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MFH-1 is required for bone morphogenetic protein-2-induced osteoblastic differentiation of C2C12 myoblasts

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Abstract Mesenchyme forkhead-1 (MFH-1), a winged helix/forkhead transcription factor, is expressed in developing cartilaginous tissues, kidney and arch arteries, and is essential for the normal development of the axial skeleton and aortic arch formation of mice. To investigate the possible role of MFH-1 in osteogenesis and osteoblast differentiation, we examined expression of MFH-1 induced by bone morphogenetic protein-2 (BMP-2) in C2C12 myoblasts, and found that MFH-1 protein and also MFH-1 mRNA increased markedly in C2C12 cells after treatment with BMP-2. To confirm the hypothesis that BMP-2 induced osteoblastic differentiation of C2C12 cells by increasing MFH-1 expression, we lowered the endogenous MFH-1 level by stably transfecting C2C12 cells with antisense MFH-1 sequence, and found that in antisense MFH-1 cell lines, both alkaline phosphatase (ALP) activity and production of osteocalcin induced by BMP-2 decreased markedly in comparison with control cell lines. Our results suggest that the BMP-2-induced MFH-1 protein may play a key role in regulating the commitment to osteoblastic differentiation of C2C12 myoblasts and production of osteoblast markers including ALP and osteocalcin.

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Key words: Mesenchyme forkhead-1; Forkhead; Bone morphogenetic protein-2; Osteoblast

1. Introduction

Bone morphogenic protein-2 (BMP-2), a prototype of the BMP subgroup [1], promotes the development of bone and cartilage by inducing the differentiation of multipotent mesenchymal progenitor cells into osteoblastic cells or cartilage cells, respectively. Recombinant BMP-2 not only stimulates the osteoblastic maturation of the osteoblast progenitor ROB-C26 cells [2] and human neonatal calvaria cells [3], but also induces non-osteoblast cells, such as pluripotent fibroblastic cell lines, C3H10T1/2 [4], bone marrow stromal cell lines, W-20-17 [5], ST2 and MC3T3-G/PA6 cell lines [6] to differentiate into osteoblasts, as assessed by induction of alkaline phosphatase (ALP) activity and expression of osteocalcin [2–6].

C2C12 myoblasts can generate multinucleated myotubes when they are cultured in low-mitogen medium [7]. However, under the same condition, formation of myotubes could be inhibited by adding BMP-2 to the medium, which converted the differentiation pathway to the osteoblast lineage [7]. Thus, BMP-2-induced osteogenesis of C2C12 cells provides a useful experimental model for investigating the molecular mechanism of osteogenesis in vitro in culture systems. Recent studies have demonstrated that cytoplasmic Smad proteins are involved in the processes of inhibition of myoblastic differentiation and induction of osteoblast differentiation [8,9]. The effects of BMP-2 are mediated through BMP receptor IA (BMPRIA), and activation of BMPRIA causes phosphorylation of Smad5 to subsequently form a complex with DPC4 (Smad4) and this complex is then translocated to the nucleus. When Smads are transiently transfected into C2C12 cells, both Smad1 and Smad5 induce ALP activity and decrease the activity of the myogenic promoter without BMP-2 [9].

Mesenchyme forkhead-1 (MFH-1) protein is a member of the winged helix/forkhead transcription factors. We have isolated it as a 62-kDa protein [10], and identified its expression in developing cartilaginous tissues, kidney and arch arteries of mice [11–13]. Mice deficient in MFH-1 gene display defective formation in the aortic arches, multiple craniofacial bones and vertebral column, thus implying that it plays an essential role in the normal development of the axial skeleton and aortic arch formation [12,14,15]. However, it is still unknown how this transcriptional factor functions in the regulated proliferation, survival and differentiation of the cell. In the present study, we investigated the role of MFH-1 protein in the in vitro osteogenic pathway, and found that MFH-1 responded to BMP-2 and subsequently promoted osteoblastic differentiation of C2C12 myoblasts.

2. Materials and methods

2.1. Plasmid construction

The *Not*I/*Eco*RI fragment of MFH-1 cDNA [11] was filled in, ligated with the *Eco*RI linker and inserted into the *Eco*RI-cut pGEX-2T to make the GST-MFH-1 plasmid. The CX-MFH-1 plasmid was described previously [10]. The CX-MFH-1 antisense (as) plasmid was made by introducing the *Eco*RI fragment of the CX-MFH-1 plasmid into the *Eco*RI site of the CX-N2 vector [10] and selecting the antisense orientation.

2.2. Production of anti-mouse MFH-1 monoclonal antibody

The GST-MFH-1 plasmid was transformed in *Escherichia coli* LE 392. After induction with 0.5–1.0 mM isopropyl β-D-thiogalactopyr-

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Abbreviations: ALP, alkaline phosphatase; BMP-2, bone morphogenetic protein-2; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione *S*-transferase; MFH-1, mesenchyme forkhead-1; PVDF, polyvinylidene difluoride; RIA, radioimmunoassay; TGF-β, transforming growth factor-β

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anoside, the bacterial lysates were prepared and applied to glutathione-Sepharose 4B (Pharmacia Biotech, Sweden). Washing and elution of the GST-MFH-1 protein (amino acids 290–494) were performed according to the supplier's directions. Purified GST-MFH-1 protein was used to immunize Wistar rats. After 3 days of boosters, isolated spleen cells were fused with P3U1 myeloma cells using polyethylene glycol 4000 (Nakarai Tesque Inc., Tokyo, Japan), as described elsewhere [16]. Hybridoma cells secreting anti-MFH-1 antibody were isolated and cloned by immunostaining the CX-MFH-1 gene-transfected L cells with culture supernatants. Cloned anti-MFH-1 hybridoma cell lines were injected into the abdomen of pristane-treated nude mice to obtain ascites. Monoclonal anti-MFH-1 antibody was purified from ascites using protein A-Sepharose 4B (Pharmacia).

2.3. Cell culture and transfection

The mouse myoblast C2C12 cells and L cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical Co., St. Louis, MO, USA) containing 10% fetal calf serum (FCS). Human bladder carcinoma HTB9 cells were maintained in RPMI 1640 (Bio-Rad) containing 10% FCS. To examine the effects of BMP-2 and transforming growth factor-β (TGF-β), C2C12 cells were inoculated in 12-well tissue culture plates at a density of 2×10^4 cells per cm^2. After overnight incubation, the medium was replaced with DMEM containing 5% FCS and 300 ng/ml of BMP-2 or 10 ng/ml of TGF-β, and then the cells were cultured for 0.5, 1, 2, 3 and 6 days. For antisense studies, 20 μg of CX-MFH-1(as) plasmid and CX-N2 empty vector as control were transfected into cultured C2C12 cells using the calcium phosphate precipitation method [17]. Forty-eight hours after transfection, the medium was replaced with DMEM containing 400 μg/ml G418 (Gibco/BRL) for 3 weeks. Three antisense clonal cell lines (named AS 7, AS 20 and AS 23) and two control clonal cell lines (named V 14 and V 16) were established.

2.4. Western blot analysis

Cells were solubilized in RIPA lysis buffer (50 mM Tris–HCl, pH 6.8, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM dithiothreitol and 2 mM phenylmethylsulfonyl fluoride). The lysates were separated by SDS–PAGE and transferred to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore) [18]. Immunoblotting with anti-mouse MFH-1 monoclonal antibody (1:1000) was carried out. The proteins were visualized with horseradish peroxidase-conjugated swine anti-rat IgG antibody (Dako, Glostrup, Denmark), and enhanced by the ECL detection kit (Amersham, Buckinghamshire, UK).

2.5. RNA isolation and Northern blot analysis

Total RNA was isolated from C2C12 cells treated with BMP-2 (300 ng/ml) for 2 days by the guanidine thiocyanate and cesium chloride ultracentrifugation extraction method. For Northern hybridization, total RNA (12 μg) was electrophoresed in 1.2% agarose gels containing 2.2 M formaldehyde, and blotted to GeneScreen Plus Hybridization Transfer Membrane (NEN Life Science, Billerica, MA, USA). The 1.1-kb *Not*I/*Eco*RI fragment of mouse MFH-1 cDNA was labeled with [^{32}P]dCTP (Amersham Pharmacia Biotech, Buckinghamshire, UK) using the random primer technique (Multiprime DNA Labeling Systems, Amersham Pharmacia Biotech). Hybridization was performed in a solution containing 5×SSPE, 50% (w/v) deionized formamide, 5×Denhardt's solution, 1% SDS, 10% dextran sulfate (MW 500 000) and 10 μg/ml of denatured salmon sperm DNA at 42°C overnight. The hybridized probe was removed by boiling in 0.1×SSPE, 50% formamide and 0.1% SDS for 1 h at 65°C. The amounts of mRNA were verified by rehybridizing the filter with elongation factor-1 (EF-1α) cDNA [19] probe, and the signals were detected by autoradiography.

2.6. ALP activity assay

ALP activity in the cell lysate was measured as described by Katagiri et al. [7]. The enzyme activity was expressed as nmol of *p*-nitrophenol produced per min per mg of protein.

2.7. Quantitation of osteocalcin production

The amount of osteocalcin secreted into the culture medium was measured by radioimmunoassay (RIA) using a mouse osteocalcin assay kit (Biomedical Technologies Inc., Stoughton, MA, USA).

2.8. Immunofluorescent analysis

The cells were seeded on coverslips in 24-well plates. On the next day, the cells were fixed and immunofluorescently stained with anti-mouse MFH-1 monoclonal antibody (1:1000), and FITC-conjugated anti-rat IgG antibody (Dako) was used as the secondary antibody, as described previously [20].

3. Results

3.1. MFH-1 protein was detected specifically by monoclonal antibody

To study the role of MFH-1 protein, we produced anti-mouse MFH-1 monoclonal antibody. We transfected the CX-MFH-1 plasmid and CX-N2 empty vector into human bladder carcinoma HTB9 cells. Two days after transfection, the cell lysates were processed for Western blot analysis. In the HTB9 cells transiently transfected with MFH-1 gene, a 62-kDa band was detected with the anti-mouse MFH-1 antibody (Fig. 1A, lane 2). No signals were detected in cells transfected with the control vector (Fig. 1A, lane 1). In addition, immunofluorescent staining with anti-mouse MFH-1 antibody showed that overexpressed MFH-1 protein was present in the nucleus of the transfected cells (Fig. 1B, b) and endogenous MFH-1 protein was detected in the mouse myoblast C2C12 cells (Fig. 1B, c). These results indicated that our antibody specifically recognized the mouse MFH-1 protein.

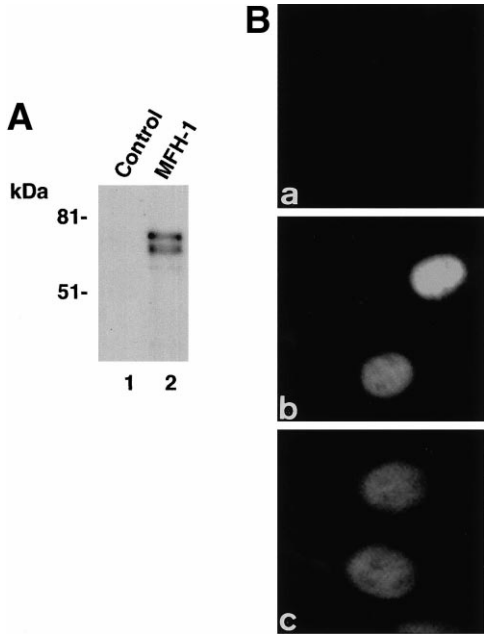

Fig. 1. A: Western blot analysis of MFH-1 protein in transfected cells with monoclonal antibody. HTB9 cells were transfected with 20 μg of CX-MFH-1 (lane 2) plasmid and CX-N2 empty vector (lane 1). Two days after the transfection, the cells were solubilized in RIPA lysis buffer. Equivalent amounts of lysates (20 μg) were subjected to SDS–PAGE (10% gel) and transferred to a PVDF membrane, which was immunoblotted with anti-mouse MFH-1 monoclonal antibody. B: Expression of MFH-1 proteins in cultured cell lines. L cells transfected with CX-N2 empty vector (a) and CX-MFH-1 gene (b), and C2C12 cells (c) were fixed and immunostained with anti-mouse MFH-1 monoclonal antibody, followed by incubation with FITC-conjugated anti-rat IgG antibody. Magnification: ×490.

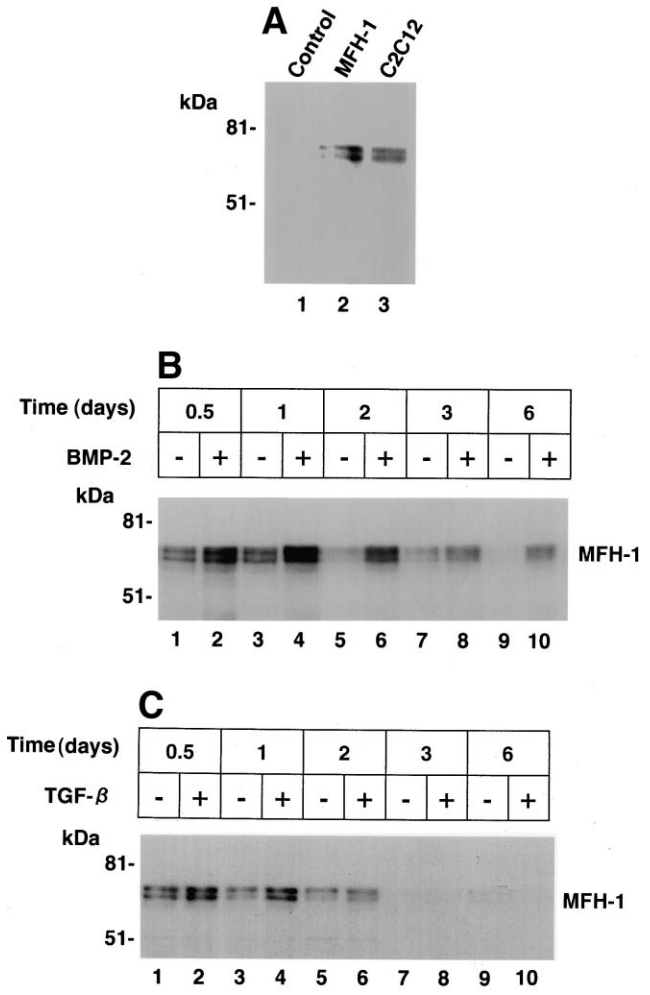

Fig. 2. Effects of BMP-2 and TGF-β on MFH-1 protein levels in C2C12 cells. A: Basal expression of MFH-1 protein in cultured C2C12 cells. HTB9 cells transfected with the CX-N2 empty vector (lane 1) and CX-MFH-1 gene (lane 2) and C2C12 cells (lane 3) were cultured in medium containing 10% FCS. 20 μg of lysates from transfected cells and 50 μg of lysates from C2C12 cells were analyzed by Western blot as described in Fig. 1A. B and C: C2C12 cells were inoculated in 12-well tissue culture plates at a density of 2×10^4 cells/cm^2. After overnight incubation, the medium was replaced with DMEM containing 5% FCS in the absence (−) or presence (+) of BMP-2 (300 ng/ml) (B) and TGF-β (10 ng/ml) (C) for the indicated times. The cells were solubilized in lysis buffer and the MFH-1 protein levels were determined by Western blot analysis (50 μg of lysates/lane) with anti-mouse MFH-1 monoclonal antibody.

3.2. BMP-2-induced expression of MFH-1 protein in C2C12 cells

We examined the relationship between the expression of MFH-1 protein and osteogenesis by BMP-2 in C2C12 cells. First, we determined the basal expression of MFH-1 protein in C2C12 cells. MFH-1 protein was constitutively expressed in C2C12 cells (Fig. 2A, lane 3 and Fig. 1B, c), and its mobility was the same as that for HTB9 cells transfected with the CX-MFH-1 expression plasmid (Fig. 2A, lane 2). Then, we examined the time course of the expression level of MFH-1 protein in C2C12 cells after BMP-2 treatment. After replating C2C12 cells in the absence of BMP-2, the expression of MFH-1 protein was increased in 0.5 and 1 day and then decreased gradually from 2 to 6 days (Fig. 2B, lanes 1, 3, 5, 7 and 9), However, when C2C12 cells were treated with BMP-2, the MFH-1 protein sustained prominently higher levels from 12 h to 6 days in comparison with untreated cells (Fig. 2B, lanes 2, 4, 6, 8 and 10). In contrast, in C2C12 cells treated with TGF-β, a short-term slight increase of MFH-1 protein was detected in 12–24 h (Fig. 2C). These results suggest that MFH-1 may be a target for the BMP-2 signaling pathway in C2C12 cells, and may mediate the effects of BMP-2 in promoting the osteogenic differentiation pathway in C2C12 myoblasts.

3.3. Expression of MFH-1 mRNA was induced by BMP-2 in C2C12 cells

Given the marked enhancement of the MFH-1 protein level by BMP-2 in C2C12 cells, we next determined whether this increase might reflect changes in the MFH-1 mRNA level. By Northern blot analysis of total RNA from C2C12 cells after 2 days of incubation with low-mitogen medium containing 300 ng/ml of BMP-2 or lacking BMP-2, MFH-1 mRNA was detected as a weak band of 3.0 kb in the BMP-2-untreated cells (Fig. 3A, lane 1), but was enhanced dramatically in the BMP-2-treated cells (Fig. 3A, lane 2), which showed an approximately eight-fold higher level than in the untreated cells (Fig. 3B). This result suggests that BMP-2-induced up-regulation of MFH-1 occurred at the pretranslational level.

3.4. Decreasing MFH-1 level by transfection of antisense MFH-1-inhibited osteoblastic differentiation of C2C12 cells

Our results demonstrated that BMP-2 markedly enhanced MFH-1 mRNA and protein levels in C2C12 cells. We hypothesized that if BMP-2-induced MFH-1 plays a key role in promoting osteoblastic differentiation from C2C12 cells, a lowered MFH-1 level by transfection of the antisense MFH-1 sequence would block the effects of BMP-2, and thereby, the osteoblastic differentiation of C2C12 cells would be inhibited. To address this issue, we transfected C2C12 cells with the antisense MFH-1 expression construct, CX-MFH-1(as). After

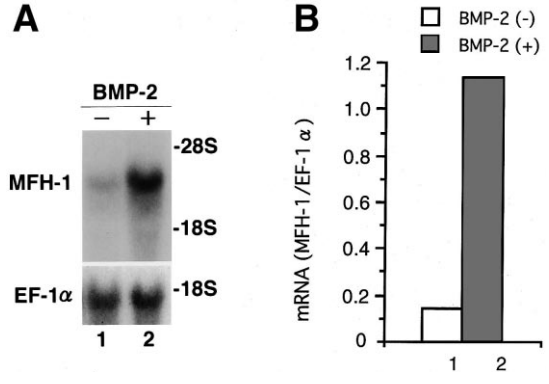

Fig. 3. Effect of BMP-2 on expression of MFH-1 mRNA in C2C12 cells. A: Northern blot analysis of total RNA from C2C12 cells. The cells were cultured in DMEM containing 5% FCS in the absence (lane 1) and presence of 300 ng/ml of BMP-2 (lane 2) for 2 days. Total RNA from each culture (12 μg) was analyzed by Northern blotting using a ^{32}P-labeled mouse MFH-1 cDNA probe. The same blot was reprobed with elongation factor-1 (EF-1α) cDNA to assess the quantity of RNA (bottom). B: The levels of MFH-1 mRNA expression were quantified using NIH Image software and normalized to the EF-1α levels. The values are expressed as arbitrary units.

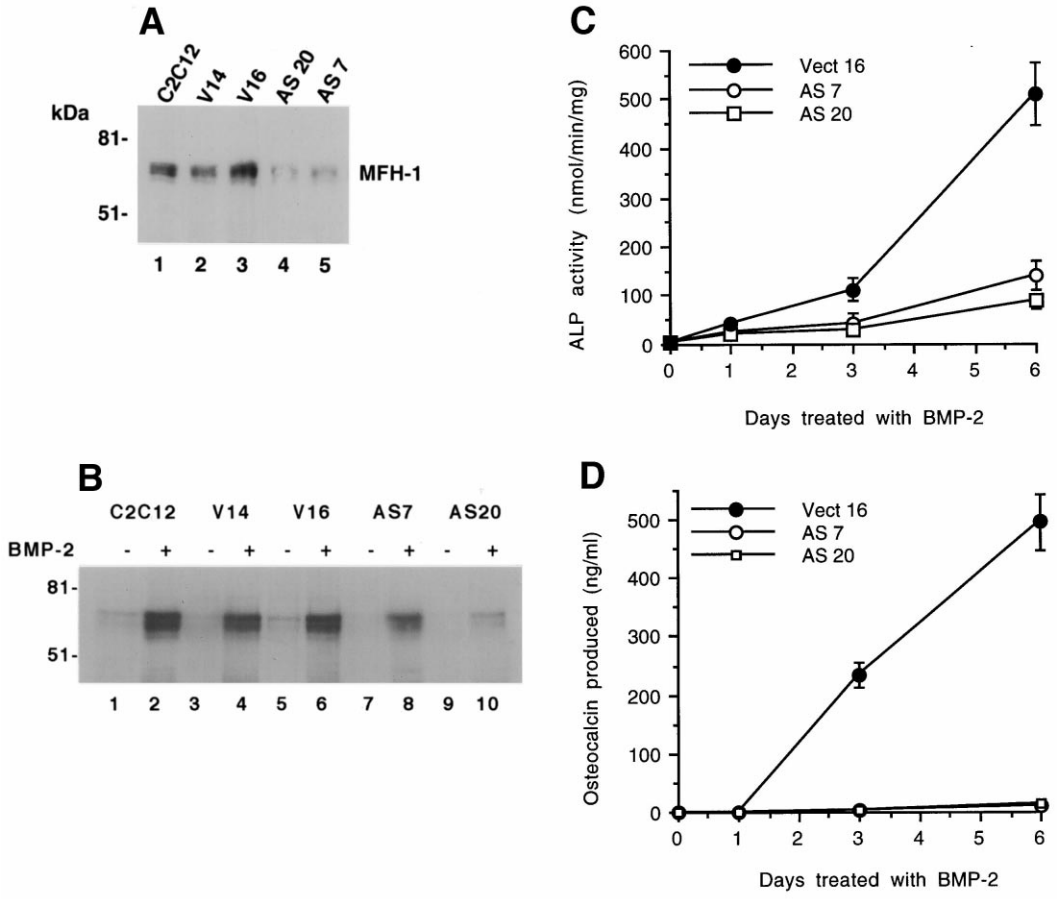

Fig. 4. Antisense MFH-1 inhibited osteoblastic differentiation of C2C12 cells. A: Antisense MFH-1 attenuated the expression of endogenous MFH-1 protein. C2C12 cells (lane 1), control cell lines (lanes 2 and 3) and antisense cell lines (lanes 4 and 5) were seeded in a 6-cm tissue culture plate at a density of 5×10^4 cells/cm^2 and cultured in DMEM containing 10% FCS. On the next day, cells were harvested and MFH-1 protein levels were determined by Western blot analysis (50 μg of lysates/lane). B: Antisense MFH-1 inhibited the BMP-2-induced expression of MFH-1 protein. C2C12 cells (lanes 1 and 2), control cell lines (lanes 3–6) and antisense cell lines (lanes 7–10) were cultured in DMEM containing 5% FCS in the absence (lanes 1, 3, 5, 7 and 9) and presence of 300 ng/ml of BMP-2 (lanes 2, 4, 6, 8 and 10) for 2 days. MFH-1 protein levels were determined by Western blot analysis (50 μg of lysates/lane). C and D: Antisense MFH-1 inhibited BMP-2-induced ALP activity and osteocalcin production in C2C12 cells. The control cell line (V 16, closed circles) and the antisense cell lines (AS 7, open circles and AS 20, open squares) were seeded in 12-well tissue culture plates at a density of 2×10^4 cells/cm^2 and cultured in DMEM containing 10% FCS. After overnight incubation, the medium was replaced with DMEM containing 5% FCS and 300 ng/ml of BMP-2 for the indicated times. The ALP activity of the cell lysate was measured using *p*-nitrophenyl phosphate as a substrate. The unit of ALP activity is nmol of *p*-nitrophenol formed per mg of protein per min (C). The amounts of osteocalcin secreted into the culture medium were determined by RIA (D). Values are the means ± S.D. of three individual experiments.

selection of stable clones, three independent lines of antisense cells (named AS 7, AS 20 and AS 23) showed a marked reduction in endogenous MFH-1 protein level by Western blot analysis (Fig. 4A, lanes 4 and 5 and data not shown). The expression level of the MFH-1 protein in C2C12 cells transfected with an empty vector (named V 14 and V 16) was the same as that of the parental C2C12 cells (Fig. 4A, lanes 1–3). Next, we investigated the expression levels of MFH-1 protein in antisense cells after BMP-2 treatment, and found that the MFH-1 expression in antisense cell lines (Fig. 4B, lanes 8 and 10) was lower than those in the control cell lines (Fig. 4B, lanes 4 and 6) and parental C2C12 cells (Fig. 4B, lane 2). This result suggests that antisense MFH-1 gene would inhibit the expression of the MFH-1 protein after treatment with BMP-2.

Finally, we examined whether reducing the MFH-1 levels would inhibit the osteoblastic differentiation of C2C12 cells. ALP activity and production of osteocalcin, which are well known markers of the osteoblast lineage, were measured in control cells and antisense cells cultured in low-mitogen medium containing 300 ng/ml of BMP-2 for 1, 3 and 6 days. As shown in Fig. 4C,D, in the control cell line V 16, ALP activity and production of osteocalcin were detected from 1 day after BMP-2 treatment, and then markedly increased from 3 days onwards. In contrast, in both the antisense cell lines AS 7 and AS 20, ALP activity was lower than that of the control cells (Fig. 4C), and almost no production of osteocalcin was detected until day 6 (Fig. 4D). Also, ALP activity and production of osteocalcin were not detected in untreated cells over the 6-day period examined (data not shown). This means that a lowered level of MFH-1 inhibits the BMP-2-induced ALP activity and production of osteocalcin in antisense cells. These results suggest that the MFH-1 protein may mediate osteoblastic differentiation of C2C12 cells by BMP-2.

4. Discussion

In this study, we found that BMP-2 induced a sustained increase of MFH-1 expression during C2C12 myoblast transdifferentiation to osteoblast lineage. Furthermore, lowering the endogenous MFH-1 level by stably transfecting C2C12 cells with antisense MFH-1 markedly decreased ALP activity and inhibited production of osteocalcin in the presence of BMP-2. These findings suggest that the MFH-1 protein response to BMP-2 signaling contributes to the osteoblast differentiation of C2C12 cells.

The MFH-1 gene is expressed in developing vertebrae, cartilaginous tissues, kidney and arch arteries of mice [11–13]. We examined more than 20 cultured cell lines and found that MFH-1 protein was expressed in human bone, cartilage, blood vessel and nephroblast cell lines, and also in mesenchymal progenitor cells such as C2C12 cells and bone marrow stromal ST2 cells of mice (Fig. 1 and data not shown). These results indicate that the expression of MFH-1 in vitro in cultured cell lines corresponds well to that in mice embryos. In vitro studies using these cultured cells may clarify the differentiation process in embryos. In MFH-1-deficient mice, defective formation of skeleton and aortic arches was observed [12,14,15]. The defects of skeletogenesis were mainly observed in the craniofacial bones and vertebral column [12,15], implying that MFH-1 plays an essential role in the normal development of the axial skeleton and skull of mice. Thus, we are interested in investigating the possible roles of MFH-1 in skeletogenesis and osteoblast differentiation in culture systems.

It is well known that BMP-2 not only induces the osteogenesis of osteoblast and multipotent mesenchymal progenitor cells, but also converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage [7]. We found that MFH-1 was expressed in C2C12 cells, and its expression gradually decreased after 2 days of culture in low-mitogen medium, and only a very weak band was detected at 6 days (Fig. 2B,C, lanes 1, 3, 5, 7 and 9). However, in BMP-2-treated cells, the MFH-1 protein expression displayed sustained increased levels from 12 h to 6 days in comparison with untreated cells (Fig. 2B). In contrast, a short-term slight increase of MFH-1 protein was found in 12–24 h with TGF-β treatment (Fig. 2C), which is consistent with the fact that neither an increase of ALP activity nor production of osteocalcin was found in TGF-β-treated C2C12 cells [7,8]. This result indicates that different pathways may exist for the effects of BMP-2 and TGF-β in C2C12 cells. Our results strongly support the idea that MFH-1 responds to the BMP-2 signaling pathway in C2C12 cells and promotes the osteoblastic fate. The possibility that MFH-1 might respond to TGF-β to favor other cell fates in another differentiation system could not be excluded.

We hypothesized that MFH-1 protein might play a key role in the BMP-2-mediated commitment to osteoblastic differentiation of C2C12 myoblasts. As demonstrated in Fig. 4C,D, ALP activity decreased dramatically and only a trace amount of osteocalcin production was detected in antisense MFH-1 cells, in which the endogenous MFH-1 level was lowered. This suggests that BMP-2-induced MFH-1 protein may regulate the osteoblast differentiation of C2C12 cells and the synthesis of osteoblast markers.

We have no direct evidence for the involvement of MFH-1 in myogenic differentiation from the C2C12 cells. However, it has been reported that myotubes were first detected on day 3 and increased in number until day 6 in the low-mitogen medium [7]. Under the same culture condition, our present results showed that expression of MFH-1 was decreased obviously on day 2, and only a minimal amount of MFH protein was detected on day 6 (Fig. 2B, lanes 1, 3, 5, 7 and 9). These results indicated that MFH-1 might have an inhibitory effect on myogenic differentiation of the myoblasts.

Little is known about the molecular mechanism that MFH-1 is induced with BMP-2 signaling. A current study on the BMP-2 signaling pathway in C2C12 cells demonstrated that, as in the case of BMPs, BMP-2 exerts its diverse biological effects through two types of transmembrane receptors, BMP type I (BMPRI) and BMP type II (BMPRII) receptors [21]. BMPRI is further subclassified into BMPRIA and BMPRIB [21]. The effects of BMP-2 are mediated through BMPRIA in C2C12 cells [8,9]. This signal causes phosphorylation of cytoplasmic Smad1 protein as well as Smad5 protein, and forms a complex with Smad4, and then translocates to the nucleus [8,9]. The functions of Smads are known to be associated with various nuclear transcription factors that bind to the specific DNA sequence. *Mix.2*, an activin-responsive gene, is involved in the early frog embryogenesis [22]. By analysis of the *Mix.2* promoter, it has been clarified that FAST-1, a winged helix transcription factor, forms a complex with Smad2 and Smad4, and then binds to DNA [23]. The binding regions of FAST-1 protein to Smad2 and Smad4 have been determined [24]. We compared the amino acid sequences of the Smad-associating regions of FAST-1 protein and that of MFH-1 protein and could not find any homologous sequences in the MFH-1 protein. Taken together with the finding that MFH-1 responds to BMP-2, it is not likely that MFH-1 works cooperatively with Smads like FAST-1, but might be a downstream gene of the BMP-2/Smad signal pathway. In order to understand the molecular mechanism that MFH-1 mediates osteoblastic differentiation, the target genes that MFH-1 regulates must also be determined in the future.

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