Reciprocal Roles of Msx2 in Regulation of Osteoblast and Adipocyte Differentiation*

Received for publication, October 15, 2003, and in revised form, June 1, 2004
Published, JBC Papers in Press, June 1, 2004, DOI 10.1074/jbc.M403621200

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Mice deficient in the Msx2 gene manifest defects in skull ossification and a marked reduction in bone formation associated with decreases in osteoblast numbers, thus suggesting that Msx2 is involved in bone formation. However, the precise role of Msx2 during osteoblast differentiation is not fully understood. In the present study, we investigated the role of Msx2 in the regulation of osteoblast differentiation in the multipotent mesenchymal cell lines C3H10T1/2 and C2C12 and in murine primary osteoblasts. Introduction of Msx2 induced alkaline phosphatase activity in C3H10T1/2 and C2C12 cells and promoted the calcification of mesenchymal primary osteoblasts. This effect of Msx2 was also observed in mesenchymal cells isolated from Runx2-deficient mice. Interestingly the expression of Msx2 was induced by bone morphogenetic protein 2 treatment in Runx2-deficient mesenchymal cells. In contrast, Msx2 diminished peroxisome proliferator-activated receptor γ (PPARγ) expression and adipogenesis of the preadipocytic cell line 3T3-F442A. Moreover Msx2 inhibited the transcriptional activity of PPARγ, CCAAT/enhancer-binding protein β (C/EBPβ), and C/EBPα and blocked adipocyte differentiation of mesenchymal cells induced by overexpression of PPARγ, C/EBPα, C/EBPβ, or C/EBPδ. These data indicate that Msx2 promotes osteoblast differentiation independently of Runx2 and negatively regulates adipocyte differentiation through inhibition of PPARγ and the C/EBP family.

These factors conduct the transcriptional events that are necessary for the differentiation process of osteoblasts. Runx2/Cbfa1, an essential transcription factor for bone formation (1, 3), promotes the differentiation of undifferentiated mesenchymal cells into osteoblasts by regulating the transcription of type I collagen, osteopontin, and osteocalcin (4). A recent study has shown that a zinc finger transcription factor, osteXis, is necessary for bone formation and osteoblast differentiation (5). However, the molecular events that regulate the process of osteoblast differentiation have not been fully clarified.

Msx2, a homeobox gene, is a mammalian homologue of the Drosophila muscle segment homeobox. Msx2 is known to be induced by BMPs, which play critical roles in bone formation and osteoblast differentiation (2). Msx2-deficient mice develop reduced bone formation, decreases in osteoblasts, impaired chondrogenesis, abnormal calvarial development, and defects in the ectodermal organs including the teeth, hair, and mammary glands (6). In contrast, transgenic mice overexpressing Msx2 show enhanced growth of calvariae (7). Mutations in the MSX2 gene in humans, which affects DNA binding activity, causes defects in skull ossification (8, 9). Furthermore an autosomal dominant disorder, Boston-type craniosynostosis, results from a gain-of-function mutation of MSX2 at proline 148 (10, 11). These findings suggest a positive role for Msx2 in bone development and formation. In contrast to these genetic studies, in vitro studies have shown that Msx2 negatively regulates the transcription of the osteoblast-specific genes such as osteocalcin (12, 13). Furthermore Msx2 has been shown to bind to Runx2 and inhibit its transcriptional activity (14). These studies provide the notion that Msx2 serves as a negative regulator for osteoblast differentiation.

To understand the complex role of Msx2 in osteoblast differentiation, we examined the effects of Msx2 on osteoblast differentiation of mesenchymal cells. We found that Msx2 promotes the differentiation of mesenchymal cells into the osteoblast lineage in a Runx2-independent fashion. In addition, we showed that Msx2 inhibits the transcriptional activity of PPARγ and the C/EBP family, thereby suppressing adipocyte differentiation of mesenchymal cells. Thus, our data provide the evidence that Msx2 is an important transcriptional regulator for the commitment of mesenchymal cells into osteoblasts and adipocytes.

These abbreviations are used: BMP, bone morphogenetic protein; ALP, alkaline phosphatase; PPAR, peroxisome proliferator-activated receptor; C/EBP, CCAAT/enhancer-binding protein; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; PPRe, PPARγ-binding element; TK, thymidine kinase; m.o.i., multiplicity of infection.
EXPERIMENTAL PROCEDURES

Cells and Reagents—C3H10T1/2, C2C12, and CHO-K1 cell lines were purchased from RIKEN cell bank and cultured in α-modified Eagle’s medium containing 10% fetal bovine serum. The 3T3-F442A cell line was kindly provided by Dr. Minoru Morikawa. Anti-Msx2, anti-PPARγ, and anti-Myc antibodies were purchased from Santa Cruz Biotechnology, Inc. Recombinant BMP2 was obtained from the conditioned medium of CHO-K1 cells infected with BMP2 adenovirus. The activity of BMP2 was determined by comparison with human recombinant BMP2.

Isolation of Primary Osteoblasts and Mesenchymal Cells—The calvariae were isolated from 2- or 3-day neonatal mice and digested with 0.1% collagenase and 0.2% dispase for 7 min at 37 °C. The cells were then collected by centrifugation and used as the primary mesenchymal cells. These cells contained a small amount of ALP-positive cells. The digested calvariae were sequentially digested four times with 0.1% collagenase and 0.2% dispase for 7 min at 37 °C. The last three groups of fractionated cells were collected and used as the primary osteoblasts. The cells showed ALP activity. Runx2-deficient mesenchymal cells were isolated from the calvariae of Runx2-deficient embryos as described previously (15). In brief, the anterior region of calvariae from an embryo at embryonic day 18.5 was minced and cultured for 10–14 days in three-dimensional collagen gel (Cellmatrix, Nitta Gelatin Co.) with α-modified minimum Eagle’s medium containing 10% fetal bovine serum. The cells outgrowing from the explants were retrieved by incubation for 30 min with 0.2% collagenase (Wako Pure Chemical Industry, Osaka, Japan) in phosphate-buffered saline (PBS) at 37 °C and then cultured with α-modified minimum Eagle’s medium containing 10% fetal bovine serum.

Constructs and Transfection—Msx2 cDNA was isolated from BMP2-treated C2C12 cells by reverse transcribed PCR. The sequence of the cDNA was confirmed by DNA sequence analysis, and then the cDNA was subcloned into a pcDNA3 expression vector (Invitrogen) tagged with Myc epitope at the N terminus. A mutant of Msx2 was generated by replacing arginine 172 by histidine using the GeneEditor in vitro site-directed mutagenesis system (Promega). The sequence of the mutant cDNA was confirmed by DNA sequence analysis. PPARγ cDNA...

FIG. 1. A, induction of Msx2 expression by BMP2. C3H10T1/2 cells cultured with or without BMP2 (300 ng/ml) for 7 days were immunoprecipitated with anti-Msx2 goat polyclonal antibody and immunblotted with anti-Msx2 rabbit polyclonal antibody. B, induction of osteoblast differentiation of C3H10T1/2 cells by BMP2. C3H10T1/2 cells were cultured with or without BMP2 (300 ng/ml) for 7 days and determined by ALP staining. C and D, introduction of Msx2 by adenovirus infection. C3H10T1/2 cells were infected with control adenovirus or adenovirus carrying Myc-tagged Msx2 at 40 m.o.i. and subjected to immunoblotting (C) or immunostaining with anti-Myc antibody (D). E, effects of Msx2 on proliferation. C3H10T1/2 cells were infected with control, wild-type, or R172H mutant of Myc-Msx2 adenovirus, and the cell numbers were counted 3, 5, or 7 days after infection. Data represent mean ± S.D. Cont, control; WT, wild type; IP, immunoprecipitation; DAPI, 4',6-diamidino-2-phenylindole.

FIG. 2. Induction of osteoblast differentiation by Msx2. A, B, and C, C3H10T1/2 cells were infected with control or Myc-Msx2 adenovirus (40 m.o.i.) with or without Runx2 adenovirus (5 m.o.i.) and cultured for 7 days. The cells were determined by immunoblotting (A), ALP staining (B), or ALP activity (C). Data represent mean ± S.D. D, C2C12 cells were infected with control or Myc-Msx2 adenovirus, cultured for 7 days, and determined by ALP staining. E, primary mouse osteoblasts were infected with control or Myc-Msx2 adenovirus, cultured in the presence of ascorbic acid (100 μg/ml) and β-glycerophosphate (5 mM) for 14 days, and determined by ALP staining (top panel) or alizarin red staining (bottom panel). Cont, control.

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Regulation of Osteoblastogenesis and Adipogenesis by Msx2

34017

(16, 17), PPARy binding element (PPRE)-luciferase construct (16), C/EBPb cDNA, C/EBPb cDNA (16), and C/EBPba cDNA (18) were kindly provided by Drs. Bruce M Spiegelman, Ormond MacDougald, and Shizuo Akira. PPARy promoter fused to a luciferase construct was used as described previously (19). Transfection of C3H10T1/2 cells was carried out using FuGENE 6 (Roche Applied Science) according to the manufacturer's protocol (17).

Generation of Adenovirus—Recombinant adenoviruses carrying a wild-type or a mutant form of Msx2, C/EBPb, C/EBPb, or C/EBPb were constructed by homologous recombination between the expression cosmid cassette (pAxCAwt) and the parental virus genome in 293 cells (RIKEN) as described previously (17) using an adenovirus construction kit (Takara). The adenovirus carrying Runx2 or PPARy was used as described previously (17, 20). The viruses showed no proliferative activity due to a lack of E1A-E1B (21). Titers of the viruses were determined using a modified point assay (21). Infection of recombinant adenoviruses with C3H10T1/2 cells, C2C12 cells, 3T3-F442A cells, primary osteoblasts, or mesenchymal cells was performed by incubation with adenovirus at 40 multiplicity of infection (m.o.i.) except where specifically indicated.

Immunoprecipitation and Western Blotting—The cells were washed four times with ice-cold PBS and solubilized in lysis buffer (20 mM Hepes (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1.5 mM MgCl2, 10% glycerol, 1% Triton X-100, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate) (22). The lysates were centrifuged for 20 min at 4 °C at 16,000 × g and incubated with antibodies for 4 h at 4 °C followed by immunoprecipitation with protein A-Sepharose (Zymed Laboratories Inc.) or protein G-agarose (Roche Applied Science). Immunoprecipitates were washed five times with lysis buffer and boiled in SDS sample buffer containing 0.5 M β-mercaptoethanol. The supernatants were recovered as immunoprecipitate samples. These samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, immunoblotted with corresponding antibodies, and visualized with horseradish peroxidase coupled to protein A (Kirkegaard & Perry Laboratories, Inc.) or horseradish peroxidase-coupled anti-mouse IgG antibodies (Cappel) with enhancement by ECL detection kits (Amersham Biosciences).

Immunocytochemistry—Cultured cells were washed three times with ice-cold PBS and fixed in 3.7% paraformaldehyde in PBS for 20 min. After a 20-min incubation with 0.1% Triton X-100 in PBS, the cells were blocked with 1% bovine serum albumin-containing PBS for 30 min, incubated with anti-Myc monoclonal antibody in 1% BSA in PBS, then washed six times with PBS, and incubated with fluorescein isothiocyanate-conjugated affinity purified anti-mouse IgG antibody (Jackson

![Figure 3](image1.png)

**Figure 3. Inhibition of BMP2-induced calcification and ALP activity by a mutant of Msx2 (R172H).** A, primary mouse osteoblasts were infected with control adenovirus or adenovirus carrying the R172H mutant of Msx2 tagged with a Myc epitope, cultured for 2 days, and analyzed by immunoblotting with anti-Myc antibody. B, primary mouse osteoblasts were infected with control adenovirus or adenovirus carrying the R172H mutant of Msx2 tagged with a Myc epitope, cultured in the absence or presence of BMP2 (300 ng/ml) for 14 days, and determined by alizarin red staining.

![Figure 4](image2.png)

**Figure 4. Induction of ALP activity by Msx2 in Runx2 deficient mesenchymal cells.** A, mesenchymal cells isolated from wild-type (WT) or Runx2-deficient mice (Runx2<sup>-/-</sup>) were cultured in the absence or presence of BMP2 (300 ng/ml) for 7 days and determined by ALP staining. B, Runx2-deficient mesenchymal cells were infected with or without BMP2 (300 ng/ml) for 2 days. Total RNA isolated from the cells was determined by reverse transcribed PCR analysis for Msx2 (top panel) or β-actin (bottom panel). C and D, Runx2-deficient cells were infected with Msx2 adenovirus (40 m.o.i.), Runx2 adenovirus (5 m.o.i.), or both; cultured for 7 days; and determined by ALP staining (C) or ALP activity (D). Data represent mean ± S.D. E, C3H10T1/2 cells infected with control or Myc-Msx2 adenovirus were cultured in the absence or presence of BMP2 for 7 days. The cell lysates were analyzed by immunoblotting with anti-Runx2 antibody. Cont, control.

**Control** | **BMP2** | **BMP2**
---|---|---
**Cont** | **Runx2** | **Runx2**
**Msx2** | **Runx2** | **Runx2**

**Blot: α-Runx2**

**ALP (nmol/min/mg)**

| | Cont | Msx2 | Runx2 | Msx2 |
---|---|---|---|---|
**Control** | **BMP2** | **Msx2** | **Runx2** | **Runx2**

**Cont, control.**
Immunoresearch Laboratories, Inc.). The cells were washed extensively with PBS and visualized under a fluorescence microscope (Zeiss).

**Luciferase Assay**—PPRE-luciferase construct or PPARγ promoter construct was co-transfected with TK-Renilla luciferase construct (Promega) into C3H10T1/2 cells. Two days after transfection, cells were lysed, and luciferase activity was determined using specific substrates in a luminometer (Promega) according to the manufacturer’s protocol. Transfection efficiency was normalized by determining the activity of Renilla luciferase.

**Reverse Transcribed PCR**—Total RNA was isolated from cells using the RNeasy kit (Qiagen) and treated with DNase (Wako Pure Chemical Industry) for 30 min. After denaturation of total RNA at 70 °C for 10 min, cDNA was synthesized with oligo(dt)1 primer and reverse transcriptase (Invitrogen). PCR amplifications were performed using the specific primers for mouse Msx2 (sense primer, 5′-TCGAGGAAACACAAGCAGCA-3′; antisense primer, 5′-GTCTATGGAGGGTTAGGAT-3′).

PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. After the PCR products were subcloned into TA cloning vector, the DNA sequences of the PCR products were determined.

**Determination of Alkaline Phosphatase Activity**—ALP activity was determined as described previously (22). C3H10T1/2 or C2C12 cells were washed with PBS and lysed with 0.05% Triton X-100 solution. The ALP activity of the lysates was determined using p-nitrophenol phosphate as a substrate. The protein content of each lysate was measured using Bradford protein assay reagent (Bio-Rad). For cytochemical analysis, cells were washed with PBS, fixed in 3.7% formaldehyde, and stained with a mixture of 330 μg/ml nitro blue tetrazolium, 165 μg/ml bromochloroindolyl phosphate, 100 mM NaCl, 5 mM MgCl2, and 100 mM Tris (pH 9.5).

**Alizarin Red Staining**—The cultured calvaria cells were rinsed twice with PBS, fixed in 10% buffered formalin, and stained with 1% alizarin red solution for 5 min.

**Oil Red-O Staining**—C3H10T1/2 or 3T3-F442A cells were washed with PBS and fixed in 10% formalin for 20 min. After washing the cells twice with PBS and once with 60% isopropl alcohol, the cells were stained with Oil Red-O solution (Sigma). The area of the cells stained was determined using an Image Pro Plus analyzer (Pallmerton Inc.) (17).

**Oligonucleotide Pull-down Assay**—The nuclear extracts isolated from C3H10T1/2 cells were incubated with 1 μg of biotinylated double-stranded PPRE oligonucleotide probe (sense primer, 5′-TCGATCTCGAGGAAACACAAGCAGCA-3′; antisense primer, 5′-TCGAGGAAACACAAGCAGCAAAACGGTAGTAGTACGCTCAGGGA-3′) and 10 μg of poly(dI-dC) for 4 h (20). DNA-associated proteins were incubated with streptavidin-agarose beads for 1 h, washed with the lysis buffer, and determined by immunoblotting with anti-PPARγ antibody.

**Statistical Analysis**—All data were analyzed by analysis of variance followed by a paired t test. Data represent mean ± S.D.

**RESULTS**

**Induction of Msx2 during Osteoblast Differentiation**—To examine whether Msx2 is involved in the osteoblast differentiation of mesenchymal cells, we first determined the expression of Msx2 using a multipotent mesenchymal cell line, C3H10T1/2, which differentiates into osteoblast cells upon stimulation with BMP2 (23). C3H10T1/2 cells did not express Msx2 in an undifferentiated condition (Fig. 1A). However, when treated with BMP2, the cells showed distinct expression of Msx2 (Fig. 1A) along with the induction of ALP activity (Fig. 1B) (23). These results indicate that Msx2 expression is associated with osteoblast differentiation of mesenchymal cells and suggest the involvement of Msx2 in osteoblast differentiation.

**Induction of Osteoblast Differentiation of Mesenchymal Cells by Msx2**—To examine the potential role of Msx2 in osteoblast differentiation of mesenchymal cells, we introduced Msx2 into C3H10T1/2 cells using an adenovirus system. As shown in Fig. 1, C and D, Msx2 is efficiently expressed in C3H10T1/2 cells upon infection with Msx2 adenovirus. Since Msx2 has been implicated in the proliferation of osteoblast precursor cells (6), we therefore determined the effect of Msx2 on the growth of C3H10T1/2 cells. We observed that the introduction of Msx2 marginally stimulated the proliferation of C3H10T1/2 cells (Fig. 1E), suggesting that Msx2 is involved not only in the proliferation stage but also in the differentiation process during osteogenesis.

To confirm this hypothesis, we next determined the effect of Msx2 on the osteoblast differentiation of C3H10T1/2 cells. Introduction of Msx2 clearly induced ALP activity in C3H10T1/2 cells (Fig. 2, A, B, and C). This effect of Msx2 was also observed in a mesenchymal cell line, C2C12 (Fig. 2D), which possesses the capacity to differentiate into osteoblastic cells with BMP2 treatment (22). Notably introduction of Msx2 increased ALP activity and calcification in primary osteoblasts isolated from mouse calvariae (Fig. 2E). These data suggest that Msx2 plays an important role in the regulation of osteoblast differentiation.

To further understand the osteogenic activity of Msx2, we evaluated its effects on BMP2-induced calcification using a point mutant of Msx2 at arginine 172 that has less DNA binding activity and causes enlarged parietal foramina characterized by...
retarded or deficient ossification in humans (8). We confirmed that the R172H mutant was introduced into the primary osteoblasts using an adenovirus system (Fig. 3A). Consistent with previous reports (24), BMP2 induced calcification of primary mouse osteoblasts (Fig. 3B). Introduction of the mutant Msx2 markedly inhibited the activity of BMP2 in primary mouse osteoblasts (Fig. 3B). The mutant also blocked BMP2-induced ALP activity in C3H10T1/2 cells (Fig. 3C) but had no effect on proliferation of C3H10T1/2 cells (Fig. 1E). The data suggest that Msx2 plays a role in BMP2-regulated osteogenesis.

Runx2-independent Regulation of Expression and Function of Msx2—Msx2 has been shown to physically associate with Runx2 and suppress its transcriptional activity (14), and thus we examined whether Msx2 affects the osteogenic activity of Runx2. As we reported previously (20), overexpression of Runx2 promoted the osteoblast differentiation of C3H10T1/2 cells (Fig. 2B and C). Msx2 additively increased ALP activity in the presence of Runx2 (Fig. 2B and C). The data indicate that Msx2 does not affect the osteogenic action of Runx2 and raises the possibility that Msx2 regulates osteoblast differentiation independently of Runx2.

To address this issue, we examined the expression and function of Msx2 in Runx2-deficient mesenchymal cells that had been isolated from Runx2-deficient mice. Interestingly BMP2 clearly induced ALP activity in Runx2-deficient mesenchymal cells (Fig. 4A), although the induction of ALP activity by BMP2 treatment in Runx2-deficient mesenchymal cells was weaker than that of wild-type mesenchymal cells. These data suggest that another transcription factor(s) is involved in induction of ALP activity in Runx2-deficient cells. Treatment with BMP2 induced Msx2 expression in Runx2-deficient mesenchymal cells (Fig. 4B). However, we observed that overexpression of Runx2 did not induce Msx2 expression (data not shown). Furthermore Msx2 overexpression induced ALP activity in these cells (Fig. 4C and D). Collectively these results indicate that the expression and function of Msx2 is regulated in a Runx2-independent fashion.

Msx2 Inhibited Adipocyte Differentiation of Mesenchymal Cells by Suppressing PPARγ, C/EBPβ, and C/EBPδ—Since adipocytes share their origin with osteoblasts (25, 26) and the number of adipocytes appears to increase in the bone marrow of Msx2-deficient mice (6), we examined whether Msx2 is involved in the regulation of adipocyte differentiation. To address the
issue, we examined the effect of Msx2 on adipocyte differentiation using a preadipocytic cell line, 3T3-F442A (27). As shown in Fig. 5A, overexpression of Msx2 profoundly suppressed the adipocyte differentiation of 3T3-F442A cells. Consistent with these results, Msx2 abolished the expression of PPARγ in 3T3-F442A cells (Fig. 5B). We next asked whether Msx2 is involved in the commitment of osteoblasts and adipocytes and tested the effects of Msx2 on C3H10T1/2 cells, which are able to differentiate into both osteoblastic and adipocytic cells in the presence of BMP2 (23). As we and others have reported (17, 23), BMP2 promoted the differentiation of C3H10T1/2 cells toward osteoblastic and adipocytic cells (Fig. 5, C and D), thus allowing us to determine the balance of osteoblastogenesis and adipogenesis under this condition. The introduction of Msx2 enhanced BMP2-induced osteoblastogenesis but markedly inhibited BMP2-induced adipogenesis (Fig. 5, C and D). This result suggests that Msx2 plays a role in the commitment of mesenchymal cells.

A nuclear receptor, PPARγ, plays a central role in adipogenesis (28). Likewise we have recently demonstrated that the functional regulation of PPARγ is critical for BMP2-regulated adipocyte differentiation of C3H10T1/2 cells (17). To under-
stand the mechanisms by which Msx2 inhibits adipocyte differentiation, we examined the effect of Msx2 on the function of PPARγ. Overexpression of PPARγ using an adenovirus system induced adipocyte differentiation in C3H10T1/2 cells (Fig. 6, A, B, and C). Msx2 inhibited the adipogenic effect of PPARγ (Fig. 6, B and C). Consistent with these results, Msx2 suppressed the transcriptional activity of PPARγ (Fig. 6D). To determine whether Msx2 affects the DNA binding capacity of PPARγ, we performed an oligonucleotide pull-down assay using a biotinylated probe containing the PPRE. We found that Msx2 interfered with the binding of PPARγ to PPRE (Fig. 6E). These data strongly suggest that Msx2 negatively regulates the function of PPARγ by suppressing its DNA binding activity, thereby inhibiting adipocyte differentiation.

Msx2 has also been shown to antagonize C/EBP transcription factor in its role of regulating amelogenin gene expression (29). The C/EBP family is important for the regulation of adipogenesis in the early stage especially in the induction of PPARγ (30). We demonstrated that the introduction of Msx2 inhibited the expression of PPARγ (Fig. 5B), and thus we examined whether Msx2 is involved in the regulation of C/EBPα and C/EBPδ during adipocyte differentiation. C/EBPδ, as well as C/EBPα, promoted adipocyte differentiation of C3H10T1/2 cells (Fig. 7, A, B, C, D, E, and F). Msx2 overexpression clearly inhibited adipocyte differentiation induced by either C/EBPα or C/EBPδ (Fig. 7, B, C, D, E, and F). Furthermore Msx2 inhibited the transcriptional activity of C/EBPδ and C/EBPα on the PPARγ gene promoter, which is directly regulated by both C/EBPα and C/EBPδ (Fig. 7G). Consistent with this, Msx2 significantly inhibited PPARγ induction by C/EBPα and C/EBPδ (Fig. 7H). These results indicate that Msx2 plays a role in the early stage of adipocyte differentiation as well as in the later stages.

Because C/EBPα is known to regulate adipocyte differentiation synergistically and harmoniously with PPARγ (28), we next examined the effect of Msx2 on C/EBPα. As shown in Fig. 8, Msx2 markedly suppressed the adipogenic action of C/EBPα. Thus, Msx2 is a negative transcriptional regulator for adipogenesis at multiple steps.

**DISCUSSION**

Genetic studies in human diseases and mouse models suggest positive roles for Msx2 in osteogenesis (6–8). However, in vitro studies based on transcriptional assays have raised the possibility that Msx2 functions as a repressor during osteoblast differentiation (13, 14). To understand the complexity of osteoblastogenesis regulation by Msx2, we investigated the functional roles of Msx2 in osteoblast differentiation using mesenchymal cell lines and primary mouse osteoblasts into which Msx2 had been introduced by an adenovirus system. Our results strongly suggest that Msx2 is an important transcriptional regulator for osteoblast differentiation. First, introduction of Msx2 induced ALP activity in C3H10T1/2 and C2C12 cells in the absence of BMP2. Second, Msx2 also promoted the calcification of primary mouse osteoblasts. Third, a mutant Msx2, which shows functional haploinsufficiency of DNA binding and dominantly causes enlarged parietal foramina (8), inhibited BMP2-induced calcification in primary mouse osteoblasts. These results are consistent with the phenotype seen in Msx2-deficient mice (6). In contrast, we showed that Msx2 produced a marginal effect on the growth of C3H10T1/2 cells. Thus, Msx2 is most likely implicated in the regulation of osteoblast differentiation more than the proliferation of its precursor cells.

Msx2 has been proposed to negatively regulate Runx2 function through physical association (14). In contrast, when we co-introduced Msx2 together with Runx2, we found that Msx2 does not inhibit the osteogenic action of Runx2 but has an additive effect on Runx2. We have also observed that Msx2 does not affect the induction of osteocalcin by Runx2 (data not shown). These data suggest a Runx2-independent role for Msx2 in osteoblastogenesis. This notion is supported by our findings that Msx2 was able to induce ALP activity in Runx2-deficient mesenchymal cells and that this effect of Msx2 is additively increased by exogenous Runx2. In addition, BMP2 induced Msx2 expression in Runx2-deficient mesenchymal cells. Collectively, the data indicate that the BMP2-Msx2 axis, which plays a positive role in osteoblast differentiation, functions in a Runx2-independent fashion. The identification of the target genes of Msx2 may allow further dissection of the molecular basis by which Msx2 promotes osteoblast differentiation.

Accelerated adipogenesis in the bone marrow cavity appears to be associated with osteoporosis or aging (31, 32), and thus the identification of the molecular mechanisms that control the balance between osteoblastogenesis and adipogenesis will con-
contribute to the understanding of the pathogenesis related to metabolic bone diseases such as osteoporosis. Our finding that Msx2 stimulates osteoblastogenesis but inhibits adipogenesis suggests that Msx2 is one of the transcriptional regulators involved in the regulation of the balance of osteoblastogenesis and adipogenesis. Our data reveal that Msx2 inhibits adipocyte differentiation at multiple steps. Msx2 suppresses the function of C/EBPβ and C/EBPδ, both of which control adipogenesis in the early stage by regulating the transcription of the PPARγ gene. Consistently Msx2 inhibited the PPARγ gene promoter activity activated by C/EBPβ and C/EBPδ and abolished PPARγ expression. In the latter step, Msx2 also inhibited the transcriptional activity of PPARγ by suppressing its DNA binding ability, consequently preventing adipocyte differentiation. In addition, Msx2 also inhibited C/EBPα-induced adipocyte differentiation. To support the notion that Msx2 is a negative regulator for adipogenesis, Msx2 was not expressed during adipocyte differentiation (data not shown). Thus, our findings demonstrate a novel function of Msx2, namely regulating differentiation of mesenchymal cells into adipocytes.

In conclusion, our data suggest that Msx2 regulates the differentiation of mesenchymal cells into osteoblasts independently of Runx2, whereas Msx2 inhibits the function of C/EBPα, C/EBPβ, C/EBPδ, and PPARγ, resulting in inhibition of adipogenesis. These findings advance the understanding of the molecular mechanisms that regulate the differentiation pathway of mesenchymal cells into the osteoblastic and adipogenic lineages.

Acknowledgments—We thank Dr. Bruce M Spiegelman (Harvard Medical School) for the PPARγ expression vector and PPRE-Luc, Dr. Shizuo Akira (Osaka University) for the C/EBPβ and C/EBPδ cDNA, Dr. Ormond MacDougald for the C/EBPδ cDNA and Dr. Minoru Morikawa (National Institute of Health Sciences) for the 3T3-F442A cells.

Addendum—During the review process, Cheng et al. (33) reported that Msx2 is implicated in the regulation of osteoblast and adipocyte differentiation.

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Reciprocal Roles of Msx2 in Regulation of Osteoblast and Adipocyte Differentiation

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J. Biol. Chem. 2004, 279:34015-34022.
doi: 10.1074/jbc.M403621200 originally published online June 1, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M403621200

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