MicroRNA-378 promotes myogenic differentiation by targeting BMP4

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Abstract. MicroRNA-378 (miRNA-378) has been reported to have a crucial role in skeletal muscle differentiation; however, the underlying mechanisms have largely remained to be elucidated. The present study employed high-throughput RNA sequencing to investigate the transcriptome following transfection of miRNA-378 mimics or control RNAs into C2C12 myoblast cells. By sequencing and annotation, 2,802 transcripts that were changed by >1.5 fold were obtained and then subjected to signaling pathway enrichment and gene ontology analysis. Eight genes associated with development were subsequently selected for validation by quantitative qPCR, the results of which were highly consistent with those of the high-throughput RNA sequencing. The protein levels of bone morphogenetic protein 4 (BMP4), which was among the differentially expressed genes, were decreased following ectopic expression of miRNA-378. BMP4 was further confirmed to be a direct target of miRNA-378 by using a dual luciferase assay. Finally, treatment with miRNA-378 or small interfering RNA against BMP4 induced myogenic differentiation in C2C12 cells. In conclusion, the present study suggested that miRNA-378 is critical for the promotion of myoblast differentiation by targeting BMP4.

Introduction

The development of skeletal muscle is controlled by an evolutionarily conserved transcription factor network involving in the regulation of genes associated with muscle differentiation, contractility and growth (1,2). Myocyte enhancer factor-2 (MEF2) is the longest known myogenic transcription factor, and most muscle-associated genes are directly activated by MEF2 through combined interaction with other transcription factors (3). Studies have demonstrated that, in addition to activating genes involved in muscle contraction and differentiation, myogenic transcription factors regulate the expression of a number of conserved microRNAs (miRNAs) that function to 'fine-tune' the output of transcriptional networks, leading to precise cellular responses to pathological, physiological and developmental signals (4). As individual miRNAs can regulate hundreds of mRNAs and individual mRNAs are in turn targeted by numerous miRNAs, miRNAs add complexity and accuracy to the regulation of the core muscle transcriptional network (5).

miRNAs are endogenous, small, non-coding RNAs of ~22 nt in length that have emerged as powerful negative regulators of gene expression (6). By selectively repressing the activity of the 3'-untranslated region (3'-UTR) of target mRNAs, miRNAs confer appropriate timing and robustness in differentiation programmes. A set of miRNAs, including miRNA-1, miRNA-133, miRNA-206, miRNA-208, miRNA-486 and miRNA-499, have been identified to be highly enriched in skeletal and/or cardiac muscle (7). Certain key myogenic regulatory factors (MRFs), including myogenin and myogenic differentiation 1 (MyoD1), are known to regulate these miRNAs (8,9). Several miRNAs that are normally induced during myogenic differentiation can initiate the myogenic program in C2C12 myoblasts, even in the presence of high serum (10,11). miRNA-378, a cardiac-enriched miRNA, has been shown to directly target insulin-like growth factor 1 receptor and regulate post-natal cardiac re-modeling (12). However, the functions of miRNA-378 in the differentiation of skeletal muscle have largely remained elusive.

To reveal the underlying mechanisms of the roles of miRNA-378 in myoblast differentiation, the present study employed a gain-of-function approach by transfecting miRNA-378 mimics into C2C12 myoblast cells and analyzing the resulting gene expression profiles by means of genome-wide mRNA deep sequencing. The present study demonstrated that miRNA-378 promoted myoblast differentiation by targeting the bone morphogenetic protein 4 (BMP4) gene.

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Materials and methods

Cell culture. C2C12 myoblasts (Shanghai Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 20% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere with 5% CO_2. Transfection with miRNA mimics or plasmids was performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols.

cDNA library construction. C2C12 cells transfected with miRNA-378 mimics or control RNA (synthesized by Suzhou GenePharma Co., Ltd., Suzhou, China) were collected, and total RNA was then extracted using TRIzol reagent (Invitrogen). The sequences of the miRNA-378 mimics are as follows: Sense, 5'-CUCCUGACUCCAGGUCCUGUGU-3' and antisense, 5'-ACACAGGACCUGGAGUCAGGAG-3'. The quality of the RNA was determined with a NanoDrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA), and detected with 1% agarose gels (Beyotime Institute of Biotechnology, Haimen, China). Total RNA (10 μg) was used for sequencing with an Illumina Hiseq2000 (Illumina, San Diego, CA, USA). To construct a cDNA library, oligo (dT) beads (Qiagen GmbH, Hilden, Germany) were used to isolate mRNA. The mRNA was digested by 10 U/μl *Eco*RI (Takara Biotechnology Co., Ltd., Dalian, China) into short fragments, and first-strand cDNA was synthesized using random hexamer primers (Takara Biotechnology Co., Ltd.). Following synthesis of the second-strand cDNA, the double-stranded cDNA was purified using a QIAquick PCR Extraction kit (Qiagen GmbH) following the manufacturer's instructions. The purified fragments were enriched by polymerase chain reaction (PCR) to generate a cDNA library (13) and were then sequenced with the IlluminaHiseq2000.

Data assembly and annotation. Raw data were generated by sequencing using the Illumina Hiseq2000. The data were assembled into transcripts, unigenes and contigs with Trinity *de novo* software (version 3.1; http://www.blast2go.com), excluding the low-quality sequences, adaptors, sequences with uncertain bases and sequences of >50 bp. To identify differentially expressed genes between miRNA-378-transfected samples and control RNA-transfected samples, expression ratios were calculated using the Limma algorithm in R, applying moderated t-tests. Benjamini-Hochberg correction was then used for correcting multiple hypothesis testing, in which the q-value was calculated for each P-value. Genes with an absolute log2 expression ratio of >0.6 between miRNA-378-transfected and control RNA-transfected group and a q-value of <0.005 were considered to be significant under the corresponding treatment conditions. In addition, to identify enriched Gene Ontology (GO) terms (http://www.geneontology.org) in the sets of differentially expressed genes, the Database for Annotation, Visualization and Integrated Discovery Bioinformatics Resource (14) was used, where P<0.01 was considered to indicate a statistically significant difference. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/) was used to analyze the metabolic pathways of the genes.

Target gene prediction. Miranda databases (www.microrna.org/) and TargetScan version 4.0 (www.targetscan.org/) were employed to identify potential target genes of miRNA-378 that were downregulated in miRNA-378-transfected C2C12 cells compared with control RNA-transfected cells. Following identification of potential target genes, SigTerm software (version 7.0) was used to confirm the prediction results.

Plasmid construction. The BMP4 or transforming growth factor beta 2 (TGFB2) 3'-UTR sequences were PCR-amplified from C2C12 genomic DNA and cloned into the pGL3-control vector (Promega Corporation, Madison, WI, USA). Amplification was conducted using primers from Sangon Biotech Co., Ltd., Shanghai, China) in a PTC240 PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The reaction mixture was incubated at 95˚C for 30 sec, followed by 30 cycles of 95˚C for 30 sec, 56˚C for 30 sec and 72˚C for 60 sec. The products were sequenced to verify the results. Mutagenesis was performed by PCR (15), followed by *Dpn*I (10 U/μl; Takara Biotechnology Co., Ltd.) digestion to remove parental template DNA and obtain the mutated sequences BMP4-mut or TGFB2-mut.

Reverse-transcription quantitative (RT-q)PCR. Cells were lysed and total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer's instructions. cDNA synthesis was performed using the Superscript III first-strand synthesis system (Invitrogen). qPCR was then performed using SYBR Green PCR master mix in an ABI cycler (Thermo Fisher Scientific, Inc.). ABI 7300 software (Thermo Fisher Scientific, Inc.) was used for quantitative analysis.

Dual luciferase assay. To assess whether BMP4 or TGFB2 are direct targets of miRNA-378, firefly luciferase reporter vectors (2 ng; Promega Corporation) driven by fragments from the respective gene's 3'-UTR or their mutants together with miRNA-378 mimics and a Renilla luciferase control vector (1 ng) were co-transfected into C2C12 cells according to the manufacturer's protocols. Experiments were analyzed using a dual-luciferase reporter assay system (Promega Corporation) following the manufacturer's protocols. The luciferase signal was quantified using a luminometer (Monolight 3020; BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis. At two days following transfection with miRNA-378 mimics or control RNA (100 nM), C2C12 cells were lysed in radioimmunoprecipitation buffer [Beyotime Institute of Biotechnology; 150 mM sodium chloride, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCl (pH 8.0) and 0.5% sodium deoxycholate] supplemented with 1% protease inhibitor cocktail stock solution (Roche Diagnostics GmbH, Mannheim, Germany). Protein concentration was determined by BCA Protein assay kit (Beyotime Institute of Biotechnology). Proteins (20 μg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (Beyotime Institute of Biotechnology). After electroblotting onto a

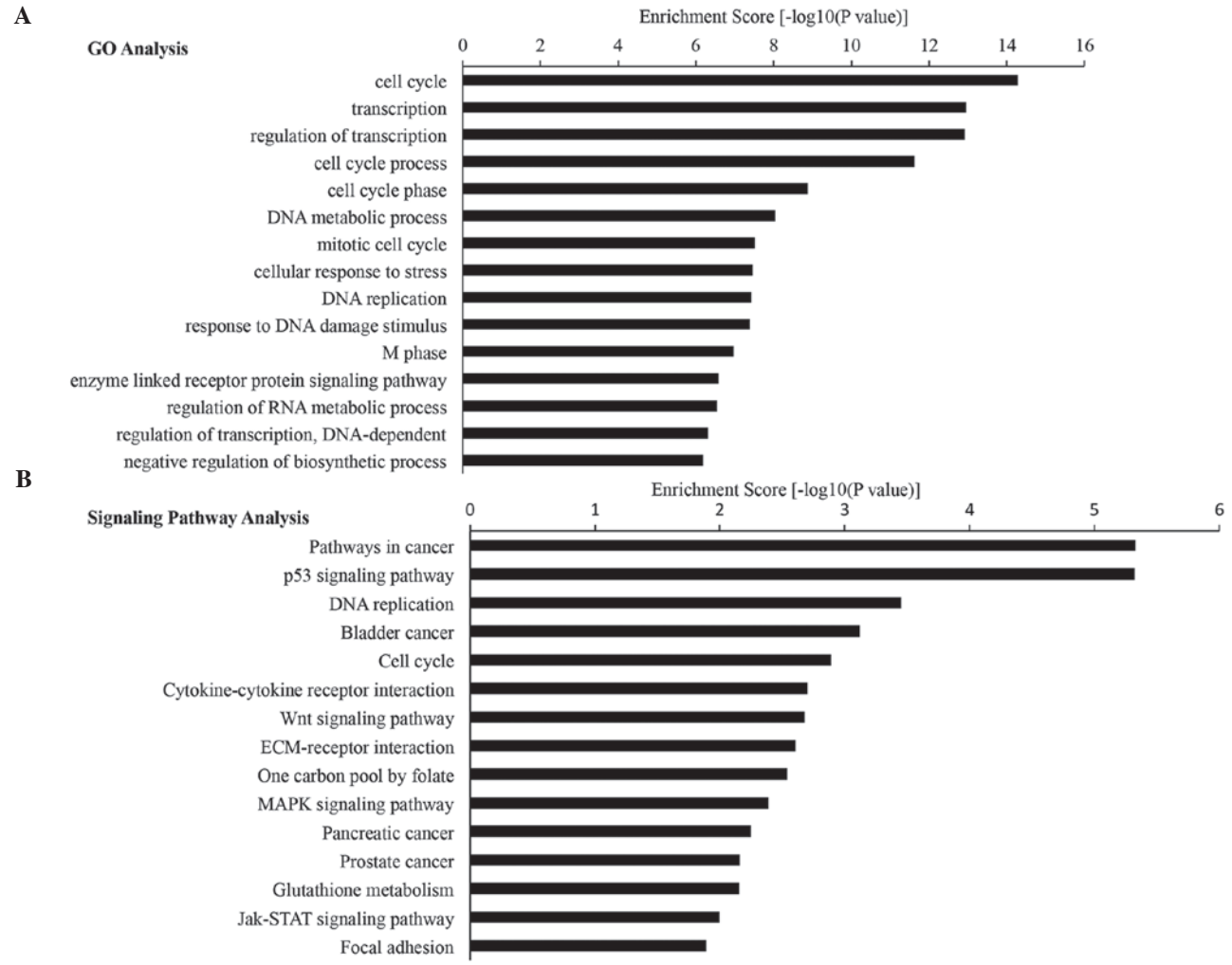

Figure 1. GO and signaling pathways of the downregulated genes. (A) Functional gene annotation of the differentially expressed genes according to the GO tool (http://www.geneontology.org). (B) Kyoto Encyclopedia of Genes and Genomes signaling pathway analysis for the differentially expressed genes (http://www.genome.jp/kegg/). GO, gene ontology. ECM, extracellular matrix; MAPK, mitogen-activated protein kinase; Jak, Janus kinase; STAT, signal transducer and enhancer of transcription.

polyvinylidene fluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA), Tris-buffered saline/0.05% Tween-20 (TBST; Beyotime Institute of Biotechnology) containing 1% skimmed milk was used to block non-specific binding sites. The PVDF membrane was then incubated with rabbit polyclonal anti-BMP4 (1:500 dilution; Abcam, Cambridge, MA, USA; cat. no. ab39973), rabbit polyclonal anti-TGFB2 (1:200 dilution; Abcam; cat. no. ab66043), rabbit polyclonal anti-MyoR (1:200 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. no. sc-366698), rabbit polyclonal anti-MyoD1 (1:200 dilution; Abcam; cat. no. ab64159), anti-MyHC (1:200 dilution; Abcam; cat. no. ab185967) or with rabbit monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (1:2,000 dilution; Sigma-Aldrich, St. Louis, MO, USA; cat. no. G9545) for 1 h at room temperature prior to washing three times for 10 min with TBST. The membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibodies (1:2,000 dilution; Abcam, cat. no. ab6721) for 1 h at room temperature. Signals were analyzed using enhanced chemiluminescence reagent, ECL Start (GE Healthcare, Little Chalfont, UK) and visualized with a Bio-Rad ChemiDoc MP system (Bio-Rad Laboratories, Inc.).

Small interfering RNA (siRNA) transfection. An siRNA against BMP4, termed si-BMP4 (Suzhou GenePharma Co., Ltd.) was used to endogenously inhibit BMP4. The sequences were as follows: Sense, 5'-AGGGCCAGGAAGAAGAAU AAUUUU-3' and antisense, 5'-AUUAUUCUUCUUCCU GGCCCUUUU-3'. Cells were transfected with 100 nM of si-BMP4, miRNA-378 or negative control (NC) RNAs using Lipofectamine 2000 according to the manufacture's protocols. After 24 h transfection, the cells were harvested and subjected to the creatine kinase assay.

Creatine kinase (Ck) assay. Ck enzymatic activity was measured in cell lysates using the EnzyChrom Creatine Kinase assay kit (ECPK-100; BioAssay Systems LLC, Hayward, CA, USA) according to the manufacturer's protocol and as described previously (16). In brief, cells were washed twice with phosphate-buffered saline, lysed on ice for 10 min and scraped to remove cellular debris. The supernatant was then enriched by centrifuging at 8,000 x g for 10 min, and a 2.5-fold volume of H_2O was added, of which 10 μl was used for Ck analysis. Evaluation was performed using the Bio-Rad BCA Protein assay kit (Bio-Rad Laboratories, Inc.). The absorbance

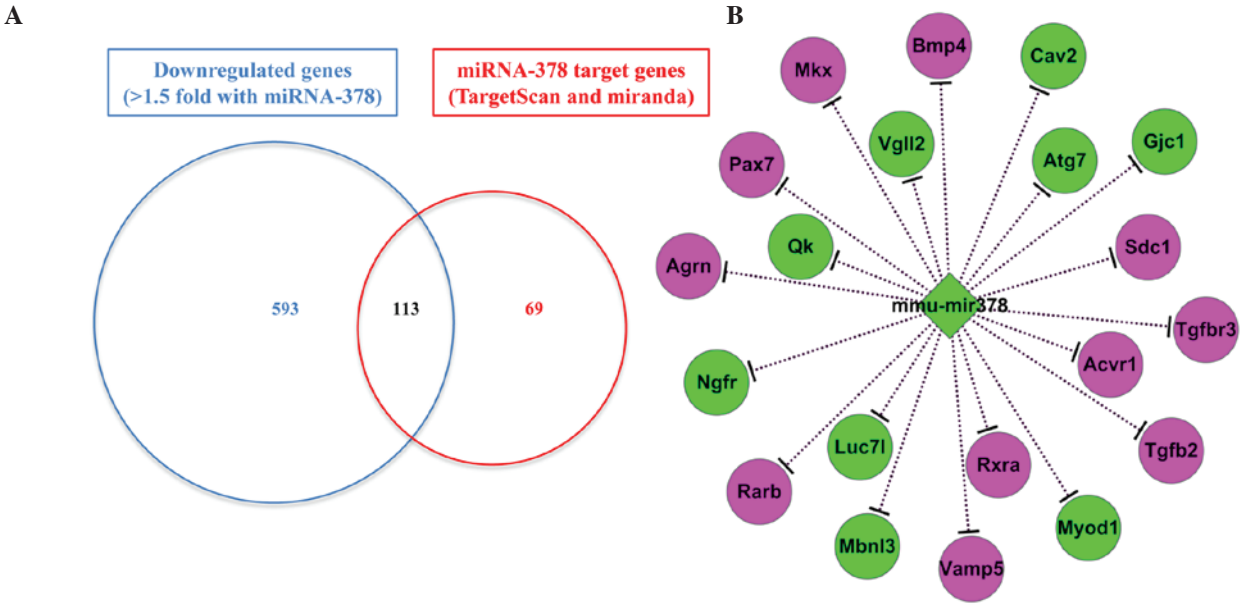

Figure 2. Transcriptome analysis the miRNA-378-regulated genes. (A) Venn diagram illustrating the association between the downregulated genes and predicted target genes by Targetscan and Miranda. (B) Network map illustrating the regulatory association between miRNA-378 and genes involved in development or differentiation. Downregulated genes are shown in magenta and upregulated genes in green. miRNA, microRNA.

was measured at 595 nm using a UV-1780 UV spectrophotometer (Shimadzu Corporation, Kyoto, Japan) and the results were expressed in arbitrary units with normalization to total protein content.

Statistical analysis. Values are expressed as the mean ± standard deviation of at least three independent experiments. The two-tailed Student's *t*-test was used for performing statistical analysis in Excel software (version 2007). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Transcriptome analysis for the assessment of aberrant genome-wide mRNA expression following miRNA-378 overexpression. To enhance the current understanding of the role of miRNA-378 in C2C12-cell differentiation, the present study performed a genome-wide gene expression analysis of miRNA-378-transfected cells in comparison with control RNA-transfected cells. A total of 2,802 genes that were differentially expressed by >1.5-fold following miRNA-378 treatment for 24 h were identified. To evaluate the potential functional significance of the differentially expressed genes, this set of genes was subjected to gene ontology (GO) pathway enrichment analysis. The results revealed a significant enrichment of a number of GO terms associated with the cell cycle (Fig. 1A). To further explore the canonical pathways involved, the genes were then subjected to signaling pathway enrichment analysis with the KEGG. As shown in Fig. 1B, the 15 most significantly upregulated pathways included the p53 signaling pathway and Wnt signaling pathway.

The 706 downregulated genes were then compared with the 182 predicted target genes of miRNA-378 obtained using the TargetScan and Miranda databases (Fig. 2A). A total of 113 candidate target genes of miRNA-378 were thereby identified, which were also significantly overrepresented in the downregulated gene sets obtained by SigTerm software (http://sigterms.sourceforge.net) analysis (17). The candidate genes included TGFB2, MKX, PAX7, RXRA and BMP4, which function in muscle development and muscle cell differentiation (10). Furthermore, of these candidate genes, the genes associated with development or differentiation were clustered as a regulatory network with miRNA-378, as illustrated in Fig. 2B generated by String software (version 9.0) (18). The interaction network showed that certain genes, including BMP4, RXRA, ACVR1 and SDC1, were potentially downregulated by miRNA-378, whereas other genes, including QK, ATG7, CAV2 and GJC1, were upregulated by miRNA-378.

BMP4 is a direct target of miRNA-378. To validate the results of the transcriptome sequencing, eight genes, GJC1, ATG7, QK, MYOD1, TGFB2, RARB, TGFBR3 and BMP4, were selected for further qPCR analysis, since the functions of these genes are involved in muscle differentiation or development. The PCR results demonstrated that the mRNA levels of four genes, GJC1, ATG7, QK and MYOD1, were significantly upregulated, while TGFB2 and BMP4 were markedly downregulated in miRNA-378-transfected cells compared with the negative control group. The mRNA expression of RARB and TGFBR3 was not markedly affected by miRNA-378 (Fig. 3A).

To further examine the potential targets of miRNA-378, western blot analysis of BMP4 and TGFB2 protein expression was performed in miRNA-378-transfected C2C12 cells. Transfection of miRNA-378 resulted in an obvious decrease in endogenous BMP4 protein, but had no effect on the protein levels of TGFB2 (Fig. 3B). These results indicated that miRNA-378 affected BMP4 expression at the mRNA as well as the protein level. To further validate whether miRNA-378 is a direct regulator of BMP4 or TGFB2, luciferase plasmids containing the BMP4 or TGFB2 3'-UTR downstream of the

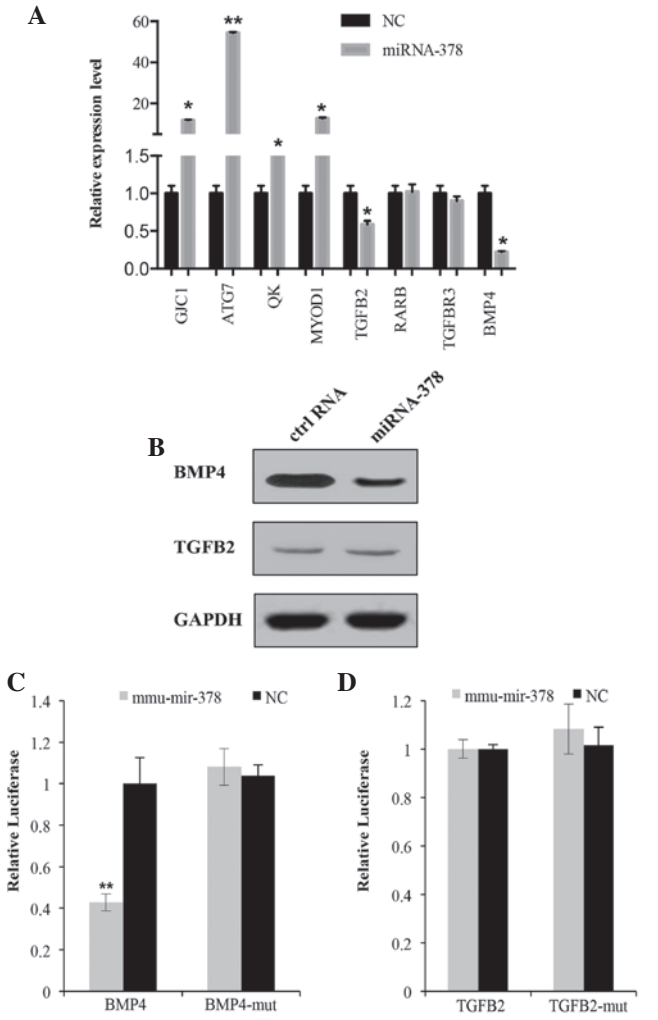

Figure 3. miR-378 directly targets BMP4. (A) mRNA expression of eight genes in C2C12 cells transfected with miR-378 mimics or control RNAs as determined by quantitative polymerase chain reaction analysis with normalization to GAPDH. Expression levels relative to those in the negative control group are shown. Values are expressed as the mean ± standard deviation from three different experiments. (B) Western blot analysis of the BMP4 and TGFB2 proteins in miR-378 mimics or control RNA-transfected C2C12 cells. GAPDH served as protein loading control. Representative blots of three independent experiments are shown. (C and D) A dual luciferase reporter assay was performed to identify direct target genes of miR-378. Cells were transfected with luciferase vectors driven by a fragment of the 3'-untranslated region of BMP4 or TGFB2, respectively, or a mutated sequence, as well as mmu-miR-378. Firefly luciferase/Renilla luciferase activity was expressed relative to that of the negative control. Values are expressed as the mean ± standard deviation (n=3) from three independent experiments. *P<0.05, **P<0.01 vs. the NC group. miR/miRNA, microRNA; BMP4, bone morphogenetic protein 4; TGFB2, transforming growth factor beta 2; NC, negative control; mut, mutated; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ctrl, control.

luciferase gene were constructed and used in a dual luciferase assay with miRNA-378. The results showed that in miRNA-378-transfected cells, the luciferase activity of the BMP4 3'-UTR-driven vector was reduced by ~60% compared with that in the negative control-transfected group; however, luciferase activity of the mutant luciferase reporter vector for BMP4 was not affected by miRNA-378 (Fig. 3C). This result confirmed that BMP4 is a direct target of miRNA-378. However, miRNA-378 did not affect the luciferase activity of the TGFB2 or TGFB2-mut reporter vectors, suggesting that miRNA-378 did not directly target the TGFB2 gene (Fig. 3D).

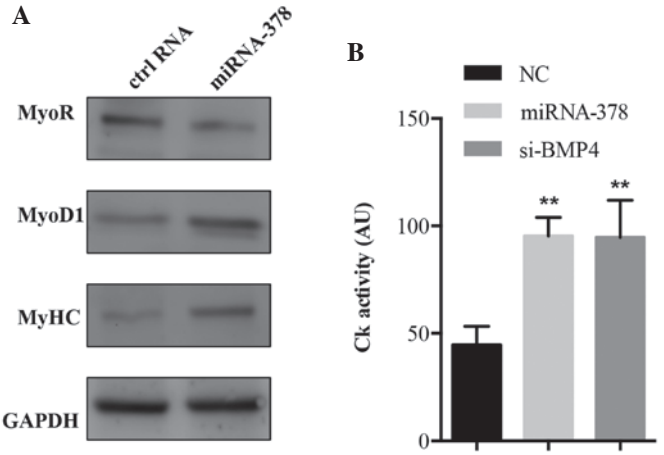

Figure 4. miRNA-378 promotes myogenic differentiation. (A) Western blot analysis of MyoR, MyoD1 and MyHC protein in the miRNA-378 mimics or control RNA-transfected C2C12 cells. GAPDH served as protein loading control. Representative blots of three experiments are shown. (B) C2C12 cells transfected with miRNA-378 mimics, si-BMP4 or control RNAs were analyzed for Ck activity. Values are expressed as the mean ± standard deviation of three independent experiments. **P<0.001 vs. the NC group. miRNA, microRNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NC, negative control; siRNA, small interfering RNA; BMP4, bone morphogenetic protein 4; Ck, creatine kinase; MyoR, myogenic repressor; MyoD1, myogenic differentiation 1; MyHC, myosin heavy chain.

Effect of miRNA-378 on myogenic differentiation. Finally, the present study investigated the overall effect of miRNA-378 overexpression on the myogenic differentiation of C2C12 by assessing the expression of a panel of differentiation biomarkers. As shown in Fig. 4A, C2C12 cells transfected with miRNA-378 mimics showed a significantly increased expression of myosin heavy chain (MyHC) and MyoD1 proteins, which are commonly detected in post-mitotic muscle cells and in committed proliferating myoblast cells, respectively, along with a concomitant decrease in the expression of myogenic repressor (MyoR), an inhibitor of myogenesis (10). Furthermore, the effect on myogenic differentiation was evaluated by comparing Ck activity in miRNA-378-transfected cells and control RNA-transfected cells. Ck activity in miRNA-378-transfected cells was significantly higher than that in control RNA-treated cells (Fig. 4B). The present study further examined whether knockdown of endogenous BMP4 was able to promote myogenic differentiation in C2C12 cells. As shown in Fig. 4B, transfection of BMP4 siRNA and miRNA-378 resulted in an increase of Ck activity compared with the control group. This indicates that the downregulation of BMP4 mimics the function of miRNA-378 on myogenic differentiation of C2C12 cells, further indicating that miRNA-378 regulates myogenic differentiation by downregulating BMP4.

Discussion

The present study performed a genome-wide mRNA deep sequencing analysis to investigate the changes in mRNA expression in miRNA-378-transfected C2C12 cells and demonstrated that miRNA-378 promoted myogenic differentiation by directly targeting the BMP4 gene.

The process of muscle differentiation occurring in embryonic as well as adult muscle precursors is accompanied by the

induction of a specific class of miRNAs, whose expression is driven by MRFs and other myogenic-dependent transcription factors (8,19). Functionally, two different groups of miRNAs are found in muscle tissue: i) Non-muscle-specific miRNAs which are present in muscles, but are also expressed in other tissues (20) and ii) muscle-specific miRNAs, which are present in cardiac and skeletal muscle tissues in greater amounts compared with other tissues (20,21). Muscle-specific miRNAs were indeed shown to act on various levels in the regulation of muscle homoeostasis and differentiation, and their expression was found to be aberrant in certain muscular disorders, including Duchenne muscular dystrophy, myocardial infarction and other types of myopathy (22,23). Among muscle-specific miRNAs, the miR-1/206 and miR-133 families, which originate from three different chromosomes (10), have been most extensively studied and have been shown to take multiple roles in the modulation of muscle differentiation (24). In addition, several non-muscle-specific miRNAs, including miRNA-24, miRNA-214 and miRNA-26a have been shown to participate in the differentiation process into muscle cells (25-27).

Studies have indicated that miRNA-378 is involved in skeletal muscle development (28,29). In the C2C12 myoblast cell line, upregulation of miRNA-378 has been shown to promote efficient myotube formation by repressing antagonists of differentiation, such as MyoR (29). Likewise, miRNA-378 expression in porcine longissimus muscles was shown to be implicated in the modulation of myogenesis, mainly with regard to fibre formation (28). Recently, miRNA-378 has been shown to induce skeletal muscle differentiation in the RH30 human alveolar rhabdomyosarcoma cell line (13). In accordance with these findings, the present study demonstrated that miRNA-378 promoted myogenic differentiation of C2C12 cells via the BMP4 gene. Aberrant expression of specific myogenic biomarkers indicated the induction of myogenic differentiation. Transfection of miRNA-378 resulted in a significant increase of MyoD1 and MyHC protein and a decrease of MyoR protein. Furthermore, transfection of C2C12 cells with siRNA against BMP4 mimicked the effect generated by miRNA-378, suggesting that miRNA-378 activated myogenic differentiation by targeting BMP4.

In conclusion, the present study demonstrated that miRNA-378 promoted myogenic differentiation by down-regulating BMP4. These findings enhanced the current understanding of the biological mechanisms underlying muscle differentiation.

Acknowledgements

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