

## Bone Morphogenetic Protein 2-Induced Osteoblast Differentiation Requires Smad-Mediated Down-Regulation of Cdk6

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Received 31 March 2004/Accepted 8 May 2004

**Because a temporal arrest in the G<sub>1</sub> phase of the cell cycle is thought to be a prerequisite for cell differentiation, we investigated cell cycle factors that critically influence the differentiation of mouse osteoblastic MC3T3-E1 cells induced by bone morphogenetic protein 2 (BMP-2), a potent inducer of osteoblast differentiation. Of the G<sub>1</sub> cell cycle factors examined, the expression of cyclin-dependent kinase 6 (Cdk6) was found to be strongly down-regulated by BMP-2/Smads signaling, mainly via transcriptional repression. The enforced expression of Cdk6 blocked BMP-2-induced osteoblast differentiation to various degrees, depending on the level of its overexpression. However, neither BMP-2 treatment nor Cdk6 overexpression significantly affected cell proliferation, suggesting that the inhibitory effect of Cdk6 on cell differentiation was exerted by a mechanism that is largely independent of its cell cycle regulation. These results indicate that Cdk6 is a critical regulator of BMP-2-induced osteoblast differentiation and that its Smads-mediated down-regulation is essential for efficient osteoblast differentiation.**

Bone morphogenetic protein 2 (BMP-2) is a potent inducer of bone formation through its stimulation of osteoblast differentiation (17). It exerts this effect via two types of serine/threonine kinase receptors: BMP-2 binds the type II receptor, which subsequently activates the type I receptor by a direct association. Signals from the activated type I receptor are transmitted to the nucleus through various mediator molecules, the most important among them being a family of proteins termed Smads. Smads are classified into three subgroups, i.e., Smad1, Smad5, and Smad8 are classified as receptor-regulated Smads (R-Smads), Smad4 is classified as a common-partner Smad (Co-Smad), and Smad6 and Smad7 are classified as inhibitory Smads (I-Smads) (6). R-Smads are directly activated by the type I receptor, form complexes with Co-Smad, and are translocated into the nucleus, where they regulate the transcription of target genes. I-Smads inhibit the activation of R-Smads by interfering with their association with the type I receptor, which results in the hindrance of the assembly of R-Smads with Co-Smad. Although the downstream signaling of the BMP-2/Smad pathway leading to osteoblast differentiation has been extensively investigated, most of the studies have focused on the bone-related transcriptional regulators Runx2/Cbfa1 (7, 31), osterix (12), SIP1 (25), Smurf1 (32), NF- $\kappa$ B (4, 9), Hoxc-8 (1, 20), and Tob (29).

The proliferation of eukaryotic cells depends on their progression through the cell cycle, and an at least temporal cell cycle arrest in the G<sub>1</sub> phase is thought to be a prerequisite for cell differentiation (18). Cell cycle progression is controlled by the action of cyclins and cyclin-dependent protein kinases (Cdks), which phosphorylate and thereby activate cell cycle

factors that are essential for the onset of the next cell cycle phase. In mammalian cells, traverse through G<sub>1</sub> and subsequent S-phase entry require the activities of the cyclin D-dependent kinase Cdk4 and/or Cdk6 (11) and the cyclin E-dependent kinase Cdk2. A key physiological substrate for Cdk4 and Cdk6 is the retinoblastoma (Rb) protein, which binds and inactivates the E2F-DP transcription complexes that are essential for S-phase entry. Phosphorylation by Cdk4/6 and additionally by Cdk2 inactivates Rb, thereby releasing E2F-DP from inactivation and consequently promoting S-phase entry and progression (5, 14). These Cdks are negatively regulated by cyclin-dependent kinase inhibitors (CKIs) via direct binding to themselves (19, 26). CKIs have been classified into two families, the INK4 family and the Cip/Kip family. INK4 family members (p16, p15, p18, and p19) inhibit only Cdk4 and Cdk6, whereas Cip/Kip family members (p21, p27, and p57) inhibit all of the Cdks except for the Cdk6-cyclin D3 complex. Because of its unique ability to evade inhibition by Cip/Kip proteins, Cdk6-D3 can control the cell's proliferative potential under growth-suppressive conditions despite its relative minority in level of expression in mesenchymal cells (8).

Cdks play crucial roles in controlling cell cycle progression. Therefore, much attention has been attracted by the view that the CKI-led inhibition of G<sub>1</sub>-specific Cdks is critical for cell differentiation. Accordingly, potential roles for CKIs in differentiation have been extensively studied, but with mixed results. Many studies revealed a certain correlation between the induction of p21<sup>CIP1</sup> and differentiation, yet many did not. Mice with a complete deletion of p21<sup>CIP1</sup> and/or p27<sup>KIP1</sup> or other major CKIs still develop normally, with proper differentiation, which calls the current view into question (3, 13). Although there is evidence for p57<sup>KIP2</sup> being involved in the differentiation of some cells (28, 30), no one has identified cell cycle factors that are controlled by differentiation signals and that critically influence the differentiation commitment process.

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Since in lower eukaryotes the control of the cell cycle factors driving the onset of S phase greatly influences the commitment to cell differentiation, we reinvestigated the possibility of the crucial participation of some cell cycle start factors in mammalian cell differentiation control as well, using BMP-2-induced osteoblast differentiation as a model system, and we found that upon BMP-2 treatment, Cdk6 expression was down-regulated primarily by BMP-2/Smad signal-invoked transcriptional repression and that its down-regulation was essential for efficient osteoblast differentiation.

## MATERIALS AND METHODS

**Reagents and antibodies.** Recombinant human BMP-2 was generously provided by Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan), and recombinant human fibroblast growth factor 2 (FGF-2) was provided by Kaken Pharmaceutical Co., Ltd. (Chiba, Japan). Antibodies against Cdk2 (H-298), Cdk4 (C-22), Cdk6 (C-21), Rb (C-15), cyclin D1 (C-20), cyclin D2 (M-20), cyclin D3 (C-16), cyclin E (M-20), p18 (M-20), p21 (H164), p27 (F-8), BMPRIA (E-16), BMPRII (T-18), and Smad6 (S-20) were obtained from Santa Cruz Biotechnology. Anti-phosphorylated Rb (G3-245) and anti- $\beta$ -actin (AC-15) antibodies were purchased from Pharmingen and Sigma, respectively.

**Osteoblast culture.** The mouse osteoblast cell line MC3T3-E1 was purchased from the Riken Cell Bank (Tsukuba, Japan). Primary osteoblasts were isolated from neonatal mouse calvariae according to international and university guidelines (33). Calvariae dissected from 1- to 4-day-old mice were digested for 10 min with 1 ml of trypsin-EDTA (Sigma) containing 10 mg of collagenase (type 7; Sigma), and the released cells were collected. This step was repeated five times, and the cells obtained by the second through fifth digestion steps were pooled as primary osteoblasts. MC3T3-E1 cells and primary osteoblasts were maintained in  $\alpha$ -modified minimum essential medium ( $\alpha$ -MEM) (Life Technologies Inc.) containing 10% fetal bovine serum (FBS) (Sigma). The cells were inoculated at  $5 \times 10^4$  cells in a six-well plate or  $5 \times 10^5$  cells in a 10-cm-diameter plate and allowed to proliferate for 20 to 24 h, and then their growth was arrested by incubation for 24 to 48 h in  $\alpha$ -MEM containing 0.5% FBS. Growth-arrested cells were then induced for differentiation or proliferation by stimulation with  $\alpha$ -MEM containing 10% FBS and BMP-2 (300 ng/ml) or FGF-2 (10 nM). To block protein degradation, we added the proteasome inhibitor MG132 (Z-Leu-Leu-Leu-aldehyde; Peptide Institute, Osaka, Japan) to the culture medium to a final concentration of 2  $\mu$ M, as described previously (15, 24).

**Construction of cell clones constitutively expressing Cdk6.** MC3T3-E1 cells were inoculated at  $5 \times 10^5$  cells per 6-cm-diameter plate, incubated for 24 h, and then transfected with the pEF/neo I vector carrying a human Cdk6 cDNA or no insert by use of the Lipofectamine reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Twenty-four hours later, the cells were split 1:10 to 1:100 and selected in  $\alpha$ -MEM containing 10% FBS and 200  $\mu$ g of G418 (Geneticin; Life Technologies, Inc.)/ml. Stable G418-resistant colonies were then isolated and expanded for analysis. The levels of Cdk6 were then quantified by Western blotting to identify several high- and low-expression-level clones for in-depth analysis.

**Smad6 expression and differentiation induction.** MC3T3-E1 cells were inoculated at  $5 \times 10^4$  cells per well in a six-well plate and incubated at 37°C for 20 h in  $\alpha$ -MEM containing 10% FBS. The cells were then infected for 2 h with a recombinant adenovirus carrying *smad6* or *lacZ* at a multiplicity of infection of 100 PFU/cell, for which >80% of the cells were infected, as determined by staining for  $\beta$ -galactosidase. The cells were washed twice with phosphate-buffered saline (PBS), their growth was arrested by incubation in  $\alpha$ -MEM containing 0.5% FBS for 48 h, and they were stimulated with  $\alpha$ -MEM containing 10% FBS with or without BMP-2 (300 ng/ml).

**Western blot analysis.** The cells were rinsed with ice-cold PBS and lysed with RIPA buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40 [NP-40], 0.1% sodium dodecyl sulfate [SDS], 10  $\mu$ g of aprotinin/ml, 0.1 M NaF, 2 mM  $\text{Na}_3\text{VO}_4$ , and 10 mM  $\beta$ -glycerophosphate). After a brief sonication, the lysed cells were centrifuged at  $15,000 \times g$  for 20 min at 4°C to obtain soluble cell extracts. The cell extracts (10  $\mu$ g of protein each) were separated by SDS-7.5, 10, or 12.5% polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Bedford, Mass.). After the blocking of nonspecific binding by soaking of the filters in 5% skim milk, the desired proteins were immunodetected with their respective antibodies, followed by visualization by use of the ECL Plus Western blotting detection

system (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) according to the manufacturer's instructions.

**In vitro kinase assay.** Cells were lysed with ice-cold IP buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol, 0.1% Tween 20, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10  $\mu$ g of aprotinin/ml, 1 mM NaF, and 0.1 mM sodium orthovanadate. The lysates were incubated at 4°C for 2 h with 0.5  $\mu$ g of the anti-Cdk4 or -Cdk6 antibody. Protein G-Sepharose (15  $\mu$ l) (Amersham-Pharmacia) was then added and incubated for an additional 1 h. Immune complexes bound to protein G-Sepharose were precipitated by centrifugation and washed with glycerol-free IP buffer. The immunopurified Cdk4 or Cdk6 protein was incubated at 30°C for 30 min in R buffer (50 mM HEPES [pH 7.5], 2.5 mM EGTA, 10 mM KCl, 10 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol) containing 0.5  $\mu$ g of truncated Rb (Santa Cruz) and 10 mM ATP. The reaction products were electrophoresed in SDS-10% polyacrylamide gels and transferred to polyvinylidene difluoride membrane filters. Phosphorylated truncated Rb was detected with an anti-Ser780-phosphorylated Rb antibody (MBL).

**Reverse transcription-PCR (RT-PCR).** Total RNAs (1  $\mu$ g) extracted from cells were reverse transcribed and amplified by PCR. The gene-specific primer pairs used were as follows: 5'-CGTGGTCAGGTTGTTTGTATG-3' and 5'-TGC GAAACATTTCTGCAAAG-3' for Cdk6, 5'-ATGAGGACCTCTCTCTGC T-3' and 5'-CCGTAGATGCGTTTGTAGGC-3' for osteocalcin, 5'-TAGCAC CAGAGATACCTTGC-3' and 5'-AATGCTTCATCCCTGTCCAAA-3' for BMPRIA, 5'-CAGAATCAAGAACGGCTATG-3' and 5'-TTGTTTACGGTC TCCTGTCA-3' for BMPRII, and 5'-CATGTAGGCATGAGGTCCACCA C-3' and 5'-TGAAGGTCGGTGTGAACGGATTGGC-3' for glyceraldehyde-3-phosphate dehydrogenase. The cycling parameters were 30 s at 94°C, 30 s at 55°C, and 1 min 30 s at 72°C for Cdk6, osteocalcin, and glyceraldehyde-3-phosphate dehydrogenase; and 30 s at 94°C, 30 s at 53°C, and 1 min 30 s at 72°C for BMPRIA and BMPRII.

**ALP activity measurement and in situ staining.** MC3T3-E1 cells were inoculated at  $5 \times 10^4$  cells per well in a six-well plate and cultured in  $\alpha$ -MEM containing 10% FBS with or without BMP-2 (300 ng/ml). After being cultured for 3 days, the cells were rinsed with PBS and lysed by sonication in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM  $\text{MgCl}_2$  and 0.5% Triton X-100. The alkaline phosphatase (ALP) activity in the lysates was measured by the hydrolysis of *p*-nitrophenyl phosphate to *p*-nitrophenol. The protein content was determined by using a protein assay kit (Bio-Rad). For in situ ALP staining, the cells were fixed with 3.7% (vol/vol) formaldehyde in PBS and were stained with naphthol AS-MX phosphate (Sigma), with *N,N*-dimethyl formamide as a substrate and Fast BB salt (Sigma) as a coupler.

**BrdU incorporation assay.** MC3T3-E1 cells were inoculated at  $10^3$  cells per well in a 96-well plate and cultured in  $\alpha$ -MEM containing 10% FBS with or without BMP-2 (300 ng/ml). After being cultured for 1 or 3 days, the cells were labeled with bromodeoxyuridine (BrdU) for 2 h, and the cell population entering S phase was determined by quantifying the incorporated BrdU (Cell Proliferation ELISA; Roche Molecular Biochemical, Mannheim, Germany).

**XTT assay.** Cells were inoculated at  $10^3$  cells per well in a 96-well plate and cultured for 5 days in  $\alpha$ -MEM containing 10% FBS with or without BMP-2 (300 ng/ml), with cell sampling every day. The sampled cells were quantified by use of an XTT {sodium 3,3-[(phenylamino)carbonyl]-3,4-tetrazolium-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate} assay kit (Roche).

**Flow cytometric analysis.** Approximately  $10^5$  cells were suspended in 0.02 ml of citrate buffer and subjected to the following serial treatments at room temperature: (i) the addition of 0.18 ml of solution A (0.03 mg of trypsin/ml, 3.4 mM trisodium citrate, 0.1% NP-40, 1.5 mM spermine 4HCl, and 0.5 mM Tris-HCl [pH 7.6]) and incubation for 10 min; (ii) the addition of 0.15 ml of solution B (3.4 mM trisodium citrate, 0.1% NP-40, 1.5 mM spermine-4HCl, 0.5 mM Tris-HCl [pH 7.6], 0.5 mg of trypsin inhibitor/ml, 0.1 mg of RNase A/ml) and incubation for 10 min; and (iii) the addition of 0.15 ml of solution C (4.16 mg of propidium iodide/ml, 3.4 mM trisodium citrate, 0.1% NP-40, 4.8 mM spermine 4HCl, 0.5 mM Tris-HCl [pH 7.6]) and incubation for 10 min. The DNA content was determined and analyzed with EPICS XL and XL EXPO32 instruments (Beckman).

**ChIP.** Chromatin immunoprecipitation (ChIP) was performed by use of a commercial kit (Upstate Cell Signaling Solutions, Lake Placid, N.Y.). Cells were inoculated at a density of  $5 \times 10^5$  cells per 10-cm-diameter dish and cultured in  $\alpha$ -MEM containing 10% FBS and BMP-2 (300 ng/ml). At days 1 and 4 of culture, the protein and DNA were cross-linked by the direct addition of formaldehyde to the culture medium to a final concentration of 1% and incubation at 37°C for 10 min. The cells were then washed twice with ice-cold PBS containing 1 mM PMSF and 1  $\mu$ g of aprotinin/ml, collected with cell scrapers, sedimented by low-speed centrifugation, resuspended, and incubated at 4°C for 10 min in 200  $\mu$ l

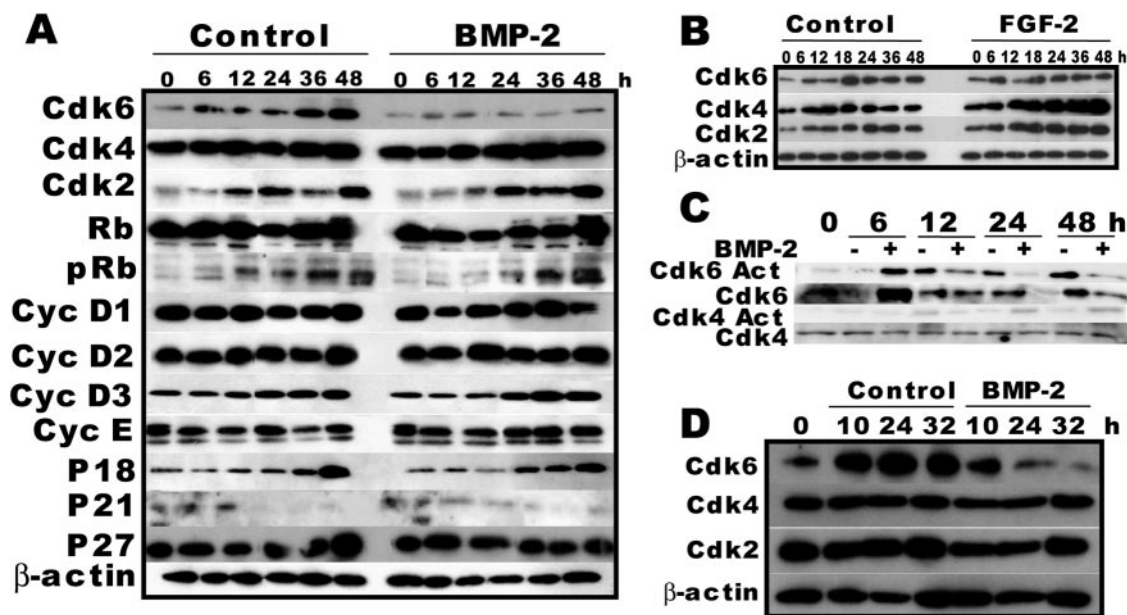


FIG. 1. BMP-2 treatment inhibits expression of Cdk6. (A) Time-dependent expression of cell cycle factors controlling the  $G_1$ -S transition in mouse osteoblastic MC3T3-E1 cells during differentiation induction. Growth-arrested MC3T3-E1 cells were stimulated with FBS in the presence or absence of BMP-2, with cell sampling done at the indicated times. The amounts of cell cycle factors expressed during stimulation were semiquantified by Western blotting.  $\beta$ -Actin was used as a loading control. Cyc, cyclin. (B) Time-dependent expression of Cdk2, 4, and 6 in MC3T3-E1 cells during FGF-2 stimulation. Growth-arrested MC3T3-E1 cells were stimulated with FBS in the presence or absence of FGF-2, with cell sampling performed at the indicated times, followed by Western blotting of Cdk2, 4, and 6. (C) Cdk6 and Cdk4 activities in MC3T3-E1 cells during stimulation with or without BMP-2. Cdk6 and Cdk4 were immunoprecipitated and assayed for kinase activity, and their amounts were determined in parallel. (D) Time-dependent expression of Cdk2, Cdk4, and Cdk6 proteins in primary neonatal mouse calvarial osteoblasts stimulated with or without BMP-2.

of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris [pH 8.1]) containing 1 mM PMSF and 1  $\mu$ g of aprotinin/ml. The cell lysates were then sonicated at 4°C with a Branson 450 sonifier at an output control of 2 and a duty cycle of 10 for 10 s to fragment the chromosomal DNA into 0.2- to 1-kb pieces. After the insoluble material was removed by centrifugation at  $15,000 \times g$  for 10 min, 1/10 aliquots of the supernatants were taken and saved as input. The rest of the supernatants were diluted appropriately with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.1], 167 mM NaCl) containing 1 mM PMSF and 1  $\mu$ g of aprotinin/ml and were pretreated with a salmon sperm DNA-protein A-50% agarose slurry at 4°C for 30 min with gentle agitation. After a brief centrifugation to remove the slurry, the supernatants were collected and incubated at 4°C overnight with antibodies (anti-mouse pRb [1  $\mu$ g each of Ab-4 and Ab-5; Neomarkers] or anti-CBFA1 [2  $\mu$ g of sc-8566; Santa Cruz]). Immunocomplexes were allowed to bind to protein A-agarose by incubation with a salmon sperm DNA-protein A-50% agarose slurry at 4°C for 1 h with gentle agitation. After a brief centrifugation, the supernatants were saved as the unbound fraction. The protein A-bound immunocomplexes were washed once with 1 ml each of the buffers shown below in sequential order: (i) low-salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 150 mM NaCl), (ii) high-salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 500 mM NaCl), (iii) LiCl immune complex wash buffer (0.25 M LiCl, 1% IGEPAL-CA630, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris, pH 8.1), and (iv) TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The protein A-bound immunocomplexes were resuspended in 250  $\mu$ l of elution buffer (1% SDS, 0.1 M NaHCO<sub>3</sub>), mixed by vortexing, and incubated at room temperature for 15 min, with gentle agitation. After a brief centrifugation, the supernatants were saved, and elution was repeated one more time. Both eluates were combined, and after the addition of 20  $\mu$ l of 5 M NaCl, were heated at 65°C for 4 h to reverse cross-linking. Ten microliters of 0.5 M EDTA, 20  $\mu$ l of 1 M Tris-HCl (pH 6.5), and 2  $\mu$ l of proteinase K (20 mg/ml) were added to the eluates, and the mixtures were incubated for 1 h at 45°C to remove proteins bound to DNA, followed by sequential extraction steps with phenol-chloroform and chloroform and by DNA precipitation with ethanol after the addition of 10  $\mu$ g of glycogen as a carrier. The precipitated DNA was recovered by centrifugation and resuspended in 100  $\mu$ l of TE buffer. The input fractions of the supernatants and the unbound

fractions were similarly treated to remove cross-links and proteins, and the DNAs were ethanol precipitated similarly and dissolved in 10 and 100  $\mu$ l of TE buffer, respectively. The osteocalcin and myogenin promoter sequences were amplified by 40 cycles of PCR from 1  $\mu$ l each of the DNA solutions, with parameters of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C and with the following primers: 5'-CTGAAGTGGCAAATGAGGACA-3' and 5'-AGGGGATGCTGCCAGGACTAAT for the mouse osteocalcin promoter (positions -67 to -471) and 5'-ACCCCTTCTTGTCCCTCCT-3' and 5'-CTCCCGCAGCCCTCAC-3' for the mouse myogenin promoter (positions -9 to -431).

**Statistical analysis.** The means of groups were compared by analysis of variance, and the significance of differences was determined by post hoc testing using the Bonferroni method.

## RESULTS

**Cdk6 is down-regulated in osteoblasts upon BMP-2 treatment.** Within 24 h after treatment with BMP-2, C2C12 cells begin to express osteocalcin mRNA and ALP, two representative markers of mature osteoblasts (21). Accordingly, the commitment to BMP-2-induced osteoblast differentiation occurs within 24 h. Since the MC3T3-E1 cells used in the present study are known to be in a stage that is one step more differentiated toward mature osteoblasts than C2C12 cells, we assumed that the commitment of this cell line to BMP-2-induced osteoblast differentiation occurred well within 24 h, and therefore we analyzed the effect of BMP-2 on the initial 48-h expression levels of cell cycle factors that critically regulate the onset of S phase in this cell line. The cells were arrested in quiescence by serum starvation, stimulated with serum in the presence or absence of BMP-2, and harvested every 6 or 12 h. The amounts of cyclins (D1, D2, D3, and E), Cdk2, Cdk4,

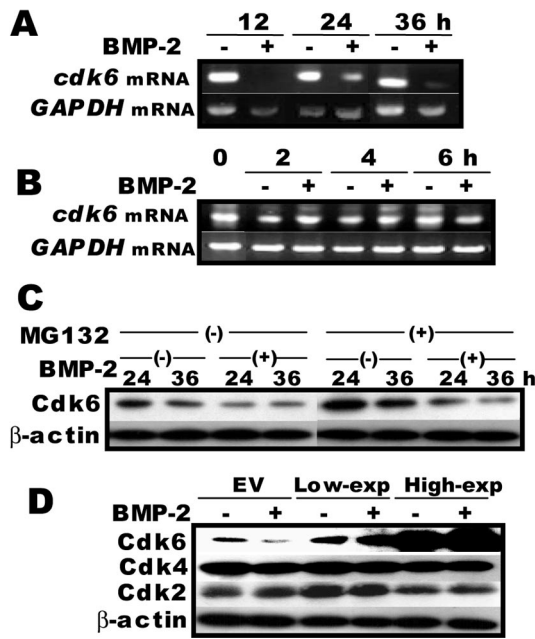


FIG. 2. BMP-2 down-regulates Cdk6 expression mainly via preventing transcription. (A) BMP-2 treatment markedly reduces *cdk6* mRNA level. Growth-arrested MC3T3-E1 cells were stimulated with or without BMP-2 for the indicated times, and RNAs were prepared. *cdk6* mRNA was semiquantified by RT-PCR. (B) BMP-2 treatment does not affect the stability of *cdk6* mRNA. Growth-arrested MC3T3-E1 cells were stimulated with serum for 6 h and then incubated with actinomycin D (Sigma) (0.1  $\mu$ g/ml) with or without the addition of BMP-2. RNAs were prepared and the Cdk6 transcript was semiquantified by RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as a control for the RNA preparations. (C) The proteasome inhibitor MG132 does not influence the action of BMP-2 on the Cdk6 protein level. Growth-arrested MC3T3-E1 cells were stimulated for 24 and 36 h with or without BMP-2 and/or MG132 (2  $\mu$ M), and the Cdk6 protein levels were determined by Western blotting. (D) BMP-2 treatment does not affect the level of forcefully expressed Cdk6 protein. Clones constitutively expressing Cdk6 from an EF1 $\alpha$ -promoter as well as empty vector-transfected MC3T3-E1 (EV) cells were growth arrested as described in the text and stimulated for 48 h with or without BMP-2. The amounts of Cdk6, Cdk4, and Cdk2 were then semiquantified by Western blotting.

Cdk6, CKIs (p18, p21, p27, and p57), and Rb and phosphorylated Rb proteins in the whole-cell lysate at each time point were analyzed by Western blotting (Fig. 1). In this cell line, the amounts of Cdk6, Cdk2, and p18 increased during serum stimulation, as early as 6 h after stimulation, and the amount of p21 decreased, whereas the amounts of the remaining factors were unchanged. This cell line did not express detectable amounts of p57 throughout the experiment (data not shown). Coinciding with the elevation of Cdk2, phosphorylated forms of the Rb protein appeared. Under these conditions, the cells grew to confluence but did not commit to differentiation into osteoblasts.

In contrast, when MC3T3-E1 cells were stimulated with BMP-2 to stimulate osteoblast differentiation, the induction of Cdk6 completely disappeared (Fig. 1A), while the levels of the remaining factors, including Cdk4, were virtually indistinguishable from those obtained by serum stimulation. The behavioral difference between Cdk6 and Cdk4 was further noticeable in another experiment. When MC3T3-E1 cells were treated with

FGF-2, a potent stimulator of osteoblast proliferation, the level of Cdk4, but not that of Cdk6, was markedly elevated (Fig. 1B).

To confirm that the blocked induction of Cdk6 was faithfully reflected by its kinase activities, we assayed the activities of Cdk6 and Cdk4 during serum stimulation in the presence and absence of BMP-2. When the cells were stimulated with serum, Cdk6 activity increased at 12 h and remained high at least until 48 h. In contrast, when the cells were stimulated with serum containing BMP-2, Cdk6 activity increased at 6 h, but rapidly decreased to the basal level by 12 and 24 h, the times expected for the commitment to differentiation to take place (Fig. 1C). These activities were roughly correlated with the amounts of immunoprecipitated Cdk6, which faithfully reflected its amounts in whole-cell lysates (Fig. 1A), except for the BMP-2-treated 6-h sample, from which Cdk6 was more efficiently immunoprecipitated. This result was reproducible, but its reason was unclear.

On the other hand, there was no significant difference in the time course and extent of Cdk4 activation between BMP-2-treated and untreated MC3T3-E1 cells. Thus, despite the fact that Cdk4 and Cdk6 are structurally homologous (11) and share D cyclins as their catalytic partners, only the expression of Cdk6 was significantly influenced by BMP-2 treatment. We did a similar experiment with proliferating MC3T3-E1 cells and observed a similar down-regulation of Cdk6 upon BMP-2 treatment, although it was less obvious, perhaps due to the lack of synchronization in their cell cycle progression (data not shown).

Cdk6 down-regulation is not specific to this cell line and is a more general phenomenon. We performed the same Western blot analysis with a culture of primary osteoblasts isolated from neonatal mouse calvariae and obtained the same results. Treatment with BMP-2 during serum stimulation completely blocked the induction of Cdk6 at 24 and 32 h, whereas Cdk4 and Cdk2 were uninfluenced (Fig. 1D). These results show that Cdk6 was specifically down-regulated during the commitment to BMP-2-induced osteoblast differentiation.

**BMP-2-led Cdk6 down-regulation is exerted at the transcriptional level.** We performed a semiquantitative RT-PCR analysis of the Cdk6 transcript and found that *cdk6* mRNA disappeared when MC3T3-E1 cells were stimulated with BMP-2 (Fig. 2A). In contrast, BMP-2 did not significantly influence the stability of either *cdk6* mRNA or protein. When the cells were stimulated with serum for 6 h and then treated with

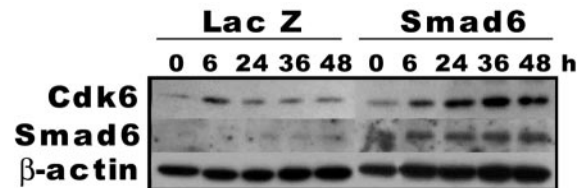


FIG. 3. Smad6 overexpression effectively blocks BMP-2-led down-regulation of Cdk6. MC3T3-E1 cells were infected with a recombinant adenovirus carrying the *smad6* or *lacZ* gene and were subsequently growth arrested by incubation in a low-serum-level medium for 48 h. The cells were then stimulated with serum in the presence or absence of BMP-2. The expression of Cdk6 and Smad6 was determined by Western blotting, with  $\beta$ -actin used as a loading control.

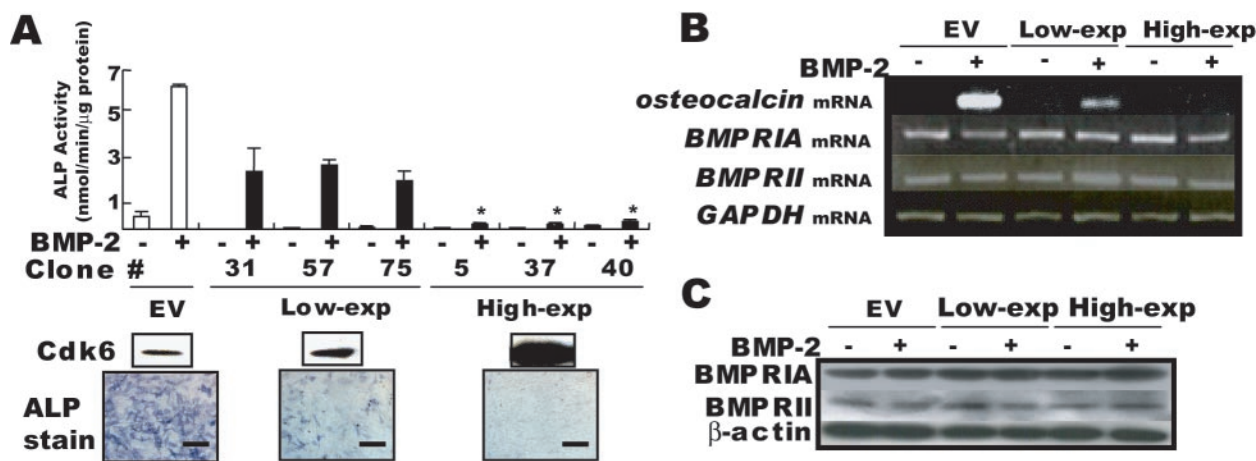


FIG. 4. Overexpression of Cdk6 inhibits BMP-2-induced osteoblast differentiation. MC3T3-E1 cell clones expressing low (no. 31, 57, and 75) or high (no. 5, 37, and 40) levels of Cdk6 and empty vector-transfected MC3T3-E1 (EV) cells were induced for osteoblast differentiation by treatment with BMP-2 according to the standard protocol. (A) After 72 h of treatment, the induced ALP activity was assayed (top). The middle panel shows Western blot data for Cdk6 in growing empty vector-transfected cells and cells from clones 31 and 40. \*,  $P < 0.1$ ; there is a significant difference from the ALP activity of BMP-2-treated empty vector-transfected cells. The bottom panels show ALP staining of empty vector-transfected cells and clones 31 and 40. Bars, 100  $\mu\text{m}$ . (B) Levels of osteocalcin, BMPRIA, and BMPRII mRNAs, as determined by semiquantitative RT-PCRs with empty vector-transfected cells and clones 31 and 40 stimulated for 48 h with or without BMP-2. GAPDH mRNA was used as a control for the RNA preparations. (C) Protein levels of BMPRIA and BMPRII in empty vector-transfected cells and clones 31 and 40 stimulated for 48 h with or without BMP-2.

actinomycin D in the presence or absence of BMP-2, there was no difference in the rate of disappearance of the Cdk6 transcript between the treatment and nontreatment groups (Fig. 2B).

Cdk4 is known to be degraded by the ubiquitin-proteasome system (27). Because Cdk6 and Cdk4 are siblings and execute similar functions, we assumed that Cdk6 was likely to be degraded by the same proteolytic system. When MC3T3-E1 cells were treated with MG132, a potent inhibitor of this proteolytic system, during serum stimulation in the absence of BMP-2, the level of Cdk6 was elevated two- to threefold, consistent with this assumption (Fig. 2C). In the presence of BMP-2, MG132 treatment elevated the level of Cdk6 by the same degree, suggesting that there is no acceleration of Cdk6 degradation by treatment with this differentiation inducer. To further confirm that BMP-2 did not affect the stability of the Cdk6 protein, we constructed and analyzed MC3T3-E1 cell clones that constitutively expressed Cdk6 at low and high levels. BMP-2 treatment did not influence the levels of Cdk6 protein expressed from the constitutive EF1 $\alpha$  promoter (Fig. 2D). These findings indicate that BMP-2 down-regulates Cdk6 expression mainly, if not exclusively, by transcriptional repression.

**Smads mediate BMP-2-induced down-regulation of Cdk6.** A differentiation signal invoked by BMP-2 is known to be mediated by Smad proteins, particularly R-Smads and Co-Smads, while I-Smads, such as Smad6 and Smad7, inhibit this signaling (6). To investigate whether Smads mediate Cdk6 down-regulation, we overexpressed Smad6 in MC3T3-E1 cells by use of an adenovirus vector and examined its effect on BMP-2-led Cdk6 down-regulation. As shown in Fig. 3, BMP-2-led Cdk6 down-regulation was completely abolished by the overexpression of Smad6, indicating that Smads do indeed mediate the BMP-2-led Cdk6 down-regulation.

**Osteoblasts overexpressing Cdk6 strongly resist BMP-2-invoked differentiation.** As discussed above, Cdk6 was down-regulated during BMP-2-invoked osteoblast differentiation. Consequently, a key question is whether this down-regulation is essential for osteoblast differentiation or not. To address this question, we constructed >100 MC3T3-E1 cell clones that stably expressed various levels of Cdk6 by transfecting MC3T3-E1 cells with an expression vector harboring *Cdk6* cDNAs and then tested their ability to respond to BMP-2 and to differentiate into mature osteoblasts. Figure 2D shows the protein levels of Cdk6, Cdk4, and Cdk2 in some representative clones that were treated with BMP-2 or were left untreated compared to the protein levels in similarly treated empty vector-transfected control MC3T3-E1 cells. The Cdk4 and Cdk2 protein levels were not affected by the overexpression of Cdk6, suggesting the absence of any particular compensatory regulation of Cdk4 or Cdk2 expression upon Cdk6 overexpression.

The ability of BMP-2 to induce osteoblast differentiation, as monitored by ALP production and osteocalcin expression, which are well-characterized early and late differentiation markers, respectively, was then studied with control cells and low (~2-fold) and high (>10-fold) Cdk6 expressers. The induction of ALP upon BMP-2 treatment was inhibited significantly in the low expressers and completely in the high expressers (Fig. 4A). Osteocalcin mRNA, as measured by semiquantitative RT-PCR, was markedly reduced in the low expressers and was undetectable in the high expressers (Fig. 4B). Thus, a twofold overexpression of Cdk6, or the same level of exogenous Cdk6 expression as endogenous expression, already showed a strong inhibitory effect on differentiation. This implies that the presence of the original level of Cdk6 expression was enough to markedly inhibit the BMP-2-invoked differentiation of MC3T3-E1 cells.

TABLE 1. Proliferation and cell cycle distribution of MC3T3-E1 cells that overexpress Cdk6<sup>a</sup>

Cell type	BrdU incorporation (OD) at indicated time (h) in presence or absence of BMP-2				Flow cytometric analysis result in presence or absence of BMP-2			
	24		72		% G <sub>0</sub> /G <sub>1</sub>		%G <sub>2</sub> /M	
	-	+	-	+	-	+	-	+
Empty vector	0.37 ± 0.01	0.37 ± 0.12	0.07 ± 0.01	0.08 ± 0.01	78 ± 0	80 ± 1	16 ± 1	15 ± 0
Low expresser	0.41 ± 0.05	0.37 ± 0.08	0.06 ± 0.02	0.08 ± 0.02	78 ± 1	76 ± 0	17 ± 1	18 ± 1
High expresser	0.55 ± 0.07	0.43 ± 0.07	0.05 ± 0.01	0.06 ± 0.01	77 ± 0	79 ± 1	15 ± 1	15 ± 1

<sup>a</sup> Cell proliferation was determined by BrdU incorporation after 24 and 72 h of culture in the presence and absence of BMP-2. Cell cycle distribution was determined by flow cytometric analysis of cells after 24 h of culture. Three representative clones (31, 57, and 75 and 5, 37, and 40, respectively) were chosen from the low- and high-expressing group. Data are expressed as means ± standard deviations after analysis of cells in eight separate wells for each clone.

The Cdk6-led resistance to osteoblast differentiation is unlikely to be caused by an unexpected interference of the BMP-2/Smads signaling pathway by the overexpressed Cdk6. Neither the mRNA nor the protein levels of the BMP receptors BMPRIA and BMPRII were significantly affected by Cdk6 expression (Fig. 4B and C).

**Overexpression of Cdk6 does not influence proliferation of osteoblasts.** Because Cdk6 promotes the G<sub>1</sub>-S transition, the suppression of osteoblast differentiation by overexpressed Cdk6 could be a mere consequence of its execution of this role. We therefore examined the effects of both BMP-2 and Cdk6 overexpression on the G<sub>1</sub>-S transition and the proliferation of MC3T3-E1 cells. Empty vector-transfected MC3T3-E1 cells, as well as the low and high Cdk6 expressers, were similarly arrested in quiescence, stimulated with serum in the presence or absence of BMP-2, and analyzed for S-phase entry as well as for cell populations in G<sub>0</sub>-G<sub>1</sub> and G<sub>2</sub>-M by BrdU incorporation and flow cytometric analyses (Table 1). Cell proliferation was also monitored in the presence and absence of BMP-2 by an XTT assay (Fig. 5). The overexpression of Cdk6 did not cause any significant changes in either the cell cycle distribution or the proliferation rate of the cells, regardless of whether the cells were stimulated with BMP-2 or not. These results suggest that the inhibitory effect of Cdk6 on osteoblast differentiation is not exerted via cell cycle regulation.

**The Cdk6-exerted differentiation block is correlated with a loss of the binding of Runx2/Cbfa1, but not Rb, to the osteocalcin promoter.** Rb, a potent repressor of the E2F-DP transcriptional factor that is essential for the onset of S phase, has been reported to also act as a transcriptional coactivator for Runx2/Cbfa1, a key transcriptional factor for osteoblast differentiation (23). Since Rb is a direct target of Cdk6 for cell cycle control, we examined whether Rb mediates the Cdk6-exerted inhibition of osteoblastic differentiation by determining the effects of overexpressed Cdk6 on the *in vivo* binding of Runx2/Cbfa1 and Rb to the osteocalcin promoter during BMP-2 treatment. To this end, we performed a ChIP assay. The specificities of the anti-Runx2/Cbfa1 and anti-Rb antibodies used for this assay were confirmed by the lack of precipitation of the irrelevant myogenin promoter, but the clear precipitation of the osteocalcin promoter, by both antibodies (Fig. 6).

Using these antibodies, we compared the binding levels of Runx2/Cbfa1 and Rb to the osteocalcin promoter in empty vector-transfected MC3T3-E1 cells and in low and high Cdk6 expressers at day 1 (a time just after the differentiation commitment) and day 4 (well after the onset of full differentiation phenotypes for MC3T3-E1 cells) post-BMP-2 treatment. As

shown in Fig. 6A, on day 1 Runx2/Cbfa1 bound slightly to the osteocalcin promoter in the empty vector-transfected MC3T3-E1 cells, but not in either the low or high Cdk6 expressers, whereas no binding of Rb to this promoter was detected in any of these cells. On day 4, when osteocalcin was fully expressed, the binding of Runx2/Cbfa1 to this promoter was clearly detected in the empty vector-transfected MC3T3-E1 cells and the low Cdk6 expresser, but not in the high Cdk6 expresser, in which osteoblast differentiation was completely blocked (Fig. 4) despite a high level of Runx2/Cbfa1 expression (Fig. 6B). Thus, there was a close correlation between osteoblast differentiation and the binding of Runx2/Cbfa1 to the osteocalcin promoter in Cdk6-led differentiation inhibition. In

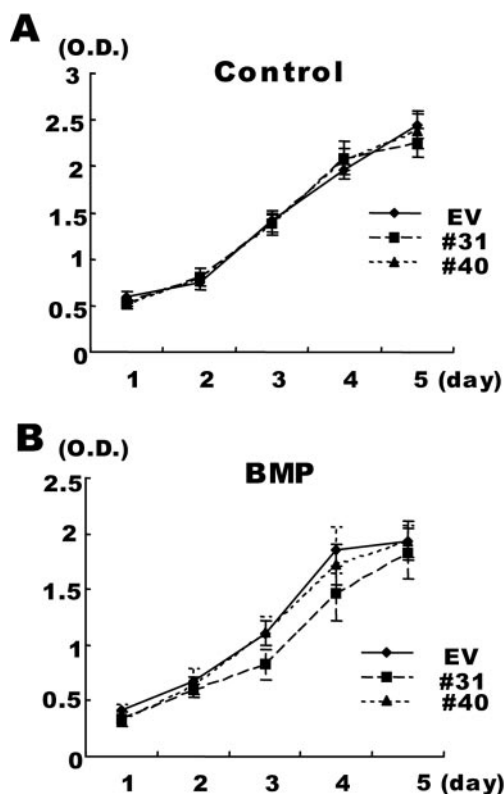


FIG. 5. Overexpression of Cdk6 does not influence proliferation of MC3T3-E1 cells. Proliferation rates were determined by XTT staining of empty vector-transfected cells (EV) and clones 31 and 40 cultured in the presence (B) or absence (A) of BMP-2, with cell sampling done every day.

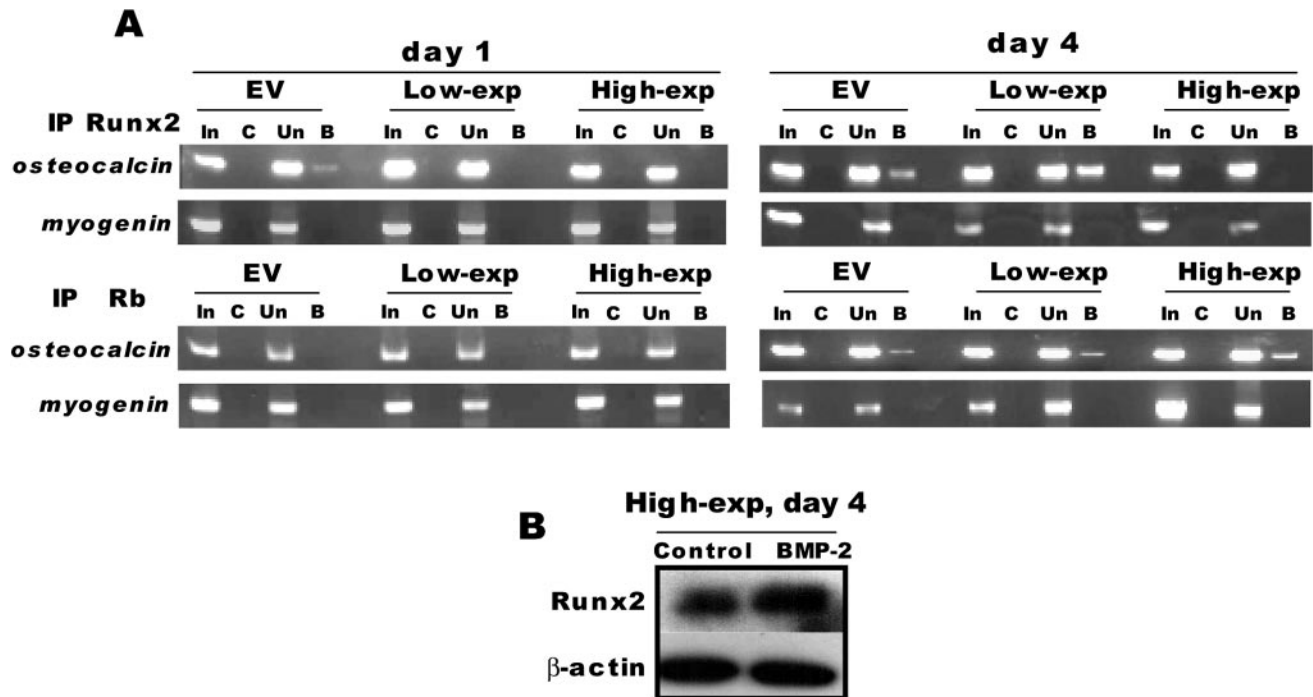


FIG. 6. Cdk6-exerted differentiation block is correlated with a loss of binding of Runx2/Cbfa1, but not Rb, to the osteocalcin promoter. (A) ChIP assay performed with empty vector-transfected MC3T3-E1 cells (EV) and clones 31 (low Cdk6 expresser) and 40 (high Cdk6 expresser) cultured for 1 and 4 days with BMP-2, as described in Materials and Methods. IP Rb, immunoprecipitation with an anti-Rb antibody; IP Runx2, immunoprecipitation with an anti-Runx2/Cbfa1 antibody; In, total DNA input for each sample; C, immunoprecipitation with control serum; Un, PCR from the supernatant after immunoprecipitation; B, PCR from immunoprecipitation product. The primer sets for PCR of the osteocalcin and myogenin promoter regions are described in Materials and Methods. (B) Runx2/Cbfa1 is expressed in a high Cdk6 expresser cultured for 4 days with or without BMP-2. The level of Runx2/Cbfa1 was determined by Western blotting. The loading control is β-actin.

contrast, there was no apparent correlation between Rb binding and osteoblastic differentiation. On day 4, the binding of Rb to the osteocalcin promoter was detected, as reported previously (23), but it was present in all of the cells, despite the fact that osteoblastic differentiation in the high Cdk6 expresser was completely blocked. Moreover, Rb seemed to bind the promoter independently of Runx2/Cbfa1, because in the high Cdk6 expresser, Rb bound the promoter without the binding of Runx2/Cbfa1.

These ChIP assay results indicate that Rb is unlikely to mediate the Cdk6-led differentiation inhibition, although it is certainly required for the efficient osteoblast differentiation of MC3T3-E1 cells (23). In addition, the loss of Runx2/Cbfa1 binding to the osteocalcin promoter during Cdk6-exerted differentiation inhibition raises the possibility that Cdk6 may inhibit osteoblastic differentiation by blocking the promoter-binding ability of the Runx2/Cbfa1 transcriptional factor.

**DISCUSSION**

In the present study, we have shown that Cdk6 expression is shut down mainly at transcription upon BMP-2 treatment and that this shutdown, mediated by BMP-2-activated Smad signaling, is required for efficient osteoblast differentiation. Osteoblastic cells, including the presently used MC3T3-E1 cell line and primary mouse osteoblasts, express both Cdk4 and Cdk6, yet only Cdk6 is critically involved in the commitment to dif-

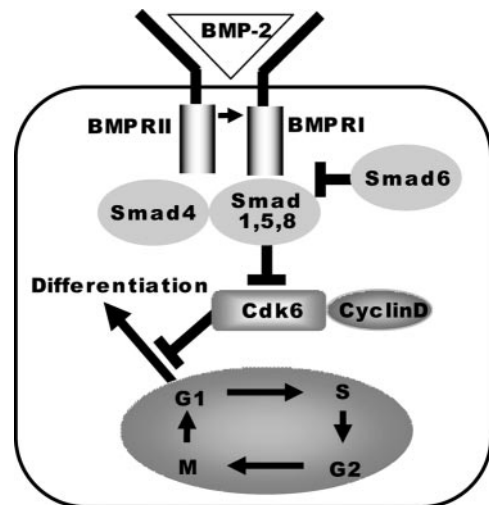


FIG. 7. Schematic presentation of the mechanism by which Cdk6 inhibits osteoblastic differentiation. BMP-2 binds the type II receptor, which subsequently activates the type I receptor by direct association. The activated type I receptor directly phosphorylates R-Smads (Smads 1, 5, and 8) and promotes their complex formation with Co-Smad (Smad4). The R-Smads/Co-Smad complexes are then translocated into the nucleus, where they repress the transcription of the *cdk6* gene and permit osteoblastic differentiation to take place.

ferentiation. This study demonstrates that Cdk6 is a key molecule determining the differentiation rate of osteoblasts as a downstream effector of BMP-2/Smad signaling. Figure 7 depicts a schematic presentation of the mechanism by which BMP-2 induces osteoblast differentiation, as revealed by the present and previous studies. BMP-2 binds to the type II receptor and activates the type I receptor, leading to the formation of R-Smads/Co-Smad complexes, which are imported into the nucleus. The R-Smads/Co-Smad complexes then repress the *cdk6* promoter, thereby removing Cdk6-exerted blocking of differentiation.

The Cdk6-cyclin D3 complex is unique among cyclin D-cognate kinase combinations and evades inhibition by CKIs (8). Therefore, it can greatly enhance the proliferative potential of fibroblasts under growth-suppressive conditions and consequently sensitizes cells to physical and chemical transformation (2). This unique ability of Cdk6, however, does not seem to be responsible for the requirement of Cdk6 down-regulation for efficient osteoblast differentiation because we did not find any noticeable effect of BMP-2 and Cdk6 overexpression on the proliferation or even the cell cycle progression of MC3T3-E1 cells under the experimental conditions we employed. This apparent lack of a growth-stimulating function for Cdk6 in this cell line is consistent with the observation that Cdk4, but not Cdk6, was up-regulated by FGF-2, a potent stimulator of osteoblast proliferation.

The Rb protein has been implicated in osteoblast differentiation. The incidence of osteosarcoma increases 500-fold in patients inheriting *Rb* gene mutations. Recently, the Rb protein was reported to physically interact with Runx2/Cbfa1, which transactivates osteoblast-specific promoters (23). This transactivation is lost in tumor-derived Rb protein mutants, underscoring its potential role in osteoblast differentiation. The possibility that Rb directly mediates the role of Cdk6 as a differentiation inhibitor, however, is remote because unlike for Runx2/Cbfa1, there was no apparent correlation between the Cdk6-exerted differentiation block and the binding of Rb to the osteocalcin promoter.

How could Cdk6 control differentiation without influencing cell cycling? One possibility is that Cdk6 directly controls a factor(s) that is critically involved in differentiation. This possibility may not be as remote as is generally thought. In *Schizosaccharomyces pombe*, Pas1 cyclin and its partner kinase Pef1 activate a transcriptional factor complex that is functionally equivalent to E2F-DP of mammals, thereby promoting S-phase entry, just like Cdk6, yet they independently inhibit mating pheromone signaling, whose activation is essential for the differentiation of yeast cells (22). Thus, this may be a good model for the situation of Cdk6 in BMP-2-induced osteoblast differentiation, highlighting a potential functional similarity between Cdk6 and Pef1.

This study demonstrates for the first time that Cdk6, a G<sub>1</sub> cell cycle factor, plays a critical role in controlling BMP-2-induced osteoblast differentiation. Several transcription factors, such as Runx2/Cbfa1, osterix, and low-density lipoprotein receptor protein 5, have been reported to be involved in bone formation (12, 16). Consequently, one of these factors may be responsible for the BMP-2-induced repression of Cdk6 transcription. The identification of the transcriptional repressor as well as key targets of Cdk6 will definitely be required for a

deeper understanding of the molecular basis of bone formation.

Finally, it is appropriate to stress that our finding is not specific to BMP-2-induced osteoblast differentiation. Very recently, Matushansky et al. reported a similar role for Cdk6 in the erythroid differentiation of a murine leukemia cell line (10).

#### ACKNOWLEDGMENTS

We thank Kohei Miyazono and Takeshi Imamura for kindly providing an adenovirus vector carrying *smad6* and Izumu Saito for an adenovirus vector carrying *lacZ*. We also thank H. Chikuda, M. Tsuji, and K. Baba for helpful discussion and support.

This work was supported by grants from the Department of Science, Education and Culture of Japan.

#### REFERENCES

- Bai, S., X. Shi, X. Yang, and X. Cao. 2000. Smad6 as a transcriptional corepressor. *J. Biol. Chem.* **275**:8267–8270.
- Chen, Q., J. Lin, S. Jinno, and H. Okayama. 2003. Overexpression of Cdk6-cyclin D3 highly sensitizes cells to physical and chemical transformation. *Oncogene* **22**:992–1001.
- Deng, C., P. Zhang, J. W. Harper, S. J. Elledge, and P. Leder. 1995. Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell* **82**:675–684.
- DiChiara, M. R., J. M. Kiely, M. A. Gimbrone, Jr., M. E. Lee, M. A. Perrella, and J. N. Topper. 2000. Inhibition of E-selectin gene expression by transforming growth factor beta in endothelial cells involves coactivator integration of Smad and nuclear factor kappaB-mediated signals. *J. Exp. Med.* **192**:695–704.
- Hinds, P. W., and R. A. Weinberg. 1994. Tumor suppressor genes. *Curr. Opin. Genet. Dev.* **4**:135–141.
- Kretzschmar, M., and J. Massague. 1998. SMADs: mediators and regulators of TGF-beta signaling. *Curr. Opin. Genet. Dev.* **8**:103–111.
- Lee, K. S., H. J. Kim, Q. L. Li, X. Z. Chi, C. Ueta, T. Komori, J. M. Wozney, E. G. Kim, J. Y. Choi, H. M. Ryoo, and S. C. Bae. 2000. Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. *Mol. Cell. Biol.* **20**:8783–8792.
- Lin, J., S. Jinno, and H. Okayama. 2001. Cdk6-cyclin D3 complex evades inhibition by inhibitor proteins and uniquely controls cell's proliferation competence. *Oncogene* **20**:2000–2009.
- Lopez-Rovira, T., E. Chalaux, J. L. Rosa, R. Bartrons, and F. Ventura. 2000. Interaction and functional cooperation of NF-kappa B with Smads. Transcriptional regulation of the JunB promoter. *J. Biol. Chem.* **275**:28937–28946.
- Matushansky, I., F. Radparvar, and A. I. Skoultschi. 2003. CDK6 blocks differentiation: coupling cell proliferation to the block to differentiation in leukemic cells. *Oncogene* **22**:4143–4149.
- Meyerson, M., G. H. Enders, C. L. Wu, L. K. Su, C. Gorka, C. Nelson, E. Harlow, and L. H. Tsai. 1992. A family of human cdc2-related protein kinases. *EMBO J.* **11**:2909–2917.
- Nakashima, K., X. Zhou, G. Kunkel, Z. Zhang, J. M. Deng, R. R. Behringer, and B. de Crombrughe. 2002. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* **108**:17–29.
- Nakayama, K., N. Ishida, M. Shirane, A. Inomata, T. Inoue, N. Shishido, I. Horii, and D. Y. Loh. 1996. Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* **85**:707–720.
- Nevins, J. R. 1992. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. *Science* **258**:424–429.
- Nishimori, S., Y. Tanaka, T. Chiba, M. Fujii, T. Imamura, K. Miyazono, T. Ogasawara, H. Kawaguchi, T. Igarashi, T. Fujita, K. Tanaka, and H. Toyoshima. 2001. Smad-mediated transcription is required for transforming growth factor-beta 1-induced p57(Kip2) proteolysis in osteoblastic cells. *J. Biol. Chem.* **276**:10700–10705.
- Patel, M. S., and G. Karsenty. 2002. Regulation of bone formation and vision by LRP5. *N. Engl. J. Med.* **346**:1572–1574.
- Reddi, A. H. 1998. Role of morphogenetic proteins in skeletal tissue engineering and regeneration. *Nat. Biotechnol.* **16**:247–252.
- Sherr, C. J. 1994. G1 phase progression: cycling on cue. *Cell* **79**:551–555.
- Sherr, C. J., and J. M. Roberts. 1999. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* **13**:1501–1512.
- Shi, X., X. Yang, D. Chen, Z. Chang, and X. Cao. 1999. Smad1 interacts with homeobox DNA-binding proteins in bone morphogenetic protein signaling. *J. Biol. Chem.* **274**:13711–13717.



21. Takeda, K., H. Ichijo, M. Fujii, Y. Mochida, M. Saitoh, H. Nishitoh, T. K. Sampath, and K. Miyazono. 1998. Identification of a novel bone morphogenetic protein-responsive gene that may function as a noncoding RNA. *J. Biol. Chem.* **273**:17079–17085.
22. Tanaka, K., and H. Okayama. 2000. A Pcl-like cyclin activates the Res2p-Cdc10p cell cycle “start” transcriptional factor complex in fission yeast. *Mol. Biol. Cell* **11**:2845–2862.
23. Thomas, D. M., S. A. Carty, D. M. Piscopo, J. S. Lee, W. F. Wang, W. C. Forrester, and P. W. Hinds. 2001. The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. *Mol. Cell* **8**:303–316.
24. Urano, T., H. Yashiroda, M. Muraoka, K. Tanaka, T. Hosoi, S. Inoue, Y. Ouchi, and H. Toyoshima. 1999. p57(Kip2) is degraded through the proteasome in osteoblasts stimulated to proliferation by transforming growth factor beta1. *J. Biol. Chem.* **274**:12197–12200.
25. Verschuere, K., J. E. Remacle, C. Collart, H. Kraft, B. S. Baker, P. Tylzanowski, L. Nelles, G. Wuytens, M. T. Su, R. Bodmer, J. C. Smith, and D. Huylebroeck. 1999. SIP1, a novel zinc finger/homeodomain repressor, interacts with Smad proteins and binds to 5'-CACCT sequences in candidate target genes. *J. Biol. Chem.* **274**:20489–20498.
26. Vidal, A., and A. Koff. 2000. Cell-cycle inhibitors: three families united by a common cause. *Gene* **247**:1–15.
27. Wang, H., T. Goode, P. Iakova, J. H. Albrecht, and N. A. Timchenko. 2002. C/EBPalpha triggers proteasome-dependent degradation of cdk4 during growth arrest. *EMBO J.* **21**:930–941.
28. Yan, Y., J. Frisen, M. H. Lee, J. Massague, and M. Barbacid. 1997. Ablation of the CDK inhibitor p57Kip2 results in increased apoptosis and delayed differentiation during mouse development. *Genes Dev.* **11**:973–983.
29. Yoshida, Y., S. Tanaka, H. Umemori, O. Minowa, M. Usui, N. Ikematsu, E. Hosoda, T. Imamura, J. Kuno, T. Yamashita, K. Miyazono, M. Noda, T. Noda, and T. Yamamoto. 2000. Negative regulation of BMP/Smad signaling by Tob in osteoblasts. *Cell* **103**:1085–1097.
30. Zhang, P., N. J. Liegeois, C. Wong, M. Finegold, H. Hou, J. C. Thompson, A. Silverman, J. W. Harper, R. A. DePinho, and S. J. Elledge. 1997. Altered cell differentiation and proliferation in mice lacking p57KIP2 indicates a role in Beckwith-Wiedemann syndrome. *Nature* **387**:151–158.
31. Zhang, Y. W., N. Yasui, K. Ito, G. Huang, M. Fujii, J. Hanai, H. Nogami, T. Ochi, K. Miyazono, and Y. Ito. 2000. A RUNX2/PEBP2alphaA/CBFA1 mutation displaying impaired transactivation and Smad interaction in cleidocranial dysplasia. *Proc. Natl. Acad. Sci. USA* **97**:10549–10554.
32. Zhu, H., P. Kavsak, S. Abdollah, J. L. Wrana, and G. H. Thomsen. 1999. A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. *Nature* **400**:687–693.
33. Zimmermann, M. 1983. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* **16**:109–110.BM