MyoD enhances BMP7-induced osteogenic differentiation of myogenic cell cultures

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Accepted 4 November 2003
Journal of Cell Science 117, 1457-1468 Published by The Company of Biologists 2004
doi:10.1242/jcs.00965

Summary

The muscle-specific, basic helix-loop-helix transcription factor MyoD can induce cells from other mesenchymal lineages to express a skeletal muscle phenotype. Interestingly, MyoD is initially upregulated in myogenic cells incubated with bone morphogenetic proteins (BMPs), a treatment that induces osteogenic differentiation, suggesting that MyoD has a role in BMP-induced osteogenesis of myogenic cells. This possibility is supported by our observations that muscle satellite cells derived from adult MyoD$^{-/-}$ mice show severely impaired osteogenic induction by BMP-7 (osteogenic protein 1; OP-1) as indicated by the decreased gene expression of the bone markers *alkaline phosphatase, osteocalcin, Runx2/Cbfa1*, and *Osterix*. Ectopic expression of MyoD increased alkaline phosphatase activity and *Osterix* mRNA expression in response to BMP treatment. Similarly, ectopic expression of MyoD in the pluripotent mesenchymal cell line C3H10T1/2 increased alkaline phosphatase activity induced by BMP-7. Transcription assays showed that transfection with a MyoD-expression vector, but not other myogenic basic helix-loop-helix transcription factors (Myf5, myogenin) increased Runx2/Cbfa1 transactivation of a reporter gene construct containing either six OSE sequences in tandem or a single OSE site. This effect was enhanced by BMP treatment. These studies, therefore, demonstrate that the muscle transcription factor MyoD is required for efficient BMP-induced osteogenesis of myogenic cells and indicate that MyoD might exert its effects through co-operative interactions with Runx2/Cbfa1.

Key words: FOP, myogenesis, osteogenesis, MyoD, BMPs, Runx2/Cbfa1

Introduction

Differentiation of a common mesenchymal precursor cell to specific cell lineages such as muscle, bone, cartilage and fat, occurs under the guidance of local influences that promote a cascade of gene activities leading to the generation of the mature cells. Several key genes have been identified that are essential to lineage specification. These include the MyoD family of transcription factors that are required for myogenesis (Weintraub, 1993), Runx2/Cbfa1 (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997) and Osterix (Osx) (Nakaahima et al., 2002) for osteogenesis, and PPARγ (Tontonoz et al., 1994) for adipogenesis.

The MyoD family of basic helix-loop-helix (HLH[1]) transcription factors, comprising MyoD, Myf5, myogenin and MRF4, regulate myogenic differentiation in a functional hierarchy (Rudnicki and Jaenisch, 1995; Weintraub et al., 1991). MyoD activates transcription of muscle-specific genes by binding as a heterodimer with E-proteins to a consensus sequence (E-box) present in the promoters of various muscle-specific genes, including muscle creatine kinase (MCK) (Davis et al., 1999; Lassar et al., 1989). Since forced expression of MyoD can also induce myogenic differentiation in cells from other lineages (Olson and Klein, 1994; Weintraub et al., 1989), MyoD is considered to be a 'master regulatory gene' for myogenesis (Weintraub et al., 1989). Another HLH transcription factor, inhibitor of differentiation (Id), can also form heterodimers with E-proteins but lacks the basic domain necessary for E-box binding. Id, therefore, acts as a negative regulator of muscle differentiation (Benezra et al., 1990). Notably, forced expression of the *Id* gene inhibits not only myogenesis but also adipogenesis (Moldes et al., 1997) and osteogenesis (Murray et al., 1992).

The runt-related transcription factor Runx2/Cbfa1 has been identified as a key regulator of osteoblast differentiation because mice deficient in Runx2/Cbfa1 fail to form bone (reviewed in Ducy, 2000; Karsenty et al., 1999). The gene for *Runx2/Cbfa1* encodes three isoforms that are regulated by two promoters. The type I isoform with the N-terminus 'MRIPV' is driven by one promoter whereas the type II and III isoforms with N-termini starting with 'MASN' and 'MLH', respectively, are regulated by the other promoter (Harada et al., 1999). Whereas the expression of mRNA for the type II and III isoforms is specific to mineralizing tissues, the type I isoform is more widely expressed (Banerjee et al., 2001). The DNA-binding site for Runx2/Cbfa1 has been identified as osteoblast specific element 2 (OSE2), which is required for osteoblast-specific expression of osteocalcin (Ducy and Karsenty, 1995). Notably, forced expression of Runx2/Cbfa1 can induce

osteogenic differentiation in the pluripotent mesenchymal cell line C3H10T1/2 (Ducy et al., 1997).

Recently, the C_2H_2 zinc-finger transcription factor Osterix (Osx) has been identified and shown to be required for osteoblast differentiation (Nakashima et al., 2002). *Osx*-null mice lack both endochondral and membranous bone, but in contrast with *Runx2/Cbfa1*-null mice, the formation of mineralized cartilage is not affected. Interestingly, Runx2/Cbfa1 is expressed in *Osx*-null mice suggesting that Osx acts downstream of Runx2/Cbfa1.

Expression of MyoD, Osx, and Runx2/Cbfa1 is regulated by the bone morphogenetic proteins (BMPs). BMPs, initially identified by their ability to induce ectopic bone formation (Urist, 1965), are members of the transforming growth factor-β (TGF-β) superfamily. TGF-βs and BMPs are involved in tissue morphogenesis and developmental processes and signal through cell surface serine/threonine-kinase receptors. These signals are transferred to the nucleus by the Smad family of proteins that interact with other transcription factors to regulate gene expression (reviewed in Derynck et al., 1998; Massagué and Wotton, 2000). Several BMPs are expressed during skeletogenesis and their targeted disruption generates abnormalities in bone, suggesting that BMPs have crucial roles in skeletal development and in embryonic development generally (reviewed in Hogan, 1996; Vortkamp, 1997). BMP-2, -4 and -7 [the latter is also known as osteogenic protein-1 (OP-1)] are potent osteo-inductive cytokines (Asahina et al., 1996; Katagiri et al., 1994; Wang et al., 1993) that induce osteogenic differentiation of pluripotent mesenchymal cell lines (such as C3H10T1/2 cells) and promote the maturation of osteoblastic progenitor cells (Katagiri et al., 1990; Yamaguchi et al., 1991; Yamaguchi et al., 2000). In the muscle satellite cell line C2C12, BMPs suppress expression of muscle genes including myogenin and MCK and stimulate the expression of genes involved in osteogenesis, such as *alkaline phosphatase* (*ALP*), *parathyroid-hormone/parathyroid-hormone-related protein receptor* (*PTHrp-R*), *osteocalcin* (Katagiri et al., 1994), *Runx2/Cbfa1* (Lee et al., 1999; Nakashima et al., 2002) and *Osx* (Nakashima et al., 2002).

Although both TGF-β and BMPs inhibit myogenic differentiation and stimulate the expression of *Runx2/Cbfa1*, only BMPs induce further osteogenic differentiation of myogenic cells (Katagiri et al., 1990; Katagiri et al., 1994; Katagiri et al., 1997; Lee et al., 1999). Interestingly, an initial transient increase in *MyoD* mRNA expression was observed when myogenic cells were treated with BMP-2 (Katagiri et al., 1994). Here, we report that MyoD is required for the efficient induction of the initial stages of osteogenesis by BMP in myogenic cells and that Runx2/Cbfa1 appears to be a target of MyoD activity.

Materials and Methods

Cell types

The C3H10T1/2 cell line was purchased from American Type Culture Collection (Rockville, Maryland, USA) and maintained according to the supplier's instructions. Muscle satellite cells were isolated from the hind limb muscles of 2-3 month old wild-type or $MyoD^{-/-}$ mice as previously described (Sabourin et al., 1999) and used between passages 4 and 9.

Differentiation conditions

Twenty-four hours after seeding 20,000 cells/cm^2, cultures were shifted to Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% horse serum (low mitogen medium), penicillin (1000 units/ml) and streptomycin (1000 μg/ml) (Life Technologies, Burlington, Ontario, Canada). Cells were then re-fed every 2 days (Sabourin et al., 1999). To induce osteogenic differentiation, the medium was further supplemented with recombinant human BMP-7 at 400 ng/ml (or as indicated in the figure legends). BMP-7 was kindly provided by K. Sampath (Creative Biomolecules, Hopkinton, MA, USA)

Plasmids and transfections

The following plasmids were used in this study: expression plasmids pEMSV-MyoD, pEMSV-MyoD-puro (containing a puromycin resistant cassette), pEMSV-Myf5 and pEMSV-myogenin and PGK-puro (all kindly provided by R. Perry, McMaster University, Ontario, Canada) (Perry et al., 2001); the β-galactosidase (β-Gal) vectors pCMV-β-Gal and pcDNA.3-nlacZ, which contains a nuclear localization signal, were used to normalize transcription assays and to identify transiently transfected cells, respectively; the reporter construct p6OSE2-luc and the Runx2/Osf2 expression vector encoding Runx2/Cbfa1 isoforms II and III were provided by G. Karsenty (Baylor College of Medicine, Texas); 4R-tk-luc was made by inserting the 4R-tk-fragment (Weintraub et al., 1990) into the pGL3-Basic vector (Promega, Fisher Canada); mouse osteocalcin promoter-Luc (–147/+13) (Ducy and Karsenty, 1995) was kindly provided by H. Harada (Sumitomo Pharmaceuticals Research Center, Osaka, Japan) (Harada et al., 1999). To transfect the cells, Lipfoectamine or Lipfectoamine 2000 (Life Technologies) were used as outlined in the manufacturer's instructions. For transient transfections, cells were incubated for 4 hours with a total of 2.4 μg plasmid (2 μg of expression plasmid plus 0.4 μg of β-Gal vector), and 20 μl of Lipofectamine in 2 ml of DMEM + 15% FBS (minus antibiotics) and then shifted to low mitogen medium with or without BMP-7. The cultures were analyzed 72 hours after transfection. Stable cell lines expressing MyoD were established from C3H10T1/2 cultures by co-transfecting the cells with pEMSV-MyoD and PGK-puro, followed by a selection with puromycin (4 ng/ml). Individual colonies were isolated and designated as 10TMD lines. For the control cell lines (10Tpuro), individual colonies were isolated from cells transfected with PGK-puro alone. The conditions for preparing a pool of $MyoD^{-/-}$ cells stably transfected with the MyoD expression vector have been described (Sabourin et al., 1999).

Immuno-histochemical and enzymatic assays

All incubations and washes were done at room temperature unless indicated otherwise. To detect Runx2/Cbfa1 expression, cells were fixed in 4% paraformaldehyde in PBS for 10 minutes and then incubated for 2 hours with a monoclonal anti-Cbfa1 antibody (provided by Y. Ito, Kyoto University, Japan) at 2 ng/ml in PBS-BSA. Secondary antibody incubation (1 hour) and color development (5 minutes) was done using Histofine Simple Stain MAX-PO kit (Nichirei, Japan) according to the manufacturer's instructions. The conditions used for the immunohistochemical detection of myosin heavy chain (MHC) (clone MF20, Developmental Studies Hybridoma Bank under auspices of the NICHD and maintained by the University of Iowa) and the detection of ALP activity at cellular level have been described (Katagiri et al., 1994; Sabourin et al., 1999). Anti-MyoD monoclonal antibody clone 5.8A (PharMingen, San Diego, California, USA) was used to confirm MyoD expression in the transfected cells according to published procedures (Dias et al., 1992). To stain cells for both β-Gal and ALP activity, cells were fixed for 3 minutes in 4% paraformaldehyde, washed twice with PBS and then incubated for 2 hours at 37°C with the X-Gal substrate solution (0.95 mg X-Gal/ml

of PBS containing 5 mM potassium ferricyanide [$K_3Fe(CN)_6$], 5 mM potassium ferrocyanide [$K_4Fe(CN)_6$] and 2 mM $MgCl_2$). Cultures were then washed once with PBS, once with water and then stained for ALP activity. Digital photographs of the stained cultures were captured using a SPOT digital camera (Diagnostic Instruments, USA). To estimate the extent of differentiation, three randomly selected areas per image were analyzed using NIH Image version 1.6 (NIH, USA). The results of two independent experiments were combined and the standard deviation was calculated. The total ALP activity in detergent extracts of cells was measured, with minor modifications, as described previously (Sodek and Berkman, 1987). The absorbance at 405 nm was measured after a 20 minute incubation at 37°C. Protein concentration was determined using DC Protein Assay kit (BioRad-Laboratories, Canada) and the results expressed as $OD_{405\ nm}$/mg protein/minute.

RNA isolation and reverse transcriptase (RT)-PCR

Total RNA was isolated using Trizol (Life Technologies) according to the supplier's instructions. cDNA templates were prepared from aliquots of total RNA using MuLV-RT (Applied Biosystems, Foster City, California) and the targets amplified using Gibco Taq polymerase (Life Technologies, Canada). To control for genomic contamination, parallel aliquots of RNA were incubated without reverse transcriptase (RT) and subjected to PCR amplification. PCR conditions for each target gene (Table 1) were checked to ensure that the cycle number was not within the plateau region of the amplification curve. PCR products were analyzed by electrophoresis on 2% agarose gels in TRIS-borate-EDTA buffer containing ethidium bromide.

Luciferase reporter gene assays

Near confluent cultures of C3H10T1/2 cells in 24-well multicluster plates were incubated for 4 hours with 0.55 ml of serum-free OPTI-MEM (Life Technologies, Canada), containing the reporter plasmid (0.2 μg), expression plasmid or corresponding empty vector (0.3 μg), pCMV-β-Gal (0.1 μg) and 1 μl of Lipofectamine 2000, followed by an additional 12 hours in growth medium. The cultures were then shifted to low mitogen medium with or without BMP-7 (400 ng/ml) and incubated a further 48 hours before harvesting the cells for analyses of β-Gal and luciferase activity using Galacto-Light Plus™ (Tropix, Bedford, MA, USA) and Luciferase assay systems (Promega, Fisher, Canada), respectively. Luciferase activity was normalized to β-Gal activity to correct for differences in transfection efficiency. Each test condition was done in triplicate and experiments were repeated at least three times. The conditions used for the analyses of (–147/+13) osteocalcin-luciferase promoter were as described above with the exception that pCMV-β-Gal was replaced with pRL-SV40 (0.015 μg) and the luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega, Fisher, Canada). *Renilla* luciferase activity from pRL-SV40 was used to correct for differences in transfection efficiency.

Results

MyoD$^{-/-}$ cells have a decreased osteogenic response to BMP-7

In low mitogen medium, cultures of wild-type and MyoD$^{-/-}$ primary muscle satellite cells differentiated into myotubes (Fig. 1). Under the higher seeding densities used in this study, the delayed myogenic differentiation of MyoD$^{-/-}$ cells (Sabourin et al., 1999) was partially overcome. This allowed sufficient myotube formation in both cell types after a 3-day incubation in low mitogen medium to directly compare the effects of BMP treatment on cell differentiation. Inclusion of BMP-7 (100 or 400 ng/ml) inhibited myogenic differentiation of both wild-type and MyoD$^{-/-}$ cells, as reflected in the decreased number of cells expressing MHC (Fig. 1, brown/black staining). Based on estimates of the relative area covered by MHC-positive cells, BMP-7 appeared to be more potent at inhibiting myogenesis in MyoD$^{-/-}$ satellite cells compared with the wild-type cells (Fig. 1).

Contrasting the enhanced inhibition of myogenesis in BMP-7-treated MyoD$^{-/-}$ cultures, the analyses of three independent preparations of cells showed that BMP-7 was considerably less effective at inducing osteogenesis in MyoD$^{-/-}$ cultures when compared with wild-type cultures. Thus, when cultures were stained for ALP activity (that is a marker for early stages of osteogenesis), BMP-7 induced ALP expression in a large number of wild-type cells in both a dose- (Fig. 1) and time-dependent (Fig. 2) manner. By contrast, few cells of the MyoD$^{-/-}$ cultures were induced to express ALP. Consistent with the staining pattern, measurement of ALP activity in cell extracts revealed a dose-dependent increase in wild-type cells that were incubated with BMP-7, whereas a minimal induction was seen in extracts prepared from MyoD$^{-/-}$ cultures (Fig. 1). Moreover, although exposure of wild-type cells to BMP-7 during the last day of a 6-day culture period in differentiation medium was sufficient to induce ALP activity in many of them, few MyoD$^{-/-}$ cells were positive for ALP activity even after a 6-day incubation with BMP-7 (Fig. 2).

Table 1. PCR targets and conditions

Group	Target	Cycles	°C	Region	Accession number	Source
Muscle-associated	MyoD	25	63	386-831	M84918	This study
	Myf5	30	60			Borycki et al., 1999
	Myogenin	25	60			Cornelison and Wold, 1997
	Id1	28	54			Hernandez et al., 1996
	AchR	20	60			Rohwedel et al., 1995
Bone-associated	Runx2/Osf2	50	60	1-1017	AF010284	Harada et al., 1999*
	Osx	30	60	2408-2858	AF184902	This study
	ALP	30	54			Studer et al., 1991
	PTH/PTHrp-R	32	52	558-965	X78936	This study
	Osteocalcin	40	59			Fleet and Hock, 1994
	Osteopontin	30	62			Sodek et al., 1995
Housekeeping	β-actin	25	59	381-820	X03672	This study

*5′-primer from Harada et al. (Harada et al., 1999). PCR-buffer contained 10% DMSO.

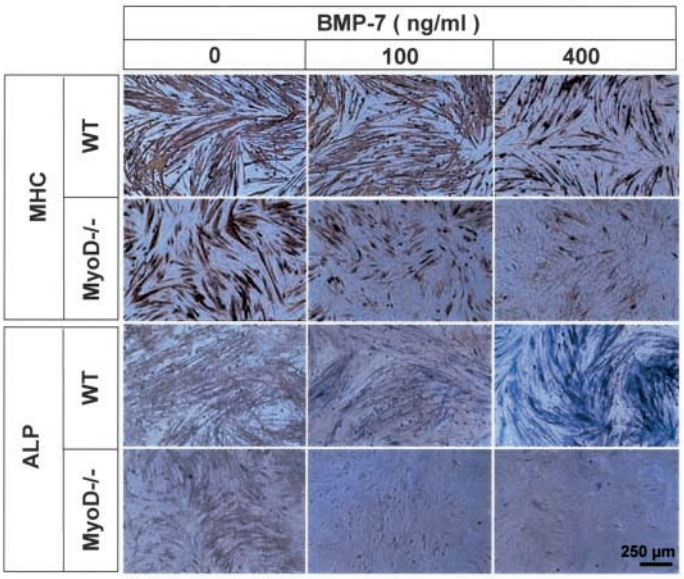
To determine whether the reduction in ALP activity reflected a suppression of BMP-induced osteogenic differentiation in Myo$^{-/-}$ cells, total RNA was prepared from cultures that were incubated for 3 days in low-mitogen medium with (+3) and without (–3) BMP-7 at 400 ng/ml, and from cultures not exposed to low-mitogen medium (day 0). Samples were analyzed by RT-PCR for the expression of selected genes associated with osteogenic and myogenic differentiation. Expression of the housekeeping gene *β-actin* was also analyzed as an internal control to provide a semi-quantitative analysis. At least three independent preparations of cells were analyzed to confirm reproducibility of the observed trends.

Expression of muscle-associated genes

As expected, *MyoD* mRNA was only detected in wild-type cells and the signal increased under differentiation conditions (Fig. 3 upper panel). Other muscle-associated genes, such as *Myf5*, *myogenin* and *AchR*, present in both MyoD$^{-/-}$ and wild-type cells, were also upregulated under myogenic differentiation conditions. Notably, whereas the constitutive levels of *Myf5* were elevated in MyoD$^{-/-}$ cells (Rudnicki et al., 1993; Sabourin et al., 1999) (Fig. 3), mRNA levels for *myogenin* and *AchR* (that are expressed later than MyoD during myogenesis) were decreased relative to wild-type cells. Consistent with its ability to suppress myogenesis, BMP-7 decreased the expression of *myogenin* and *AchR* in both cell types. However, a 3-day incubation with BMP-7 increased *Myf5* expression in wild-type cells, whereas *MyoD* appeared to be unaffected. Although *Id1* (a dominant-negative regulator of bHLH transcription factors such as MyoD), was only detected in wild-type cultures treated with BMP-7, MyoD$^{-/-}$ cells that were maintained in growth medium (day 0) or cultured with BMP-7 also expressed *Id1*, albeit at lower levels than in cultures maintained under myogenic differentiation conditions.

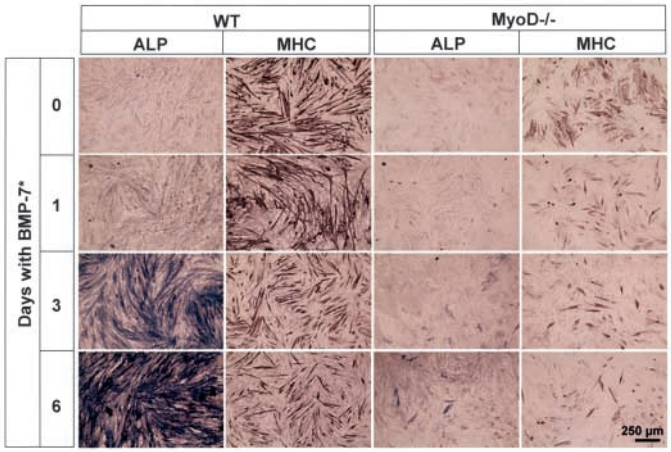
Expression of bone-associated genes

The bone-associated genes, *ALP*, *Runx2/Cbfa1*, *PTHrp-R* and *osteocalcin*, were expressed in wild-type and MyoD$^{-/-}$ satellite cell cultures that were incubated for 3 days in low mitogen medium containing BMP-7 (Fig. 3 upper panel). However, consistent with the decreased osteogenic differentiation of MyoD$^{-/-}$ cultures incubated with BMP-7, the levels of *ALP*, *osteocalcin* and *Runx2/Cbfa1* mRNAs were clearly much lower than in wild-type cell cultures. The decrease in *ALP* mRNA is consistent with the observed decrease in enzyme activity (Figs 1, 2). Notably, wild- type cells showed a marked induction of *Osx* whereas in MyoD$^{-/-}$ cells only a faint signal was detected (Fig. 3 lower panel). Curiously, low levels of *osteocalcin* mRNA were detected in day-0 wild-type cultures and in wild-type and MyoD$^{-/-}$cultures after 3 days in low mitogen medium.



		BMP-7 (ng / ml)		
		0	100	400
MHC	WT	33.3 ± 1.03	31.8 ± 4.80	19.8 ± 1.33
	MD-/-	30.1 ± 2.07	16.9 ± 1.09	8.51 ± 0.76
ALP	WT	5.75 ± 1.90	46.7 ± 3.38	264 ± 26.9
	MD-/-	3.82 ± 1.19	1.44 ± 0.26	6.96 ± 1.06

Fig. 1. Dose-response effects of BMP-7 on muscle satellite cell differentiation. Primary muscle satellite cells derived from either wild-type (WT) or MyoD$^{-/-}$ mice were treated for 3 days with 0, 100 or 400 ng/ml of BMP-7. Upper panel, cultures stained for MHC (brown) or ALP activity (blue). Lower panel, the percentage area of MHC-positive cells was measured using NIH image software (n=6). Cell lysates from parallel cultures were assayed for total ALP activity using p-nitrophenylphosphate as a substrate. The ALP activity is expressed as $OD_{405\ nm}$/min/mg protein±s.d. (n=3). Scale bar, 250 µm.



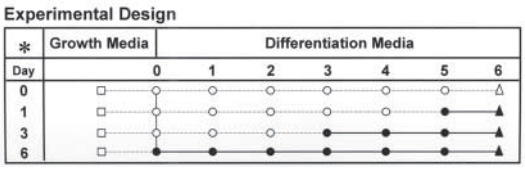


Fig. 2. Temporal effects of BMP-7 on muscle satellite cell differentiation. Twenty-four hours after seeding, at 2×10^4/cm^2, cultures of primary muscle satellite cells were shifted to differentiation media (day 0, d0). BMP-7 (400 ng/ml) was added at various times over the 6-day culture period as indicated by the solid line in the outline of the experimental design. At the end of 6 days, the cultures were stained for the expression of MHC (black/brown) or for ALP activity (blue). Results from a representative experiment are shown in the upper panel. Scale bar, 250 µm.

Whereas MyoD$^{-/-}$ cells did not express *osteocalcin* under growth conditions (day 0), *osteocalcin* expression was induced upon BMP-7 treatment and was increased further after 6 days of incubation, although not to the same level as that seen in wild-type cells (data not shown). Notably, the transcription factor *Runx2/Cbfa1* was detected in MyoD$^{-/-}$ cells that were maintained in growth medium, but not in the wild-type cells. However, expression of *Runx2/Cbfa1* in MyoD$^{-/-}$ cells was lost following a 3-day incubation in low mitogen medium without BMP-7. Moreover, the PCR primers used to amplify *Runx2/Cbfa1* generated two products (1017 bp and 630 bp) in MyoD$^{-/-}$ cells. Restriction enzyme analyses (data not shown) confirmed that the smaller product was an alternatively spliced version of *Runx2/Cbfa1* lacking exon 1 (262-648 nt) (Xiao et al., 1998). In cultures treated with BMP-7 the intensity of the larger product, corresponding to the major amplicon in the wild-type cells, decreased significantly whereas the smaller product was induced. Under growth conditions, the mRNA of *osteopontin* (*OPN*), a multifunctional protein that is highly expressed by osteogenic cells but is not specific to bone (Sodek et al., 2000), was produced at higher levels by wild-type cultures than by MyoD$^{-/-}$ cultures. Although incubation in low-mitogen medium increased the relative levels of *OPN* mRNA in MyoD$^{-/-}$ cultures, the increase was lower for BMP-treated cultures. In comparison, *OPN* mRNA levels in wild-type cultures were not altered when the cells were cultured under either of the differentiation conditions.

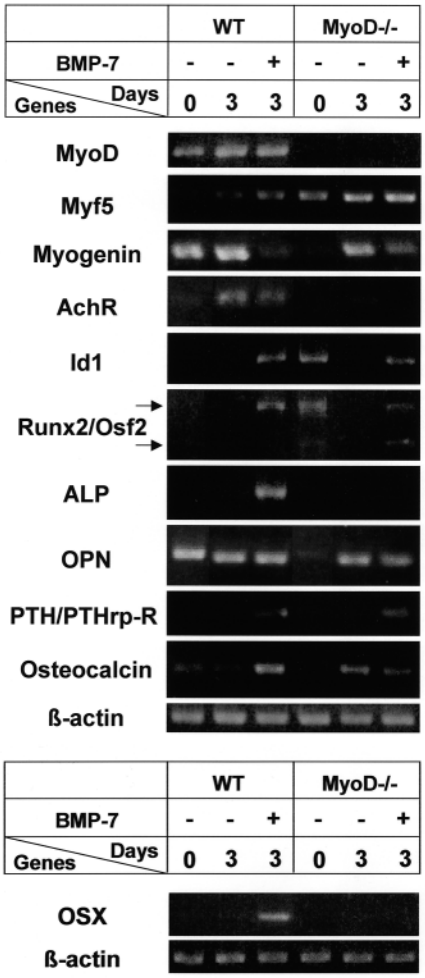


Fig. 3. RT-PCR analyses of muscle- and bone-associated genes. Upper panel, total RNA was purified from MyoD$^{-/-}$ and WT cells either before shifting them to differentiation media (day 0) or after a 3-day treatment with or without BMP-7 (400 ng/ml). Target mRNAs were analyzed by RT-PCR. β-Actin levels indicate that similar amounts of RNA were used for each time point. Arrows indicate *Runx2* transcripts. Lower panel, expression of *Osx* and the housekeeping gene *β-actin* in a second cDNA synthesis from the RNA used in the upper panel.

Ectopic expression of MyoD increases osteogenic response to BMP-7

PCR analyses indicated that wild type and MyoD$^{-/-}$ cells expressed the same profile of type I and II receptors for BMP-7 (data not shown), thus eliminating the absence of receptors as the source of the difference in responsiveness of the cell types. We therefore determined whether re-introducing MyoD into MyoD$^{-/-}$ cells affects the osteogenic response to BMP-7. Immunohistochemical analyses showed that MyoD was expressed by more than 70% of the final pool of stably-transfected cells (Fig. 4D insert). Cultures of wild-type and MyoD$^{-/-}$ cells, and MyoD$^{-/-}$ cells transfected with either the MyoD expression-vector or the empty-vector, were treated for 3 days with BMP-7 (400 ng/ml) and then double-stained for MHC expression (brown/black staining) and ALP activity (blue staining) (Fig. 4). Whereas BMP-7 treatment decreased myotube formation in all cell cultures (indicated by the decrease in MHC staining and the change in morphological appearance of the cultures) transfection of MyoD$^{-/-}$ cells with the MyoD expression vector (Fig. 4D), but not the empty vector (Fig. 4C), markedly increased the number of cells

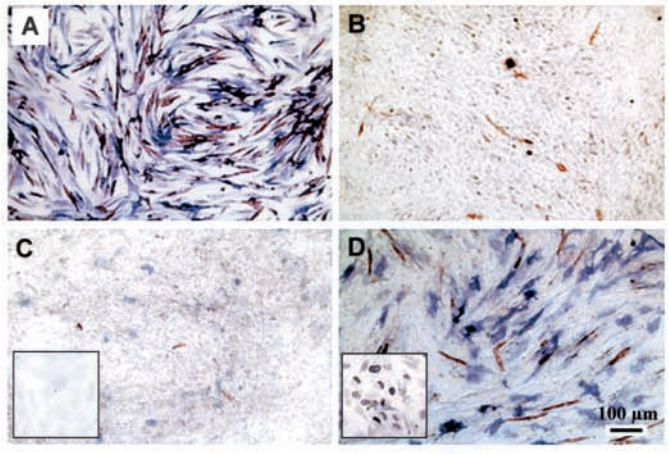


Fig. 4. Transfection with MyoD expression vector partially rescues the osteogenic response of MyoD$^{-/-}$ cells to BMP-7. The phenotype of MyoD$^{-/-}$ cells, stably transfected with either a MyoD-expression vector or its empty vector, was compared to wild-type cells after a 3-day incubation with BMP-7 (400 ng/ml). Cultures were double-stained for MHC expression (brown/black) and ALP activity (blue). Wild-type cells (A), MyoD$^{-/-}$ cells (B), MyoD$^{-/-}$ cells transfected with PGK-puro (C) or pEMSV-MyoD/PGK-puro vectors (D). Immunostaining analysis of the transfected cultures shows that only the MyoD transfected cultures are positive for MyoD expression (compare insets in C and D). Scale bar, 100 µm.

staining for ALP activity following induction by BMP-7. Notably, MyoD transfection also restored the ability of BMP-7 to induce the expression of *Osx* mRNA (data not shown).

To further investigate the involvement of MyoD in BMP-7-induced oosteogenesis of myogenic cells, the pluripotent murine mesenchymal cell line C3H10T1/2, which does not express MyoD constitutively, was transfected with either a MyoD expression-vector or a control vector. BMP-7 induced markedly higher levels of ALP activity (2.7- and 4.7-fold at 3 and 6 days, respectively) in cultures transiently transfected with the MyoD expression-vector compared with empty vector controls (Fig. 5A). After a 3-day incubation with BMP-7, double-staining of the transfected cultures for ALP and β-Gal activities, the latter being a marker of transfected cells, revealed larger numbers of double-positive cells in cultures co-transfected with the MyoD-expression vector (Fig. 5B). The intensity of ALP staining also tended to be higher than that observed in non-transfected cells. Similar trends were seen in three other independent transfections.

To study the enhanced osteogenic response in more detail, stable cell lines expressing MyoD (10TMD) were prepared from C3H10T1/2 cells by co-transfecting the cells with a MyoD expression-vector and a puromycin-resistance vector whereas control cell lines (10Tpuro) were isolated from cultures transfected with only the puromycin-resistant vector. Multiple stable clones were isolated from each transfection and initially screened for their ability to form myotubes (10TMD) in the absence of BMP, or to express ALP (10Tpuro) in response to BMP treatment. Two 10Tpuro cell lines (10Tpuro-9, 10Tpuro-13) that had a BMP-7-induced ALP activity comparable to that of the parental cells, were selected for further analyses. Of three 10TMD cell lines chosen for further analyses, two cell lines (10TMD-9 and -12) differentiated into thick, elongated multinucleated myotubes under conditions of myogenic differentiation whereas the third cell line, 10TMD-18, formed fewer and thinner myotubes (Fig. 6A, Control). BMP-7 treatment decreased myogenic conversion of the 10TMD cell lines, as reflected by decreased MyoD staining. However, BMP-7 consistently induced higher ALP activity in the 10TMD cell lines compared to the 10Tpuro cell lines (Fig. 6A, BMP), indicating that MyoD expression increased the early osteogenic response of these cells to BMP. Interestingly, the 10TMD-18 cell line showed a lower level of ALP induction relative to the other two 10TMD cell lines, suggesting a possible correlation between levels of functional MyoD and the degree of responsiveness of cultures to BMP-induced osteogenesis.

To examine the gene expression profiles of these cell lines, total RNA prepared from 10Tpuro-13 and 10TMD-12 cultures after a 3-day incubation in low mitogen medium, with or without BMP-7, was analyzed by RT PCR for the expression of *MyoD*, *AchR*, *ALP*, *Runx2/Osf2*, *osteocalcin*, and β-*actin* mRNAs. As expected, 10Tpuro-13 cultures were negative for the myogenic markers (*MyoD* and *AchR*) under all culture conditions. However, mRNAs for both markers were expressed in 10TMD-12 cultures incubated for 3 days in low mitogen medium and their expression was markedly decreased by incubation with BMP-7 (Fig. 6B). Higher levels of *ALP* mRNA were detected in 10TMD-12 cultures treated with BMP-7 relative to 10Tpuro-13 cultures. This was consistent with the results of the analyses of total ALP

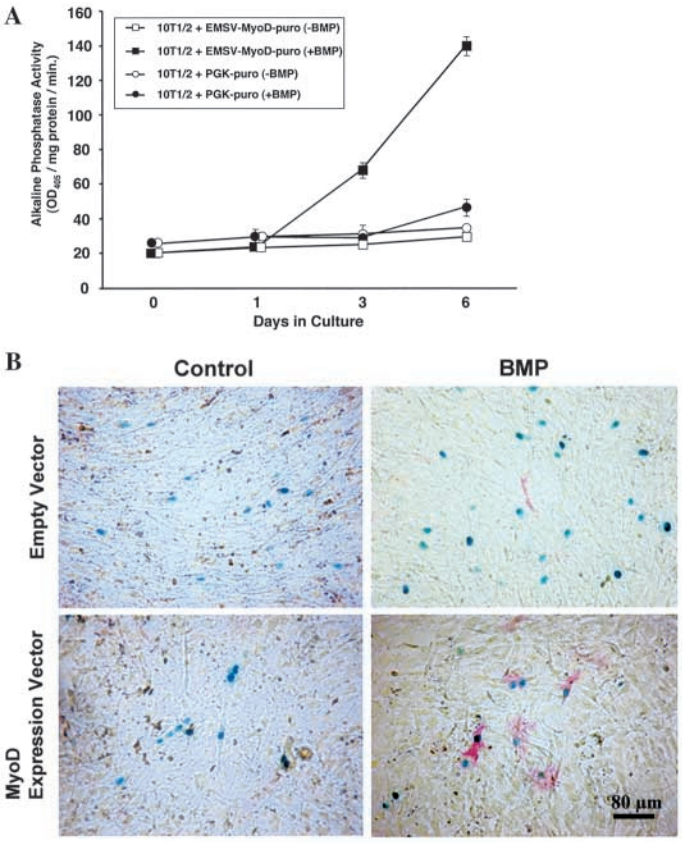


Fig. 5. Transient transfection with pMyoD increases ALP activity in C3H10T1/2 cells. (A) C3H10T1/2 cells were transiently transfected with MyoD expression vector (pEMSV-MyoD/PGK-puro) and empty vector (PGK-puro) and treated for 0, 1, 3 and 6 days with or without BMP-7 (400 ng/ml). ALP activity in the cell lysate was measured using p-nitrophenylphosphate as a substrate. An increase in BMP-7 induced ALP activity in MyoD transfected cultures above that induced in the control cultures, was evident after 3 days and was further increased at 6 days post-transfection. (B) Representative cultures of transfected cells were double-stained for β-Gal (green) and for ALP activity (red) after a 3-day incubation in differentiation media with or without BMP-7 (400 ng/ml). β-Gal staining was used to identify the transfected cells independently of any response to BMP-7 treatment. Double-stained cells are evident in the cultures transfected with MyoD and treated with BMP-7. Scale bar, 80 μm.

activity, showing that BMP-7 induced higher activity in 10TMD-12 compared to 10Tpuro-13 cultures (78.5±4.05 versus 19.9±0.57 OD_{450nm}/min/mg protein, n=3). BMP-7 treatment also stimulated *Runx2/Cbfa1* expression in 10TMD12 cells. However, little or no *osteocalcin* signal was detected, possibly due to the continuous expression of MyoD blocking further osteogenic differentiation. By contrast, the 10Tpuro-13 cultures had elevated *Runx2/Cbfa1* in the presence and absence of BMP treatment and a detectable *osteocalcin* signal after a 3-day incubation with BMP-7.

MyoD enhances osteogenic differentiation induced by Runx2/Cbfa1

Before examining in more detail whether Runx2/Cbfa1, which is required for osteogenic differentiation (Karsenty et al.,

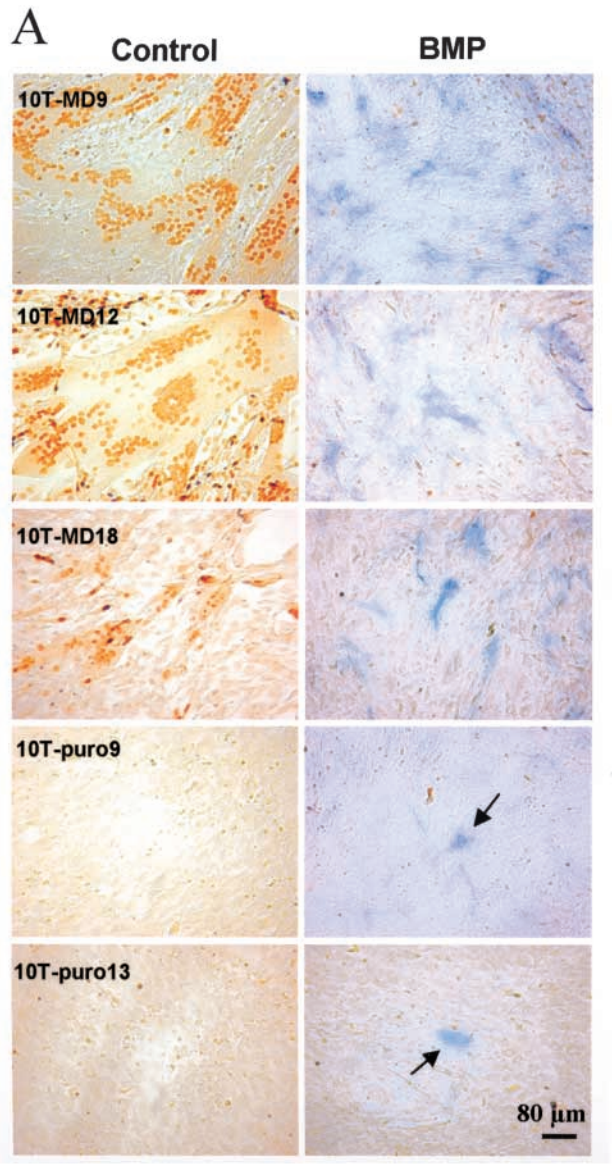


Fig. 6. Stable transfection of MyoD enhances osteogenic response of C3H10T1/2 cells to BMP. (A) Isolated clones of C3H10T1/2 cells, stably transfected with either MyoD-expression vector (10T-MD) or its empty vector (10T-puro), were cultured for 3 days in differentiation media with or without BMP-7 (400 ng/ml) and then double-stained for MyoD expression (black/brown) and ALP activity (blue). MyoD was only detected in the 10TMD lines incubated without BMP. BMP-treatment decreases MyoD expression and increases ALP-positive cells. More ALP-positive cells are evident in 10TMD cell lines than in the 10Tpuro cell lines (arrows). Scale bar, 80 µm. (B) RT-PCR analyses of selected genes in clonal cell lines incubated for 3 days with or without BMP-7 (400 ng/ml). BMP-treatment decreased the expression of MyoD and AchR in 10TMD-12 and increased the expression of ALP, consistent with the inhibition of myogenesis and the stimulation of osteogenesis. Higher ALP activity (A, BMP) is reflected in the increased *ALP* mRNA in the MyoD-transfected culture incubated with BMP-7.

1999), is involved in the effect of MyoD on BMP-7-induced osteogenesis, we confirmed that Runx2/Cbfa1 is expressed in C3H10T1/2 cultures because the expression of this protein has been shown to be under translational control (Sudhakar et al., 2001). Whereas Runx2/Cbfa1 was not detected in cultures at day zero (D0), protein was expressed after 3 days in low mitogen media with or without BMP-7 treatment (Fig. 7B, and data not shown). Thus, low mitogen media appears to release a translational block. Interestingly, BMP7-treatment did not dramatically increase staining intensity relative to untreated cultures (data not shown), yet only BMP-treated cultures showed increased ALP-activity (Fig. 5) suggesting that whereas overexpression of Runx2/Cbfa1 supports osteogenic differentiaion (see below) (Ducy et al., 1997; Harada et al., 1999), the endogenous levels alone are insufficient to do so.

The effect of overexpressing Runx2/Cbfa1 and MyoD on ALP activity was examined by transiently transfecting C3H10T1/2 cells with various combinations of these expression vectors or the corresponding empty vectors. All cultures were also transfected with a β-Gal-expression vector to identify the transfected cells. Whereas transfection with the empty vectors did not affect ALP activity, transfection with the Runx2/Cbfa1 vector induced a weak but detectable level of ALP expression in β-Gal-positive cells in the cultures maintained for 3 days in low-mitogen medium (Fig. 7A). This observation was consistent with previous reports of Runx2/Cbfa1 stimulating osteogenesis in C3H10T1/2 cells (Ducy et al., 1997; Harada et al., 1999). Whereas the number and/or staining intensity of ALP-positive cells tended to be higher in cultures co-transfected with Runx2/Cbfa1 and MyoD expression vectors relative to cultures transfected with Runx2/Cbfa1 vector alone, the difference did not reach statistical significance (data not shown) suggesting again that activation of the BMP-signaling pathway is required to maximize the ALP-response.

A series of more sensitive transcription assays were used to test for the possible interaction between Runx2/Cbfa1 and MyoD. The reporter plasmid 4R-tk-luc, containing four repeats

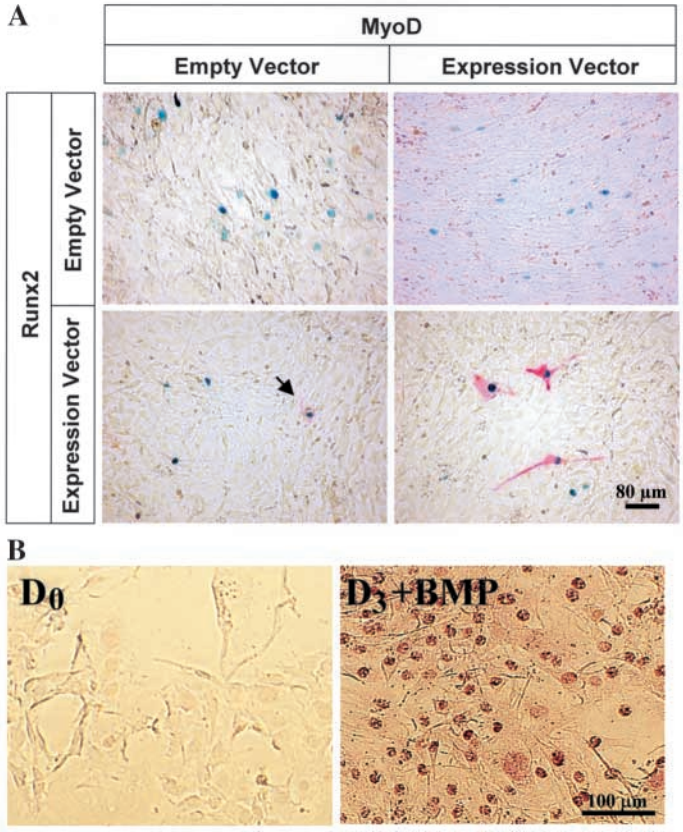


Fig. 7. Effect of co-transfection of MyoD with Runx2/Cbfa1 on ALP activity. (A) C3H10T1/2 cells were transiently transfected with MyoD- or Runx2/Cbfa1-expression vectors alone or in combination. Co-transfection with β-Gal-expression vector allowed identification of transfected cells. Cultures were double-stained for β-Gal and ALP activity after a 3-day incubation in differentiation media. MyoD and Runx2/Cbfa1 transfected cells tended to show more double-stained cells than cells transfected with Runx2/Cbfa1 alone (arrow in A); however, the difference did not reach statistical significance. (B) Immunohistochemical analyses of Runx2/Cbfa1 expression in C3H10T1/2 cells at day 0 and after 3 days in low mitogen media containing 400 ng/ml of BMP-7, revealed protein expression in BMP-7 treated cultures.

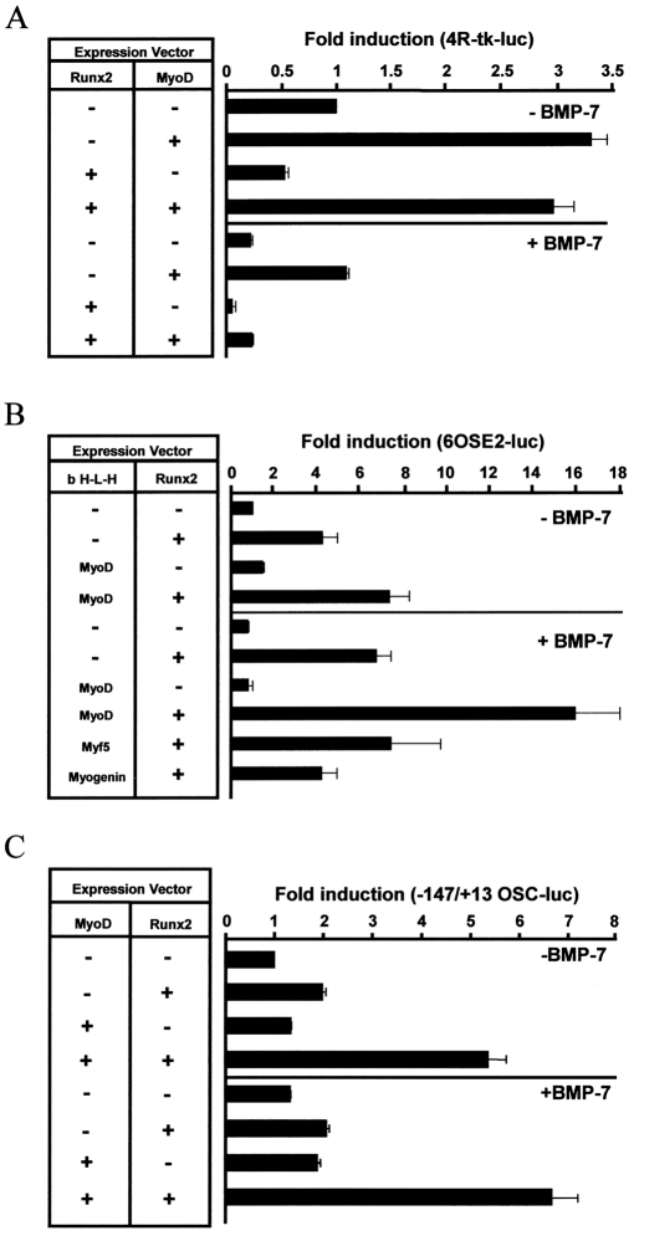


Fig. 8. MyoD affects transcriptional activity of Runx2/Cbfa1. (A) The effect of Runx2/Cbfa1 on MyoD transcriptional activity was determined by using the 4Rtk-luc reporter construct transiently transfected into C3H10T1/2 cells. Luciferase activity in extracts of cultures incubated for 48 hours with or without BMP-7 (400 ng/ml) was normalized to β-Gal activity to correct for transfection efficiency. The results are expressed relative to the activity in cultures transfected with empty vectors and incubated without BMP-7. Each transfection was done in triplicate. In the absence of BMP-7, Runx2/Cbfa1 has little effect on MyoD transactivation. However in the presence of BMP-7 the transactivation was decreased, consistent with the inhibition of myogenesis. (B) The effect of bHLH-factors on Runx2/Cbfa1 transcriptional activity was determined using the p6OSE-luc reporter construct as described in A. MyoD transfection increased the Runx2/Cbfa1 transactivation and the effect was markedly increased by incubating the transfected cultures with BMP-7. None of the other bHLH factors could synergize with Runx2/Cbfa1 to increase luciferase expression. (C) The effect of MyoD on transactivation of a reporter construct containing the –147/+13 fragment of the osteocalcin promoter.

of the E-box enhancer from the *MCK* gene (Weintraub et al., 1990), was first used to monitor the effect of Runx2/Cbfa1 on the transcriptional activity of MyoD. Forced expression of Runx2/Cbfa1 caused only a slight suppression of MyoD-induced transcription of 4R-tk-luc in transiently-transfected cells incubated for 2 days in BMP-free low mitogen medium (Fig. 8A, –BMP-7). Although there was a decrease in the overall activity relative to the control samples in cultures treated with BMP-7 (400 ng/ml), MyoD transfection alone was still able to increase luciferase expression. However, co-transfection of the cells with MyoD and Runx2/Cbfa1 expression vectors, followed by a 2-day incubation in low mitogen medium containing BMP-7, markedly suppressed MyoD transcriptional activity (Fig. 8A, BMP-7). This suppression was in contrast with the results obtained in BMP-free medium.

The effect of MyoD on the transcriptional activity of Runx2/Cbfa1 was assessed using the reporter plasmid p6OSE2-luc

containing six repeats of the Runx2/Cbfa1 binding region from the *osteocalcin* promoter (Ducy et al., 1997). As expected, transfection with Runx2/Cbfa1 expression vector alone increased luciferase activity from the p6OSE2 reporter construct. In comparison, the MyoD expression vector alone had little effect on luciferase activity in cells cultured in either the absence (Fig. 8B, –BMP-7) or presence (Fig. 8B, BMP-7) of BMP-7. However, when both expression vectors were co-transfected into C3H10T1/2 cells, MyoD further increased the transcriptional activity seen after the transfection with Runx2/Cbfa1 expression vector alone. This co-operative enhancement was more pronounced in cultures treated with BMP-7. That the enhanced transcriptional activity was selective for MyoD was indicated by the lack of a response in cells transfected with expression vectors for other myogenic bHLH transcription factors (e.g. Myf5 or myogenin) (Fig. 8B), even though these factors had potencies similar to MyoD in transactivating the 4R-tk-luc construct (data not shown). Notably, bHLH factors involved in neurogenesis (NeuroD1, NeuroD2, Neurogenin/NeuroD3) (Lee et al., 1995; Ma et al., 1996; McCormick et al., 1996) were also unable to enhance the transcriptional activity of p6OSE2 (M.K., unpublished), further suggesting that the co-operative effects on Runx2/Cbfa1-mediated transcription are selective for MyoD.

To determine whether MyoD can exert its effects in the context of a natural promoter, transcription assays were repeated using a *luciferase* reporter construct containing the (–147/+13) region of the *osteocalcin* promoter. This fragment contains a single OSE site and has been shown to be responsive to Runx2/Cbfa1 (Harada et al., 1999). Co-transfection with MyoD and Runx2/Cbfa1 expression vectors resulted in significantly higher transcriptional activity than did transfections with the individual expression vectors (Fig. 8C), supporting the co-operative effects of these transcription factors.

Discussion

Although the role of bHLH transcription factors in regulating myogenic differentiation has been extensively studied, little is known about the involvement of these proteins in osteogenic differentiation. Our study suggests that the transient stimulation in the expression of *MyoD* mRNA (Katagiri et al., 1994) and protein (M.K., unpublished) that occurs after BMP treatment of myogenic cells are an integral parts of the osteogenic differentiation of these cells, because the ability of BMP-7 to induce differentiation is markedly impaired in muscle satellite cells derived from MyoD$^{-/-}$ mice. Importantly, this inductive activity of MyoD is restored and enhanced by forced expression in MyoD$^{-/-}$ cells and in pluripotent C3H10T1/2 cells. Because both *MyoD* and the master gene in osteogenesis, *Runx2/Cbfa1*, are induced following BMP stimulation, the interactive effects of these transcription factors were also studied. Whereas Runx2/Cbfa1 suppressed MyoD-mediated transcription, particularly in the presence of BMP, MyoD enhanced Runx2/Cbfa1-mediated transcriptional regulation through an OSE. These results suggest that MyoD might act co-operatively with Runx2/Cbfa1 in promoting osteogenic differentiation of myogenic cells.

Whereas the results from the studies of MyoD$^{-/-}$ primary satellite cells strongly suggest a role for MyoD in BMP-induced osteogenesis, the gene expression profiles of wild-type and MyoD$^{-/-}$ cultures revealed several factors, in addition to the lack of MyoD, which might contribute to the delayed or decreased osteogenic response of the Myo D$^{-/-}$ cells to BMP treatment. For example, elevated levels of the potent mitogen insulin-like growth factor 1 (IGF1) (Sabourin et al., 1999) could delay differentiation by stimulating cell proliferation (reviewed in Hayden et al., 1995; Seale and Rudnicki, 2000). Similarly, increased basal levels of Id1 (Fig. 3) could also contribute to the observed delay because overexpression of Id1 inhibits both myogenic (Jen et al., 1992; Neuhold and Wold, 1993) and osteogenic (Murray et al., 1992) differentiation. Interpreting the role of Id1, however, is complicated by the observation that BMP-7 stimulated osteogenic differentiation of the wild-type cultures, even though it also increased the expression of Id1 (Fig. 3A). BMP-7 treatment also increased *Id1* levels in the Myo D$^{-/-}$ cells (Fig. 3A) consistent with the ability of BMPs to stimulate Id expression in various cell types (Clement et al., 2000; Hollnagel et al., 1999; Katagiri et al., 1994; Lee et al., 2000). Whether the myogenic pathway is more sensitive to the inhibitory effects of Id1 than is the osteogenic pathway is unknown. Finally, the inability of BMP-7 to cause a significant increase in *Osx* expression in the knock-out cells is likely to be a major factor in the poor osteogenic response of these cells to BMP-treatment. Whereas the mechanism for the MyoD effect on *Osx* expression is beyond the scope of this study, it is important to note that re-introduction of MyoD into the knock-out cells restored the expression of *Osx* suggesting that MyoD or a bHLH factor regulates *Osx* expression.

To confirm that MyoD can support BMP-induced osteogenic commitment in non-myogenic cells, MyoD was expressed in the pluripotential C3H10T1/2 cell line. Because BMP-treatment stimulates osteogenic differentiation of this cell line (Ducy et al., 1997; Katagiri et al., 1990) and this line does not normally express MyoD (Taylor and Jones, 1979), the effect of introducing exogenous MyoD on BMP-induced osteogenesis could be more easily addressed. Forced expression of MyoD in C3H10T1/2 cells induces myogenic differentiation (Katagiri et al., 1997) and, despite constitutive expression of exogenous MyoD, myogenic differentiation is inhibited by BMP treatment (Katagiri et al., 1997) (Fig. 6). Moreover, the early osteogenic response, measured by relative ALP activity, was enhanced in the transfected cultures (Fig. 5, 6) suggesting that MyoD directly influences the BMP-induced osteogenic commitment. This is further supported by the increased transactivation of OSE2-containing reporter constructs in cells co-transfected with MyoD and Runx2/Cbfa1. That BMP-7 treatment augments this increase in transactivation is indicative of an additional level of regulation, probably reflecting the involvement of Smad activation.

The mechanism by which MyoD enhances Runx2/Cbfa1 transactivation is currently under investigation. Since the p60SE2-luc construct does not contain an E-box, MyoD cannot be affecting transcription by binding directly to the promoter. The results are not an anomaly associated with a multimerized OSE, as similar results were obtained using the –147/+13 fragment of the osteocalcin promoter with only one OSE2 site. Whereas MyoD could enhance transactivation by direct interaction with Runx2/Cbfa1 to produce a more stable complex or a more favorable structural conformation, neither electrophoretic mobility shift assays nor immunoprecipitation

experiments have convincingly demonstrated any direct binding of these two proteins (M.K., unpublished). Whereas the interaction might be too weak to be maintained under the in vitro assay conditions, it is also possible that additional cofactors are involved, or that MyoD sequesters an inhibitory protein such as the bHLH factor HES1, which was shown to bind to Runx proteins and regulate transcription (McLarren et al., 2000; McLarren et al., 2001). Indeed, both MyoD and Runx2/Cbfa1 were shown to interact with other transcription factors and co-factors that can affect their transactivating abilities (reviewed in Ducy, 2000; Ito, 1999; Perry and Rudnick, 2000). In particular, reports that Runx family members bind to Smads (Hanai et al., 1999; Massagué and Wotton, 2000; Zhang et al., 2000) are relevant to our observation that BMP treatment further increases the transactivation of the OSE-containing constructs in cultures transfected with MyoD.

Whereas the current study has focused on the transactivating effects of the type II/III isoforms of Runx2/Cbfa1, which are rapidly induced by BMP treatment of myogenic cells (Lee et al., 2000), myogenic cells also express the type I isoform (Lee et al., 2000; Banerjee et al., 2001). Notably, preliminary studies indicate that myoD will also augment transactivation mediated by the type I isoform (M.K. and S.C., unpublished). Although the early gene targets of Runx2/Cbfa1 during osteoblastic differentiation are unknown, our results suggest that the presence of Runx2/Cbfa1 in myogenic cells, in conjunction with MyoD, will enhance the osteogenic response to BMP.

An intriguing question raised by this study is, whether bHLH transcription factors are generally involved in BMP-induced osteogenesis (indicated by MyoD effects in C3H10T1/2 cells) or whether this is unique to osteoblastic differentiation of myogenic cells. If bHLH proteins are more generally involved, what is the nature of the protein and what characteristics might it share with MyoD? A role for bHLH transcription factors in regulating osteogenic differentiation is supported by the demonstration that forced expression of *Id1* inhibits osteoblastic differentiation of the pre-osteoblastic MC3T3E1 cell line (Murray et al., 1992). In addition, in the promoter of the bone-specific gene *osteocalcin*, a functional E-box (OCE1) is required for efficient gene transcription and this transcriptional activity can be suppressed by overexpressing *Id1* (Tamura and Noda, 1994). Furthermore, gel-shift assays have been used to show that osteogenic cells contain OCE-1-binding activity (Kazhdan et al., 1997; Tamura and Noda, 1994) and that BMP treatment increases this activity (Tamura and Noda, 1994).

We now show that the myogenic bHLH transcription factor MyoD enhances the initial stages of osteogenic differentiation induced by BMP treatment. Although BMP-induced osteogenesis in vivo generally follows an endochondral pathway, there have been reports of direct bone induction depending on the nature of the carrier used to deliver the BMP (Matura et al., 2002 and references therein). This is important because an intermediate cartilage phenotype has not been demonstrated for muscle cells in vitro. Despite these differences, the in vitro studies coupled with the in vivo work indicate that muscle is a permissive environment for BMP-induced osteogenesis and that the satellite and stem cells within the muscle might contribute to the in vivo formation of bone. This might in part account for the ectopic bone formation that occurs in humans with the disease *Fibrodysplasia Ossificans Progressiva* following any injury to the muscle (Shafritz et al., 1996; Kaplan and Shore, 1998). Because BMP-4 was shown to be overexpressed in lymphocytes of these patients (Shafritz et al., 1998), recruitment of lymphocytes to the injured muscle would elevate the BMP concentration and perhaps stimulate some of the myogenic cells to undergo osteogenic differentiation, a fate that would be enhanced by MyoD.

In summary, we have shown that MyoD acts co-operatively with the osteogenic 'master gene' Runx2/Cbfa1 in mediating efficient induction of osteogenesis by BMP-7 in cultured myogenic cells in vitro.

This work was supported by grants from Canadian Institutes of Health Research (MA 13186 to SC; MOP37786 to JS & SC). MAR is supported by grants from the Canadian Institutes of Health Research, the National Institutes of Health and the Muscular Dystrophy Association. MAR is the Canada Research Chair in Molecular Genetics and a member of the Canadian Genetic Disease Network. M.K expresses special gratitude to I. Ishikawa (Tokyo Medical and Dental University) for encouragement and advice and to O. Shigeru for technical support.

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