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RESEARCH PAPER

miR-378a-3p promotes differentiation and inhibits proliferation of myoblasts by targeting HDAC4 in skeletal muscle development

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ABSTRACT

Muscle development, or myogenesis, is a highly regulated, complex process. A subset of microRNAs (miRNAs) have been identified as critical regulators of myogenesis. Recently, miR-378a was found to be involved in myogenesis, but the mechanism of how miR-378a regulates the proliferation and differentiation of myoblasts has not been determined. We found that miR-378a-3p expression in muscle was significantly higher than in other tissues, suggesting an important effect on muscle development. Overexpression of miR-378a-3p increased the expression of MyoD and MHC in C2C12 myoblasts both at the level of mRNA and protein, confirming that miR-378a-3p promoted muscle cell differentiation. The forced expression of miR-378a-3p promoted apoptosis of C2C12 cells as evidenced by CCK-8 assay and Annexin V-FITC/PI staining results. Through TargetScan, histone acetylation enzyme 4 (HDAC4) was identified as a potential target of miR-378a-3p. We confirmed targeting of HDAC4 by miR-378a-3p using a dual luciferase assay and western blotting. Our RNAi analysis results also showed that HDAC4 significantly promoted differentiation of C2C12 cells and inhibited cell survival through Bcl-2. Therefore, we conclude that miR-378a-3p regulates skeletal muscle growth and promotes the differentiation of myoblasts through the post-transcriptional down-regulation of HDAC4.

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Introduction

Skeletal muscle growth and maintenance are essential for animal and human health, providing structural support that allows the control of motor movements and also allows energy storage.[20] Therefore, muscle development plays a crucial role in overall body metabolism. Although many factors have been found to importantly contribute to the complex process of muscle development, the different mechanisms are not fully elucidated. MicroRNAs (miRNAs), 18–25 nucleotides, small single-stranded non-coding RNAs, negatively regulate gene expression and play crucial roles in many biological processes, especially muscle development.[16] In animals, miRNAs are transcribed in the nucleus, are subsequently processed by 2 RNase III proteins (Drosha and Dicer), and are finally incorporated into RNA-induced silencing complexes that mediate translational inhibition or degradation of target mRNAs[23] by base pairing with the 3′ untranslated region (UTR) of target mRNAs. By silencing the transcription of different target mRNAs, miRNAs are involved in nearly all developmental and pathological processes in animals including cell proliferation,[6] cell differentiation,[44] apoptosis,[9,40,46] fat metabolism,[12,28] and others.

These miRNAs also play roles in muscle development in mammals. Previous studies reported that several miRNAs, such as miR-1,[47] miR-133,[31,45,49] and miR-206,[3,19,42] are muscle-specific miRNAs because they are key regulators in muscle development in human, pigs, and rats. Others miRNAs may also contribute to muscle development. For example, miR-1/206 may regulate prenatal skeletal muscle development by inhibiting SFRP1.[48] Another miRNA, miR-27b, may affect bovine skeletal muscle growth and hypertrophy by targeting the muscle-specific gene MSTN.[29] The overexpression of miR-29 in C2C12 myoblast represses proliferation but promotes myotube formation.[39]

Another miRNA, miR-378, affects cell survival, tumor growth, angiogenesis, and cell differentiation.[7] Specifically, miR-378 can enhance tumor cell survival by repressing SuFu and Fus-1,[24] increase the transcriptional activity of MyoD in part by repressing MyoR,[13] promote BMP2-induced osteogenic differentiation,[17] and also regulate osteoblast differentiation by targeting GalNT-7 in MC3T3-E1 cells.[21] Previous studies also demonstrated that miR-378 suppressed cell migration and promoted cell apoptosis in prostate cancer[8] and increased the size of lipid droplets and the incorporation of acetate into triacylglycerol.[14] In mice, miR-378 participates in the regulation of mitochondrial metabolism and energy homeostasis.[4] miR-378 suppress expression of Gli3 to inhibit activation of hepatic stellate cells and liver fibrosis.[18] However, a role of miR-378 in the regulation of skeletal muscle development has not been described.

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HDAC4, a member of the HDACs family, is as a crucial controller of cell growth,[1] differentiation,[43] and migration[37] in various cell types. HDAC4 facilitates proliferation and migration of vascular smooth muscle cell during neointimal hyperplasia.[37] HDAC4 in human glioblastoma leads to cell proliferation arrest and tumor growth impairment via inducing p21 expression.[30,41] These reports demonstrate that HDAC4 plays a significant role in regulation of cell differentiation and proliferation. Nevertheless, the mechanism by which HDAC4 controls the differentiation and proliferation of myoblasts is unclear.

In this study, we investigated the role of miR-378a-3p in regulating muscle cell differentiation and proliferation. We found that miR-378a-3p showed a high expression level in skeletal muscle, promoted differentiation, and repressed proliferation of C2C12 cells. Additionally, we determined that miR-378a-3p overexpression can regulate the differentiation and proliferation of skeletal muscle myoblasts by targeting HDAC4.

Results

miRNAs expression profile in different tissues

To study the function of miRNAs in muscle development, 8 miRNAs including miR-378a-3p were selected as potential regulatory candidates based on our previous Solexa SBS technology sequencing results.[36] The expression profile assay showed that miR-378a-3p expression was significantly higher than other miRNAs in the longissimus muscle (Fig. 1a), suggesting a potential role in muscle. We found that miR-378a-3p was expressed predominantly in muscle and weakly expressed in other tissues (Fig. 1b). Based on this, we speculated that miR-378a-3p could be a regulator in skeletal muscle development. Mature bta-miR-378 derives from the first intron of *PPARG1B* in chromosome 7 (Fig. 1c). A similarity analysis showed that miR-378a-3p was conserved among cattle, mouse, and humans (Fig. 1d). Thus, we chose mouse C2C12 cells as the experiment model.

miR-378a-3p inhibited C2C12 cell proliferation

In order to explore an effect of miR-378a-3p on myoblast proliferation, C2C12 myoblasts were transfected with pcDNA3.1-miR-378a-3p, pcDNA3.1 (+) (control plasmid with no insert), and miR-378a-3p inhibitor. The transfection efficiency was detected by RT-qPCR and the results showed significantly different expression of miR-378a-3p (Fig. S1). Flow cytometry was used to analyze the cell cycle phase distribution, and the results showed that the number of cells at G0/G1 and G2 phases increased and there were reduced number of cells in S phase after transfection with pcDNA3.1-miR-378a-3p (Fig. 2a-c), which strongly suggests that miR-378a-3p arrests cells in the G0/G1 stage. This effect was reversed when the miR-378a-3p inhibitor was used (Fig. S2). To determine cell proliferation, we used the Cell Counting Kit-8 (CCK-8) assay and found that miR-378a-3p significantly inhibited cell proliferation (Fig. 2d). Cell proliferation was also detected using a 5-Ethynyl-2′-deoxyuridine (EdU) incorporation assay. Similarly, C2C12 cells showed significantly less mitotic activity when miR-378a-3p

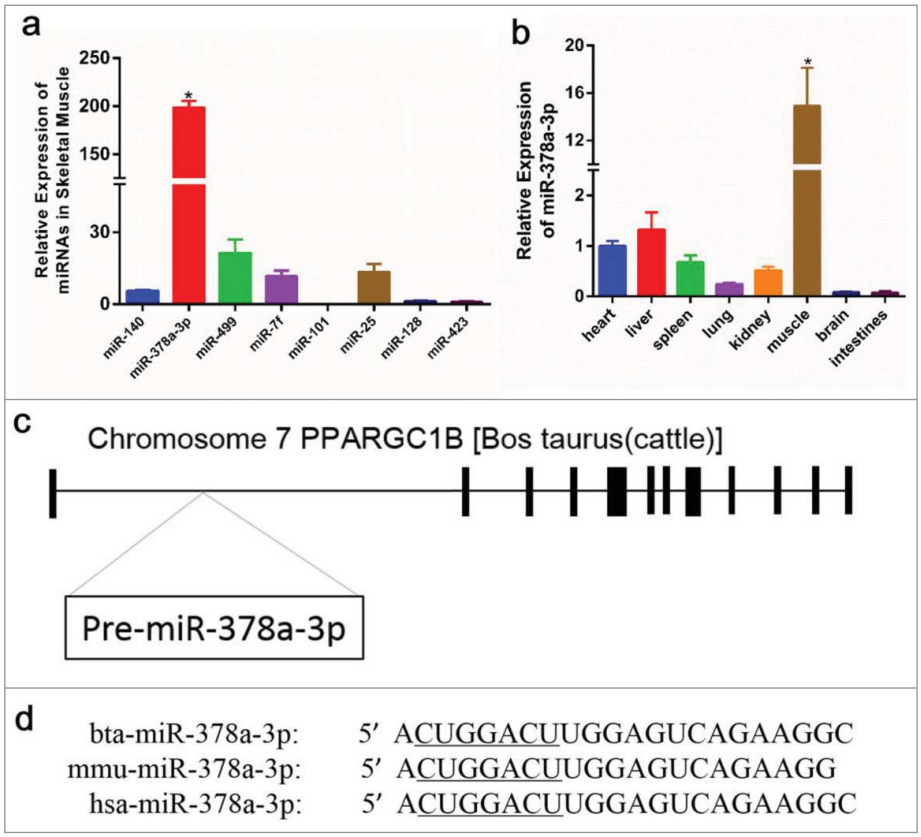

Figure 1. Expression of miRNAs in Qinchuan cattle detected by QRT-PCR. (a) The expression of different miRNAs in skeletal muscle. (b) The expression of miR-378a-3p in different tissues of Qinchuan cattle. (c) pre-miR-378a-3p is located in the first intron of *PPARGC1B* gene in chromosome 7. (d) miR-378a-3p has high conservative in different species of mammal. The expression of miRNAs was normalized to *U6*. Values are mean ± SEM for 3 biological replicates, $^{*}P < 0.05$, $^{**}P < 0.01$.

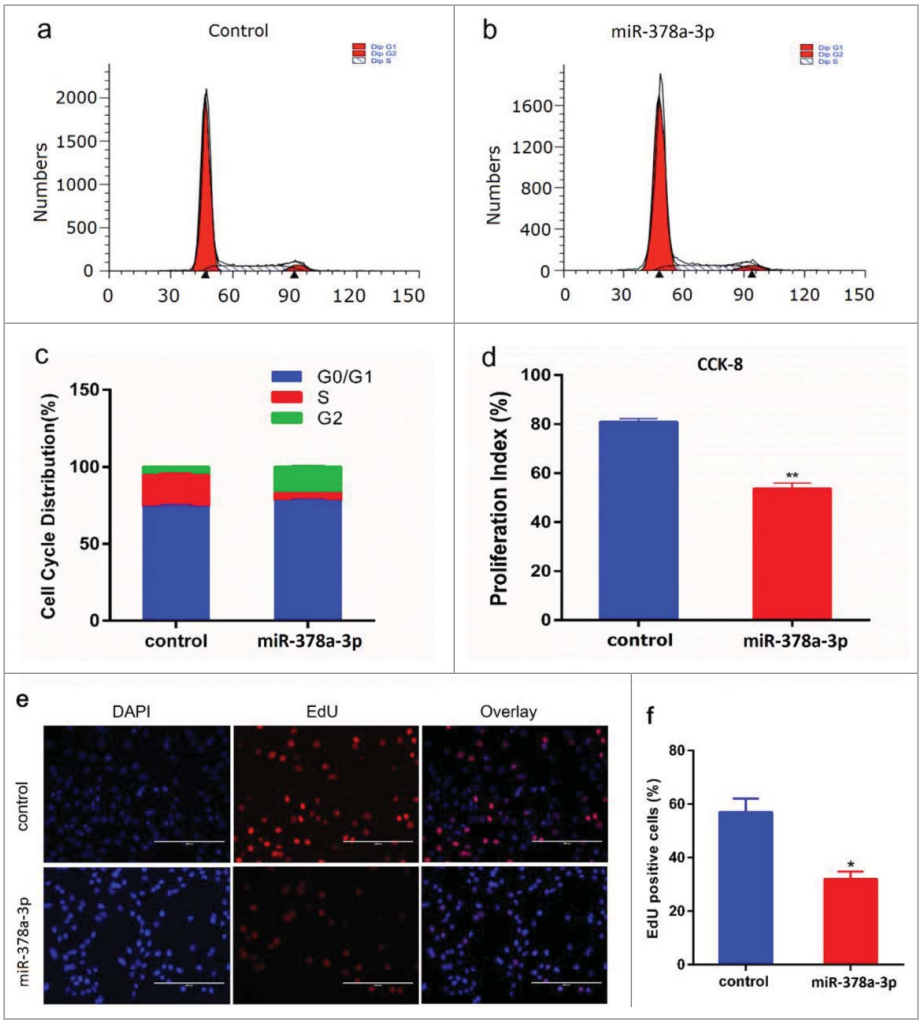

Figure 2. miR-378a-3p represses the proliferation of C2C12 cell. (a, b, c) Cell were transfected by pcDNA3.1 (+) and pcDNA3.1-miR-378a-3p vector, and the cell cycle phase and proliferation index were analyzed by flow cytomctry. (d, e) Cell counting kit-8 (CCK-8) detected cell proliferation index. (e) 5-Ethynyl-2′-deoxyuridine (EdU) detected cell proliferation index, the scale bar stand 200μm. (f) Analysis results of EdU positive cells. Values are mean ± SEM for 3 biological replicates, $^{*}P < 0.05$, $^{**}P < 0.01$.

was overexpressed (Fig. 2e, f). Taken together, miR-378a-3p was shown to inhibit the proliferation of C2C12 cells.

miR-378a-3p promoted C2C12 cell apoptosis

To confirm the effect of miR-378a-3p on cell apoptosis, pcDNA3.1-miR-378a-3p was transfected into the C2C12 cells. The Annexin V- FITC/PtdIns staining assay showed the apoptosis index in the group with miR-378a-3p overexpression was significantly increased (Fig. 3a-c). We also detected the expression of cell survival related genes *Bcl-2, Caspase 3*, and *BAX*, and found that miR-378a-3p upregulated mRNA expression of these genes (Fig. 3d-g, S3), but showed the opposite effect when miR-378a-3p was inhibited (Fig. S2). These results demonstrate that miR-378a-3p promotes C2C12 cells apoptosis.

miR-378a-3p promoted C2C12 cell differentiation

To determine whether miR-378a-3p affects cell differentiation, the expression of miR-378a-3p was detected at different stages of differentiation. We found that the expression of miR-378a-3p generally increased in a time-dependent manner (Fig. 4a). The C2C12 cells transfected with pcDNA3.1-miR-378a-3p were induced to differentiate for 6 days and myoblast differentiation marker genes were detected by RT-qPCR and protein gel blot. We found that expression of *MyoD* and *MHC* was significantly upregulated in C2C12 cells that overexpressed miR-378a-3p, both at the mRNA and protein levels (Fig. 4b-d, S5). Taken together, these results revealed that miR-378a-3p promoted C2C12 cell differentiation.

miR-378a-3p directly targeted the 3′UTR of the HDAC4 gene

To determine the mechanism by which miR-378a-3p promotes cell differentiation, we predicted potential target genes of miR-378a-3p using TargetScan6.2, miRanda, and the starBase database. *HDAC4*, one of the candidate genes, is related to muscle development and has a highly conserved binding site in the mRNA 3′UTR that is complementary to the miR-378a-3p seed. We found that this predicted binding site is highly conserved among vertebrates.

To establish the relationship between miR-378a-3p and *HDAC4*, a 500 bp fragment of *HDAC4* 3′ UTR containing the miR-378a-3p binding site was cloned into the psiCHECK-2

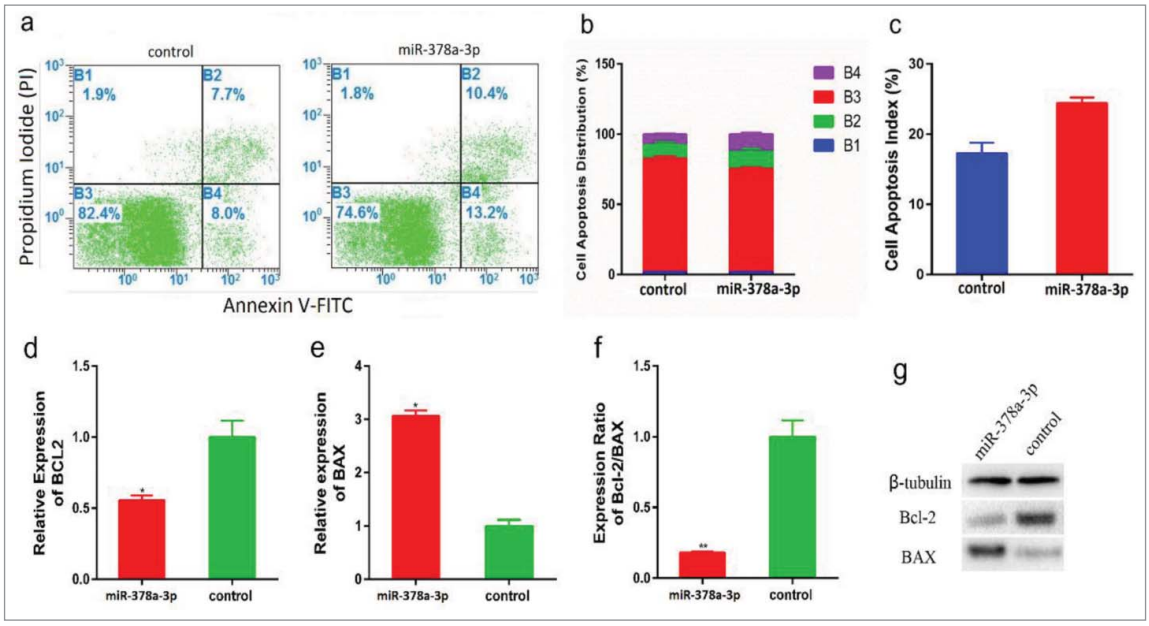

Figure 3. miR378a-3p promotes C2C12 cell apoptosis. (a, b) C2C12 cells were transfected by pcDNA3.1 (+) and pcDNA3.1-miR-378a-3p, collected and stained by Annexin V-FITC/PtdIns. Cell apoptosis phase distribution were analyzed by flow cytomctry. (c) Cell apoptosis index were analyzed between the 2 groups. (d-g) Expression of cell apoptosis relative genes were detected by QRT-PCR and western blot. Values are mean ± SEM for 3 biological replicates, $^{*}P < 0.05$, $^{**}P < 0.01$.

vector to yield psiCHECK-2-3′UTR-W. A separate fragment containing a 2-base mutation in the seed binding site of the *HDAC4* 3′ UTR was also moved into the psiCHECK-2 plasmid vector as psiCHECK-2-3′UTR-Mut (Fig.5a, b). The pcDNA3.1-miR-378a-3p was co-transfected with psiCHECK-2-3′UTR-W or psiCHECK-2-3′UTR-Mut into 293T cells. The Renilla

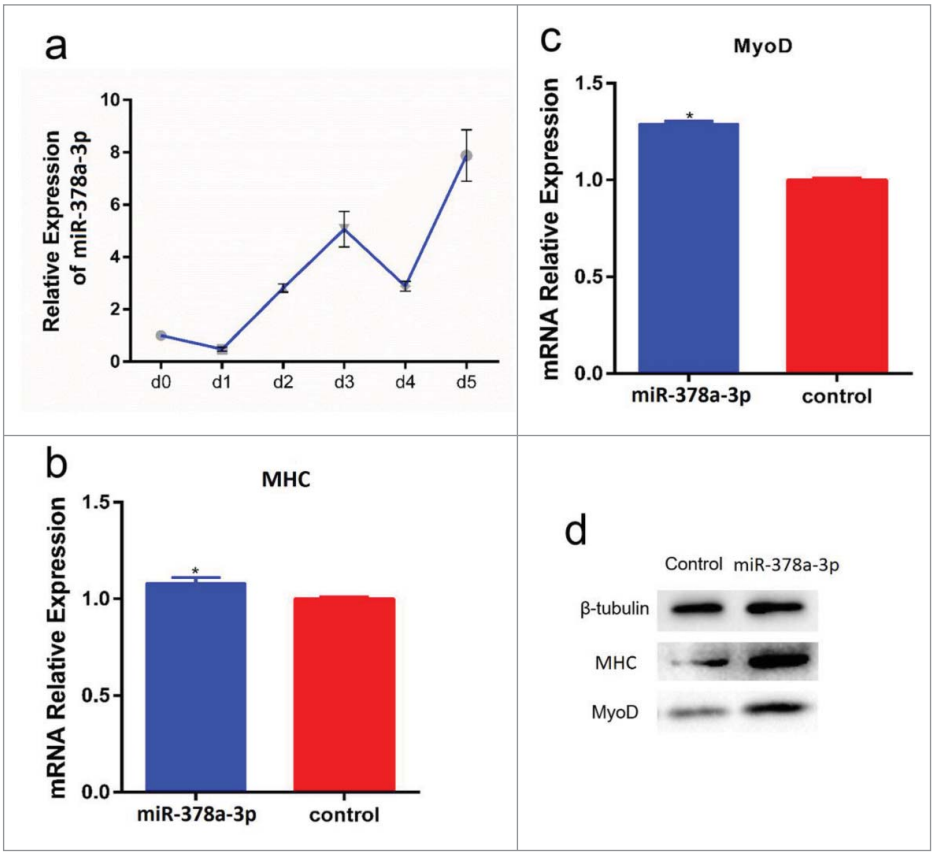

Figure 4. miR-378a-3p promotes C2C12 cell differentiation. (a) miR-378a-3p expression in different stages of differentiation. (b, c) Overexpression of miR-378a-3p in C2C12 cell and continues induced to undergo differentiation for 6 days, and subsequent detection the mRNA expression of MHC and MyoD by RT-PCR compared with control group. (d) The protein expression of MHC and MyoD were detected by protein gel blot. Days (d) indicate the time of cells in differentiation medium. Values are mean ± SEM for 3 biological replicates, $^{*}P < 0.05$, $^{**}P < 0.01$.

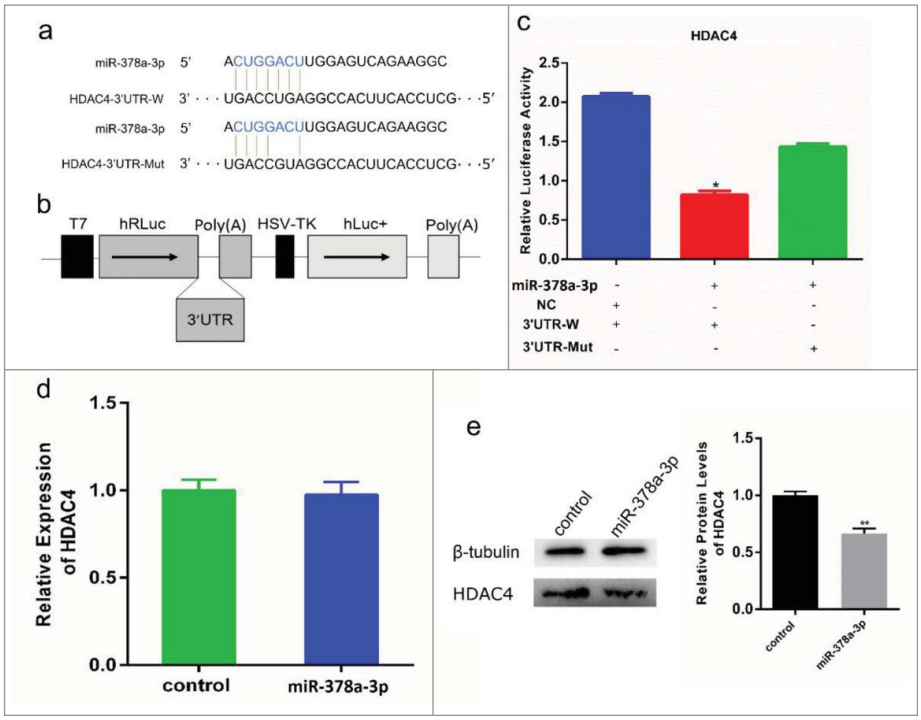

Figure 5. miR-378a-3p directly targets HDAC4 gene. (a) Sequence of miR-378a-3p and its predicted binding site in HDAC4 3′UTR and mutation 3′UTR. (b) The sketch map of psiCHECK2 vector in which HDAC4 3′UTR and mutation 3′UTR were inserted into the 3′ end of Renilla luciferase gene (hRluc). (c) miR-378a-3p/NC co-transfected with psiCHECK2-3′UTR-W and psiCHECK2-3′UTR-Mut into C2C12 cells individually, and Renilla luciferase activity was normalized to the firefly luciferase (hLuc+) activity. (d) HDAC4 mRNA expression in C2C12 myoblasts was detected by RT-PCR at 48 h post-transfection with miR-378a-3p and control. (e) HDAC4 protein expression was detected by western blot and gray value analysis. Values are mean ± SEM for 3 biological replicates, $^{*}P < 0.05$, $^{**}P < 0.01$.

luciferase activity was not reduced in cells containing the psi-CHECK-2-3′UTR-Mut compared to the cells containing the psi-CHECK-2-3′UTR-W (Fig. 5c). These results demonstrated the specific targeting relationship of miR-378a-3p and *HDAC4* gene.

To confirm the effect of miR-378a-3p on *HDAC4* in myoblasts, we examined the *HDAC4* protein level following miR-378a-3p transfection. Western blot analysis indicated that miR-378a-3p overexpression suppressed HDAC4 expression (Fig. 5e); no effects were detected when miR-378a-3p interference was used to decrease activity (Fig. S6). Interestingly, we found the mRNA level of *HDAC4* was not significantly changed between C2C12 cells that overexpressed miR-378a-3p or the control cells (Fig. 5d, S7). Therefore, we concluded that miR-378a-3p was directly targeting the 3′UTR of *HDAC4* to inhibit its protein expression.

HDAC4 inhibited myogenic differentiation and promoted C2C12 cell proliferation

To determine the role of *HDAC4* in myoblasts differentiation, C2C12 cells were transfected with siRNA against HDAC4. The results showed that *HDAC4* mRNA expression was notably reduced in C2C12 cells transfected with siHDAC4 (Fig. 6a). The mRNA expression of *MyoD, MHC*, and *MEF2C* significantly increased in the C2C12 cells transfected with siHDAC4 (Fig. 6b), confirming the role of miR-378a-3p in muscle differentiation. To confirm the effects of HDCA4 on cell survival, cell proliferation and apoptosis were detected in C2C12 cells. Cell proliferation was detected by CCK-8 and EdU staining analysis. We found that siHDAC4 inhibited proliferation of C2C12 cells (Fig. 6d-f). Also, we analyzed the effect of siHDAC4 on cell apoptosis by flow cytometry, and found no significant difference in the distribution of cell apoptosis (Fig. 6g, h). In conclusion, these results demonstrated that *HDAC4* promoted myogenic differentiation and suppressed myoblast proliferation.

Discussion

Our previous high-throughput sequencing results on miRNAs in Qinchuan bovine skeletal muscle showed that miR-378a-3p was one of the highest expression miRNAs.[36] We verified that the expression levels of miR-378a-3p was markedly higher in skeletal muscle than the levels in other tissues. The results suggested that miR-378a-3p might be a muscle-related miRNA, similar to other muscle-specific miRNAs that were reported previously.

Previous studies showed that miR-378 inhibits prostatic carcinoma cell proliferation[8] and enhanced apoptosis of cardiomyocytes by reduced signaling in the Akt cascade.[38] Additionally, miR-378-5p could suppress cell proliferation and promote cell apoptosis in CRC cells.[22] In this research, we found that miR-378a-3p inhibited C2C12 cell proliferation and arrested cells in G0/G1 phase. The overexpression of miR-378a-3p promoted cell apoptosis. Cell proliferation and apoptosis are complicated processes, and our research showed that miR-378a-3p inhibits cell proliferation and promotes apoptosis. However, more studies are warranted to determine how miR-378a-3p regulates cell proliferation and apoptosis.

Our data demonstrated that miR-378a-3p reduced the expression of *Bcl-2* and increased the expression of *Bax* and *caspase-3*. Bcl-2 family proteins regulate caspase activation and

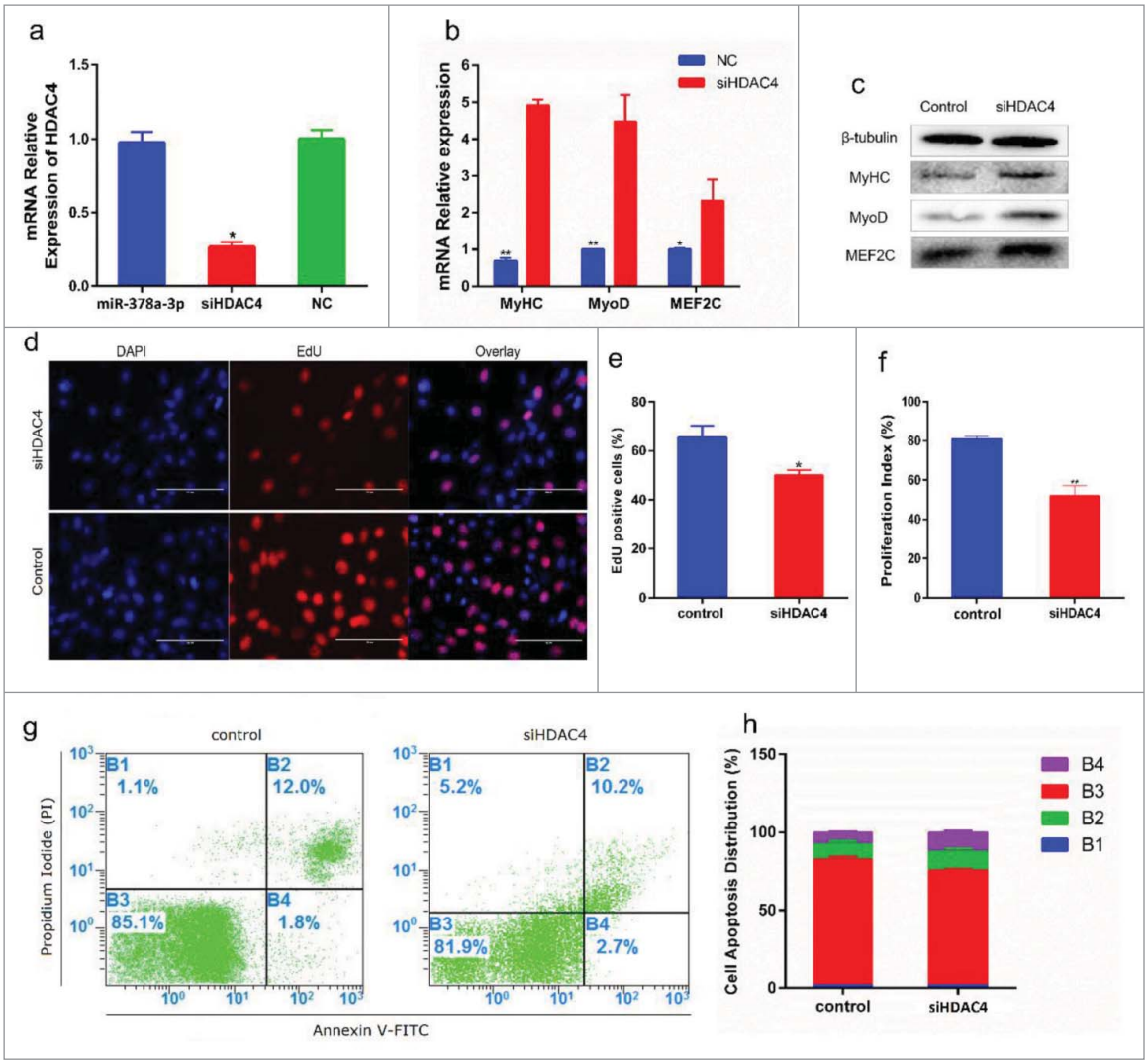

Figure 6. Knock down HDAC4 promotes differentiation and inhibits proliferation of C2C12 cells. (a) Transfection of siHDAC4 into C2C12 myoblasts to knock down the expression of HDAC4 mRNA and overexpression of miR-378a-3p to inhibited HDAC4 mRNA, detected by RT-PCR. (b) The mRNA expression of MHC, MyoD and MEF2C were detected by RT-PCR in siHDAC4 transfected and control group. (c) MHC and MyoD protein detected by protein gel blot. (d) Cell proliferation were detected with EdU stained in siHDAC4 and NC transfected of C2C12 cell. Nuclei were stained blue with DAPI, EdU were detected in red luciferase. Scale bar stand 200 μm. (e) EdU positive cells analysis. (f) CCK-8 were used to detected cell proliferation, and analyzed the absorbance value at 450 nm with Automatic microplate reader. (g, h) Cells were collected and stained by Annexin V-FITC/PI in siHDAC4 and NC groups, cell apoptosis distribution were analyzed by flow cytomctry. Values are mean ± SEM for 3 biological replicates, * $P < 0.05$, ** $P < 0.01$.

apoptosis by regulating mitochondrial outer membrane permeabilization, which releases mitochondrial proteins and promote apoptosis.[15, 26] Caspase-3 is a crucial inducer of apoptosis, acting to induce apoptosis of human colon cancer cells.[33,51] Down-regulation of Bcl-2 expression and Caspase-3 activation could suppress proliferation and induce apoptosis in cancer cell lines.[34] Usually, cell proliferation and apoptosis are regulated through complex interactions between caspase-3 and the Bcl-2/Bax pathway.[26,34] Therefore, we concluded that miR-378 promotes apoptosis through regulating the expression of *Bcl-2* and *Bax*.

Previous studies showed that miR-378 overexpression can increase *MyoD* expression which induces myoblast differentiation.[13] Similarly, we demonstrated that miR-378a-3p overexpression significantly increased the expression of the mRNA and protein levels of *MyoD* and *MHC* in C2C12 cells, indicating that miR-378a-3p is an important regulator in myocyte differentiation. We also determined the effects of miR-378 to stimulate differentiation and apoptosis occurred by regulating the expression of HDAC4 at the post-transcriptional level. HDAC4 promotes proliferation in colon cancer cells[41] and osteosarcoma cells.[35] We showed that knocking down HDAC4 markedly inhibits C2C12 cell proliferation. *HDAC4* is specifically involved in muscle development. HDAC4 reduced the ability of the myocyte enhancer factor 2 (MEF2) to access the regulatory regions of genes, resulting in repressed MEF2-dependent transcription.[5,10,11,27] Moreover, members of the MEF2 family cooperate with the *MyoD* family to control the expression of muscle–specific genes.[2,32] In the present study, we found that both *HDAC4* knockdown and miR-378 overexpression promoted differentiation, inhibited the proliferation of C2C12 cells, and significantly increased the expression of *MHC* and *MyoD*. These findings may explain how miR-378a-3p promotes differentiation of C2C12 cells.

We also found that the knockdown of *HDAC4* increased the mRNA level of *MEF2C*. *MEF2C* is a crucial transcriptional

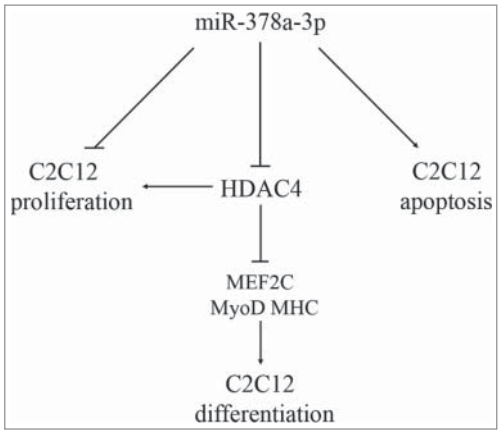

Figure 7. Model of miR-378a-3p functioning in C2C12 cells. miR-378a-3p promoted differentiation and repressed proliferation through targets HDAC4 in C2C12 cells, and promoted cell apoptosis.

factors in regulating gene expression. A previous study reported that *MEF2C* expression is limited to the skeletal muscle[25] and that the *MEF2C* protein was required in the p38 MAPK pathway that regulates the transcription of genes in skeletal muscle differentiation.[50] Thus, miR-378a-3p regulated muscle differentiation by inhibiting *HDAC4* expression, which could strengthen *MEF2C* expression of the activating p38 MAPK signal pathway.

In conclusion, our findings showed that miR-378a-3p may promote muscle differentiation by inhibiting *HDAC4* at the post-transcriptional level. Moreover, miR-378a-3p suppressed cell proliferation and induced apoptosis in C2C12 cells (Fig. 7). However, this effect of miR-378a-3p on cell differentiation requires further studies.

Materials and methods

Tissues collected and RNA isolation

All animal samples used in this study were approved by the Animal Care and Use Committee of the College of Animal Science and Technology, Northwest A&F University. Bovine tissue samples included muscle, liver, heart, lung, spleen, kidney, brain, and fat. All samples were collected at a local slaughterhouse in xi'an. The bovines were slaughtered by exsanguination (n = 3), and all tissues were obtained under sterile conditions, washed with diethypyrocarbonate (DEPC) treated water, and immediately frozen in liquid nitrogen. The samples were stored at −80°C until RNA isolation. Total RNA was extracted from tissues or cells with Trizol reagent (TaKaRa, Japan).

Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from tissues or cells, and then 500 ng total RNA was converted to cDNA using the PrimeScript RT regent Kit (TaKaRa, Japan). Random primers, oligo (dT) or miRNA-specific stem-loop primers were designed by us and used for reverse-transcribed cDNA (Table S1). RT-qPCR was performed with the SYBR Green PCR Master Mix Reagent Kit (TaKaRa, Japan) and GAPDH and U6 were used for normalization of the data. The RT-qPCR procedure was as follows: cycle 1, 94°C for 3 min; cycle 2, 94°C for 5 s, 60°C for 25 s for 39 cycles, melt curve was generated using the cycle: 95°C for 10s, 65°C for 5s and 95°C for 5s. The fold-change of expression of the transcript mRNA or miRNA were analyzed using the $2^{-\Delta\Delta CT}$ method. The RT-qPCR primers listed in Table S2 were designed by Beacon Designer 8.

Vector construction

The overexpression vector pcDNA3.1-miR-378a-3p was obtained by PCR amplification of a fragment about 380 bp including the pre-miR-378a-3p complete sequence from bovine genomic DNA using primers containing restriction enzyme sites *Hind* III/*Kpn* I (TaKaRa, Japan); the forward primer was 5′-CCCAAGCTTTAGAAGGCTCCGAGAACCAG-3′ and reverse primer was 5′- GGGGTACCGAAGTTACAGGAAGGACCAGACA-3′. T4 DNA ligase was used to ligate the pre-miR-378a-3p fragment into the pcDNA3.1 (+) vector. miR-378a-3p inhibitor was compound by GenePharma, the sequence was GCCUUCUGACUCCAAGUCCAGU.

The HDAC4-3′UTR sequence including the miRNA binding site was amplified using a forward primer 5′-CCGCTCGAGCTGAACTTTGAAGCCTGTGG-3′ and reverse primer 5′-ATA AGAATGCGGCCGCAAGACCTTCCTGTCCTGCTC-3′. Separately, a 2-base mutagen in the miR-378a-3p-binding site of HDAC4 3′UTR (HDAC4-3′UTR-Mut) was generated with a pair of mutagenic primers: 5′-GACCAAAAGATGCCAGATTCTTGGACCG-3′ and 5′-CGGTCCAAGAATCTGGCATCTTTTGGTC-3′. The two fragments were ligated into the 3′-end of the Renilla gene in the psi-CHECK-2 dual-luciferase reporter vector (Promega, USA) using restriction enzymes *Xho* I and *Not* I (TaKaRa, Japan) and then ligated by T4 DNA ligase (TaKaRa, Japan).

Cell culture

HEK293T cells (ATCC, USA) and mouse C2C12 myoblasts were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, USA) and 1% double antibiotics (penicillin and streptomycin) (GM) at 37°C with 5% CO_2. To induce myogenic differentiation, culture medium was switched to differential medium (DMEM with 2% horse serum, DM) after cell growth had reached nearly 80% confluence.

Transfection

To detect the transfection efficiency of the recombinant vector, pcDNA3.1-miR-378a-3p was transfected into HEK293T cells. The cells at 90% confluence were plated at 5×10^5 cells/well in 6-well plates. When growth reached approximately 80% confluence, 2 μg of pcDNA3.1-miR-378a-3p was transfected into the HEK293T cells using Lipofectamine 2000 (Invitrogen, USA).

To confirm the effect of miR-378a-3p on muscle differentiation, pcDNA3.1-miR-378a-3p was transfected into C2C12 cells cultured in a 6-well plates. The cells were cultured in DM medium for 6 days, the cells were collected daily, and a group of cells transfected with pcDNA3.1 (+) vector were grown as a control. The siRNA of HDAC4 (siHDAC4) was transfected into C2C12 cells to explore the HDAC4 effect on

cell differentiation; 50 nM siRNA was used for each well of the 6-well plates. The sequence of the siRNAs used in the transfection are 5′-GAGCAGCAGAGGAUCCACCAGUU AA-3′ and 5′-CACCGGAACCUGAACCACUGCAUUU-3′, and a negative control (NC) siRNA that did not target HDAC4was transfected into C2C12 cells as the control group; the sequence was 5′-UUCUCCGAACGUGUCAC-GUTT-3′.

Cell proliferation assay

Cell counting kit-8 assay

C2C12 cells were transferred to 96-well plates at a density of 1×10^4 cells/well and then 100μL GM were added to each well. When the cells reached approximately 80% confluence, they were transfected with either the pcDNA3.1-miR-378a-3p or the pcDNA3.1 (+) vectors, respectively. After 24h of culture at 37°C, 10μL of CCK-8 reagent (Multisciences; China) was added to each well and incubation was continued for 4 h. The absorbance value of all samples were detected using an automatic microplate reader (Molecular Devices, USA) at 450 nm.

EdU proliferation assay

The cell proliferation was also assessed using the Cell-Light EdU DNA cell proliferation kit (RiboBio, China). C2C12 cells was seeded into 96-well culture plates containing 100μL GM. After 24 h, the cells were incubated into EdU medium. After 2 h, the test was performed according to the manufacturer's protocol.

Flow cytometry for the cell cycle assay

C2C12 cells were grown in 6-well plates (1×10^6 cells/well) with 2mL GM. The cells were treated with pcDNA3.1-miR-378a-3p and pcDNA3.1 (+). After 24 h, the Cell Cycle Assay Kit (Multisciences, China) was used to treat the cells. The cells were washed in PBS buffer and the supernatant was removed after centrifuging. Next, 1mL of DNA strain solution and 10 μL of permeabilization solution were added to the resuspended cells, and then blended by vortex shaking for 15 s. After incubating for 30 min in the dark at room temperature, the cell cycle was analyzed by Flow Cytometry (FACS CantoTM II, BD BioSciences, USA).

Cell apoptosis assay

Cell apoptosis was measured by Annexin V-FITC/PI staining assays. C2C12 cells were cultured in 6-well plates with 2 mL GM. When the cells reached a confluence of 80%-90%, the cells were transfected with pcDNA3.1-miR-378a-3p or pcDNA3.1 (+). After 24 h of incubation, the C2C12 cells were washed 3 times with PBS buffer. Cells were then harvested cells into 1.5 mL centrifuge tube and washed again, resuspending in 500 μL 1× binding buffer. Cells were then treated with Cell Apoptosis Assay Kit (Multisciences, China), incubated in the dark for 10 min at room temperature, then cell apoptosis was immediately analyzed using a Flow Cytometer.

Luciferase activity assay

Cells were cultured in 48-well plates when the cell growth reached about 80% confluence. The pcDNA3.1-miR-378a-3p and psiCHECK-2-HDAC4-3′UTR (HDAC4-UTR-W) or psi-CHECK-2-HDAC4-mut-3′UTR (HDAC4-UTR-Mut) were co-transfected into cells by Lipofectamine2000. The transfection reagent was replaced with fresh growth medium (DMEM with 10% FBS) after transfection for 4∼6 h. Next, the cells were washed with PBS and harvested using 200μL Passive Lysis Buffer (PLB) and rocked for 30 min at room temperature. Dual-luciferase activity was measured by MPPC luminescence analyzer (HAMAMATSU; Beijing, China) and the Renilla Luciferase activity was normalized against Firefly Luciferase activity.

Western blot

The total proteins were extracted from cells using protein Lysis buffer RIPA containing 1mM PMSF (Solarbio; Beijing, China). The extracts were boiled with 4×SDS loading buffer (150 mM Tris-HCL (pH = 6.8), 12% SDS, 30% glycerol, 0.02% bromophenol blue, and 6% 2-mercaptoethanol) at 98°C for 10 min and then 20 μg total proteins were loaded and separated on 10% SDS-PAGE gels. After electrophoresis, the proteins were transferred to a 0.2 μm PVDF membrane that was soaked in formaldehyde, and then blocked with 5% skim milk in Tris Saline with Tween (TBST) buffer for about 2 h at room temperature. The membrane was then incubated overnight with primary antibodies specific for anti-HDAC4 (Dilution 1:1000; ab32534; Abcam, England), anti-MyoD (Dilution 1:1000; ab16148; Abcam, England), anti-MHC (Dilution 1:1000; ab24648; Abcam, England), anti-Bcl-2 (Dilution 1:1000; ab32124; Abcam, England), anti-Bax (Dilution 1:1000; ab32503; Abcam, England), anti-MEF2C (Dilution 1:1000; sc-365862; SANTA, USA) and anti-β-tubulin (Dilution 1:1000; KDM9003; Sungene Biotech, China) at 4°C. The PVDF membrane was washed 3 times with TBST buffer and then incubated with secondary antibody for the anti-immune rabbit IgG-HRP (Dilution 1:1000; LK2001; Sungene Biotech, China) 2 h at room temperature. β-tubulin was used as the internal control with a secondary antibody that was HRP-labeled anti-mouse IgG (Dilution 1:1000; LK2003; Sungene Biotech, China). Finally, antibody reacting bands were detected using ECL luminous fluid (Solarbio, China).

Statistical analysis

The quantitative results are presented as mean ± standard error of the mean (SEM) based on at least 3 independent experiments. All data in this study were analyzed by one-way analysis of variance (ANOVA) for P-value calculations using SPSS v17.0 software. $P < 0.05$ was considered statistically significant differences among means. The software Image J was utilized for gels image gray value analysis.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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