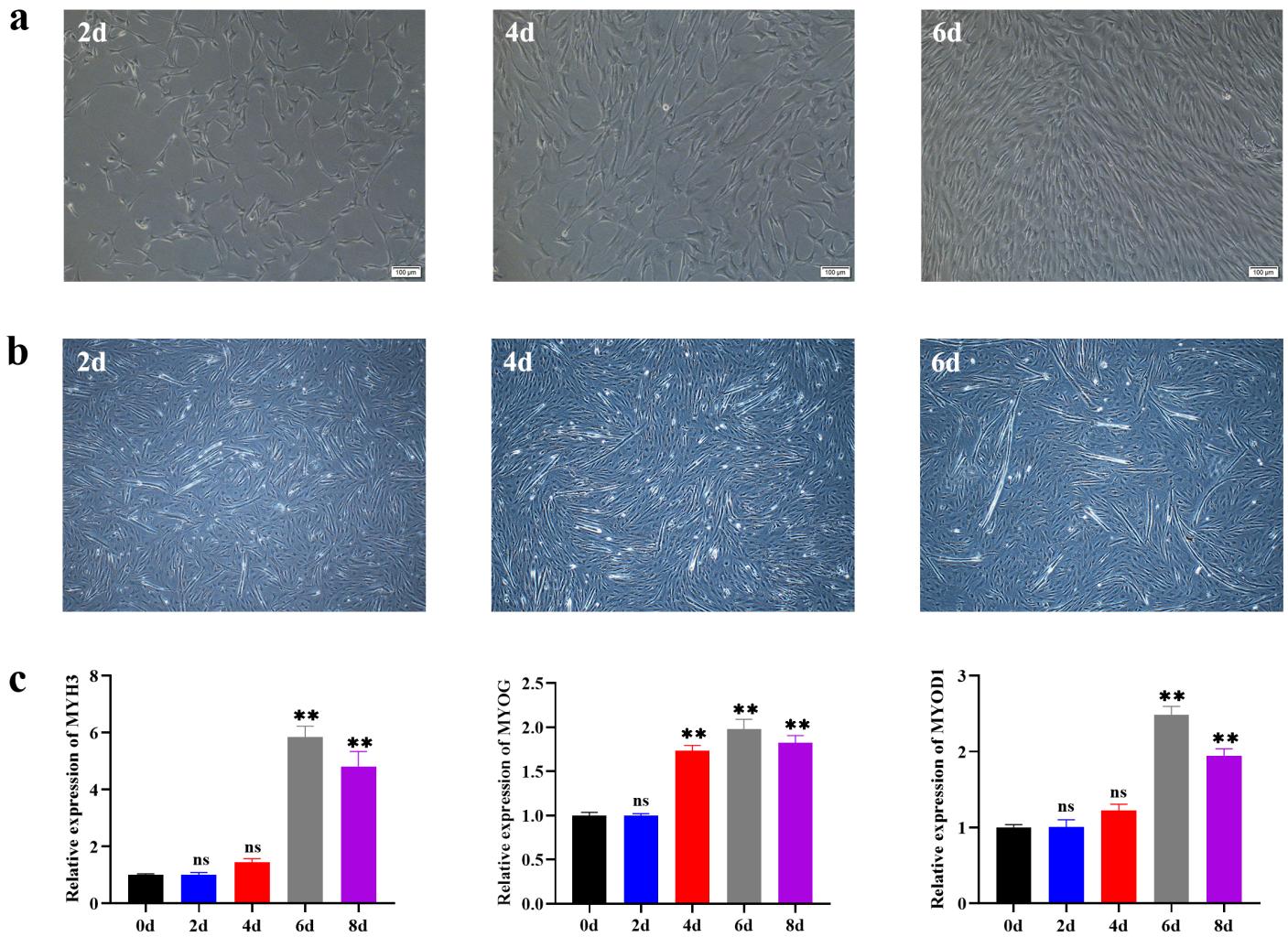
17. Wu N, Gu T, Lu L, Cao Z, Song Q, Wang Z, Zhang Y, Chang G, Xu Q, Chen GJJJoCP: Roles of miRNA-1 and miRNA-133 in the proliferation and differentiation of myoblasts in duck skeletal muscle. 2019, 234(4):3490-3499. <https://doi.org/10.1002/jcp.26857>
18. Sui M, Zheng Q, Wu H, Zhu L, Ling Y, Wang L, Fang F, Liu Y, Zhang Z, Chu MJAB: The expression and regulation of miR-1 in goat skeletal muscle and satellite cell during muscle growth and development. 2020, 31(5):455-462. <https://doi.org/10.1080/10495398.2019.1622555>
19. Cheng X, Du JJ, Shen LY, Tan ZD, Jiang DM, Jiang AA, Li Q, Tang GQ, Jiang YZ, Wang JY *et al*: MiR-204-5p regulates C2C12 myoblast differentiation by targeting MEF2C and ERRy. *Biomed Pharmacother* 2018, 101:528-535. <https://doi.org/10.1016/j.biopha.2018.02.096>
20. Cai R, Qimuge N, Ma ML, Wang YQ, Tang GR, Zhang Q, Sun YM, Chen XC, Yu TY, Dong WZ *et al*: MicroRNA-664-5p promotes myoblast proliferation and inhibits myoblast differentiation by targeting serum response factor and. *J Biol Chem* 2018, 293(50):19177-19190. <https://doi.org/10.1074/jbc.RA118.003198>
21. Emch MJ, Wicik Z, Aspros KGM, Vukajlović T, Pitel KS, Narum AK, Weivoda MM, Tang XJ, Kalari KR, Turner RT *et al*: Estrogen-regulated miRs in bone enhance osteoblast differentiation and matrix mineralization. *Mol Ther-Nucl Acids* 2023, 33:28-41. <https://doi.org/10.1016/j.omtn.2023.05.026>
22. Liu Q, Yuan W, Yan Y, Jin B, You M, Liu T, Gao M, Li J, Gokulnath P, Vulugundam GJMT-NA: Identification of a novel small-molecule inhibitor of miR-29b attenuates muscle atrophy. 2023, 31:527-540. <https://doi.org/10.1016/j.omtn.2023.02.003>
23. Sun N, Zhang L, Zhang C, Yuan Y: miR-144-3p inhibits cell proliferation of colorectal cancer cells by targeting BCL6 via inhibition of Wnt/beta-catenin signaling. *Cell Mol Biol Lett* 2020, 25:19. <https://doi.org/10.1186/s11658-020-00210-3>
24. Gao ZY, Tan C, Sha RL: Berberine Promotes A549 Cell Apoptosis and Autophagy via miR-144. *Nat Prod Commun* 2022, 17(9). <https://doi.org/10.1177/1934578x221124752>
25. Karolina DS, Armugam A, Tavintharan S, Wong MTK, Lim SC, Sum CF, Jeyaseelan K: microRNA 144 Impairs Insulin Signaling by Inhibiting the Expression of insulin Receptor Substrate 1 in Type 2 Diabetes Mellitus. *Plos One* 2011, 6(8). <https://doi.org/10.1371/journal.pone.0022839>
26. Mercken EM, Majounie E, Ding J, Guo R, Kim J, Bernier M, Mattison J, Cookson MR, Gorospe M, de Cabo RJA: Age-associated miRNA alterations in skeletal muscle from rhesus monkeys reversed by caloric restriction. 2013, 5(9):692. <https://doi.org/10.18632/aging.100598>
27. Xie Q, Tong C, Xiong XY: An overview of the co-transcription factor NACC1: Beyond its pro-tumor effects. *Life Sci* 2024, 336. <https://doi.org/10.1016/j.lfs.2023.122314>
28. Gu LX, Ren XC, Ngule C, Xiong XF, Song JX, Li ZG, Yang JM: Co-Targeting Nucleus Accumbens Associate 1 and NF- $\kappa$ B Signaling Synergistically Inhibits Melanoma Growth. *Biomedicines* 2023, 11(8). <https://doi.org/10.3390/biomedicines11082221>
29. Takeuchi Y, Mineharu Y, Arakawa Y, Hara M, Oichi Y, Kamata T, Fukuyama K, Yamamoto Y, Yamanaka T, Kakiuchi N *et al*: A novel gene fusion in an unclassifiable intracranial tumour. *Neuropath Appl Neuro* 2022, 48(7). <https://doi.org/10.1111/nan.12843>

30. Daniel JA, Elizarova S, Shaib AH, Chouaib AA, Magnussen HM, Wang J, Brose N, Rhee J, Tirard MJFiMN: An intellectual-disability-associated mutation of the transcriptional regulator NACC1 impairs glutamatergic neurotransmission. 2023, 16. <https://doi.org/10.3389/fnmol.2023.1115880>
31. Ruan Y, He J, Wu W, He P, Tian Y, Xiao L, Liu G, Wang J, Cheng Y, Zhang SJO: Nac1 promotes self-renewal of embryonic stem cells through direct transcriptional regulation of c-Myc. 2017, 8(29):47607. <https://doi.org/10.18632/oncotarget.17744>
32. Moon SH, Dilthey BG, Guan SP, Sims HF, Pittman SK, Keith AL, Jenkins CM, Weihl CC, Gross RW: Genetic deletion of skeletal muscle iPLA2g results in mitochondrial dysfunction, muscle atrophy and alterations in whole-body energy metabolism. *Iscience* 2023, 26(6). <https://doi.org/10.1016/j.isci.2023.106895>
33. Yang CY, Han LY, Li P, Ding YL, Zhu Y, Huang ZW, Dan XA, Shi YG, Kang XL: Characterization and Duodenal Transcriptome Analysis of Chinese Beef Cattle With Divergent Feed Efficiency Using RNA-Seq. *Front Genet* 2021, 12. <https://doi.org/10.3389/fgene.2021.741878>
34. Ding Y, Wang P, Li C, Zhang Y, Yang C, Zhou X, Wang X, Su Z, Ming W, Zeng L: Sodium butyrate induces mitophagy and apoptosis of bovine skeletal muscle satellite cells through the mammalian target of rapamycin signaling pathway. *International Journal of Molecular Sciences* 2023, 24(17):13474. <https://doi.org/10.3390/ijms241713474>
35. Zhao L, Ding Y, Yang C, Wang P, Zhao Z, Ma Y, Shi Y, Kang XJG: Identification and characterization of hypothalamic circular RNAs associated with bovine residual feed intake. 2023, 851:147017. <https://doi.org/10.1016/j.gene.2022.147017>
36. Li C, Li S, Yang C, Wang X, Zhou X, Shi Y, Kang XJFiG: Blood transcriptome reveals immune and metabolic-related genes involved in growth of pasteurized colostrum-fed calves. 2023, 14:1075950. <https://doi.org/10.3389/fgene.2023.1075950>
37. Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, Salzberg SL: StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nat Biotechnol* 2015, 33(3):290-295. <https://doi.org/10.1038/nbt.3122>
38. Yang C, Ding Y, Dan X, Shi Y, Kang XJFiVS: Multi-transcriptomics reveals RLMF axis-mediated signaling molecules associated with bovine feed efficiency. 2023, 10:1090517. <https://doi.org/10.3389/fvets.2023.1090517>
39. Wu T, Hu E, Xu S, Chen M, Guo P, Dai Z, Feng T, Zhou L, Tang W, Zhan L *et al.*: clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. *Innovation (Camb)* 2021, 2(3):100141. <https://doi.org/10.1016/j.xinn.2021.100141>
40. Ogura Y, Sato S, Kurosaka M, Kotani T, Fujiya H, Funabashi TJMBR: Age-related decrease in muscle satellite cells is accompanied with diminished expression of early growth response 3 in mice. 2020, 47:977-986. <https://doi.org/10.1007/s11033-019-05189-5>
41. Feng J, Han X, Yuan Y, Cho CK, Janečková E, Guo T, Pareek S, Rahman MS, Zheng B, Bi JJE: TGF-β signaling and Creb5 cooperatively regulate Fgf18 to control pharyngeal muscle development. 2022, 11:e80405. <https://doi.org/10.7554/eLife.80405>

42. Pan Y, Zhang J, Fu H, Shen L: miR-144 functions as a tumor suppressor in breast cancer through inhibiting ZEB1/2-mediated epithelial mesenchymal transition process. *Onco Targets Ther* 2016, 9:6247-6255. <https://doi.org/10.2147/OTT.S103650>
43. Yin Y, Cai J, Meng F, Sui C, Jiang Y: MiR-144 suppresses proliferation, invasion, and migration of breast cancer cells through inhibiting CEP55. *Cancer Biol Ther* 2018, 19(4):306-315. <https://doi.org/10.1080/15384047.2017.1416934>
44. Bahudhanapati H, Tan J, Dutta JA, Strock SB, Sembrat J, Alvarez D, Rojas M, Jager B, Prasse A, Zhang Y et al: MicroRNA-144-3p targets relaxin/insulin-like family peptide receptor 1 (RXFP1) expression in lung fibroblasts from patients with idiopathic pulmonary fibrosis. *J Biol Chem* 2019, 294(13):5008-5022. <https://doi.org/10.1074/jbc.RA118.004910>
45. Cao ML, Zhu BL, Sun YY, Qiu GR, Fu WN, Jiang HK: MicroRNA-144 Regulates Cardiomyocyte Proliferation and Apoptosis by Targeting TBX1 through the JAK2/STAT1 Pathway. *Cytogenet Genome Res* 2019, 159(4):190-200. <https://doi.org/10.1159/000505143>
46. Yang Z, Zhang L, Liu Y, Zeng W, Wang K: Potency of miR-144-3p in promoting abdominal aortic aneurysm progression in mice correlates with apoptosis of smooth muscle cells. *Vascul Pharmacol* 2022, 142:106901. <https://doi.org/10.1016/j.vph.2021.106901>
47. Huang F, Huang XY, Yan DS, Zhou X, Yang DY: [MicroRNA-144 over-expression induced myocytes apoptosis]. *Zhonghua Xin Xue Guan Bing Za Zhi* 2011, 39(4):353-357. <https://doi.org/10.3760/cma.j.issn.0253-3758.2011.04.015>
48. Azzimato V, Chen P, Barreby E, Morgantini C, Levi L, Vankova A, Jager J, Sulen A, Diotallevi M, Shen JXJG: Hepatic miR-144 drives fumarase activity preventing NRF2 activation during obesity. 2021, 161(6):1982-1997. e1911. <https://doi.org/10.1053/j.gastro.2021.08.030>
49. Cao Z, Chen H, Mei X, Li X: Silencing of NACC1 inhibits the proliferation, migration and invasion of nasopharyngeal carcinoma cells via regulating the AKT/mTOR signaling pathway. *Oncol Lett* 2021, 22(6):828. <https://doi.org/10.3892/ol.2021.13088>
50. Li L, Yu H, Ren Q: MiR-218-5p Suppresses the Progression of Retinoblastoma Through Targeting NACC1 and Inhibiting the AKT/mTOR Signaling Pathway. *Cancer Manag Res* 2020, 12:6959-6967. <https://doi.org/10.2147/CMAR.S246142>
51. Morita K, Fujii T, Itami H, Uchiyama T, Nakai T, Hatakeyama K, Sugimoto A, Miyake M, Nakai Y, Tanaka NJC: NACC1, as a target of MicroRNA-331-3p, regulates cell proliferation in urothelial carcinoma cells. 2018, 10(10):347. <https://doi.org/10.3390/cancers10100347>
52. Yap KL, Fraley SI, Thiaville MM, Jinawath N, Nakayama K, Wang J, Wang T-L, Wirtz D, Shih I-MJCr: NAC1 is an actin-binding protein that is essential for effective cytokinesis in cancer cells. 2012, 72(16):4085-4096. <https://doi.org/10.1158/0008-5472.can-12-0302>
53. Yap KL, Sysa-Shah P, Bolon B, Wu R-C, Gao M, Herlinger AL, Wang F, Faiola F, Huso D, Gabrielson KJPo: Loss of NAC1 expression is associated with defective bony patterning in the murine vertebral axis. 2013, 8(7):e69099. <https://doi.org/10.1371/journal.pone.0069099>

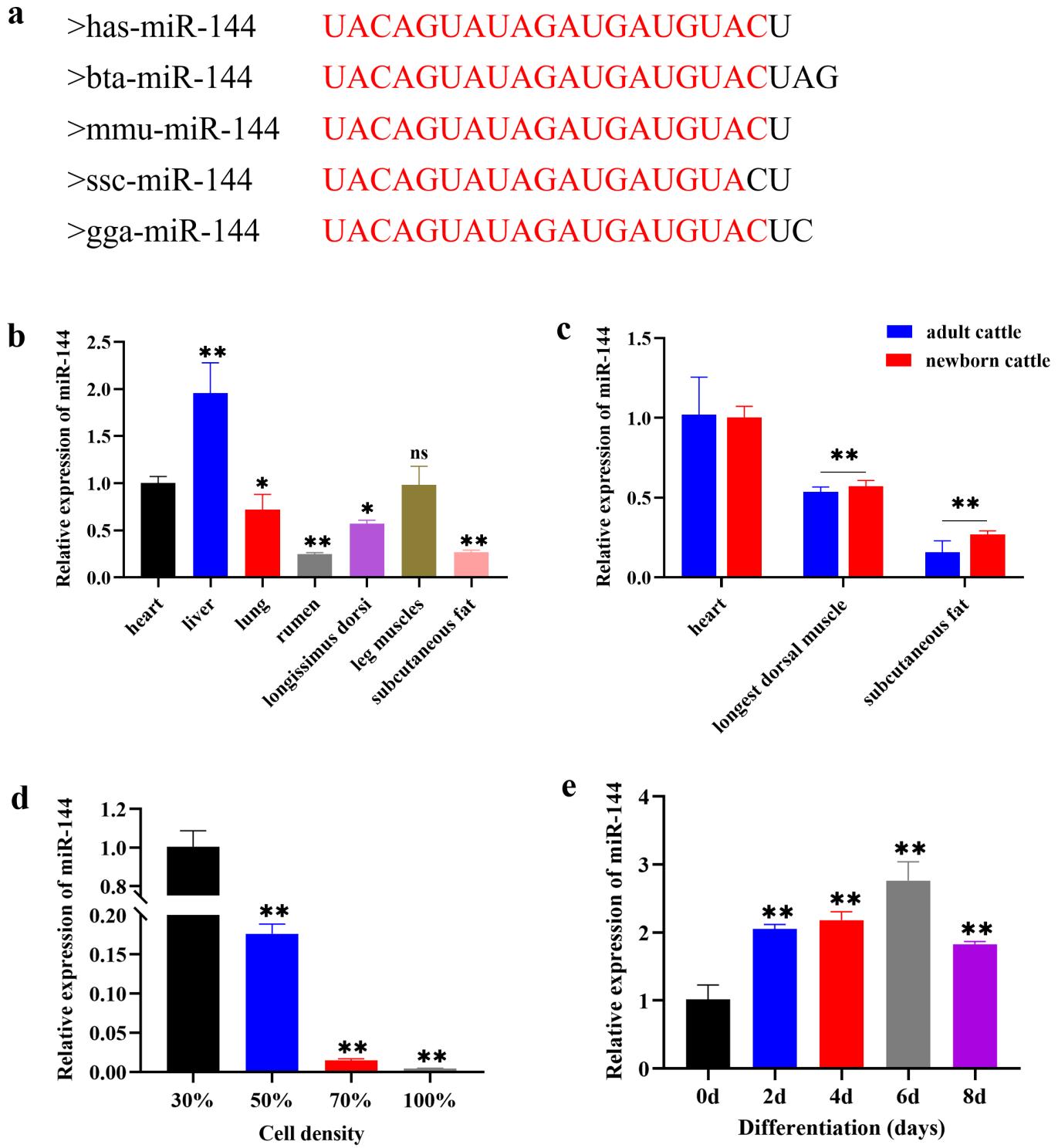
54. Komulainen-Ebrahim J, Kangas SM, López-Martín E, Feyma T, Scaglia F, Martínez-Delgado B, Kuismin O, Suo-Palosaari M, Carr L, Hinttala RJMDCP: Hyperkinetic Movement Disorder Caused by the Recurrent c. 892C> T NACC1 Variant. 2024. <https://doi.org/10.1002/mdc3.14051>
55. Jones NC, Fedorov YV, Rosenthal RS, Olwin BB: ERK1/2 is required for myoblast proliferation but is dispensable for muscle gene expression and cell fusion. *J Cell Physiol* 2001, 186(1):104-115. [https://doi.org/10.1002/1097-4652\(200101\)186:1<C104::AID-JCP1015>3E3.0.CO;2-0](https://doi.org/10.1002/1097-4652(200101)186:1<C104::AID-JCP1015>3E3.0.CO;2-0)
56. Xiao Y, Wang J, Yan W, Zhou K, Cao Y, Cai W: p38alpha MAPK antagonizing JNK to control the hepatic fat accumulation in pediatric patients onset intestinal failure. *Cell Death Dis* 2017, 8(10):e3110. <https://doi.org/10.1038/cddis.2017.523>
57. Girona J, Rosales R, Plana N, Saavedra P, Masana L, Vallve JC: Correction: FABP4 Induces Vascular Smooth Muscle Cell Proliferation and Migration through a MAPK-Dependent Pathway. *PLoS One* 2016, 11(1):e0146632. <https://doi.org/10.1371/journal.pone.0146632>
58. Kim JS, Lee YH, Chang YU, Yi HK: PPARgamma regulates inflammatory reaction by inhibiting the MAPK/NF-kappaB pathway in C2C12 skeletal muscle cells. *J Physiol Biochem* 2017, 73(1):49-57. <https://doi.org/10.1007/s13105-016-0523-3>
59. Kim S-H, Yi S-J, Lee H, Kim J-H, Oh M-j, Song E-J, Kim K, Jhun BHJAC, Systems: β2-Adrenergic receptor (β2-AR) agonist formoterol suppresses differentiation of L6 myogenic cells by blocking PI3K-AKT pathway. 2019, 23(1):18-25. <https://doi.org/10.1080/19768354.2018.1561516>
60. Zhang W, Liu X-H, Zhou J-T, Cheng C, Xu J, Yu J, Li XJAoP, Biochemistry: Apolipoprotein A-IV restrains fat accumulation in skeletal and myocardial muscles by inhibiting lipogenesis and activating PI3K-AKT signalling. 2023:1-11. <https://doi.org/10.1080/13813455.2022.2163261>

## Figures



**Figure 1**

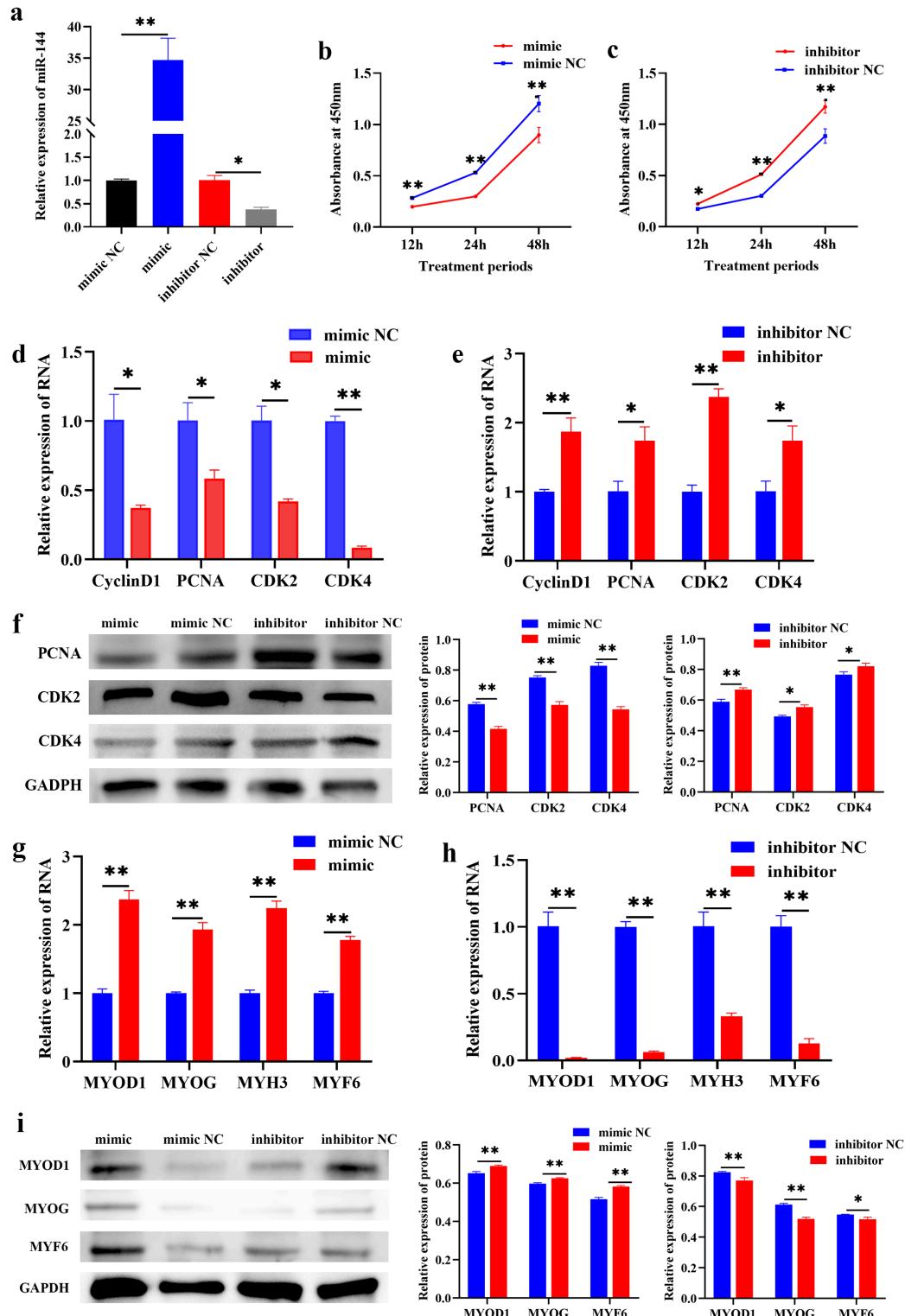
Construction and identification of proliferation and differentiation model of primary BSMSCs. a: BSMSCs diagram at different proliferation periods. b: BSMSCs diagram at different differentiation periods. c: The *MYH3*, *MYOD1*, and *MYOG* gene expression levels at BSMSCs differentiation on days 2, 4, 6, and 8. \*\*  $P < 0.01$ , ns  $P > 0.05$ .



**Figure 2**

Bta-miR-144 expression pattern analysis. a: The mature sequence of miR-144 in humans (has), cattle (bta), mice (mmu), pigs (ssc), and chickens (gga). b: The miR-144 expression level in newborn calf heart, liver, lung, rumen, longissimus dorsi, leg muscles, and subcutaneous fat. c: The miR-144 expression in main tissues at newborn calf and adult cattle. d: Expression of bta-miR-144 during

BSMSCs confluence at 30%, 50%, 70%, and 100% confluence. e: Bta-miR-144 expression levels during BSMSCs differentiation on days 2, 4, 6, and 8. \*  $P < 0.05$ , \*\*  $P < 0.01$ .

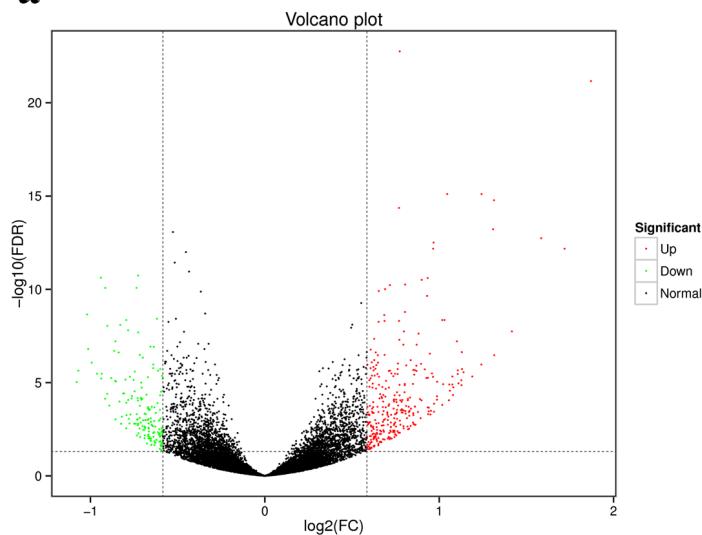


**Figure 3**

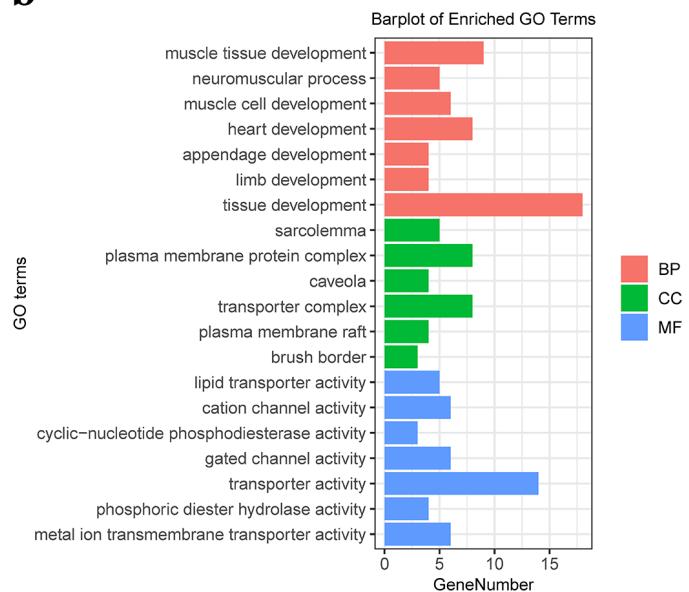
Effect of miR-144 on BSMSCs proliferation and differentiation. a: Determination of bta-miR-144 transfection efficiency. b-c: Cell viability after transfection of miR-144. d: The *CyclinD1*, *PCNA*, *CDK2* and *CDK4* gene expression levels after transfecting miR-144 mimic. e: The *CyclinD1*, *PCNA*, *CDK2* and *CDK4*

gene expression levels after transfecting miR-144 inhibitor. f: The PCNA, CDK2 and CDK4 protein expression levels after transfecting miR-144 mimic/inhibitor. g-i: The MYOD1, MYOG, MYH3 and MYF6 expression levels after transfecting miR-144mimic/ inhibitor. \*  $P < 0.01$ , \*\*  $P < 0.05$ .

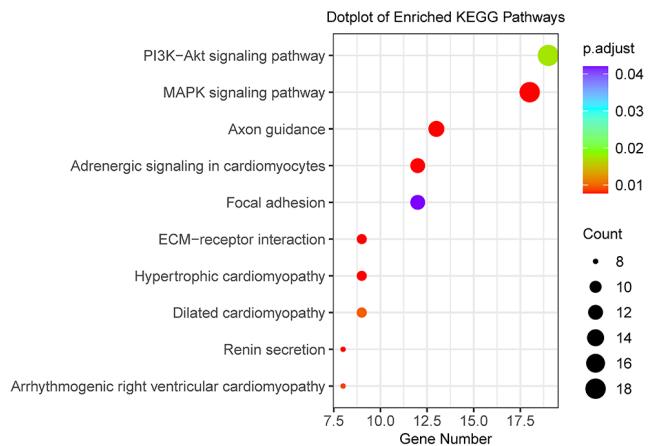
**a**



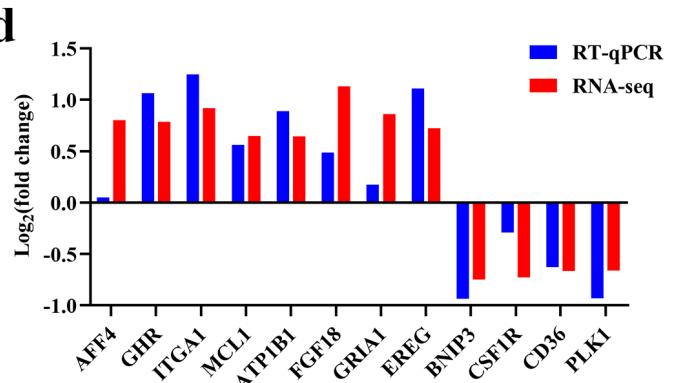
**b**



**c**

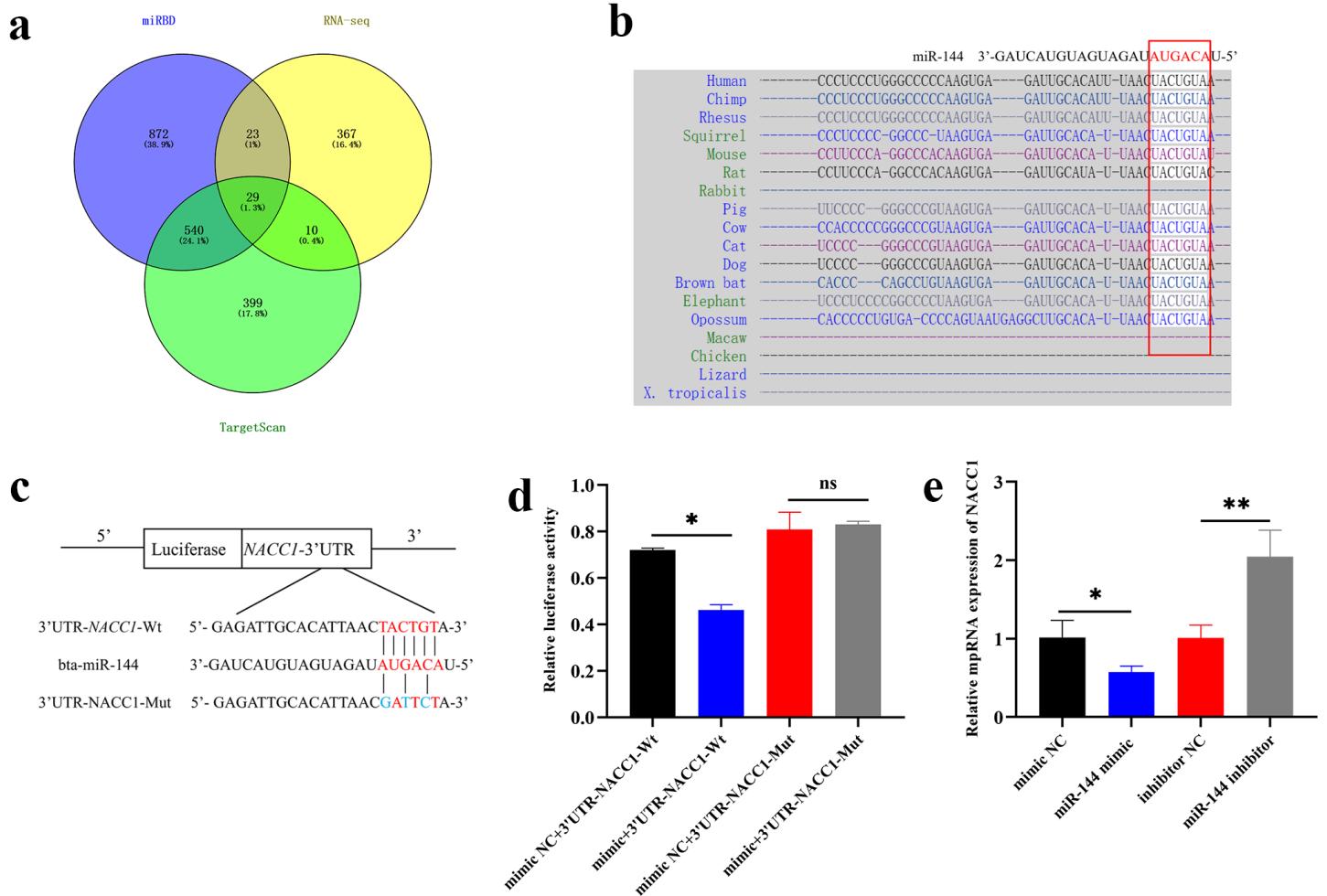


**d**



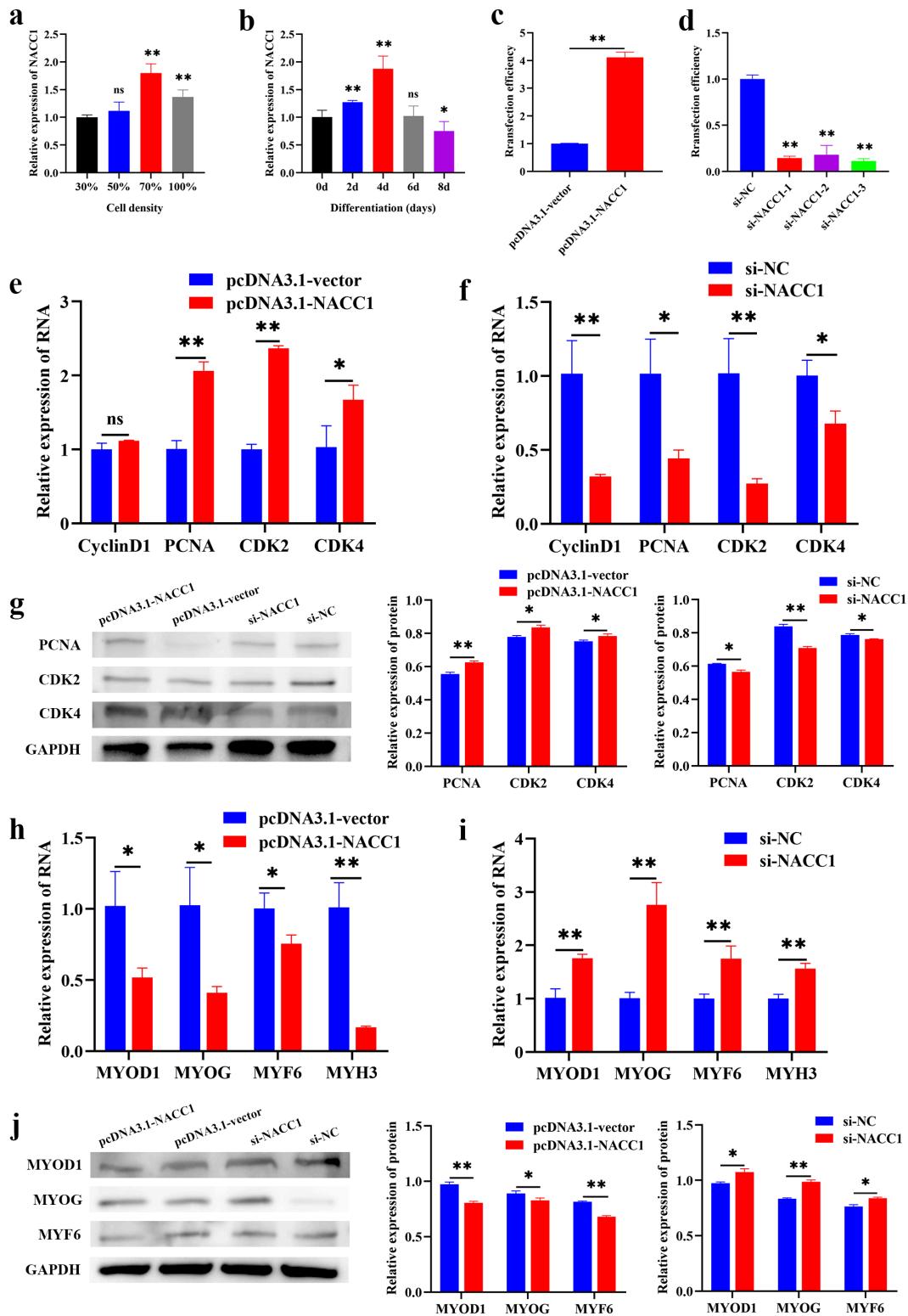
**Figure 4**

Enrichment analysis of DEGs. a: Volcano map of DEGs. b: GO terms analysis of DEGs. c: KEGG pathway analysis of DEGs. d: RT-qPCR verification of DEGs.



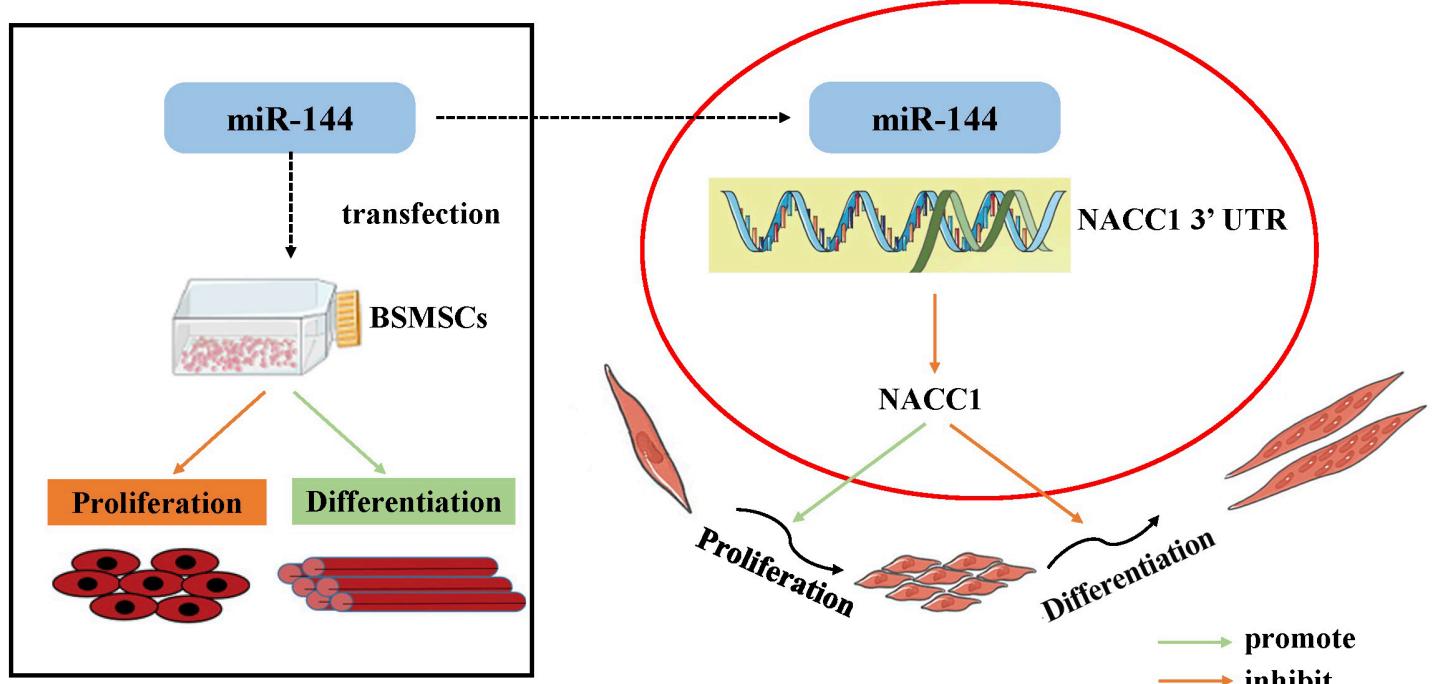
**Figure 5**

Bta-miR-144 target gene prediction and validation. a: Set of target genes predicted by TargetScan, miRDB, and RNA-seq. b: miR-144 seed region sequence targets the *NACC1* 3'-UTR in humans, bovine, mice, pigs, and chickens. c: Construction of the psiCHECK2-*NACC1*-3'UTR. d: The targeting relationship validation between the *NACC1* gene and bta-miR-144. e: The *NACC1* gene expression levels after transfecting miR-144. \*  $P < 0.05$ , \*\*  $P < 0.01$ , ns  $P > 0.05$ .



**Figure 6**

Effect of NACC1 on BSMSCs proliferation and differentiation. a: Expression of *NACC1* during BSMSCs proliferation at 30%, 50%, 70%, and 100% confluence b: Expression of *NACC1* during differentiation of BSMSCs on days 2, 4, 6, and 8. c-d: Determination of *NACC1* transfection efficiency. e-g: The PCNA, CDK2 and CDK4 expression levels after transfecting NACC1. h-j: The MYOD1, MYOG, and MYF6 expression levels after transfecting NACC1. \*  $P < 0.05$ , \*\*  $P < 0.01$ , ns  $P > 0.05$ .



**Figure 7**

The mechanism of miR-144 regulates BSMSCs development by targeting the NACC1.

## Supplementary Files

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