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Myogenin is a positive regulator of MEGF10 expression in skeletal muscle

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ABSTRACT

MEGF10 is known to function as a myogenic regulator of satellite cells in skeletal muscle. Mutations in MEGF10 gene cause a congenital myopathy called early onset myopathy, areflexia, respiratory distress and dysphagia (EMARDD). Despite its biological importance in muscle physiology, transcriptional regulation of the MEGF10 gene is unknown. Here, we characterized the 5′ flanking region of the human MEGF10 gene and showed that the role of myogenic basic helix-loop-helix factor (bHLH) myogenin in MEGF10 transcription in muscle cells. Myogenin was found to share a similar expression pattern with MEGF10 during muscle regeneration and to increase the promoter activity of the MEGF10 gene in C2C12 cells. Overexpression of myogenin led to upregulation of MEGF10 mRNA in C2C12 cells. Site-directed mutagenesis assays revealed that the conserved E-box element at the region −114/−108 serves as a myogenin-binding motif. Promoter enzyme immunoassays and chromatin immunoprecipitation analysis showed direct interaction between myogenin and the myogenin-binding motif in the MEGF10 promoter. Taken together, these results indicate that myogenin is a positive regulator in transcriptional regulation of MEGF10 in skeletal muscle.

1. Introduction

Multiple EGF-like domain 10 (MEGF10), a type I transmembrane protein consisting of 17 EGF-like domains in the extracellular region, is known as a mammalian ortholog of nematode CED-1 [1,2]. MEGF10 is predominantly expressed in the brain and skeletal muscle [1,3,4]. MEGF10 acts as a phagocytic receptor for amyloid β-peptide (Aβ42) clearance [5] and apoptotic cell engulfment [6,7]. Involvement of MEGF10 and MERTK pathways in synapse elimination in astrocytes has recently been reported [8]. MEGF10 is required for mosaic spacing of retinal neurons via homotypic interaction with MEGF11 [9]. In skeletal muscle, high expression of MEGF10 in activated satellite cells as well as regulation of satellite cell functions by MEGF10 have been reported [10]. Recent studies have reported an association of MEGF10 with congenital myopathy in human. Mutations in the MEGF10 gene caused early onset myopathy, areflexia, respiratory distress and dysphagia (EMARDD) [11,12]. Among mutations in the MEGF10 gene, two mutations in the extracellular EGF-like domains of MEGF10 (C326R and C774R) showed an association with decreased tyrosine phosphorylation of MEGF10 [13]. More recently, novel SNP array analysis and exome sequencing found that a homozygous exon 7 deletion of MEGF10 resulted in EMARDD [12]. These findings indicated that expression of functional MEGF10 plays an important role in muscle physiology. However, the basis for transcriptional regulation of MEGF10 expression in skeletal muscle is largely unknown. In this study, the potential role of myogenin in transcriptional regulation of MEGF10 in skeletal muscle is addressed and supported by the evidence reported herein.

2. Materials and methods

2.1. Reagents

Polyclonal anti-myogenin antibody and horseradish peroxidase (HRP)-conjugated anti-mouse IgG were purchased from Santa Cruz Biotechnology. Anti-FLAG- or anti-Myc-conjugated agarose beads

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were obtained from Sigma. Protein G-conjugated agaroge beads were purchased from Millipore.

2.2. Cell culture

C2C12 and 293FT cells were grown in Dulbecco's modified Eagle medium (DMEM, glucose 4.5 g/L) supplemented with 10% (v/v) fetal bovine serum (FBS) and the appropriate antibiotics.

2.3. RNA isolation from regenerating muscles

Nine-week-old male mice were anaesthetized with isoflurane, and 100 μl of 10 μM cardiotoxin (CalBiochem) in PBS was injected into the right tibialis anterior (TA) muscle. The mice were sacrificed by cervical dislocation at the indicated time after cardiotoxin injection. TA muscles were harvested tendon-to-tendon, and total RNAs were isolated using Trizol reagent, in accordance with the manufacturer's instructions (Invitrogen).

2.4. Quantitative real-time PCR

Reverse transcription was performed with M-MLV reverse transcriptase (Promega), and the resulting cDNA was diluted 2.5-fold and used to amplify MEGF10 and myogenic bHLH factors (myogenin, MyoD, Myf5, and MRF4). Real-time PCR amplification was carried out using SYBR green master mix (Roche Applied Science) in a LightCycler 480 (Roche Applied Science) as follows: initial denaturation at 95 °C for 5 min; 45 cycles of amplification with denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s; 1 cycle of melting curves at 95 °C for 5 s, 65 °C for 1 min, and 97 °C continuous; and a final cooling step at 40 °C for 30 s. The qRT-PCR results were analyzed using the comparative cycle threshold (C_T) method as previously described [14]. The primers for real-time PCR are listed in Table 1.

2.5. Plasmid constructs

For generation of myogenin, MyoD, and Myf5 expression vectors, coding region of each gene was amplified by PCR using cDNA from rhabdomyosarcoma RD cells as the template and cloned into the EcoRI–XhoI sites of pcDNA3.1 vector (Invitrogen) with the FLAG epitope sequence. Plasmid encoding MEF2C under the control of the CMV promoter were obtained from 21C Frontier Human Gene Bank (Seoul, Korea). Plasmids encoding NFATc1, NFATc2, and NFATc3 under control of the CMV promoter were described previously [15]. For generation of the human MEGF10 promoter construct, the transcriptional initiation site of the human MEGF10 gene was determined by 5′ rapid amplification of cDNA ends (RACE) using cDNA isolated from RD cells. The 5′-flanking region of the human MEGF10 gene (pMEGF10 −1334/+226) was amplified by PCR and cloned into the Asp718I–XhoI sites of the pGL3/basic vector (Promega). For generation of 5′ deletion constructs of the human MEGF10 promoter, a PCR reaction was performed using the primers listed in Table 1. The PCR products were cloned into the Asp718I−XhoI sites of the pGL3/basic vector. MEGF10 promoter constructs with a point mutation in each putative myogenin-binding motif were generated using the Quikchange II site-directed mutagenesis kit (Agilent Technologies) with the primers listed in Table 1, in accordance with the manufacturer's instructions.

2.6. Reporter gene assays

C2C12 cells were seeded in 12-well plates at a density of 7×10^4 cells/well. The cells were transfected with 0.2 μg of pGL3/basic or pMEGF10 vector, 0.1 μg of *Renilla* luciferase reporter pRL-TK (Promega), and 30 ng of myogenin expression vector using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, cells were harvested and lysed using passive lysis buffer (Promega). Luciferase activities in cell lysates were measured using a Dual Luciferase assay kit (Promega), in accordance with the manufacturer's instructions. Firefly luciferase activity was normalized to *Renilla* luciferase activity for transfection efficiency.

Table 1
DNA sequences of oligonucleotides used in this study.

Name	Nucleotide sequence
5′ RACE-PCR	
5′ GSP (RACE)	5′-ACGCTGCTTGGACGCTAACCG-3′
3′ GSP-1 (RACE)	5′-ACCAGTTTAGAATGTCAGTGC-3′
3′ GSP-2 (RACE)	5′-AGTGGCTACACACATTAGGGTC-3′
3′ GSP-3 (RACE)	5′-AGAACAACCTCTCTGAAATCCG-3′
Cloning of MEGF10 promoter and its deletion constructs	
5′ pMEGF10	5′-AAAAGGTACCATCCTCTTGCTTCCTCAC-3′
5′ pMEGF10–887	5′-AAATGGTACCGGTCCTGTAAAAGCTGG-3′
5′ pMEGF10–201	5′-AAATGGTACCTCAGGCCGGGAAGATCC-3′
5′ pMEGF10–102	5′-AAATGGTACCCGGCAACTGCTCGAATCC-3′
3′ pMEGF10	5′-AAAACTCGAGCCAGGTCCAGAGATCTTG-3′
Primers for quantitative real-time PCR	
5′ MEGF10	5′-ACTACACAAGCTGCACCGACATC-3′
3′ MEGF10	5′-AACCAGAAGGGCAAGAGCACT-3′
5′ Myogenin	5′-GCAAGGTGTGTAAGAGGAAG-3′
3′ Myogenin	5′-TGTGGGAGTTGCATTCACTG-3′
5′ MyoD	5′-GCACTACAGTGGCGACTCAGAT-3′
3′ MyoD	5′-TAGTAGGCGGTGTCGTAGCCAT-3′
5′ Myf5	5′-ATGCCATCCGCTACATTGAG-3′
3′ Myf5	5′-GGGTAGCAGGCTGTGAGTTG-3′
5′ MRF4	5′-CTGCGCGAAAGGAGGAGACTAAAG-3′
3′ MRF4	5′-ATGGAAGAAAGCCGCTGAAGACTG-3′
5′ GAPDH	5′-AACATCAAATGGGGTGAGGCC-3′
3′ GAPDH	5′-GTTGTCATGGATGACCTTGGC-3′
Primers for site-directed mutagenesis	
5′ pMEGF10-M1	5′-CACTGCCCCGGCACCAGCAGCTGCAGC-3′
3′ pMEGF10-M1	5′-CGTGCAGCTGCTGGTGCCGGGGCAGTG-3′
5′ pMEGF10-M2	5′-CTGCCCCGGCAGCACCAGCTGCAGCAG-3′
3′ pMEGF10-M2	5′-CTGCTGCAGCTGGTGCTGCCGGGGCAG-3′
5′ pMEGF10-M3	5′-GGCAGCAGGTGCAGCAGCGGCAAC-3′
3′ pMEGF10-M3	5′-GTTGCCGCTGCTGCACCTGCTGCTGCC-3′
5′ pMEGF10-M4	5′-CAGCAGCAGCTGCACCAGCGGCAACTG-3′
3′ pMEGF10-M4	5′-CAGTTGCCGCTGGTGCAGCTGCTGCTG-3′
Probes for promoter enzyme immunoassays	
pMEGF10-wt (S)	5′-Biotin-AGCAGCAGCTGCAGCAG-3′ (−119/−103)
pMEGF10-wt (AS)	5′-CTG CTG CAG CTG CTG CT-3′
pMEGF10-mut (S)	5′-Biotin-AGCAGCAGGTGCAGCAG-3′ (−119/−103)
pMEGF10-mut (AS)	5′-CTGCTGCACCTGCTGCT-3′
Primers for ChIP assays	
5′ pMEGF10-ChIP	5′-CCAACATTTGCTAACTTCGC-3′ (−300/−281)
3′ pMEGF10-ChIP	5′-TGAACAAGGTTTTCTCTGTCTG-3′ (−36/−15)
5′ GAPDH-ChIP	5′-TGCCACCCAGAAGACTGTG-3′
3′ GAPDH-ChIP	5′-ATGTAGGCCATGAGGTCCAC-3′

2.7. Promoter enzyme immunoassays

Promoter enzyme immunoassays were performed as previously described [16]. For biotin-labeled probe containing wild-type or mutated myogenin-binding motif, sense and antisense oligonucleotides were synthesized and annealed in annealing buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris–HCl, pH 8.0). The sequences of the oligonucleotides are listed in Table 1. Streptavidin-coated 96-well plates were conjugated with biotin-labeled probes (5 pmol per well) by incubation at room temperature for 1 h. After three washes with PBS-T buffer, the plates were incubated with the nuclear extract (20 μg/100 μl) from 293FT cells transfected with myogenin expression vector and poly-dI/dC (Sigma) at 4 °C for 2 h. After three washes with wash buffer, the plate was incubated with anti-myogenin antibody at 4 °C for 2 h, followed by incubation with HRP-conjugated anti-mouse IgG. After three washes with

wash buffer, a substrate reagent (R&D Systems) was added, and the plates were then incubated for 1 h at 4 °C. The colorimetric reaction was quenched by addition of 2 M H_2SO_4. Absorbance at 450 nm was measured using a microplate reader (BioRad).

2.8. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using the Upstate Biotechnology ChIP assay kit as previously described [17] with slight modifications. 293FT cells transfected with myogenin-FLAG expression vector were crosslinked in culture media with formaldehyde (a final concentration of 1%) at room temperature for 10 min. Then, cells were lysed and sonicated in order to shear the chromatin, ranging in size from 200 to 1000 base pairs, followed by centrifugation for 10 min at 12,000 rpm for removal of cell debris. The supernatants were diluted 10-fold into ChIP dilution buffer, precleared by incubation with salmon sperm DNA–protein G-agarose for 30 min at 4 °C with gentle agitation, and then immunoprecipitated with anti-FLAG antibody-conjugated agarose beads, anti-Myc antibody-conjugated beads, or unconjugated protein G beads. Immunoprecipitated complexes were eluted from the beads using elution buffer (1% SDS, 0.1 M $NaHCO_3$), and incubated at 65 °C for 4 h in order to reverse the formaldehyde crosslinks. After digestion with proteinase K for removal of proteins, associated DNA was purified by phenol/chloroform extraction and ethanol precipitation, and then used as templates for PCR reactions. The primers used in ChIP assays are listed in Table 1.

2.9. Statistical analysis

Statistical significance was assessed using Student *t*-test. A *P* value of <0.05 was considered statistically significant.

3. Results and discussion

3.1. MEGF10 and bHLH myogenic factors share a similar expression pattern during muscle regeneration

A previous study showed that MEGF10 is highly expressed in activating satellite cells [10]. Because activation of satellite cells during muscle regeneration is coordinated by myogenic basic helix-loop-helix (bHLH) factors such as Myf5, MyoD, myogenin, and MRF4/Myf6 [18], we examined expression of MEGF10 and myogenic bHLH factors during muscle regeneration using a model of cardiotoxin-induced muscle injury. MEGF10 mRNA was up-regulated during muscle regeneration and peaked at three days after cardiotoxin injury, when satellite cells were activated and proliferated (Fig. 1A and B). Expression of myogenin, Myf5, and MyoD showed an increase three days after muscle injury and declined thereafter, similar to the expression pattern of MEGF10 (Fig. 1C–E), whereas MRF4 expression showed a transient increase five days after muscle injury (Fig. 1F). These findings suggest possible involvement of myogenic bHLH factors in transcriptional regulation of MEGF10 during muscle regeneration.

3.2. Characterization of the human MEGF10 promoter region

Human MEGF10 gene consists of 26 exons, and the translational start codon (AUG) is localized on exon 2. The transcription start site of the MEGF10 gene was determined by 5′ rapid amplification of cDNA ends (RACE)-PCR analysis (Fig. 2A). A band with approximately 300 bp was amplified using specific primer for the MEGF10 gene (Fig. 2B, lane 2), and the transcription initiation site of the human MEGF10 gene was identified by DNA sequencing (Fig. 2C). In sequence analysis for cis-acting regulatory elements that have been implicated in muscle-specific gene regulation, four E-box elements that are known to serve as binding sites for basic helix-loop-helix (bHLH) transcription factors were found in the promoter region of the MEGF10 gene. Three nuclear factor of activated T cells (NFAT)-binding sites and a MEF2-binding site were also found (Fig. 2D).

3.3. E-box element at region −114/−108 serves as a myogenin-binding motif

To investigate the molecular mechanism for regulation of MEGF10 expression in skeletal muscle, reporter gene construct containing the 5′-flanking region of the human MEGF10 gene was generated, and the effects of myogenic bHLH factors, MEF2C, and NFAT isoforms on the MEGF10 promoter activity were examined in C2C12 cells. Myogenin enhanced the promoter activity of

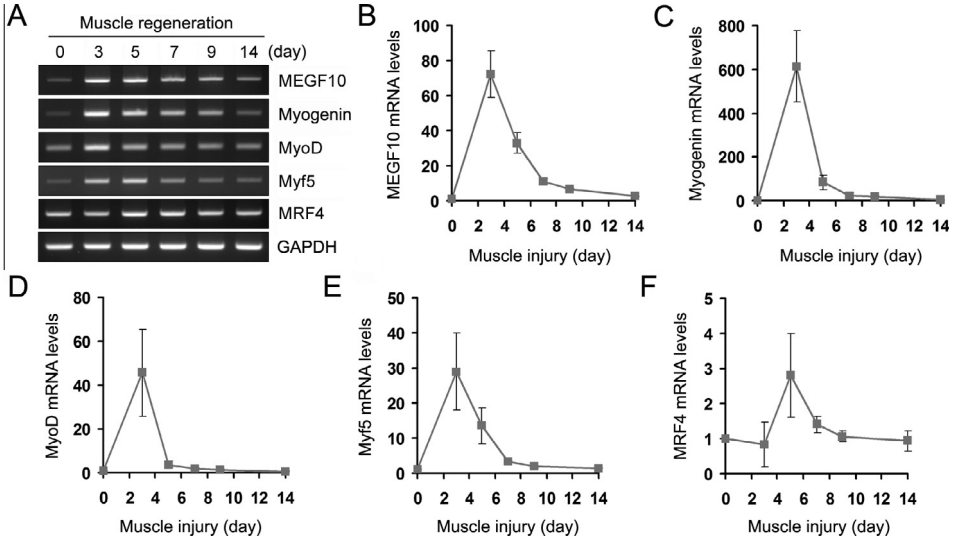

Fig. 1. Expression patterns of MEGF10 and myogenic bHLH factors during muscle regeneration after muscle injury. (A) mRNA expressions of MEGF10 and myogenic bHLH factors during muscle regeneration after cardiotoxin injection were analyzed by RT-PCR. (B–F) mRNA levels of MEGF10 (B), Myogenin (C), MyoD (D), Myf5 (E), and MRF4 (F) during muscle regeneration were quantified using real-time PCR. The relative expression level was plotted against that of each gene before cardiotoxin injury, which was set as 1. Results are expressed as the mean ± SD of three independent experiments.

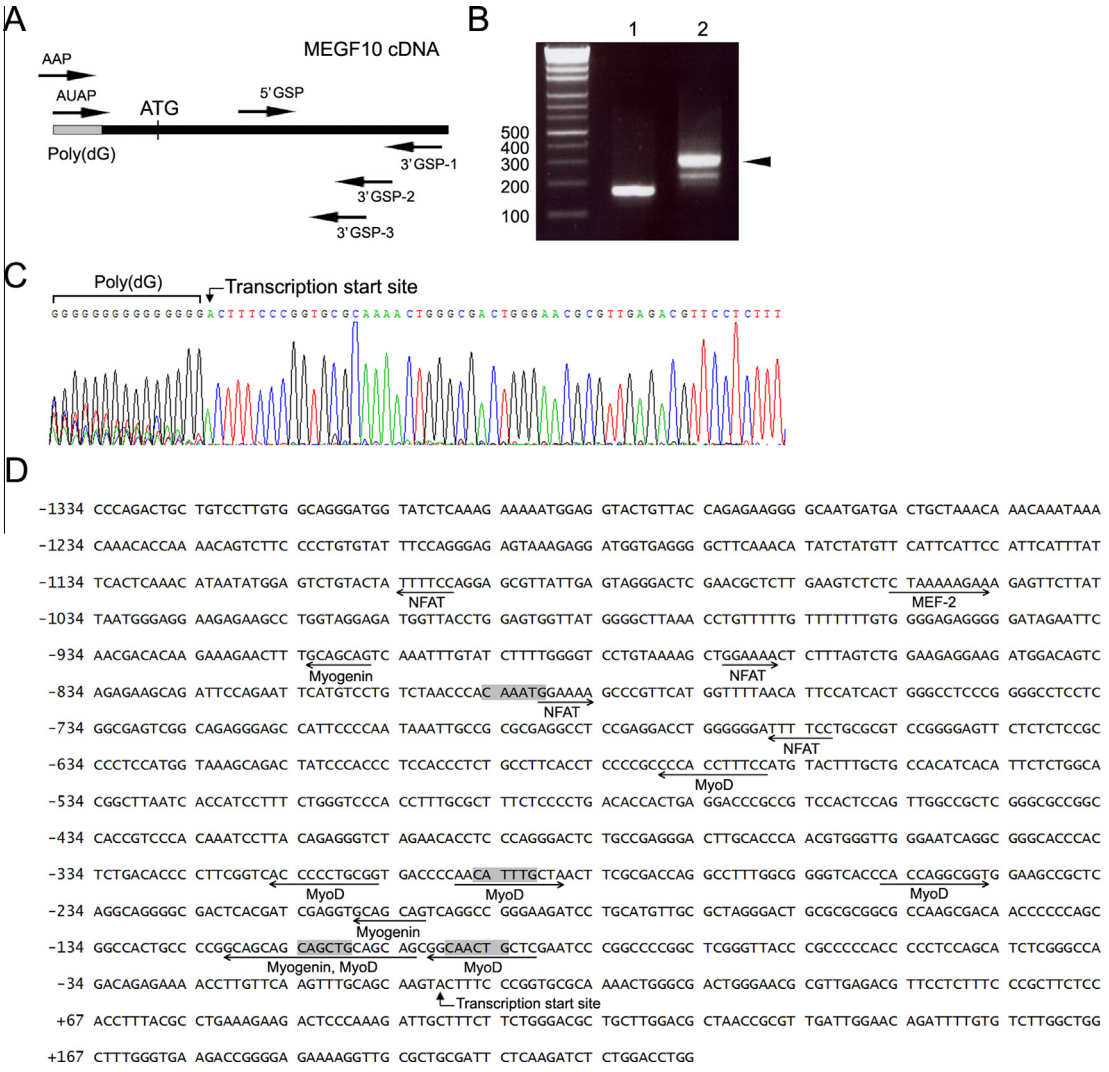

Fig. 2. Characterization of promoter region of the human MEGF10 gene. (A) The strategy for 5′ rapid amplification of cDNA ends (RACE)-PCR amplification is shown. The locations of the gene-specific primers (GSP) used for 5′ RACE-PCR are indicated by arrows. (B) The RACE-PCR product was resolved on a 2% agarose gel with a specific band (lane 2). Lane 1: 5′ GSP & 3′ GSP-2; Lane 2: AAP & 3′ GSP-3. (C) The transcriptional initiation site was determined by DNA sequencing of the RACE-PCR product. (D) Nucleotide sequences of the MEGF10 promoter region (−1334/+226). Numbers indicate position from the transcriptional start site, which is designated as +1 in the numbering of the nucleotide sequence. E-box elements are indicated by gray boxes. The TFSEARCH program was used to search the cloned MEGF10 promoter region (−1334/+226) for putative transcription factor binding sites (*arrows* indicate the direction).

MEGF10 by 11-fold (Fig. 3A). Myf5 and NFATc1 also increased MEGF10 promoter activity by 2.8 and 2.7-fold, respectively. Myogenin induced a dose-dependent increase in MEGF10 promoter activity (Fig. 3B). Overexpression of myogenin resulted in significant upregulation of MEGF10 mRNA expression in C2C12 cells (Fig. 3C). Thus, these results indicate that myogenin is a positive regulator of MEGF10 expression. Sequence analysis of the MEGF10 promoter region (−1334/+226) showed that there are six putative myogenin-binding motifs ($CA^{C}/_{G}CTGC$) in the human MEGF10 promoter. To determine which region is important for activation of MEGF10 promoter, we generated a series of 5′ deletion constructs of the MEGF10 promoter reporter (Fig. 3D). These constructs were cotransfected with myogenin expression vector into C2C12 cells, and their effects on promoter activity were measured by performance of luciferase assays. As shown in Fig. 3E, a significant decrease was observed in myogenin-mediated activation of the MEGF10 promoter when the promoter was deleted from −201 to −102, indicating the presence of a myogenin-responsive element in region −201/−103 of the human MEGF10 promoter. Sequence analysis of the myogenin-binding motif revealed the existence of four overlapping motifs, three of which are conserved in human and mouse MEGF10 promoters (Fig. 4A). To further determine which motif is a functional element for MEGF10 activation, we generated mutant MEGF10 promoter constructs containing a point mutation in each myogenin-binding motif using site-directed mutagenesis (Fig. 4B). Point mutation in the third myogenin-binding motif (pMEGF10-M3), which was localized in conserved E-box element, caused a significant decrease in myogenin-mediated activation of MEGF10 promoter, whereas mutations of other myogenin-binding motifs (pMEGF10-M1, -M2, and -M4) had no effect on MEGF10 promoter activity, indicating that the myogenin-binding motif at region −114/−108 plays a critical role in myogenin-responsive transcription of MEGF10 (Fig. 4C).

3.4. Myogenin binds directly to the myogenin binding motif in the human MEGF10 promoter

In order to examine the association between myogenin and myogenin-binding motif at −114/−108 of the MEGF10 promoter, promoter enzyme immunoassays were performed using nuclear

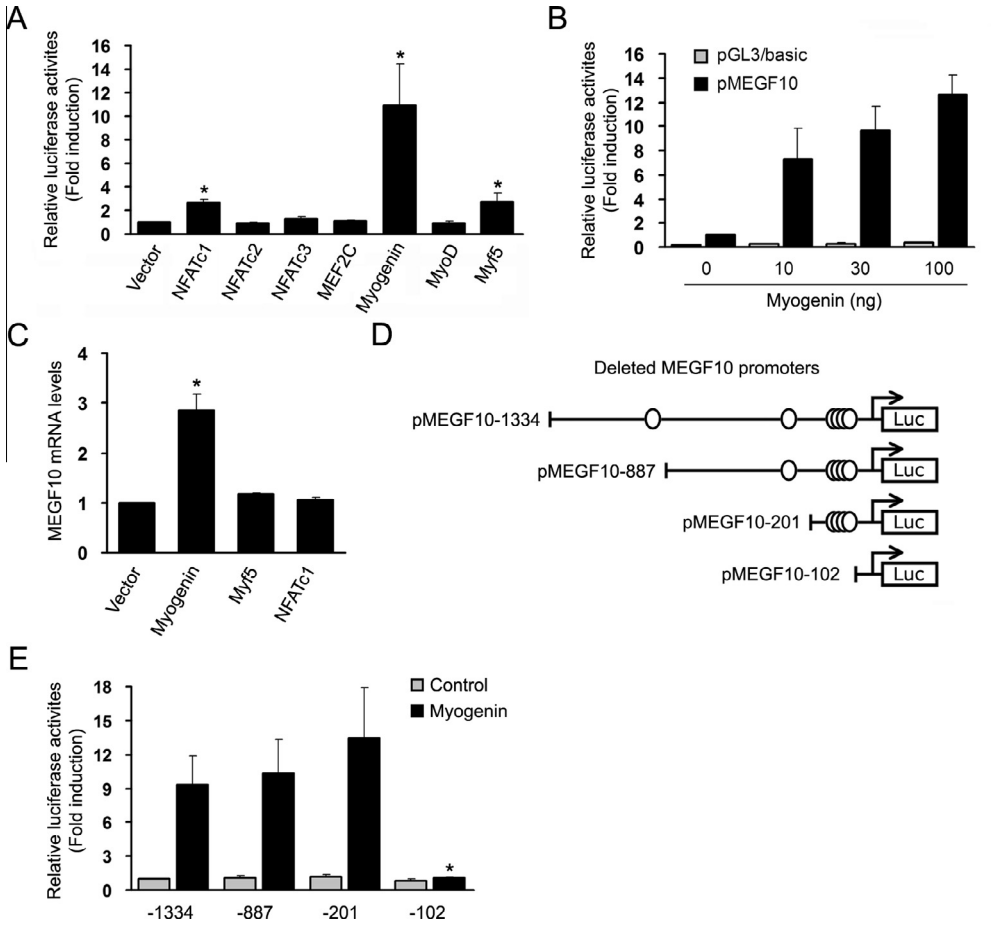

Fig. 3. Myogenin acts a positive regulator of the MEGF10 gene in muscle cells. (A) The MEGF10 promoter construct (−1334/+226) was cotransfected with the plasmid encoding the indicated transcription factor or control pcDNA3.1 vector into C2C12 cells. The relative luciferase activities were plotted against that of the control pcDNA3.1 vector, which is set as 1. Bars represent the mean ± SD of at least three independent experiments. *t*-test: $^{*}P < 0.01$. (B) The MEGF10 promoter construct (−1334/+226) was cotransfected with the indicated amount of myogenin expression vector or pcDNA3.1 vector into C2C12 cells. Relative luciferase activities were plotted against MEGF10 promoter activity in the absence of myogenin, which is set as 1. Bars represent the mean ± SD of three independent experiments. (C) C2C12 cells were transfected with the plasmid encoding myogenin, Myf5, or NFATc1. 48 h posttransfection, total RNAs were isolated from the cells, and MEGF10 expression was evaluated using quantitative real-time PCR. Bars represent the mean ± SD of at least three independent experiments. *t*-test: $^{*}P < 0.01$. (D) The schematic representation of the full-length and deleted MEGF10 promoters. The putative myogenin-binding sites are indicated by ovals. (E) The full-length (−1334/+226) and a series of 5′ deletion mutants of the human MEGF10 promoter constructs were cotransfected with the plasmid encoding myogenin or control pcDNA3.1 vector into C2C12 cells. The relative luciferase activities were plotted against that of the full-length promoter in the absence of myogenin, which is set as 1. Bars represent the mean ± SD of at least three independent experiments. *t*-test: $^{*}P < 0.01$.

extracts from 293FT cells transfected with myogenin expression vector. Biotin-labeled oligonucleotides containing wild-type or mutated myogenin-binding motif were generated (Fig. 4D), and then conjugated on streptavidin-coated microplates. After incubation of the nuclear extracts with the plate, the interaction of myogenin with the oligonucleotide probe was observed using anti-myogenin antibody. Myogenin proteins bound to oligonucleotides containing the myogenin-binding motif (Fig. 4E). A point mutation of the myogenin-binding motif caused a significant decrease in myogenin binding to oligonucleotide probes (Fig. 4E), indicating that myogenin binds specifically to the myogenin-binding motif at region −114/−108 in the MEGF10 promoter. Association of myogenin with the MEGF10 promoter was further investigated in 293FT cells transfected with myogenin-FLAG expression vector by chromatin immunoprecipitation (ChIP) analysis. Using specific primers to the MEGF10 promoter, the PCR product encompassing the myogenin-binding motif was obtained from the DNA precipitated using anti-FLAG antibody (Fig. 4F). The PCR product was only slightly amplified from DNA precipitated using control antibody (anti-Myc). When GAPDH primers were used as a negative control, no signal was observed in the PCR amplification. Taken together, these results demonstrate that myogenin binds specifically to the myogenin-binding motif in the human MEGF10 promoter.

Myogenin is a basic helix-loop-helix (bHLH) transcription factor required for regulation of myogenesis. Its expression is restricted to cells of skeletal muscle origin. Mice with a targeted mutation in the myogenin gene die at birth due to severe skeletal muscle deficiency [19]. Transcription factors of MRF family, such as myogenin, MyoD, Myf5, and MRF4, bind to E-box elements (CANNTG) in the promoter region of muscle-specific genes [20] and regulate their expression in skeletal muscle. The DNA-binding domain of myogenin protein interacts preferentially with CAGCTG-containing DNA elements [21]. In this study, we found conserved myogenin-binding motifs in the human MEGF10 promoter. Mutation in core sequence of the myogenin-binding motif resulted in abrogation of both myogenin-mediated activation of the MEGF10 promoter and successful binding between myogenin and the myogenin-binding motif of the human MEGF10 promoter.

Satellite cell activation during muscle regeneration is coordinated by myogenic transcription factors such as myogenin, Myf5, MyoD, and MRF4/Myf6 [18]. Myogenin mRNA expression occurred

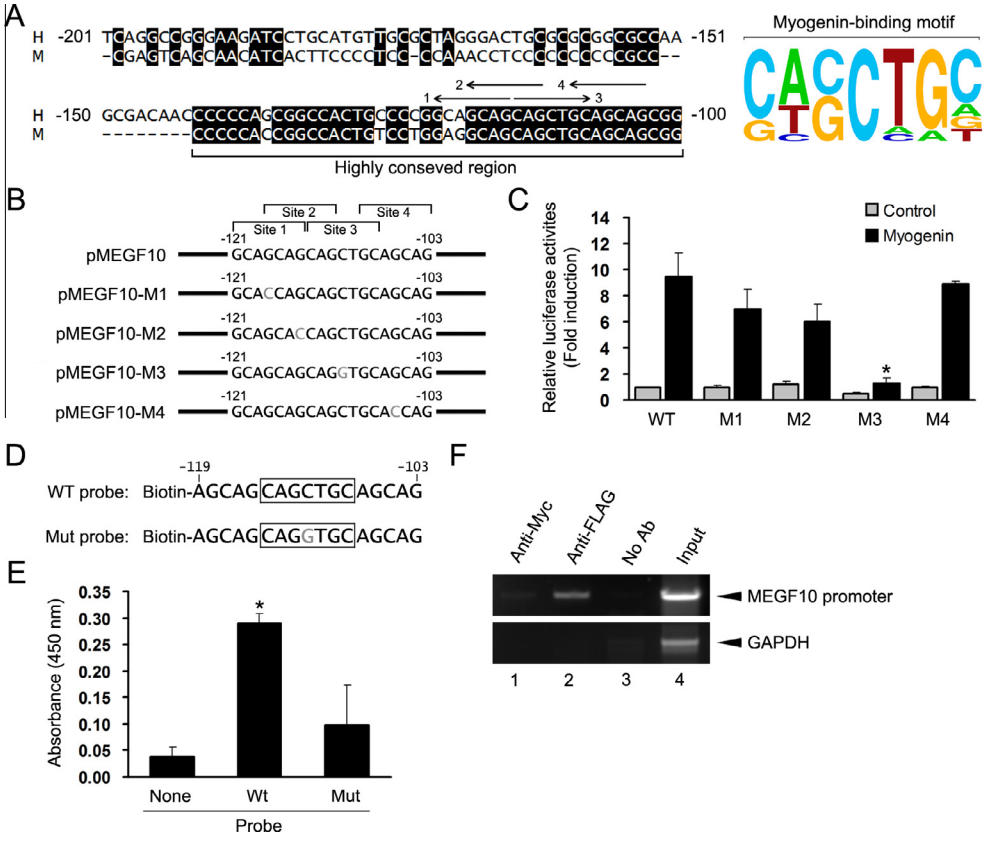

Fig. 4. Myogenin regulates MEGF10 expression via region −114/−108 of the human MEGF10 promoter. (A) Sequence alignment of the region between −201 and −100 of the MEGF10 promoter in human and mouse was performed using the ClusterW program. Completely conserved nucleotides are indicated by black boxes. Consensus myogenin-binding motifs were indicated by upward arrows, with orientation of arrows indicating that the sites were found in sense and antisense. (B) Schematic representation of four mutant MEGF10 promoters containing a point mutation in each putative myogenin-binding motif. Numbers indicate position from the transcriptional start site. Point mutations in putative myogenin-binding motifs are shown in gray. (C) Wild-type (−1334/+226) and mutants (M1–M4) of the human MEGF10 promoter constructs were cotransfected with myogenin expression vector or control pcDNA3.1 vector into C2C12 cells. The relative luciferase activities were plotted against that of the wild-type promoter in the absence of myogenin, which is set as 1. Bars represent the mean ± SD of at least three independent experiments. *t*-test: $^{*}P < 0.01$. (D) Oligonucleotide probes encompassing wild-type or mutated myogenin-binding motif. Myogenin-binding motifs are boxed. Point mutation in conserved cytidine in putative myogenin-binding motif is shown in gray. (E) Nuclear extracts from 293FT cells transfected with myogenin expression vector were incubated with biotinylated probes immobilized on streptavidin-coated plates for 2 h at 4 °C. Interaction of myogenin with oligonucleotide probes was analyzed as described in "Section 2". Bars represent the mean ± SD of four independent experiments. *t*-test: $^{*}P < 0.05$. (F) Soluble chromatin was prepared from 293FT cells transfected with myogenin-FLAG expression vector and immunoprecipitated with anti-FLAG antibody- or anti-Myc antibody-conjugated agarose beads. Immunoprecipitated DNA was subjected to PCR with primers specific to the MEGF10 promoter flanking the myogenin-binding motif. GAPDH primers were used as a negative control.

4–8 h after injury in regenerating muscle and showed correlation with the mitotic activity of satellite cells [22]. MEGF10 is highly expressed in activated satellite cells and regulates myogenesis though promotion of satellite cell proliferation [10]. In agreement with these findings, the results in the present study showed that myogenin and MEGF10 shared a similar expression pattern during regeneration. Overexpression of myogenin led to an increase of both MEGF10 promoter activity and MEGF10 mRNA expression in C2C12 cells. These observations suggest that myogenin could regulate MEGF10 expression during satellite cell activation. Although the results of the current study showed that myogenin directly regulates transcriptional activation of the MEGF10 gene, it is possible that myogenin regulates MEGF10 expression in the presence of a specific factor or through cooperation with other myogenic factors. In a previous study, MEGF10 expression was increased in MyoD$^{-/-}$ myoblasts during myogenic differentiation compared with wild-type myoblasts [10]. We found that Myf5 and NFATc1 activated promoter activity of MEGF10 by 2.8 and 2.7-fold, respectively. The mechanism of cooperation between myogenin and other myogenic factors for MEGF10 regulation remains to be investigated.

In summary, we demonstrated that myogenin plays a critical role in transcriptional regulation of the MEGF10 gene in muscle cells. Considering the role of MEGF10 as a regulator of the myogenic program, our results will be helpful in elucidation of the molecular mechanism underlying satellite cell activation and proliferation during muscle regeneration.

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