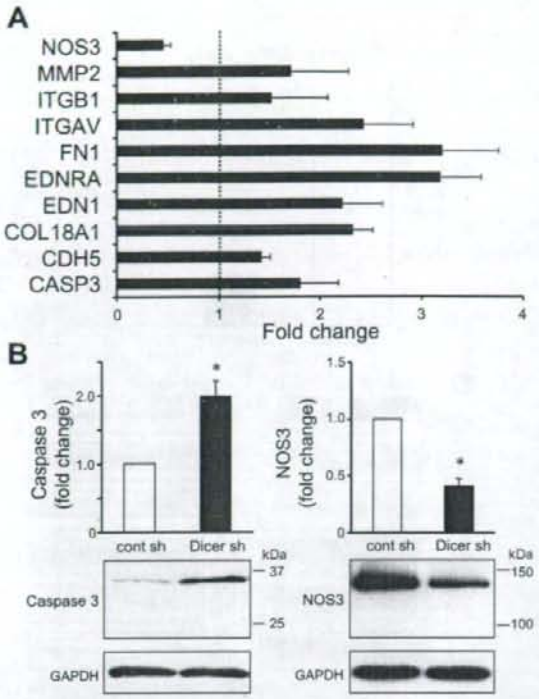


Fig. 4. Dicer silencing alters the expression profiles of genes related to endothelial biology. *A*: real-time PCR-based gene expression profiling was used to compare the gene expression in control and Dicer shRNA HUVECs. Data were expressed as fold change from control to Dicer-silenced cells. Genes with $P < 0.05$ vs. control were listed. MMP2, matrix metalloproteinase-2; ITGB1, integrin-$\beta_1$; ITGAV, integrin-$\alpha_v$; FN1, fibronectin-1; EDNRA, endothelin receptor type A; EDN1, endothelin-1; COL18A1, collagen type XVIII-$\alpha_1$; CDH5, cadherin 5. *B*: expression of caspase-3 (Casp3) and nitric oxide synthase 3 (NOS3) was further verified at mRNA level by real-time kinetic PCR with other sets of primers than those used in PCR array and at protein level by immunoblotting with specific antibodies. *$P < 0.05$ vs. control shRNA cells.

downregulation of Dicer expression induced by serum withdrawal. VEGF and S1P are well-known polypeptide and lipid mediators that modulate many important endothelial functions including proliferation, chemotactic responses, angiogenic morphogenesis, and inhibition of apoptosis (12, 25, 39). While bFGF and LPA are also shown to have similar properties in endothelial cells (34, 39, 43), VEGF and S1P specifically maintained Dicer expression in HUVECs. The elucidation of the molecular mechanisms responsible for the maintenance of Dicer expression specifically by VEGF and S1P will be an important issue to fully clarify how Dicer expression is regulated.

Serum withdrawal is an established technique for apoptosis induction in nontransformed cells and has been widely used as an initiator of endothelial apoptosis because of its reproducible effects. Although the pathophysiological conditions correlating to serum withdrawal-induced endothelial apoptosis is uncertain, it is also unclear whether endothelial apoptosis in the settings of pathophysiological states is induced by proapoptotic factors or by the absence of protective, survival factors (32, 46). Therefore, in this study, we focused on the role of a repressed expression of Dicer by serum withdrawal in apoptosis in endothelial cells. However, a diminished expression of Dicer may not be a general feature induced by apoptosis-inducing stimuli in endothelial cells. We observed that oxidized LDL upregulated Dicer expression (S. Asada and T. Takahashi, unpublished observations), whereas oxidized LDL induces not only apoptotic cell death but also inflammatory responses in endothelial cells (10, 14). Thus the regulation of Dicer expression is stimulus specific, which could have a different functional significance in endothelial pathophysiology.

Our results indicated that silencing Dicer resulted in a greater apoptotic rate in response to serum withdrawal, whereas an overexpression of Dicer markedly reduced serum deprivation-induced apoptosis in endothelial cells. These results revealed that the expression level of Dicer plays an important role in determining the endothelial cell fate. However, neither silencing nor overexpression of Dicer influenced endothelial apoptosis in the serum-containing condition. This observation was in agreement with the previous report in which Dicer knockdown is shown not to impair the endothelial cell viability in the presence of serum (26). In several models such as Dicer inactivation in limb mesoderm, epidermis, and myoblasts in vivo, the inactivation of Dicer has been shown to result in an increased apoptosis (1, 18, 38). In vivo, the cells are continuously exposed to diverse stresses from their local microenvironment such as mechanical and chemical stresses and exposure to various extracellular factors, and programmed cell death occurring in a variety of cell types during embryogenesis is thought to be required for proper developmental processes and maintaining homeostasis (20). A very recent study demonstrated that a targeted deletion of Dicer in cardiac myocytes results in the development of heart failure in a short period of time after birth, whereas new born mice are indistinguishable from wild-type littermates (6). The increased apoptosis is also observed in the Dicer knockout hearts of P2 mice, although no increase in apoptosis is detected before P0 (6). These results and ours suggested that the reduced expression of Dicer sensitizes the cells to apoptosis under stresses rather than initiates or triggers the apoptotic response.

Our gene expression analysis revealed that Dicer silencing in endothelial cells modulated the expression of several genes involved in endothelial biology. These included NOS3, matrix metalloproteinase (MMP) 2, integrins-$\alpha_v$ and -$\beta_1$, fibronectin, endothelin receptor type A, endothelin 1, vascular endothelial-cadherin, and caspase-3. Both integrins-$\alpha_v$ and -$\beta_1$ are implicated in angiogenesis and endothelial survival (46), and MMP-2 participates in autocrine processes that determine the migration as well as the apoptotic death of endothelial cells induced by hypoxia (3). Vascular endothelial-cadherin is also shown to be involved in endothelial survival through intercellular interactions (11). Our results showed that the migratory activities of HUVECs were impaired by Dicer knockdown, whereas the adhesive properties to gelatin or fibronectin were preserved. Furthermore, cell adhesion-mediated signaling pathways such as tyrosine phosphorylation of FAK and PYK2 in adhesive cells were also diminished by Dicer silencing. Although these adhesion-mediated signaling pathways are shown to participate in cell survival through

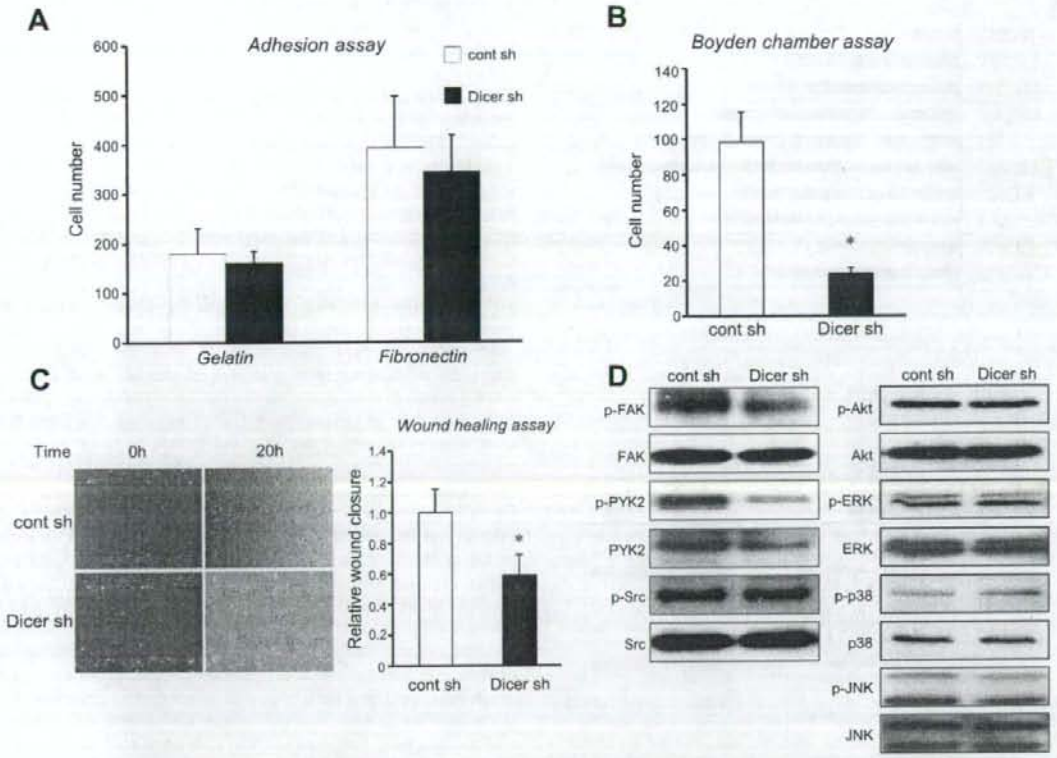


Fig. 5. Dicer silencing impairs migratory functions and adhesion-mediated phosphorylation of focal adhesion kinase (FAK) and proline-rich tyrosine kinase 2 (PYK2). *A*: cell adhesion to gelatin- or fibronectin-coated dishes was analyzed in control and Dicer shRNA HUVECs. *B*: chemotactic activity to VEGF was assessed by modified Boyden chamber assay in control and Dicer shRNA HUVECs. $*P < 0.05$ vs. control shRNA cells. *C*: lateral migration was assessed by wound healing assay in control and Dicer shRNA HUVECs. $*P < 0.05$ vs. control shRNA cells. *D*: phosphorylation of FAK, PYK2, Src, Akt, ERK, p38 MAPK, and JNK was analyzed by immunoblot analysis with phosphospecific and total antibodies. Blots shown represent 1 of at least 3 independent trials that gave nearly identical results.

downstream kinases such as Akt (44, 51, 52), Dicer knockdown did not alter the phosphorylation and activation of Src, Akt, and MAPKs such as ERK, p38-MAPK, and JNK. Thus, although a disregulated expression of adhesion and matrix-related molecules might be involved in the reduced phosphorylation of FAK and PYK2, it remains to be determined whether the modulation of adhesion-mediated signaling pathways participates in an increased susceptibility to apoptosis induced by serum withdrawal.

In this study, we demonstrated that the expression of NOS3 and caspase-3, two proteins critically involved in endothelial biology and apoptosis, was modulated by serum withdrawal. Caspase-3 is a member of the cysteine-aspartic acid protease (caspase) family, which is considered to be central in the execution of apoptosis, whereas the requirement of caspase-3 for apoptosis is tissue and stimulus dependent (49). In endothelial cells, caspase-3 activation is an important mechanism in serum withdrawal-induced en-

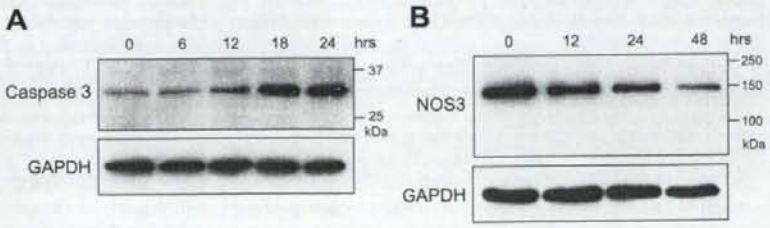


Fig. 6. Serum withdrawal increases caspase-3 and decreases NOS3 expression in endothelial cells. *A*: protein expression of caspase-3 (*A*) or NOS3 (*B*) was analyzed by immunoblot analysis with anti-caspase-3 or NOS3 antibody. GAPDH was served as a loading control.

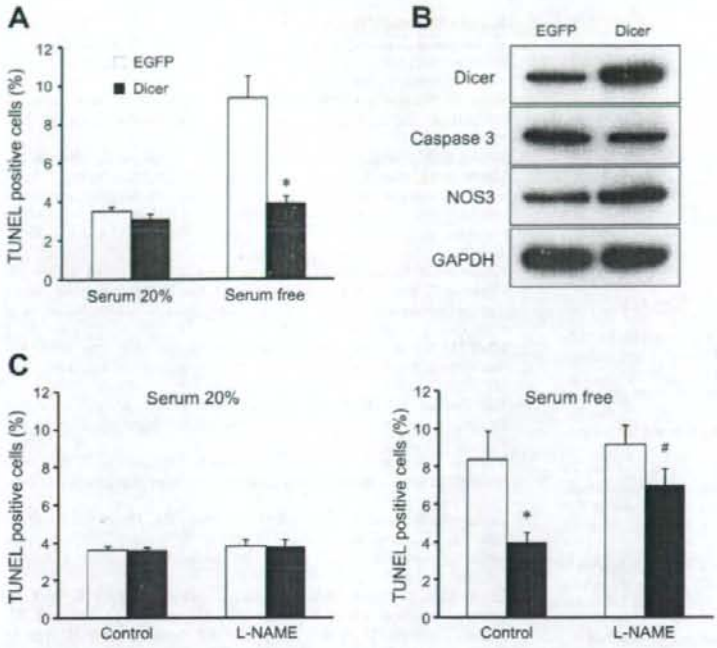


Fig. 7. Overexpression of Dicer protects endothelial cells from apoptosis induced by serum withdrawal and alters caspase-3 and NOS3 expression. *A*: HUVECs overexpressing enhanced green fluorescent protein (EGFP) or Dicer was cultured with or without serum for 24 h, and apoptotic rate was analyzed by TUNEL staining. $^*P < 0.05$ vs. EGFP-expressing cells. *B*: protein expression of caspase-3 and NOS3 was examined in HUVECs overexpressing EGFP or Dicer. *C*: cells were transfected with the vector expressing EGFP or Dicer and, after 48 h, treated with or without 1 mM $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME) for 24 h in the presence or absence of serum. Apoptotic rate was examined by TUNEL staining and plotted. $^*P < 0.05$ vs. EGFP-expressing cells. $^{\#}P < 0.05$ vs. Dicer-overexpressing cells without L-NAME.

dothelial apoptosis (28). In Dicer-silenced cells, caspase-3 expression is upregulated, and Dicer overexpression suppressed the expression of caspase-3. Furthermore, serum withdrawal, which induced the repression of Dicer expression, indeed increased the expression of caspase-3. It has been reported that mice overexpressing caspase-3 are phenotypically normal under physiological conditions, while exhibiting increased apoptosis in response to ischemia-reperfusion injury, implying the role of an increased expression of caspase-3 in the increased susceptibility to degenerative insults (23). These results indicated the causative role of Dicer repression in caspase-3 upregulation in response to serum withdrawal and suggested that the upregulation of caspase-3 in the settings of Dicer downregulation involves an increased sensitivity to apoptosis.

Our results also demonstrated that the knockdown of Dicer reduced the expression of NOS3. This seems conflicting to the previous report by Suárez et al. (47) that Dicer silencing results in an increased expression of NOS3, although Dicer silencing showed a similar increase in collagen type XVIII-$\alpha_1$, integrin-$\alpha_v$, and MMP-2 expression in both our and their studies. While the precious mechanism producing the difference in NOS3 expression is unknown, the discrepancy might be due to the selecting of different methods to silence Dicer expression. In our study, we generated retrovirus expressing shRNA against Dicer, and the gene expression was analyzed after an antibiotic selection of cells stably expressing shRNA, whereas Suárez et al. used a transient transfection of synthetic siRNA against Dicer, and NOS3 expression was analyzed up to 72 h after transfection, which could produce different levels and kinetics of Dicer silencing. Since we did not see a transient upregulation of NOS3 expression after infecting retrovirus expressing shRNA against Dicer (data not shown), analyzing the gene expression at different time points between studies might not account for the difference in the results. Our results with the overexpression of Dicer showed the upregulation of NOS3 expression, and NOS3 expression was downregulated upon serum withdrawal in concomitance with the downregulation of Dicer expression, indicating that Dicer expression is critically involved in the regulation of NOS3 expression. Further studies are needed to clarify the molecular mechanisms responsible for the regulation of NOS3 expression by Dicer expression, which might help explain the difference in NOS3 expression in Dicer-silenced cells.

In this study, we demonstrated for the first time that serum withdrawal reduced the expression of Dicer, which was involved in the regulation of caspase-3 and NOS3 expression and apoptosis in human endothelial cells. As previous reports identified Dicer as an important component to regulate angiogenesis (26, 47, 50), Dicer regulates diverse aspects of endothelial cell biology from a life and death decision and a proliferation to the morphological differentiation to form capillary-like structure. An identification of the regulatory mechanisms of Dicer expression and activity and the downstream targets of Dicer will be important issues to fully clarify the roles of Dicer in endothelial functions.

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

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

**Masashi Tagawa,[1,2] Tomomi Ueyama,[1] Takehiro Ogata,[1] Naofumi Takehara,[1] Norio Nakajima,[1,2] Koji Isodono,[1,2] Satoshi Asada,[1,2] Tomosaburo Takahashi,[1,2] Hiroaki Matsubara,[1,2] and Hidemasa Oh[1]**

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**Tagawa M, Ueyama T, Ogata T, Takehara N, Nakajima N, Isodono K, Asada S, Takahashi T, Matsubara H, Oh H.** MURC, a muscle-restricted coiled-coil protein, is involved in the regulation of skeletal myogenesis. *Am J Physiol Cell Physiol* 295: C490–C498, 2008. First published May 28, 2008; doi:10.1152/ajpcell.00188.2008.—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 cardiomyocyes, 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 Hybridoma 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 myotube and the fusion index were analyzed as previously described (21). Briefly, the average number of nuclei per myotube 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 myotube 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′-ACA GTC ACA CAG CAA TAC GGG CTA-3′ and reverse primer 5′-TTC TCG GGC AGG CTT CTG TCT TTA-3′; myogenin, forward primer 5′-TAC GTC CAT CGT GGA CAG CAT-3′ and reverse primer 5′-TCA GCT AAA TTC CCT CGC TGG-3′; MyoD, forward primer 5′-TGA GCA AAG TGA ATG AGG CCT TCG-3′ and reverse primer 5′-TGC AGA CCT TCG ATG TAG CGG AT-3′; muscle creatine kinase (MCK), forward primer 5′-CAC CTC CAC AGC ACA GAC AG-3′ and reverse primer 5′-ACC TTG GCC ATG TGA TTG TT-3′; and GAPDH, forward primer 5′-TTG TGA TGG GTG TGA ACC ACG AGA-3′ and reverse primer 5′-CAT GAG CCC TTC CAC AAT GCC 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, 1× protease inhibitor cocktail (Pierce), 1 mM $Na_3VO_4$, 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, F1.652, MF20, Flag (Sigma), phospho-ERK ($Thr^{202}/Tyr^{204}$), 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′-CTA TTT GTA GTC TGA GGA CTG CTT TAG CTC CA-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 ACG TTG TCA ACA AGC TG-3′ and 5′-AGA AAG TGA GTG GGA TTA GAA-3′) were cloned into the *Bam*HI-*Eco*RI 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 pVSV-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 adenoviruses and gene transfer.* Recombinant adenoviruses 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 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.

## RESULTS

*Subcellular localization of MURC in skeletal muscle.* We have shown previously that MURC was localized to the cytoplasm with an accumulation in the Z-line of the sarcomere in the murine adult heart (22). To determine the subcellular localization of MURC in skeletal muscle, adult mouse gastrocnemius muscle sections were immunostained with an anti-MURC antibody. Consistent with our previous data in the heart, MURC staining was detected in the cytoplasm with a striated and periodic staining pattern and partly colocalized with α-actinin in the Z-line (Fig. 1).

*MURC expression in C2C12 myoblasts during myogenesis.* We used C2C12 myoblasts that could be induced to differentiate into myotubes by serum starvation and assessed MURC expression during differentiation into myotubes. In C2C12 myoblasts, MURC mRNA expression was induced as early as *day 1* after the induction of differentiation and then reached a maximum on *day 2*, and its level continued up to *day 6* (Fig. 2*A*). The expression pattern of myogenin mRNA was similar to that of MURC mRNA because the induction of myogenin mRNA expression was detectable on *day 1* and its expression peaked on *day 2*, but the expression level declined on *day 4*. On the other hand, MyoD mRNA expression was already detectable in undifferentiated myoblasts, and its level was almost constant during myogenesis. MURC protein expression was then examined by Western blot analysis. MURC protein expression was not detected in undifferentiated myoblasts, but its expression was activated after 1 day of serum starvation and increased gradually up to *day 6* (Fig. 2*B*). Sarcomeric myosin protein expression followed MURC and embryonic myosin protein expression. As shown in Fig. 2*C*, immunostaining analysis showed that myotubes stained with an anti-sarcomeric myosin antibody expressed MURC. Thus, the induction of MURC expression occurred in differentiating muscle cells and preceded that of sarcomeric myosin expression.

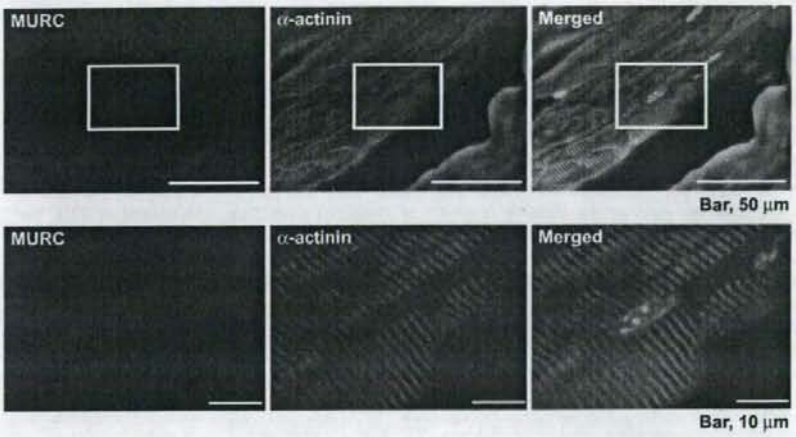


Fig. 1. Subcellular localization of muscle-restricted coiled-coil (MURC) protein in skeletal muscle. Immunostaining was performed using adult mouse gastrocnemius muscle sections with anti-MURC and anti-α-actinin antibodies. Nuclei were stained by 4′,6-diamino-2-phenylindole (DAPI; blue). *Bottom*: higher magnification images.

*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. 3*A*). 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. 3*B*, 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-

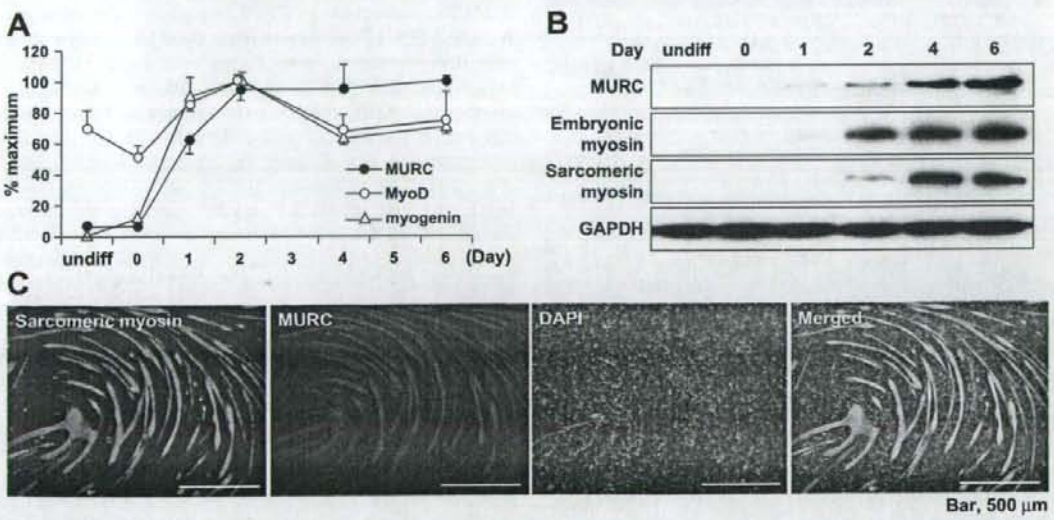


Fig. 2. MURC expression in C2C12 cells during myogenesis. *A*: expression of MURC mRNA during myogenesis in C2C12 myoblasts. Real-time RT-PCR was performed with cDNAs from C2C12 cells during myogenesis. *B*: expression of MURC protein during myogenesis in C2C12 cells. Lysates of C2C12 cells were immunoblotted with antibodies recognizing embryonic myosin, MURC, sarcomeric myosin, and GAPDH as an internal control. *C*: MURC expression in differentiated myotubes. Immunostaining was performed using C2C12 cells at 6 days after the induction of differentiation with anti-MURC and anti-embryonic myosin antibodies. Nuclei were stained by DAPI (blue).

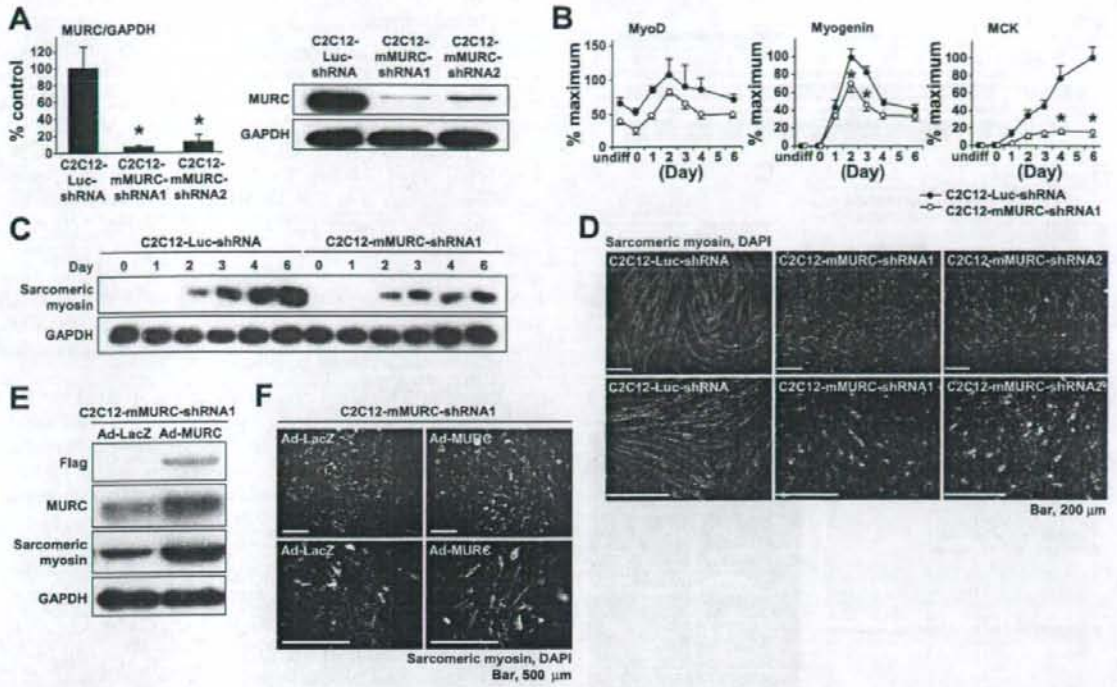


Fig. 3. Impaired myogenesis by MURC RNA interference (RNAi). *A*: expression of endogenous MURC mRNA (*left*) and protein (*right*) in differentiated C2C12 cells. Real-time RT-PCR was performed with cDNAs from C2C12-Luc-shRNA, C2C12-mMURC-shRNA1, and C2C12-mMURC-shRNA2 cells at 6 days after the induction 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 or Ad-MURC. Cell lysates from C2C12-mMURC-shRNA1 cells 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.

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. 3*C*). 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 (Fig. 3*D*). Furthermore, to exclude off-target effects of the mMURC-shRNA used, C2C12-mMURC-shRNA1 cells were infected with Ad-MURC. As shown in Fig. 3*E*, compared with C2C12-mMURC-shRNA1 cells infected with Ad-MURC and C2C12-mMURC-shRNA1 cells infected with Ad-LacZ at *day 6* after the induction of differentiation, the forced expression of Flag-tagged MURC using Ad-MURC resulted in increases in MURC protein expression and sarcomeric myosin protein expression. In accordance with this finding, myotube formation in C2C12-mMURC-shRNA1 cells infected with Ad-MURC was promoted compared with that in C2C12-mMURC-shRNA1 cells infected with Ad-LacZ (Fig. 3*F*). These results indicate that MURC has at least an important permissive role in myogenesis in C2C12 cells.

*MURC RNAi impaired ERK activation during myogenesis in C2C12 myoblasts.* ERK has been reported to have a key role in the differentiation into myotubes (1, 10, 30, 38). To reveal the role of MURC in the signaling pathway involved in myogenic differentiation, we investigated ERK activation in C2C12 myoblasts. Consistent with a previous report (38), ERK phosphorylation decreased at the onset of differentiation, reached a minimum at *day 2* after differentiation, and increased at later stages of differentiation in C2C12-Luc-shRNA cells (Fig. 4*A*). We obtained a similar pattern of ERK phosphorylation in naïve C2C12 cells (data not shown). In C2C12-mMURC-shRNA1 cells, ERK phosphorylation was impaired at the later stages of differentiation (Fig. 4, *A* and *B*). To examine whether ERK activation at the later stages is required for differentiation in C2C12 cells, we used PD-98059, a MEK inhibitor. Upon the induction of differentiation in C2C12 cells treated with PD-98059 at 25 μM for 3 days, inhibition of ERK phosphorylation

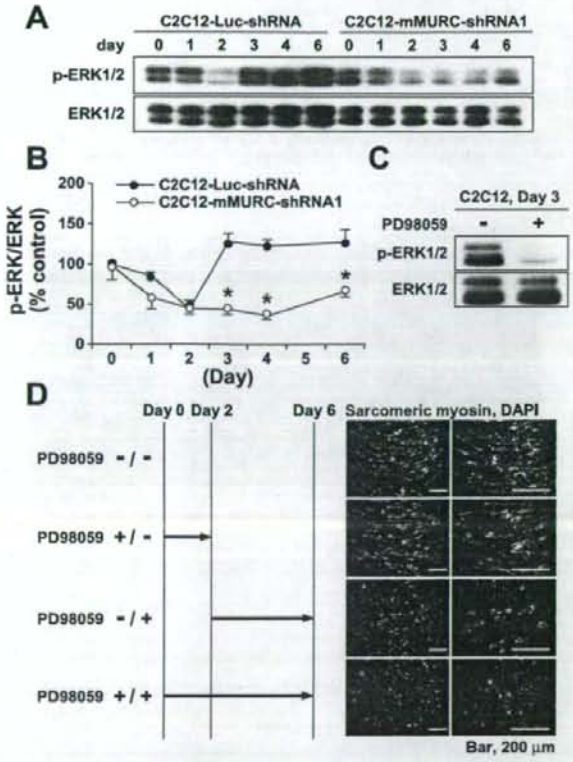


Fig. 4. Impairment of ERK activation by MURC RNAi during myogenesis. *A* and *B*: ERK phosphorylation during myogenesis in C2C12 cells. Cell lysates from C2C12-Luc-shRNA and C2C12-mMURC-shRNA1 cells at the indicated days after the induction of differentiation were immunoblotted with antibodies recognizing phosphorylated ERK (p-ERK) and ERK. $*P < 0.05$ compared with C2C12-Luc-shRNA cells. *C*: effect of PD-98059 on ERK phosphorylation in C2C12 cells. C2C12 cells were treated with or without PD-98059 at 25 μM for 3 days. Cell lysates were immunoblotted with antibodies recognizing p-ERK and ERK. *D*: effect of ERK inhibition on myogenesis in C2C12 cells. C2C12 cells were treated with or without PD-98059 at 25 μM for the indicated periods of time. Cell lysates were immunoblotted with antibodies recognizing p-ERK and ERK. Immunostaining was performed 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. 4*C*). 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. 4*D*). 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. 5*A*). 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. 5*B*). 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. 5*D*). 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. 5*E*). 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. 6*C*).

*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. 7*A*, 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. 7*B*). 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. 7*B*). To examine which cells account for ERK activation, immunostaining was performed. As shown in Fig. 7*D*, 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

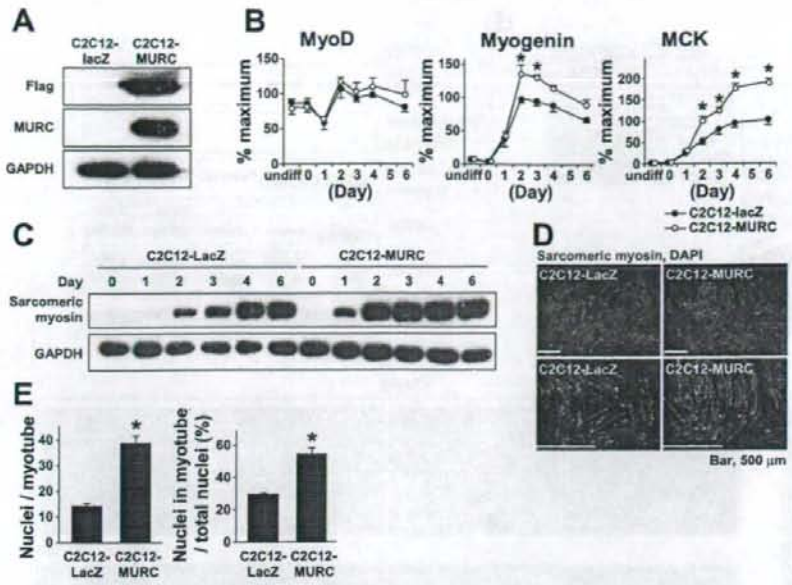


Fig. 5. Promotion of myogenesis by MURC overexpression. *A*: expression of Flag-tagged MURC in C2C12-MURC cells. Cell lysates from C2C12-LacZ and C2C12-MURC cells were immunoblotted with antibodies recognizing Flag (*top*), MURC (*middle*), and GAPDH as an internal control (*bottom*). *B*: mRNA expression of MyoD, myogenin, and MCK during myogenesis in C2C12 cells. Real-time RT-PCR was performed with cDNAs from C2C12-LacZ and C2C12-MURC cells during myogenesis. $^*P < 0.05$ compared with C2C12-LacZ cells. *C*: expression of sarcomeric myosin protein during myogenesis in C2C12 cells. Cell lysates from C2C12-LacZ and C2C12-MURC cells were immunoblotted with antibodies recognizing sarcomeric myosin (*top*) and GAPDH (*bottom*). *D*: immunostaining of differentiated C2C12 cells. Immunostaining was performed using C2C12-LacZ and C2C12-MURC 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*: numbers of nuclei per myotube (*left*) and percentages of all nuclei present in myotubes (fusion index; *right*) of differentiated C2C12 cells. The numbers of nuclei and myotubes were counted in C2C12-LacZ and C2C12-MURC cells at 6 days after the induction of differentiation. $^*P < 0.05$ compared with C2C12-LacZ cells.

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-

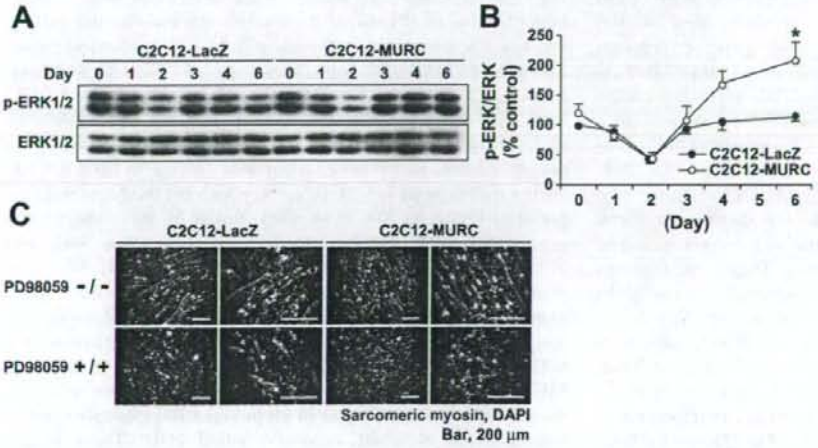


Fig. 6. Promotion of ERK activation by MURC overexpression during myogenesis. *A* and *B*: ERK phosphorylation during myogenesis in C2C12 cells. Cell lysates from C2C12-LacZ and C2C12-MURC cells at the indicated days after the induction of differentiation were immunoblotted with antibodies recognizing p-ERK and ERK. $^*P < 0.05$ compared with C2C12-LacZ cells. *C*: effect of ERK inhibition on myogenesis in C2C12-MURC cells. C2C12-MURC cells were treated with or without PD-98059 at 25 μM for 6 days. Cell lysates were immunoblotted with antibodies recognizing p-ERK and ERK. Immunostaining was performed with an anti-sarcomeric myosin antibody. Nuclei were stained by DAPI (blue). *Bottom*, higher magnification images.

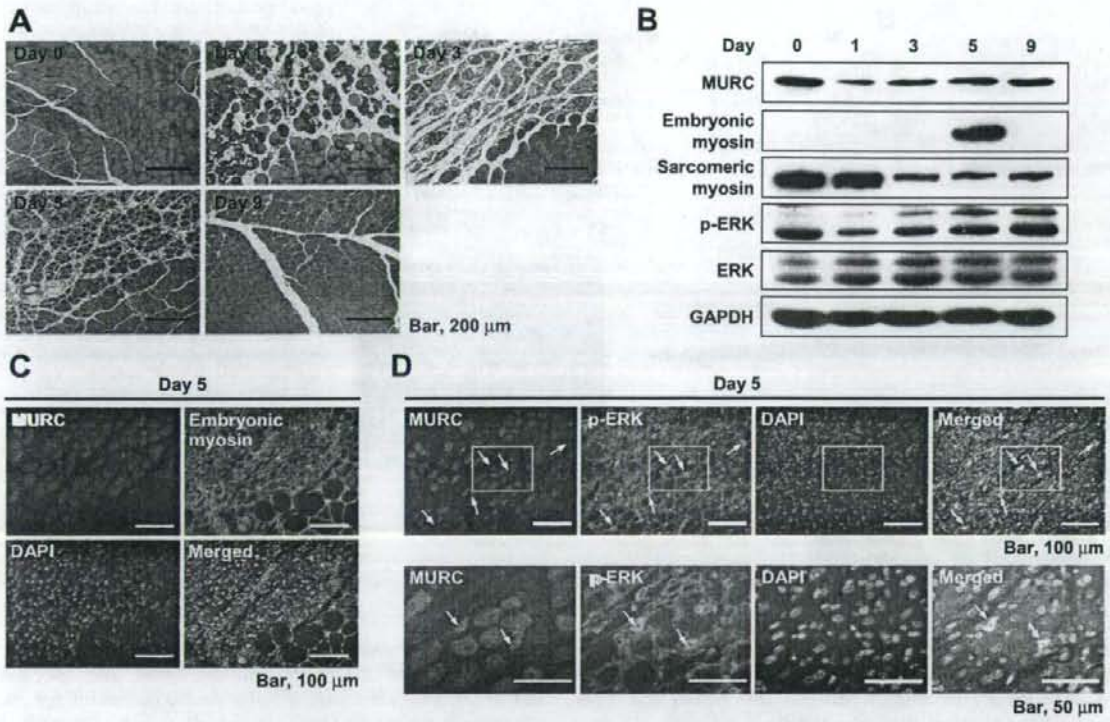


Fig. 7. MURC expression and ERK activation during injury-induced muscle regeneration. *A*: muscle regeneration induced by cryoinjury. Hematoxylin and eosin staining was performed using sections of the tibialis anterior muscle of the adult mouse with or without cryoinjury. *B*: expression of MURC and 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. *D*: ERK activation in regenerating muscle. Immunostaining was performed using sections of the tibialis anterior muscle muscle at 5 days after cryoinjury with anti-MURC and anti-p-ERK antibodies. Nuclei were stained by DAPI (blue). *Bottom*, higher magnification images.

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/ROCK pathway contributes to the SRF-mediated gene transcription in MURC signaling. We found that the overexpression of MURC increased RhoA activity in undifferentiated C2C12 cells (C2C12-MURC cells, 4.70 ± 0.89-fold, $P < 0.05$ compared with C2C12-LacZ cells). However, myogenin expression was not induced in undifferentiated C2C12-MURC cells, as shown in Fig. 5*B*. These findings 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.

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 myogenesis in C2C12 cells. However, p38 MAPK activation during differentiation was not detected in either naïve 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 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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# Crossveinless-2 Controls Bone Morphogenetic Protein Signaling during Early Cardiomyocyte Differentiation in P19 Cells*

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**Increasing evidence indicates that bone morphogenetic proteins (BMPs) are crucial for cardiac induction, specification, and development. Although signaling of BMPs is tightly regulated through soluble BMP-binding proteins, how they regulate BMP signaling during cardiac differentiation remains unknown. To identify molecules responsible for BMP signaling during early cardiomyocyte differentiation of P19 cells, cDNA subtraction was performed. We found a bimodal expression of the BMP-binding protein Crossveinless-2 (Cv2) during cardiomyocyte differentiation; Cv2 is temporally expressed earlier than cardiac transcription factors such as *Nkx2.5* and *Tbx5* and acts as a suppressor for BMP signaling in P19 cells. We established a P19 clonal cell line harboring a cardiac alpha-myosin heavy chain promoter-driven enhanced green fluorescent protein gene to monitor cardiac differentiation by flow cytometry. Treatment with BMP2 during the first 2 days of differentiation suppressed cardiomyocyte differentiation through activation of downstream targets Smad1/5/8 protein and *Id1* gene, whereas treatment with Cv2 conversely inhibited Smad1/5/8 activation and *Id1* expression, leading to increased generation of cardiac cells. RNA interference-mediated knockdown (KD) of endogenous Cv2 showed increased Smad1/5/8 activation and impaired cardiomyocyte differentiation. Expression of cardiac mesoderm markers was reduced, whereas expression of *Id1* and endoderm markers such as *Sox7*, *Hnf4*, and E-cadherin was induced in *Cv2*-kinase dead cells. These phenotypes were rescued by the addition of Cv2 protein to the culture media during the first 2 days of differentiation or co-culture with parental cells. These data suggest that Cv2 may specify cardiac mesodermal lineage through inhibition of BMP signaling at early stage of cardiogenesis.**

Heart development during embryogenesis is a multistep process that involves cardiac induction of mesodermal progenitor cells into the cardiac lineage. Although the genetic blueprint for cardiac differentiation and development is rapidly being elucidated (1–3), there is still uncertainty about cardiac-inducing factors which might be involved in cardiogenic induction and specification.

Increasing evidence indicates that BMPs[2] are crucial for cardiac induction, specification, and development (2–7). Conventional deletion of BMP receptor 1a in mice showed no formation of mesoderm and heart (8). Studies with an *in vitro* cardiac differentiation model demonstrated an essential role of BMP signals in cardiomyogenesis (9). In contrast, functional disruption of the different BMPs in mice displayed a role in late but not in early cardiogenesis (10). For example, although deletion of BMP2 in the heart resulted in abnormal heart development, specification of cardiac mesoderm occurred normally (11). Explant cultures in chicken revealed that activation of BMP signaling inhibits cardiogenesis at early developmental stages (7). Until now, whether activation or suppression of BMP signaling is required during early cardiogenesis has not been resolved. It is also not known how the inhibitory effect of BMP signaling on early cardiogenesis is regulated.

BMPs belong to the transforming growth factor-$\beta$ superfamily of secreted growth factors. The BMP family signals have been shown to play multiple roles in the control of embryogenesis, including cell-type specification, maturation, and dorsoventral axis determination (12). These pleiotropic functions of BMPs implicate a need for tight regulation of their activities. One way by which this is achieved is via soluble BMP-binding proteins, which tightly regulate BMP signaling (12, 13). During gastrulation of early embryogenesis, collaboration with BMPs and their binding proteins specifies the patterning of mesoderm by limiting the spatial extent of each other (13, 14).

In *Drosophila*, genetic analyses have identified a five cysteine-rich (CR) domain-containing molecule, Crossveinless-2 (Cv2), which promotes decapentaplegic (homologous to vertebrate BMP) activity in wings (15). *Cv2* transcripts emerge first at gastrulation and are detected both in the precardiac mesoderm and in the posterior primitive streak in mouse embryos (16), suggesting the developmental role of Cv2 in cardiogenesis. Cv2 loss-of-function studies demonstrated the essential role of Cv2 as a

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[2] The abbreviations used are: BMP, bone morphogenetic protein; Cv2, Crossveinless-2; pSmad1/5/8, phosphorylated Smad1/5/8; FACS, fluorescent-assisted cell sorting; Id1, Inhibitor of DNA binding/differentiation 1; KD, knockdown; RNAi, RNA interference; $\alpha$MHC, $\alpha$-myosin heavy chain; EGFP, enhanced green fluorescent protein; CK, cytokeratin 18; KD, knockdown; RT, reverse transcription.

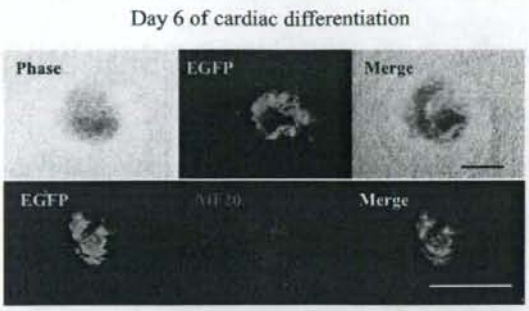


FIGURE 1. **Cardiac differentiation of P19EN8 cells harboring αMHC promoter-driven *EGFP* gene.** Cells showing bright fluorescence matching beating areas have immunoreactivities for the primary antibody against sarcomeric myosin MF20 after 6 days of cardiac differentiation with DMSO. *Scale bars* equal 250 μm in panels.

pro-BMP factor in development (17, 18). However, either antagonistic or agonistic effects of Cv2 on BMP signal have been reported in vertebrates (17–22). Thus, the molecular mechanism by which Cv2 regulates BMP signal remains unclear.

In the present study we established a novel P19 embryonal carcinoma (P19)-derived clonal cell line EN8 cells harboring a cardiac α-myosin heavy chain (αMHC) promoter-driven enhanced green fluorescent protein (EGFP) gene to monitor the generation of cardiac cells by flow cytometric analysis (Fig. 1) and found a unique requirement for Cv2 in cardiac lineage decision through the temporal suppression of BMP activity during the first 2 days of differentiation. These findings imply that cardiac fate decision requires the temporal suppression of BMP signals at the early stages of development.

## EXPERIMENTAL PROCEDURES

*Cells, Differentiation, and Transfection*—Maintenance of P19 cells and dimethyl sulfoxide (DMSO)-induced differentiation were achieved as described previously (23). Briefly, cell aggregates were formed in hanging drops of 1000 cells in 50 μl of medium with 1% DMSO for the first 4 days. Aggregates were plated 2 days later onto tissue culture grade surfaces and maintained in DMSO-free medium. P19 EN8 cells were developed by transfecting the αMHC-green fluorescent protein vector (24) into P19Cl6 cells. P19Cl6 cells stably expressing FLAG-tagged Cv2 cDNA were also isolated. The BMP-responsive luciferase reporter (25) was from Dr. T. Katagiri (Saitama Medical School). pRL-TK was from Promega. Epitope-tag Cv2 was generated by amplifying *Cv2* lacking the signal peptide and by subcloning the fragment in-frame into the pSec-Tag2 vector (Invitrogen). To develop the Cv2 knockdown cell and control cell lines, mouse Cv2 short hairpin RNA and control construct were developed by subcloning the target sequence (GCATAATGTGTGTGTGTT-TGA) and GC%-matched scramble sequence into the pBA-puro vector (Takara, Japan), respectively.

*cDNA Subtraction*—Poly (A)-RNA (1 μg) from undifferentiated (driver) or differentiated P 19 cells at day 6 (tester) was used for double-stranded cDNA synthesis. The tester cDNA was digested with EcoRV and HincII. The driver cDNA library was amplified with the biotin-14-dCTP (Invitrogen) using the cDNA PCR library kit (Takara). Digested tester cDNA and biotin-labeled driver cDNA library were dissolved in 20 μl of a hybridization solution (0.5 mol/liter NaCl, 50 mmol/liter Tris, pH 7.5, 0.15% SDS, 40% formamide). After hybridizing for 48 h at 42 °C, the mixture was extracted, precipitated, and dissolved in 10 μl of $H_2O$. After the mixture was incubated with 1.2 mg of streptavidin paramagnetic particles (Promega) in 100 μl of binding solution (10 mmol/liter Tris, pH 7.5, 1 mmol/liter EDTA, 100 mmol/liter NaCl), the supernatant was precipitated. Part of the products was ligated into pCR4Blunt-TOPO plasmids (Invitrogen). Purified plasmids went through sequence analysis, and the obtained data were compared with the GenBank™ databases with the BLAST program.

*RT-PCR and Real-time PCR*—*T*, *Gata4*, E-cadherin, *Foxa2*, *NeuroD1*, *Pax6*, *Hnf4*, *Sox7*, and cytokeratin 18 (*CK*) primer sequences can be found in Tada *et al.* (26). The PCR cycling conditions were as follows: 1 cycle of 94 °C for 4 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; 1 cycle of 72 °C for 30 s. Primers used for the PCR are as follows (forward and reverse): Cv2, 5-GGGTAAAATTCTCAACA-GGA-3 and 5-CCACCAATCAAGTCATCACG-3; Mesp1, 5-CTGGCCATCCGCTACATTGG-3 and 5-CGTTGCAT-TGTCCCCTCCAC-3; Nkx2.5, 5-CAGTGGAGCTGGACA-AAGCC-3 and 5-TAGCGACGGTTCTGGAACCA-3; Tbx5, 5-GCAGGGCCTGAGTACCTCTT-3 and 5-GGCTGATG-GGCCACTGAGGT-3; Bmp2, 5-CGGGAACAGATACAGG-AAGC-3 and 5-GCAAGGGGAAAAGGACACTC-3; Bmp4, 5-TGTGAGGAGTTTCCATCACG-3 and 5-TTATTCTTCTT-CCTGGACCG-3; Inhibitor of DNA binding/differentiation 1 (Id1), 5-GGTACCGTACAACCTTTCTCCAACTTC-3 and 5-GGCTGGAGTCCATCTGGTCCCTCAGTGC-3. For quantitative analysis of gene expression levels, real-time PCRs were done using the ABI Prism 7700 sequence detection system. Data were normalized to glyceraldehyde-3-phosphate dehydrogenase. Primers used for the real-time PCR were as follows: T, Mm00436877_m1; Mesp1, Mm00801883_g1; Nkx2.5, Mm00657783_m1; Tbx5, Mm00803521_m1; and αMHC, Mm00440354_m1 (Applied Biosystems).

*Whole-mount in Situ Analysis*—Whole-mount *in situ* hybridization was performed using digoxigenin-UTP-labeled RNA probes according to manufacturers' protocols. Probes used were cDNAs for mouse *Cv2*. All experiments were performed in accordance with local institutional guidelines for animal experiments.

*Flow Cytometry and Fluorescent-assisted Cell Sorting*—Cells were prepared into a single-cell suspension by treatment with trypsin/EDTA. Flow cytometric analysis was performed with a fluorescent-assisted cell-sorting (FACS) machine (FACSCalibur, BD Biosciences). Sorted cells were collected by FACS Aria (BD Biosciences).

*Western Blotting and Immunoprecipitation*—Standard Western blot methods were used. FLAG-tagged secreted proteins were obtained by transient transfection. For immunoprecipitation analysis, an aliquot of the supernatant was incubated with anti-FLAG M2 antibody overnight at 4 °C. The immune complexes were collected with Protein G plus-agarose beads (Promega). For the *in vitro* receptor-ligand assays, BMP was incubated for 2 h at 4 °C with BMPRIa-Fc protein (R&D Systems) and then incubated with Cv2 (R&D Systems) for 2 h at 4 °C. The protein-A-agarose was

**TABLE 1**
List of genes expressed during early cardiomyocyte differentiation in P19 cells identified by cDNA subtraction analysis

| Gene symbol | Gene name | NCBI GenBank™ |
|---|---|---|
| Cv2/Bmper | Crossveinless-2/BMP binding endothelial regulator | AF454954/NM028472 |
| Mapkap1 | Mitogen-activated kinase-associated protein 1 | NM177345 |
| Igf2bp3 | Insulin-like growth factor 2 mRNA-binding protein 3 | NM023670 |
| Sparc | Secreted acidic cysteine-rich glycoprotein/osteonectin | NM009242 |
| Fkhl18 | Forkhead-like 18 | NM010226 |
| Wnt3a | Wingless-related MMTV integration site 3A | NM009522 |

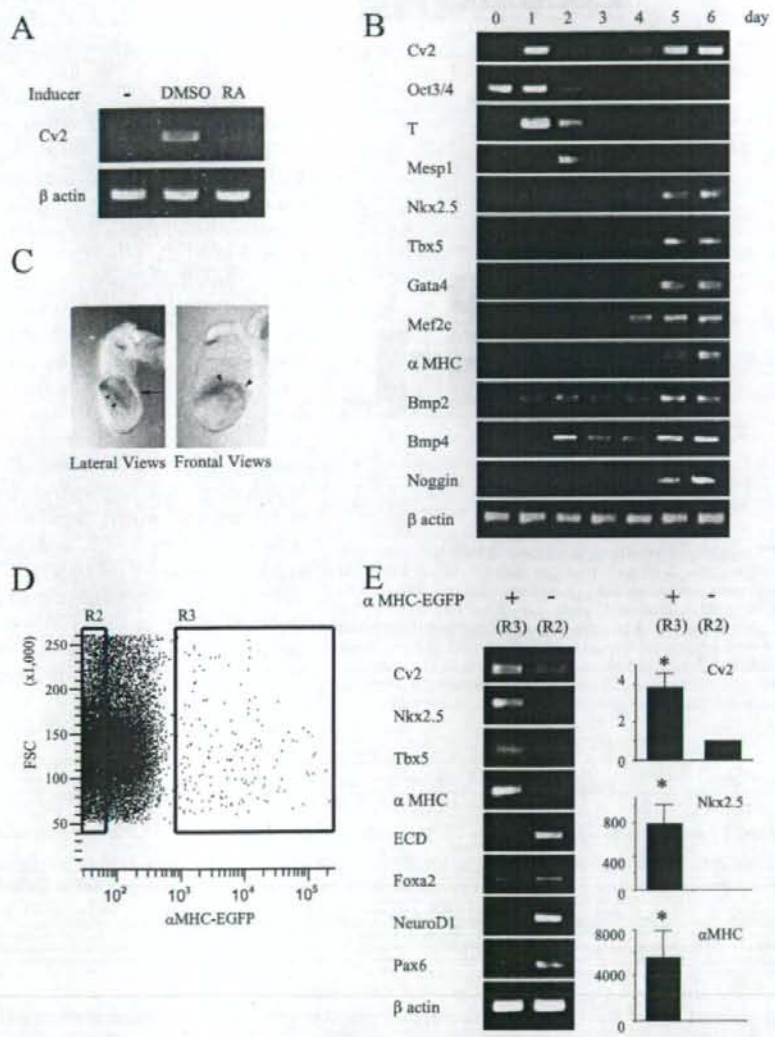


FIGURE 2. **Gene expression during an *in vitro* cardiac differentiation model of P19 cells.** *A*, RT-PCR analysis confirmed *Cv2* expression in differentiating P19 cells (day 6) induced by DMSO but not retinoic acid (*RA*) or no-treatment control. *B*, kinetic analysis of gene expression in differentiating P19 cells showing that the progressive loss of an undifferentiating cell marker and the sequential acquisition of transcripts indicative of specific stages of embryonic development. RNA was isolated from undifferentiated P19 cells (day 0), aggregates harvested at daily interval (days 1–4), and adherent cultures (days 5 and 6). *C*, whole-mount *in situ* hybridization with *Cv2* probes at 7.5 post-coitum. Lateral view and frontal view are shown. *Cv2* transcripts were detected in the cardiac crescent (*arrowhead*) and the posterior primitive streak (*arrow*). *D*, purification of EGFP-positive cells (*R3*) and negative cells (*R2*) by FACS at day 7. *FSC*, forward scatter. *E*, RT-PCR and real-time PCR analysis of various lineage markers in each population. EGFP-expressing cells are cells differentiating into cardiac myocytes ($n = 3$; *, $p < 0.05$, R2 *versus* R3).

preblocked with 1 mg/ml bovine serum albumin and then used to pull down the BMPR1A-Fc.

*Data Analysis*—Results are expressed as the mean ± S.D. Statistical significance was determined by Student's $t$ test or one-way analysis of variance. $p < 0.05$ was used to determine a significant difference.

## RESULTS

*Identification of Genes Expressed during Early Cardiomyocyte Differentiation in P19 Cells*—P19 cells are pluripotent stem cells that can mimic *in vitro* the first stages of cellular differentiation, which occur during normal mouse embryogenesis (23). To identify molecules responsible for BMP signaling during early cardiomyocyte differentiation of P19 cells, cDNA subtraction was employed. This analysis (performed by searching GenBank™ (NCBI) using the BLAST program) resulted in the identification of six distinct products including three genes that encode partial cDNA sequences of secreted proteins (Table 1). They are *Wnt-3a*, *Sparc*, and *Cv2*. Except for *Cv2*, these candidate genes have been reported on in detail elsewhere (27, 28). Among them, BMP-binding protein Cv2 was selected for further analysis based on the established role of BMPs in the regulation of cardiogenesis. A full-length *Cv2* cDNA was obtained by using 5′ and 3′ rapid amplification of cDNA end technique. RT-PCR using cDNA from differentiated P19 cells at day 6 confirmed a substantial increase in *Cv2* mRNA induced by DMSO (1%) but not retinoic acid (0.3 μmol/liter) nor a no-treatment control (Fig. 2*A*), suggesting that Cv2 is a DMSO-inducible factor in cardiomyocyte differentiation of P19 cells.

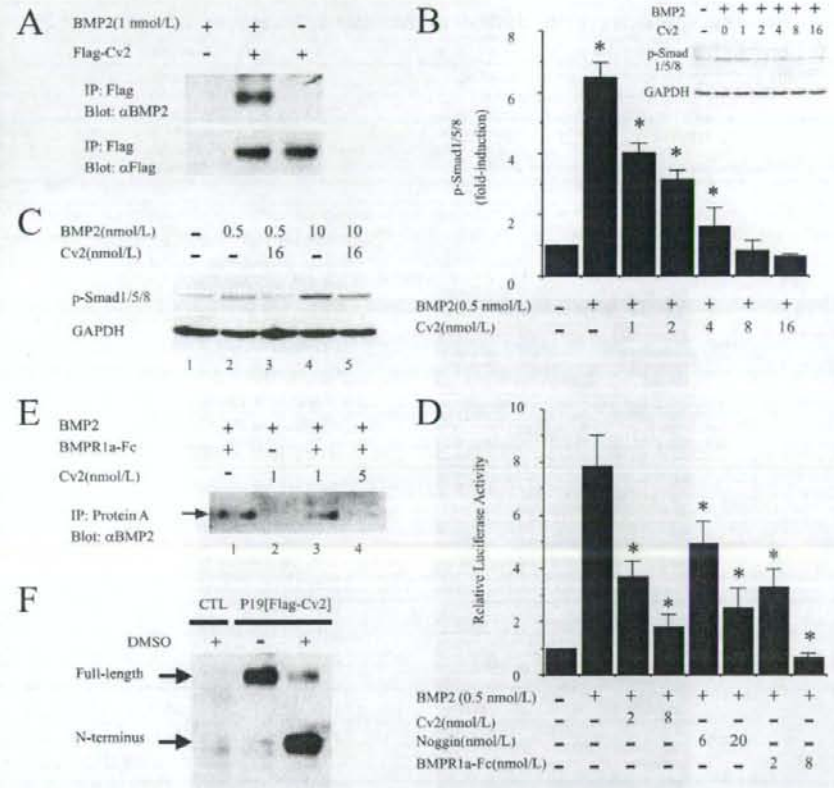


FIGURE 3. **Cv2 binds BMP2, and antagonizes BMP2 signaling by inhibiting receptor binding.** *A*, representative Western blot (*IB*) analysis of BMP2 bound to full-length Cv2 after immunoprecipitation (*IP*). *B*, Cv2 inhibited pSmad1/5/8 by BMP2 (0.5 nmol/liter) in a dose-dependent manner in P19 cells ($n = 3$; *, $p < 0.05$ *versus* 0 nmol/liter Cv2). *C*, representative Western blot showing that inhibition of pSmad1/5/8 in P19 cells by Cv2 at low, but not high, BMP2 concentrations. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. *D*, reporter analysis of P19 cells transfected with BRE reporter ($n = 3$–4; *, $p < 0.05$ *versus* 0.5 nmol/liter BMP2). *E*, pulldown assay with protein A beads shows Cv2 inhibition of BMP2 (1 nmol/liter) binding to BMPRIa-Fc (1 nmol/liter). *F*, representative Western blot analysis of conditioned medium of P19 cells stably expressing N terminus FLAG-tagged *Cv2* (designated as P19[FLAG-Cv2]) before and after cardiac differentiation. *CTL*, control.

*Bimodal Expression of Cv2 during Cardiomyocyte Differentiation*—RT-PCR analysis of differentiating P19 cells as aggregates demonstrated the progressive down-regulation of a stem cell marker such as *Oct3/4* accompanied by the sequential acquisition of mesodermal markers of *T*, *Mesp1*, *Nkx2.5*, and *Tbx5* (Fig. 2*B*). Expression of *T* and *Mesp1* peaked at day 1 and day 2, respectively. Transcription of *Nkx2.5*, *Tbx5*, *Gata4*, and *Mef2c* was also up-regulated from day 4, and αMHC was expressed from day 5. The fractions of aggregates with beating activity at day 6 are 80% in culture with DMSO and 0% in culture without DMSO (data not shown). *Bmp2* and *Bmp4* were expressed from day 1 and day 2, respectively. *Noggin*, the specific BMP antagonist, was expressed from day 5, consistent with the previous findings (29). These data indicate that our *in vitro* cardiac differentiation model of P19 cells reproduces the natural course of differentiation as described in the literature (23, 25), and this model is able to follow the *Cv2* expression during the primary steps of cardiomyocyte differentiation. The kinetics of *Cv2* expression showed a bimodal pattern; Cv2 is initially expressed earlier than cardiac transcription factors such as *Nkx2.5* and *Tbx5*, and the second expression was up-regulated from day 4. This initial increase of *Cv2* would correspond to just before or during gastrulation in intact embryos, in comparison with expression of *T* as a marker of primitive streak mesoderm (30). Because cells that are fated to become cardiac mesoderm are specified during gastrulation (31), this initial expression of *Cv2* may be involved in the specification of cardiac lineage via modulating BMP activity. Whole-mount *in situ* hybridization revealed that at 7.5 days postcoitum *Cv2* transcripts were predominantly found on the cardiac mesoderm (cardiac crescent) and the posterior primitive streak (Fig. 2*C*) as described previously (16).

*Differentiating Cardiomyocytes Autologously Express Cv2*—Next we analyzed *Cv2* expression in differentiating P19EN8 cells (Fig. 2, *D* and *E*), because *Cv2* may be expressed in a lineage-restricted manner. The EGFP-positive (differentiating cardiomyocytes) and EGFP-negative sorted populations were isolated by FACS, and gene expression was profiled by RT-PCR. *Cv2* transcripts were determined to be 4-fold higher in the EGFP-positive population compared with the EGFP-negative population. These results indicate that *Cv2* re-expresses autologously in the population of differentiating cardiac lineage.

*Cv2 Antagonizes BMP2 Signaling by Blocking Receptor Binding*—First, we confirmed the BMP binding to Cv2 (Fig. 3*A*) using the FLAG-epitope-tagged construct of Cv2, in agreement with previous studies (19–22). This binding was inhibited in the presence of 10-fold molar excess of BMP4, but not activin A, transforming growth factor-β1 and epidermal growth factor (data not shown). Next, to examine whether Cv2 can antagonize BMP activity, we performed two independent experiments; the phosphorylation state of Smad1/5/8 proteins and the activation of a reporter gene under the control of a BMP-responsive sequence (Fig. 3, *B* and *C*). In Western blots, increasing amounts of Cv2 inhibited BMP-induced phosphorylated Smad1/5/8 (pSmad1/5/8) in a dose-dependent manner with 50% inhibition ($IC_{50}$) at 2 nmol/liter (Fig. 3*B*). Although pSmad1/5/8 induced by 0.5 nmol/liter BMP2 was completely inhibited by the addition of more than 8 nmol/liter Cv2 (Fig. 3, *B* and *C*, *lane 3*), an excess of BMP2 (10 nmol/liter) was able to quench the antagonistic effect of Cv2 (16 nmol/liter) on BMP2 action (Fig. 3*C*, *lane 5*). Cv2 (16 nmol/liter) failed to affect

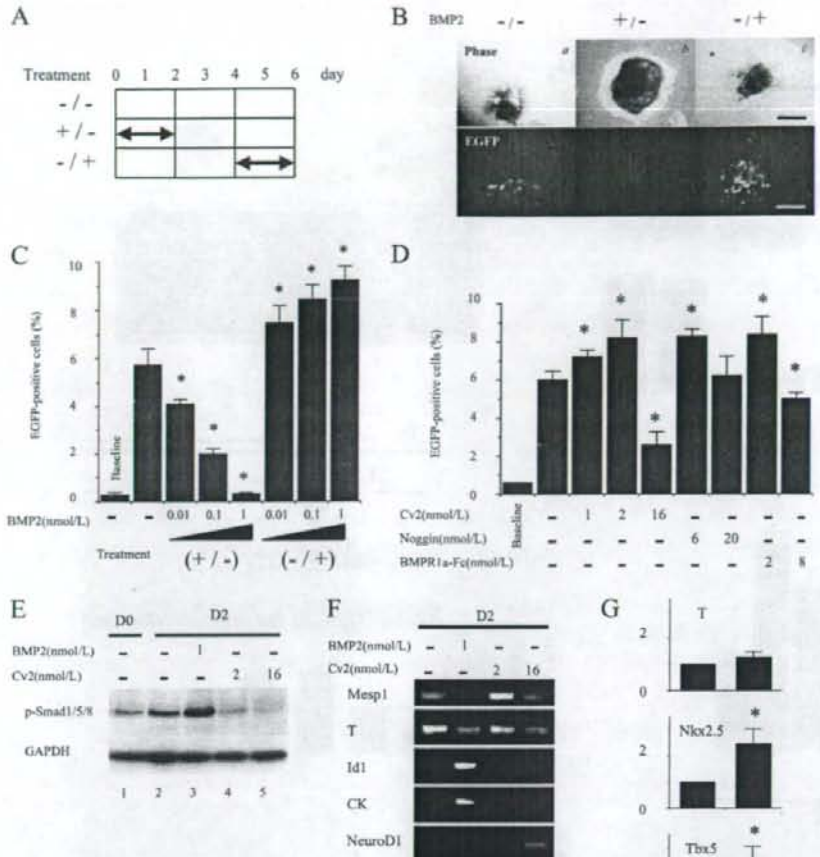


FIGURE 4. **BMP2 plays distinct roles in early and late cardiomyocyte differentiation, and Cv2 regulates BMP signaling for cardiac lineage decision.** *A*, experimental procedure of treatment. *B*, representative figures showing that effects of exposure to BMP on cardiomyocyte differentiation at day 6. Treatment with 1 nmol/liter BMP2 during the first 2 days (*b*) (+/−) resulted in no cardiac differentiation at day 6, whereas treatment without BMP2 (*a*) (−/−) generated cardiac cells. Contrarily, treatment with 1 nmol/liter BMP2 from day 5 to 6 (*c*) (−/+) increased the generation of EGFP-positive cells, suggesting enhanced cardiac differentiation. *Scale bars* equal 250 μm in panels. *C* and *D*, percentage of EGFP-positive cells assessed by FACS at day 7 ($n = 3$; *, $p < 0.05$ *versus* no treatment control). *Baseline* means no-treatment control at day 0. *E*, effects of either BMP2 or Cv2 on pSmad1/5/8 at day 2. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. *F*, effects of either BMP2 or Cv2 on gene expression at day 2. *G*, real-time PCR analysis of *T* (day 2), *Nkx2.5* (day 6), and *Tbx5* (day 6) after treatment with 2 nmol/liter Cv2 ($n = 3$; *, $p < 0.05$ *versus* no treatment control).

pSmad2 induced by 1 nmol/liter transforming growth factor-β1 or 1nmol/liter activin (data not shown). In luciferase assay of P19 cells transfected with the BMP-responsive reporter (Fig. 3*D*), Cv2 as well as Noggin and BMPRIa-Fc protein (a synthetic BMP antagonist) inhibited BMP-induced reporter expression in a dose-dependent manner. These results suggest Cv2 antagonism of BMP signals in P19 cells, consistent with the findings of others (19–21). To further address the molecular mechanism by which Cv2 antagonizes BMP2 signaling, we examined the effect of Cv2 on the binding of BMP2 to its receptor. The BMPRIa-Fc protein was immobilized on protein A-agarose, and bound BMP2 was analyzed. Increasing amounts of Cv2 (1 and 5 nmol/liter) caused a decrease in the amount of BMP2 bound to 1 nmol/liter BMPRIa-Fc protein (Fig. 3*E*, *lane 3* and *4*). We also observed an increase in the cleaved form of Cv2 in concert with cardiac differentiation using P19 cells stably expressing FLAG-tagged *Cv2* cDNA (Fig. 3*F*). Together, these results strengthen the notion that Cv2 functionally interacts with BMP signaling by blocking interaction of BMP2 with its cognate receptor.

*Distinct Roles of BMP2 during Early and Late Cardiomyocyte Differentiation*—BMP2 is an important signaling molecule for cardiac differentiation and development (2–7). To confirm this evidence, we treated differentiating P19 cells with BMP2 in a dose-dependent manner (Fig. 4*A*) and then evaluated cardiomyocyte differentiation at day 6 (Fig. 4*B*) and at day 7 (Fig. 4*C*). Contrary to our expectations, exposure to BMP2 during the first 2 days of differentiation suppressed the generation of cardiac cells (green fluorescent protein-positive cells), whereas exposure to BMP2 from day 5 to day 6 enhanced the generation of cardiac cells, as compared with the no-treatment control. The same observation applied to BMP4 (data not shown). These results suggest that at the early time frame from day 1 to day 2 of differentiation, corresponding to prior to or during gastrulation in development, BMP2 acts as an anti-cardiogenic factor. Our results agree with previous studies in chick embryos that BMPs inhibited cardiogenesis when applied at an early stage of gastrulation (7).

Progenitor cells destined to the cardiac lineage emerge from the primitive streak during gastrulation (31). Because there is a period that is sensitive to BMP concentrations in the early stage of cardiomyocyte differentiation of the P19 system, we hypothesized Cv2 modulation of BMP signaling in this period vulnerable to BMP activity. Therefore, with the focus on the time frame from day 1 to day 2 of early differentiation (Fig. 2*B*), we examined whether the suppression of BMP signals is required to generate the cardiac myocytes in further detail. For this purpose, we cultured differentiating P19 cells in the presence of Cv2, Noggin, or the BMPRIa-Fc protein during this period to evaluate cardiac differentiation by FACS. Increasing doses of