MURC, a muscle-restricted coiled-coil protein, is involved in the regulation of skeletal myogenesis

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Skeletal myogenesis is a multistep process by which multinucleated mature muscle fibers are formed from undifferentiated, mononucleated myoblasts. However, the molecular mechanisms of skeletal myogenesis have not been fully elucidated. Here, we identified muscle-restricted coiled-coil (MURC) protein as a positive regulator of myogenesis. In skeletal muscle, MURC was localized to the cytoplasm with accumulation in the Z-disc of the sarcomere. In C2C12 myoblasts, MURC expression occurred coincidentally with myogenin expression and preceded sarcomeric myosin expression during differentiation into myotubes. RNA interference (RNAi)-mediated knockdown of MURC impaired differentiation in C2C12 myoblasts, which was accompanied by impaired myogenin expression and ERK activation. Overexpression of MURC in C2C12 myoblasts resulted in the promotion of differentiation with enhanced myogenin expression and ERK activation during differentiation. During injury-induced muscle regeneration, MURC expression increased, and a higher abundance of MURC was observed in immature myofibers compared with mature myofibers. In addition, ERK was activated in regenerating tissue, and ERK activation was detected in MURC-expressing immature myofibers. These findings suggest that MURC is involved in the skeletal myogenesis that results from modulation of myogenin expression and ERK activation. MURC may play pivotal roles in the molecular mechanisms of skeletal myogenic differentiation.

myoblast; skeletal muscle; differentiation; extracellular signal-regulated kinase; myogenin

SKELETAL MYOGENESIS is characterized by a multistep process in which mononucleated, undifferentiated myoblasts proliferate (proliferation), withdraw from the cell cycle, and then differentiate into mononucleated myocytes (early differentiation), which subsequently fuse into multinucleated myotubes expressing muscle-specific proteins (late differentiation) to form the mature muscle fiber (terminal differentiation) (16). Myogenic regulatory factors (MRFs), which belong to the basic helix loop-helix family of transcription factors, in cooperation with E2A and myocyte enhancer factor (MEF)2 families, activate the differentiation program by inducing the transcription of regulatory and structural muscle-specific genes (2–4, 16, 18, 29). In addition to these regulators, skeletal myogenesis is regulated by signal transduction cascades with the complex involvement of several kinases, including ERK (1, 10, 16, 26, 30, 38, 42). In proliferating myoblasts, the ERK pathway contributes to repress myogenic transcription and maintain the undifferentiated phenotype. At the onset of differentiation, the decline of ERK activity relieves the repressed myogenic transcription. Once the activation of the myogenic program is initiated, ERK activation is required to promote myogenic differentiation. Thus, ERK shows a biphasic activation profile with peaks in undifferentiated myoblasts and postmitotic myotubes and is suggested to have a dual role during myogenic differentiation, being inhibitory at the early stage and stimulatory at the late stage (38). However, the molecular mechanisms of skeletal myogenesis have not been fully elucidated.

We have recently identified a novel muscle-restricted coiled-coil protein, MURC, which is evolutionarily conserved from the frog to human (22). MURC is expressed in cardiomyocytes, smooth muscle cells, and skeletal myocytes. In the murine adult heart, MURC was localized to the cytoplasm with accumulation in the Z-line of the sarcomere. MURC mRNA expression in the heart increased during the developmental process from the embryonic stage to adulthood. In response to pressure overload, MURC mRNA expression increased in the hypertrophied heart. Forced overexpression of MURC in cardiomyocytes induced the activation of the RhoA/Rho-associated kinase (ROCK) pathway, which modulated serum response factor (SRF)-mediated atrial natriuretic peptide (ANP) expression and myofibrillar organization. Sustained overexpression of MURC in the heart induces cardiac dysfunction and conduction disturbances with an increased vulnerability to atrial arrhythmias in mice.

In this study, we examined the role of MURC in skeletal myogenesis. MURC expression was induced during myogenic differentiation in vitro and in vivo. In C2C12 myoblasts, RNA interference (RNAi)-mediated knockdown of MURC impaired myogenic differentiation, which was accompanied by impaired myogenin expression and ERK activation at the later stages of differentiation. Overexpression of MURC in C2C12 myoblasts promoted differentiation into myotubes with enhanced myogenin expression and ERK activation during differentiation.

MATERIALS AND METHODS

Immunofluorescence microscopy. Specimens were fixed in 4% paraformaldehyde and stained with rabbit polyclonal anti-MURC antibody (22), mouse monoclonal anti-α-actinin antibody, mouse monoclonal anti-embryonic myosin antibody (F1.652, Developmental

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Studies Hyrbridoma Bank, or mouse monoclonal anti-sarcomeric myosin antibody (MF20, Developmental Studies Hybridoma Bank). Secondary antibodies were conjugated with Alexa fluor 488, 555, or 594 (Invitrogen), and nuclei were visualized using 4',6-diamino-2-phenylindole (Invitrogen). The number of nuclei per myocyte and the fusion index were analyzed as previously described (21). Briefly, the average number of nuclei per myocyte was determined from randomly chosen myosin-positive cells containing 2 or more nuclei, and 3,000 nuclei per culture were counted. The fusion index was calculated from the ratio of the number of nuclei in myotubes with 2 or more nuclei to the total number of nuclei, and 1,000 myocyte nuclei were counted.

Cell culture and induction of differentiation. C2C12 cell culture and differentiation were performed as previously described (21). Briefly, myogenesis was induced by changing the medium to DMEM supplemented with 2% horse serum after the cells were grown to confluence in growth medium.

RNA extraction and quantitative RT-PCR. Total RNA was extracted from cells using an RNeasy mini kit (QIAGEN). cDNA synthesis and quantitative real-time PCR were performed as previously described (22, 23, 33). Mouse GAPDH or β-tubulin was used for normalization, and the comparative threshold method was used to assess the relative abundance of the targets. The primers used were as follows: MURC, forward primer 5′-AGC ACA CAG CAA TAC GGG CTA-3′ and reverse primer 5′-TTC TCG GGC AGG CTG TTG TCT TTA-3′; myogenin, forward primer 5′-TAC GTC CAT CGT GGA GAG CAT-3′ and reverse primer 5′-TCT GCA AAA TCT CCT CGC TGG-3′; myoD, forward primer 5′-TGA GCA AAG TGA ATG AGG CCT-3′ and reverse primer 5′-TCC AGA CCT TCG ATG TAG CGG ATG-3′; and GAPDH, forward primer 5′-ACC TTG GCC ATG TGA TTG TT-3′ and reverse primer 5′-CAT GAG CCC TTC CAC AAT GGA AAA-3′.

Western blot analysis. Cell lysates were extracted with lysis buffer containing 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM PMSF, 1X protease inhibitor cocktail (Pierce), 1 mM Na3VO4, and 1 mM NaF. Cell lysates were electrophoresed in 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were incubated with primary antibodies against MURC, Fli652, MF20, Flag (Sigma), phospho-ERK (Thr202/Tyr204), ERK (Cell Signaling), or GAPDH (Chemicon). Horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG (GE Healthcare) were used as secondary antibodies.

Plasmid construction. The corresponding cDNA fragments for mouse MURC (mMURC) were cloned by PCR with a mouse heart cDNA template as previously described (22). PCR was performed using the following primers: mMURC, forward primer 5′-ATG GAA CAC AAC GGA TCA GCT-3′ and reverse primer 5′-CAT TTT GTT GTA GTC TGA GGA CTG CTG TAG TCA CTA-3′. cDNA encoding mMURC with a COOH-terminal Flag epitope and LacZ were cloned into the pMSCVpuro Retroviral Vector (Clontech) to generate pMSCVpuro-MURC and pMSCVpuro-LacZ, respectively. RNAi target sequences for mMURC (5′-GCT AGC TTA ACA AGC TG-3′ and 5′-AGA AAG TGA GTG GGA TTA GAA-3′) were cloned into the BamHI-EcoRI site of the RNAi-Ready-pSIREN-RetroQ vector (Clontech) as an inverted repeat with a hairpin loop spacer to generate RNAi-Ready-pSIREN-RetroQ-mMURC1 and RNAi-Ready-pSIREN-RetroQ-mMURC2, respectively.

Recombinant retroviruses and gene transfer. To generate recombinant retroviruses, GP2–293 cells (Clontech) were cotransfected with the helper vector pSVS-G, pMSCVpuro-MURC, pMSCVpuro-LacZ, RNAi-Ready-pSIREN-RetroQ- luciferase (Clontech), RNAi-Ready-pSIREN-RetroQ-mMURC1, and RNAi-Ready-pSIREN-RetroQ-mMURC2 using FuGENE6 (Roche). pMSCVpuro-LacZ and RNAi-Ready-pSIREN-RetroQ-luciferase were used as controls. The medium supernatant was collected and centrifuged to concentrate virus stocks according to the manufacturer’s instructions. Cells were infected with the retrovirus in the presence of 4 µg/ml polybrene for 24 h, and the medium was changed to fresh medium. Infected cells were selected with 2.5 µg/ml puromycin and analyzed.

Replication-defective recombinant adeno-viruses and gene transfer. Recombinant adeno-viruses expressing Flag-tagged mMURC (Ad-MURC) and LacZ (Ad-LacZ) were generated as previously described (22, 34). C2C12 cells were infected with Ad-MURC or Ad-LacZ diluted in growth medium at a multiplicity of infection of 10 and incubated at 37°C for 1 h. The viral suspension was removed, and cells were cultured with growth medium.

Induced regeneration of skeletal muscle. Induction of skeletal muscle regeneration was performed as previously described with minor modifications (25, 36). Male mice were anesthetized with 2,2,2-tribromoethanol (0.25 mg/g, Aldrich). An incision was performed to expose the tibialis anterior muscle. Muscle damage was induced by the direct application of a 5-mm metal probe precooled in liquid nitrogen to the surface of the exposed muscle for 15 s. At different times after injury, mice were euthanized, and muscles were removed. All of the aspects of animal care and experimentation performed in this study were approved by the Institutional Animal Care and Use Committee of Kyoto University.

Statistical analysis. All experiments were performed at least three times. Data are expressed as means ± SE and were analyzed by one-way ANOVA with post hoc analysis. A value of P < 0.05 was considered significant.
RNAi-mediated knockdown of MURC in C2C12 myoblasts inhibits myogenesis. The above observations prompted us to investigate whether MURC might be involved in skeletal myogenesis. To investigate the biological role of endogenous MURC expression during myogenesis, RNAi using mMURC short-hairpin RNA (shRNA) was performed in C2C12 myoblasts. We made recombinant retroviruses expressing Flag-tagged mMURC, mMURC-shRNA1, and mMURC-shRNA2. C2C12 myoblasts were infected with recombinant retrovirus expressing Flag-tagged mMURC and/or recombinant retroviruses expressing mMURC-shRNA1 or mMURC-shRNA2, which confirmed that the protein expression of Flag-tagged mMURC was reduced by mMURC-shRNA1 and mMURC-shRNA2 (data not shown). We then infected C2C12 cells with recombinant retroviruses expressing luciferase-shRNA, mMURC-shRNA1, and mMURC-shRNA2 and generated C2C12 cells expressing luciferase-shRNA (C2C12-Luc-shRNA), mMURC-shRNA1 (C2C12-mMURC-shRNA1), and mMURC-shRNA2 (C2C12-mMURC-shRNA2). Upon the induction of differentiation, endogenous MURC mRNA and protein expression were attenuated in both C2C12-mMURC-shRNA1 and C2C12-mMURC-shRNA2 cells compared with C2C12-Luc-shRNA cells (Fig. 3A). Since MURC RNAi was achieved more efficiently in C2C12-mMURC-shRNA1 cells than C2C12-mMURC-shRNA2 cells, we used C2C12-mMURC-shRNA1 cells and examined the expression levels of myogenin, MyoD, MCK, and sarcomeric myosin during myogenesis. As shown in Fig. 3B, myogenin mRNA expression was attenuated on days 2 and 3 in C2C12-mMURC-shRNA1 cells compared with C2C12-Luc-shRNA cells. MCK mRNA expression was reduced on days 4 and 6 in C2C12-mMURC-
shRNA1 cells. Consistent with these observations, sarcomeric myosin expression during myogenesis was impaired in C2C12-mMURC-shRNA1 cells compared with C2C12-Luc-shRNA cells (Fig. 3C). Immunostaining analysis showed that myotube formation was also impaired in C2C12-mMURC-shRNA1 cells compared with C2C12-Luc-shRNA cells, and this observation was confirmed in C2C12-mMURC-shRNA2 cells at 6 days after the induction of differentiation. *P < 0.05 compared with C2C12-Luc-shRNA cells. Cell lysates from C2C12-Luc-shRNA, C2C12-mMURC-shRNA1, and C2C12-mMURC-shRNA2 cells at 6 days after the induction of differentiation were immunoblotted with antibodies recognizing MURC (top right) and GAPDH (bottom right). B: mRNA expression of MyoD, myogenin, and muscle creatine kinase (MCK) during myogenesis in C2C12 cells. Real-time RT-PCR was performed with cDNAs from C2C12-Luc-shRNA and C2C12-mMURC-shRNA1 cells during myogenesis. *P < 0.05 compared with C2C12-Luc-shRNA cells. C: expression of sarcomeric myosin protein during myogenesis in C2C12 cells. Cell lysates from C2C12-Luc-shRNA and C2C12-mMURC-shRNA1 cells were immunoblotted with antibodies recognizing sarcomeric myosin (top) and GAPDH (bottom). D: immunostaining of differentiated C2C12 cells. Immunostaining was performed using C2C12-Luc-shRNA, C2C12-mMURC-shRNA1, and C2C12-mMURC-shRNA2 cells at 6 days after the induction of differentiation with an anti-sarcomeric myosin antibody. Nuclei were stained by DAPI (blue). Bottom, higher magnification images. E: expression of MURC protein and sarcomeric myosin protein in C2C12-mMURC-shRNA1 cells. C2C12-mMURC-shRNA1 cells were infected with Ad-LacZ and Ad-MURC at 6 days after the induction of differentiation were immunoblotted with antibodies recognizing Flag, MURC, sarcomeric myosin, and GAPDH. F: immunostaining of differentiated C2C12-mMURC-shRNA1 cells. Immunostaining was performed using C2C12-mMURC-shRNA1 cells infected with Ad-LacZ and Ad-MURC at 6 days after the induction of differentiation with an anti-sarcomeric myosin antibody. Nuclei were stained by DAPI (blue). Bottom, higher magnification images.

Fig. 3. Impaired myogenesis by MURC RNA interference (RNAi). A: expression of endogenous MURC mRNA (left) and protein (right) in differentiated C2C12 cells. Real-time RT-PCR was performed with cDNAs from C2C12-Luc-shRNA, C2C12-mMURC-shRNA1, and C2C12-mMURC-shRNA2 cells at 6 days after the induction of differentiation. *P < 0.05 compared with C2C12-Luc-shRNA cells. Cell lysates from C2C12-Luc-shRNA, C2C12-mMURC-shRNA1, and C2C12-mMURC-shRNA2 cells at 6 days after the induction of differentiation were immunoblotted with antibodies recognizing MURC (top right) and GAPDH (bottom right). B: mRNA expression of MyoD, myogenin, and muscle creatine kinase (MCK) during myogenesis in C2C12 cells. Real-time RT-PCR was performed with cDNAs from C2C12-Luc-shRNA and C2C12-mMURC-shRNA1 cells during myogenesis. *P < 0.05 compared with C2C12-Luc-shRNA cells. C: expression of sarcomeric myosin protein during myogenesis in C2C12 cells. Cell lysates from C2C12-Luc-shRNA and C2C12-mMURC-shRNA1 cells were immunoblotted with antibodies recognizing sarcomeric myosin (top) and GAPDH (bottom). D: immunostaining of differentiated C2C12 cells. Immunostaining was performed using C2C12-Luc-shRNA, C2C12-mMURC-shRNA1, and C2C12-mMURC-shRNA2 cells at 6 days after the induction of differentiation with an anti-sarcomeric myosin antibody. Nuclei were stained by DAPI (blue). Bottom, higher magnification images. E: expression of MURC protein and sarcomeric myosin protein in C2C12-mMURC-shRNA1 cells. C2C12-mMURC-shRNA1 cells were infected with Ad-LacZ and Ad-MURC at 6 days after the induction of differentiation were immunoblotted with antibodies recognizing Flag, MURC, sarcomeric myosin, and GAPDH. F: immunostaining of differentiated C2C12-mMURC-shRNA1 cells. Immunostaining was performed using C2C12-mMURC-shRNA1 cells infected with Ad-LacZ and Ad-MURC at 6 days after the induction of differentiation with an anti-sarcomeric myosin antibody. Nuclei were stained by DAPI (blue). Bottom, higher magnification images.
in C2C12 cells at day 3 was observed (Fig. 4C). Myogenic differentiation was impaired in C2C12 cells treated with PD-98059 during both days 0–6 and days 2–6, whereas it was not impaired in C2C12 cells treated with PD-98059 during days 0–2 (Fig. 4D). These results indicate that ERK activation at the later stages (days 2–6) is necessary for myogenic differentiation in C2C12 cells.

Overexpression of MURC in C2C12 myoblasts promotes myogenesis with enhanced ERK activation at later stages. We then examined whether MURC could promote myogenesis in C2C12 myoblasts. We used recombinant retroviruses expressing LacZ and Flag-tagged MURC and made C2C12 cells expressing LacZ (C2C12-LacZ) and MURC (C2C12-MURC), respectively (Fig. 5A). Although the myogenin mRNA expression level was not altered in undifferentiated C2C12-MURC cells compared with C2C12-LacZ cells, after the induction of differentiation, its expression level was upregulated on days 2 and 3 during differentiation in C2C12-MURC cells compared with C2C12-LacZ cells (Fig. 5B). The MyoD mRNA expression level in C2C12-MURC cells was not altered compared with C2C12-LacZ cells. The expression of MCK mRNA and sarcomeric myosin protein during myogenesis was accelerated and augmented in C2C12-MURC cells compared with C2C12-LacZ cells (Fig. 5, B and C). Morphologically, differentiated myotubes on day 6 in C2C12-MURC cells were both longer and wider than those in C2C12-LacZ cells (Fig. 5D). Consistent with this observation, the number of nuclei per myotube and the percentage of all nuclei present in myotubes (fusion index) on day 6 were significantly higher in C2C12-MURC cells than C2C12-LacZ cells (Fig. 5E). These findings suggest that MURC can promote differentiation into multinucleated myotubes in C2C12 cells.

Although ERK phosphorylation was not affected in C2C12-MURC cells on day 0 compared with that in C2C12-LacZ cells on day 0, it was enhanced at the later stages of differentiation (Fig. 6, A and B). Furthermore, myogenesis in C2C12-MURC cells was inhibited by treatment with PD-98059 during days 0–6 (Fig. 6C).

Upregulation of MURC expression and activation of ERK during injury-induced muscle regeneration in vivo. Although the cell culture system used above is a valuable tool for the identification and characterization of myogenic pathways, it may only partially recapitulate the regulation of myogenesis. Therefore, we examined MURC expression and ERK activation in regenerating muscle tissue. We induced muscle damage by cryoinjury in the tibialis anterior muscle of adult mice. Muscle tissues before injury and at 1, 3, 5, and 9 days postinjury were collected and assessed by hematoxylin and eosin staining, Western blot analysis, and immunostaining. As shown in Fig. 7A, the muscle was destroyed at 1 day postinjury, and regeneration by satellite cells resulted in the formation of small, slightly basophilic, and centronucleated myofibers at 5 and 9 days postinjury. Western blot analysis showed that the MURC protein expression level decreased at 1 day postinjury, accompanied by muscle destruction, gradually increased during muscle regeneration, and was then restored at 9 days postinjury, whereas embryonic myosin was transiently expressed at 5 days postinjury (Fig. 7B). Immunohistofluorescence analysis of regenerating muscle at 5 days postinjury showed that the abundance of MURC appeared higher in centronucleated and immature myofibers than in mature myofibers in which the nuclei occupied a peripheral position. During injury-induced muscle regeneration, Western blot analysis showed that ERK phosphorylation decreased at 1 day postinjury, accompanied by muscle destruction, and then increased at day 3 and continued up to day 9 (Fig. 7B). To examine which cells account for ERK activation, immunostaining was performed. As shown in Fig. 7D, sections of regenerating muscle at 5 days postinjury revealed that phospho-ERK appeared in small, centronucleated, MURC-expressing myofibers, suggesting that ERK activation occurred in MURC-expressing immature myofibers during myogenesis in vivo.

DISCUSSION

The Z-disc is known to be not only simply the structural border of the sarcomere but also to function in sensing and transmitting external and internal signals, because various
signaling molecules have been identified as components of the Z-disc, and a large number of the Z-disc-associated proteins have been shown to shuttle between the Z-disc and other subcellular locations to transmit signals (7, 14, 27). We have previously shown that in vascular smooth muscle cells MURC was diffusely localized to the cytoplasm and that in cardiomyocytes MURC was partly localized in the Z-line of the sarcomere and functioned as a molecule involved in Rho/ROCK signaling (22). In the present study, we showed that in skeletal muscle MURC staining was detected in the cytoplasm with a striated and periodic staining pattern and partly colocalized with α-actinin in the Z-line of the sarcomere. These findings suggest that in striated muscle MURC is a Z-disc-associated protein and functions as a molecule that shuttles between the Z-disc and other subcellular locations to transmit signals.

MURC was expressed as early as myogenin during the differentiation of myoblasts into myotubes, and knockdown and overexpression of MURC altered myogenin but not MyoD expression during myogenesis in C2C12 cells. Skeletal myogenesis is tightly controlled by the MRF family, which consists of MyoD, Myf5, myogenin, and MRF4 (2–4, 16, 29). MyoD and Myf5 are required for the commitment of proliferating somitic cells to the myogenic lineage (28), whereas myogenin is required for committed cells (myoblasts) to differentiate into myocytes and mature into myofibers but is dispensable for establishing the myogenic lineage (12, 20, 29, 35). MRF4 has functions of both commitment and differentiation in myogenesis (2, 15, 31, 44). These data suggest that myogenin expression altered by MURC might be involved in the differentiation of myoblasts into myotubes. The induction of myogenin ex-
pression has been reported to require an E box and a MEF2-binding site for proper expression (6, 41). Rho family proteins have been shown to be required for the transcription of the myogenin gene during myogenesis (9, 32). RhoA activates SRF-mediated gene expression (13, 19, 37), and SRF is involved in myogenin expression during myogenesis in myoblast cell lines (37). Our previous study suggested that MURC signaling regulates SRF-mediated ANP gene expression through the Rho/ROCK pathway in cardiomyocytes (22). Furthermore, we examined the effect of MURC signaling on the transcription of the skeletal α-actin (SkA) gene to perform a luciferase reporter assay using the SkA promoter (kindly provided by Michael D. Schneider, Imperial College, London, UK), which has SRF binding sites (17). MURC transactivated the SkA promoter, and MURC-induced transactivation of the SkA promoter was attenuated by the ROCK inhibitor Y-27632 (T. Ueyama, unpublished observations). Thus, our findings suggest that the Rho pathway alone is not sufficient for myogenin expression in undifferentiated C2C12 cells and that additional signaling pathways activated during myogenesis cooperate with the Rho pathway to regulate myogenin expression modulated by MURC.

p38 MAPK has been shown to activate MEF2 family members (11, 24, 38, 40, 43) and stimulate skeletal myogenesis (8, 16, 42). Therefore, we examined p38 MAPK activation during differentiation in C2C12 cells. However, p38 MAPK activation during differentiation was not detected in either naive C2C12 or C2C12-MURC cells. The system that we used might be under the sensitivity to detect the activation of p38 MAPK. On the other hand, we observed a biphasic change in ERK activity during myogenesis in C2C12 cells, which is consistent with the previous report by Wu et al. (38). Wu et al. have shown that reactivated ERK during myogenesis cooperates with p38 MAPK in promoting myogenic differentiation (38). We demonstrated that overexpression and knockdown of MURC modulated ERK reactivation in differentiating C2C12 myoblasts and that modulated ERK activation by the altered expression of MURC was correlated with myogenic responses. In addition, MURC expression was upregulated in immature muscle cells during muscle regeneration in vivo, and ERK phosphorylation was detected in small, centronucleated cells. These results suggest that the activation of the Rho pathway alone is not sufficient for myogenin expression in undifferentiated C2C12 cells and that additional signaling pathways activated during myogenesis cooperate with the Rho pathway to regulate myogenin expression modulated by MURC.

Fig. 7. MURC expression and ERK activation during injury-induced muscle regeneration. A: muscle regeneration induced by cryoinjury. Hematoxylin and eosin staining was performed using sections of the tibialis anterior muscle of the adult mouse with or without cryoinjury. B: expression of MURC and activated ERK during injury-induced muscle regeneration. Lysates of skeletal muscle with or without cryoinjury were immunoblotted with antibodies recognizing embryonic myosin, MURC, sarcomeric myosin, p-ERK, ERK, and GAPDH as an internal control. C: MURC expression in regenerating muscle. Immunostaining was performed using sections of the tibialis anterior muscle muscle at 5 days after cryoinjury with anti-MURC, anti-embryonic myosin, and anti-p-ERK antibodies. Nuclei were stained by DAPI (blue). Bottom, higher magnification images.
suggest the involvement of MURC in ERK activation during skeletal myogenesis both in vitro and in vivo. The Rho/ROCK pathway has been reported to contribute to the activation of ERK in cardiomyocytes (39). In myogenic cells, ROCK2 and its alternatively spliced isoform ROCK2m have been shown to positively control the activation of ERK1/2 during myogenesis (26). Therefore, the reactivation of ERK during myogenesis in C2C12 cells may be partly attributable to the Rho/ROCK pathway modulated by MURC. Our finding that treatment of naïve C2C12 and C2C12-MURC cells with PD-98059 impairs myogenesis suggests the requirement of the ERK/MEK1 pathway for myogenesis in both naïve C2C12 and C2C12-MURC cells. However, we also found that myogenic differentiation in C2C12-mMURC-shRNA1 cells infected with recombinant adenovirus expressing constitutively activated MEK1 (Ad-MEK1 EE) (34) at day 2 after the induction of differentiation was not promoted compared with that in C2C12-mMURC-shRNA1 cells infected with Ad-LacZ at day 2 after the induction of differentiation (M. Tagawa and T. Ueyama, unpublished observations). Since recombinant adenovirus-mediated protein expression is induced as early as 12 h after infection, reaches a maximum on day 2, and then declines (5), the activity and activation pattern of ERK in C2C12-mMURC-shRNA1 cells infected with Ad-MEK1 EE probably differs from that in naïve C2C12 cells during myogenesis. In addition, infection of C2C12-mMURC-shRNA1 cells with Ad-MEK1 EE on day 2 should induce ERK activation in cells not committed to the myocyte lineage as well, which may affect the commitment to the myocyte lineage through paracrine effects. These might be due to the failure of forced activation of endogenous ERK by ectopic expression of MEK1 EE to rescue myogenic differentiation in C2C12-mMURC-shRNA1 cells. Collectively, our findings suggest that the activation of the ERK/MEK1 pathway is necessary but not sufficient for MURC-mediated myogenesis in C2C12 cells or that fine regulation of ERK/MEK1 signaling during myogenesis is required for MURC-mediated differentiation in C2C12 cells. Further studies are needed to clarify how MURC is involved in ERK reactivation during skeletal myogenesis and how the reactivation of ERK modulated by MURC is involved in skeletal myogenesis.

In conclusion, the present study demonstrates that MURC is expressed as early as myogenin and is upregulated in immature differentiating muscle cells during myogenesis. MURC regulates skeletal myogenesis accompanied by the modulation of myogenin expression and ERK activation. Further investigation of the role of MURC will provide insights into the molecular mechanisms that regulate skeletal muscle development and muscle degenerating diseases.

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