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Histone Deacetylase 1-Mediated Histone Modification Regulates Osteoblast Differentiation

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Osteogenesis is a complex process associated with dramatic changes in gene expression. To elucidate whether modifications in chromatin structure are involved in osteoblast differentiation, we examined the expression levels of histone deacetylases (HDACs) and the degree of histone acetylation at the promoter regions of osteogenic genes. During osteogenesis, total HDAC enzymatic activity was decreased with significant reduction in HDAC1 expression. Consistently, recruitment of HDAC1 to the promoters of osteoblast marker genes, including osterix and osteocalcin, was down-regulated, whereas histone H3 and H4 were hyperacetylated at those promoters during osteoblast differentiation. Moreover, suppression of HDAC activity with a HDAC inhibitor, sodium butyrate, accelerated osteogenesis by inducing osteoblast marker genes including osteopontin and alkaline phosphatase. Consistently, knockdown of HDAC1 by the short interference RNA system stimulated osteoblast differentiation. Taken together, these data propose that down-regulation of HDAC1 is an important process for osteogenesis. (*Molecular Endocrinology* 20: 2432–2443, 2006)

WITH AGING, BONE mass tends to decrease, resulting in the onset of many diseases associated with bone leakage. For example, osteoporosis, characterized by low bone mass and structural deterioration of bone tissue with an increased susceptibility to fractures, is a major public health threat to the elderly (1, 2). Bone is continuously destroyed and reformed in vertebrates through tight balance between osteoblastic bone formation and osteoclastic bone resorption to maintain a certain level of bone mass and calcium homeostasis (2).

Depending on various cellular and environmental signals, multipotent mesenchymal progenitor cells are able to differentiate into osteoblasts (3, 4). For example, bone morphogenetic protein-2 and -7, members of the TGF-β superfamily, promote osteoblast differentiation (5–7). These factors transduce their signals through activation of osteogenic transcription factors such as Runx2 (also called Cbfa1, Pebp2αA, and AML3) and osterix (5, 6, 8). Runx2, a mammalian homolog of the *Drosophila* runt (9), is a master transcription factor for osteoblast differentiation by regulating the expression of most osteogenic marker genes including osterix, osteocalcin, alkaline phosphatase (ALP), and osteopontin, which are required for producing bone extracellular matrix (10, 11). Overexpression of Runx2 leads to osteoblast-specific gene expression in fibroblasts and myoblasts *in vitro*, demonstrating that Runx2 is a sufficient factor to initiate osteogenesis (12, 13). Furthermore, Runx2-null mice show no bone tissue or osteoblasts, indicating that Runx2 plays a pivotal role in osteoblast differentiation (14, 15). In addition, genetic studies have exhibited that osterix, a zinc finger-containing transcription factor, is also essential for osteoblast differentiation (16, 17).

Nucleosomal complexes formed by the histone proteins and associated DNA are the fundamental units of eukaryotic chromatin (18). Dynamic changes in chromatin architecture include several modifications of histone proteins, such as acetylation, phosphorylation, ubiquitination, and methylation, which are closely linked to regulation of eukaryotic gene expression (19–21). For instance, histone acetylation contributes to the formation of a transcriptionally competent environment by relaxing the chromatin structure and allowing general transcription factors to access to the target DNA sequences (22, 23). On the contrary, histone deacetylation makes the chromatin structure compact and leads to transcriptional repression.

Transcription activators are often associated with histone acetyltransferases (HATs) to increase target gene expression, whereas transcription repressors frequently interact with histone deacetylases (HDACs) to down-regulate target gene expression (18, 24). There are several families of HATs including p300/cAMP response element-binding protein (CREB)-binding protein, p300/CREB binding protein-associated factor (P/CAF), and Gcn-related acetyltransferases, which are present as components of multisubunit protein complexes (21), whereas HDACs fall into two major classes, class I and

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Abbreviations: ALP, Alkaline phosphatase; CDK, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; CKI, CDK inhibitor; FBS, fetal bovine serum; HAT, histone deacetylase; HDAC, histone deacetylase; HEK, human embryonic kidney; NaB, sodium butyrate; SDS, sodium dodecyl sulfate; siRNA, small interference RNA; TSA, trichostatin A.

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class II. Class I HDACs (HDAC1, -2, -3, and -8) are expressed ubiquitously, whereas class II HDACs (HDAC4, -5, -6, and -7) are highly expressed in certain tissues such as heart, brain, and skeletal muscle (25). Recently, it has been reported that there are atypical HDACs including yeast Sir2-like proteins, which have NAD^+-dependent deacetylase activity (26–28). Among HDACs, HDAC1 and -2, showing high sequence homology (~82%) (25, 29), form large protein complexes with corepressors, such as mSin3A or NuRD, and suppress target gene expression by condensing the chromatin structure (24, 30). These complexes actively catalyze deacetylation of histones to impede the assembly or recruitment of transcriptional machinery to respective target promoters (24).

Although histone modifications play crucial roles in the transcriptional regulation of most eukaryotic genes, the relationship between specific cell type differentiation and histone modifications has been not completely understood. Here, we investigated the effects of chromatin modification by HDAC1 on osteogenesis. Total HDAC enzymatic activity was decreased with significant reduction in HDAC1 expression during osteoblast differentiation. Suppression of HDAC1 activity by either a HDAC inhibitor, sodium butyrate (NaB), or knockdown of HDAC1 via small interference RNA (siRNA) stimulated osteoblast differentiation with osteogenic gene expression and induced cell cycle arrest. Therefore, our data suggest that modification of chromatin structure triggered by the down-regulation of HDAC1 is critical for osteoblast differentiation.

RESULTS

Histone Proteins Are Hyperacetylated in Osteoblasts with Reduced Expression of HDAC1

Three osteogenic cells including ROS17/2.8, MC3T3-E1, and primary bone marrow cells were differentiated into osteoblasts, and expression level of osteopontin mRNA was determined to validate their *in vitro* differentiation condition (Fig. 1A). To investigate the role of chromatin remodeling in osteoblast differentiation, we performed Western blot analyses with undifferentiated and differentiated ROS17/2.8 cells. Although the total amounts of histone proteins were not changed during osteoblast differentiation, acetylation of histone H3 (K9) was increased whereas methylation of histone H3 (K9) was decreased in differentiated osteoblasts (Fig. 1B). In addition, increase of histone H3 and H4 acetylation was observed in differentiated C3H10T1/2 osteoblasts (Fig. S1A published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). The degree of histone acetylation is determined by the balance between HATs and histone deacetylases (HDACs). To understand the primary cause(s) of shift in this balance, we compared the mRNA and/or protein levels of HDAC isoforms before and after differentiation of several osteogenic cells such as ROS17/2.8, primary bone marrow, MC3T3-E1, and C3H10T1/2 cells. Interestingly, the mRNA levels of HDAC1 and -2 were decreased in differentiated osteoblasts (Fig. 1C), whereas mRNAs of other HDACs, including HDAC3 and many class II HDACs (HDAC5 and -6), were slightly increased or unchanged (data not shown). Accordingly, the protein levels of HDAC1 and -2 were markedly decreased in differentiated osteoblasts (Fig. 1D and supplemental Fig. 1B), suggesting that increase of histone acetylation is probably due to decreased HDAC1 and -2 expression. Consistently, total HDAC enzymatic activity was reduced (~40–50%) in differentiated osteoblasts compared with undifferentiated cells (Fig. 1E). Together, these results imply that decrease of total HDAC enzymatic activity by the reduction of HDAC expression, at least in part, contributes to increase of histone acetylation during osteoblast differentiation.

Involvement of HDAC1 and p300 in Hyperacetylation of Histone H3 and H4 at the Promoters of Osteogenic Genes

To determine whether hyperacetylation of histone H3 and H4 is induced preferentially at the osteoblast marker genes during osteogenesis, we performed chromatin immunoprecipitation (ChIP) assays with undifferentiated and differentiated osteoblasts. Previously, it has been demonstrated that the levels of histone H3 and H4 acetylation increased at the osteocalcin promoter during osteogenesis (31). In accord with this report, we observed that acetylation of histone H3 (K9) and H4 (pan) was greatly enhanced at the promoters of osterix and osteocalcin genes in differentiated osteoblasts (Fig. 2A). However, acetylation of histone H3 and H4 was not increased at the peroxisome proliferator-activated receptor-γ promoter, a key transcription factor for adipogenesis, indicating that histone modification occurs in a promoter-specific manner upon cell types (Fig. 2A).

During osteoblast differentiation, we also discovered that recruitment of HDAC1 to the promoter regions of osterix and osteocalcin decreased, whereas recruitment of p300 to those promoters was enhanced remarkably (Fig. 2A). This reciprocal shift HDAC1 and p300 would result in the hyperacetylation of histones at the promoters of osteoblast marker genes. To elucidate which isoform(s) of HDACs plays a key role for the hyperacetylation of histones during osteogenesis, we investigated the recruitment of several HDACs to osteoblast marker genes. Consistent with above results that the expression level of HDAC1 was decreased in most osteogenic cells (Fig. 1D), recruitment of HDAC1 to the promoters of osterix and osteocalcin indeed repressed in differentiated osteoblasts, whereas HDAC2 and -3 were barely recruited to those promoter regions in both undifferentiated and differentiated osteoblasts (Fig. 2B). Therefore, it is likely that hyperacetylation of histone H3 and H4 at the promoters of osteoblast marker genes is probably induced by

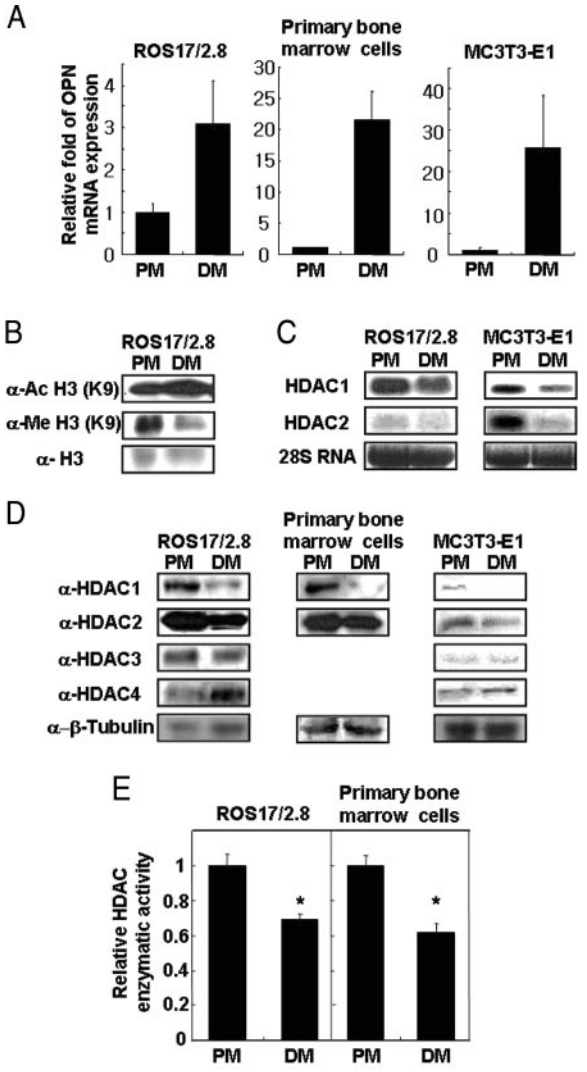

Fig. 1. Changes of Histone Modifications and Expression Patterns of HDACs and HDAC Enzymatic Activity during Osteoblast Differentiation

ROS17/2.8 cells were differentiated into osteoblasts for 5 d, MC3T3-E1 cells for 7 d, and primary bone marrow cells for 9 d. A, mRNA level of osteopontin was increased during osteogenesis. Real-time PCR analyses were independently performed three times with primer sets for osteopontin. Values are normalized to the levels of GAPDH mRNA. B, Changes of histone modifications during osteogenesis in ROS17/2.8 cells. Total histone proteins were isolated from preosteoblasts and differentiated osteoblasts by acid extraction. Western blot analyses were conducted using antibodies against K9-acetylated histone H3 [Ac-H3 (K9)], K9-methylated histone H3 [Me-H3 (K9)], and histone H3 (H3). C, Northern blot analyses were performed with each HDAC isoform cDNA. Total RNA was isolated from confluent preosteoblasts and differentiated osteoblasts. Equal loading was confirmed by 28S RNA. D, Western blot analyses were conducted using antibodies against HDAC1, HDAC2, HDAC3, and HDAC4. Total protein extracts were prepared from confluent preosteoblasts and differentiated osteoblasts. Antibodies against β-tubulin were used as loading control. E, HDAC enzymatic activity assays were performed as described in *Materials and Methods*. Total cell extracts from ROS17/2.8 cells and primary bone marrow cells were prepared from preosteoblasts and differentiated osteoblasts. HDAC activities were normalized with protein concentration, and the results were expressed as relative folds of the HDAC enzymatic activity obtained from each preosteoblast. All experiments were repeated at least three times in duplicate. Values are expressed as the mean ± SEM. *, $P < 0.05$. PM, Proliferation media; DM, differentiation media; OPN, osteopontin.

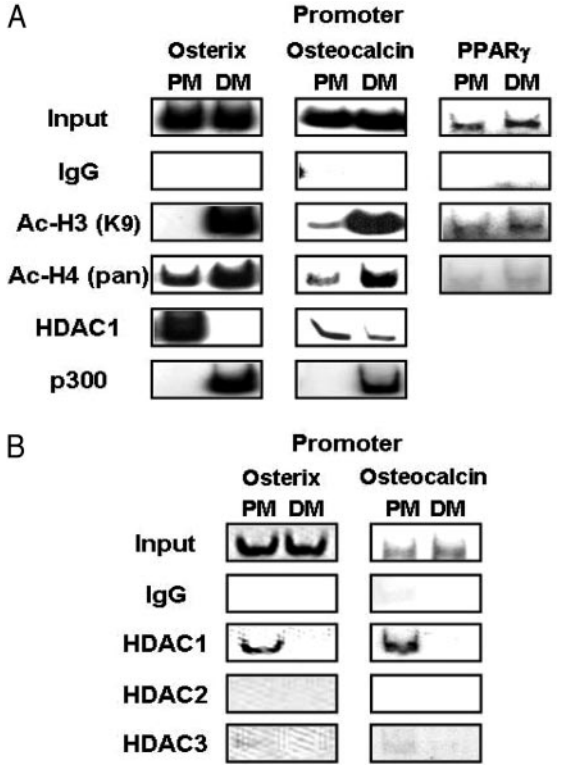

Fig. 2. Recruitment of HDAC1 and p300 to the Promoters of Osteogenic Genes during Osteogenesis

ChIP assays were performed with preosteoblasts or differentiated osteoblasts (differentiation d 9) from primary bone marrow cells. A and B, Cells were cross-linked with formaldehyde, and isolated nuclei were immumoprecipitated with the indicated antibodies. Immunoprecipitated DNA fragments were amplified by PCR at the promoter regions of the indicated genes. Input represents 10% of the total input chromatin. Rabbit preimmune serum (IgG) was used as negative control. PM, Proliferation media; DM, differentiation media. PPAR, Peroxisome proliferator-activated receptor.

the reduced association of HDAC1 and the increased association of p300 to target promoters during the execution of osteoblast differentiation.

Stimulation of Osteogenesis by HDAC Inhibitors

To clarify whether the decrease in HDAC enzymatic activity is associated with osteoblast differentiation, we investigated the effects of HDAC inhibitors such as NaB, which is a short chain fatty acid, and trichostatin

A (TSA) on osteogenesis. To assess the degree of osteoblast differentiation, we examined the morphological changes of osteogenic cells and performed ALP and alizarin red staining. As shown in Fig. 3A, NaB treatment accelerated osteoblast differentiation of ROS17/2.8, primary bone marrow, and MC3T3-E1 cells. Furthermore, calcium deposition was also increased by NaB treatment in osteogenic cells (Fig. 3B). TSA, which is a more potent HDAC inhibitor, also promoted osteoblast differentiation in C3H10T1/2 cells (supplemental Fig. 1C), although it exhibited cytotoxic effects after long-term treatment. To investigate the effects of HDAC inhibitors on the expression of osteoblast marker genes, total RNAs were isolated from cells treated with or without NaB. The mRNA level of osterix and osteopontin was significantly increased with NaB in ROS17/2.8 cells (Fig. 3C). mRNA levels of Runx2, osteocalcin, osteopontin, and ALP were increased with NaB in primary bone marrow cells (Fig. 3D), indicating that suppression of HDAC enzymatic activity with HDAC inhibitors enhances osteoblast differentiation concomitantly with stimulating osteogenic gene expression.

HDAC1 Knockdown Promotes Osteoblast Differentiation

To directly elucidate whether changes of HDAC1 expression are associated with osteoblast differentiation, we investigated the effects of HDAC1 knockdown using siRNA on osteoblast differentiation. Knockdown of HDAC1 decreased its protein level by 80% without affecting the expression of other HDACs such as HDAC2 (Fig. 4A). Accordingly, total cellular HDAC enzymatic activity was decreased by 50% (Fig. 4B), whereas total HAT enzymatic activity was not significantly affected (data not shown). Under differentiation conditions, HDAC1 knockdown cells displayed stimulated osteogenesis accompanied by increased ALP activity compared with mock cells (Fig. 4C). Furthermore, the mRNA levels of osterix and ALP were enhanced in HDAC1 knockdown cells (Fig. 4D), indicating that suppression of HDAC1 expression indeed promotes expression of osteogenic genes to induce osteogenesis.

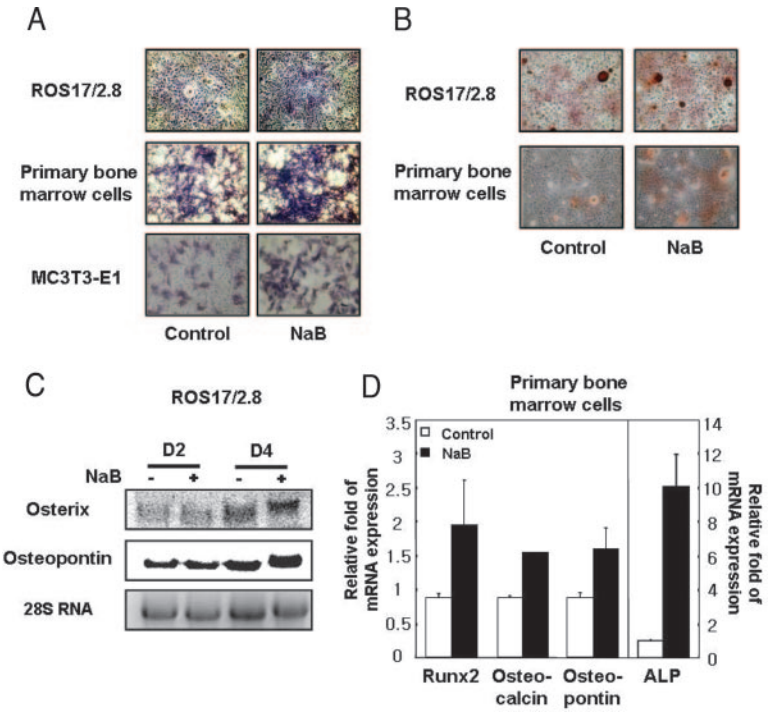

Fig. 3. Effect of HDAC Inhibitors on Osteoblast Differentiation

ROS17/2.8 cells were differentiated for 5 d, MC3T3-E1 cells for 7 d, and primary bone marrow cells for 9 d (A and B). A, HDAC inhibitor, NaB, increased ALP-positive cells in ROS17/2.8, primary bone marrow, and MC3T3-E1 cells. Confluent cells (80%) were treated with differentiation media in the presence or absence of NaB (100 μM) and then stained for ALP-positive cells using ALP staining. B, NaB also promotes matrix mineralization in ROS17/2.8 and primary bone marrow cells. Cells were treated with differentiation media in the absence or presence of NaB (100 μM), and then calcium deposition was observed using alizarin red staining. C, ROS17/2.8 cells were differentiated into osteoblasts with or without NaB (100 μM) and harvested at the indicated time points. Northern blot analyses were performed with osterix and osteopontin probes. Equal loading was confirmed by 28S RNA. D, Primary bone marrow cells were differentiated into osteoblasts with or without NaB (100 μM) for 9 d. Relative amounts of each mRNA for osteoblast marker genes including Runx2, osteocalcin, osteopontin, and ALP were determined using real-time PCR. Values are normalized to the levels of GAPDH and are represented as means $\pm$ SEs (n = 3). D2, Differentiation d 2; D4, differentiation d 4.

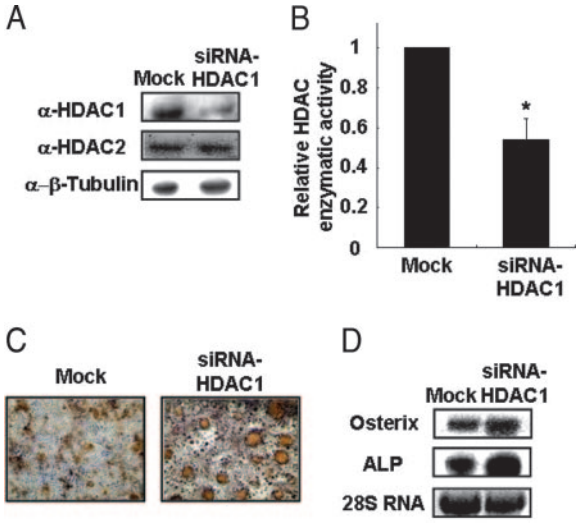

Fig. 4. Effect of HDAC1 Knockdown via siRNA on Osteogenesis

ROS17/2.8 cells were infected with pSUPER retroviruses including mock or siRNA-HDAC1 (see *Materials and Methods*). A, Infected cells were harvested for Western blot analyses. Anti-HDAC2 antibodies were used to validate specificity of HDAC1 siRNA, and anti-β-tubulin antibodies were used for loading control. B, Retroviral mock siRNA or HDAC1 siRNA infected cells were differentiated into osteoblats for 5 d, and total cell extracts were isolated from each cell type to measure HDAC activity. Results were expressed as relative folds of the HDAC activity obtained from mock siRNA-infected cells. Experiments were independently performed three times with duplicates. Values are expressed as the mean $\pm$ SEM. *, $P < 0.05$. C and D, Mock and HDAC1 siRNA-infected cells were differentiated into osteoblasts under normal differentiation conditions for 5 d and stained for mineralization (*violet*) using the ALP staining. Northern blot analyses were performed with osterix and ALP probes. Equal loading was confirmed by 28S RNA. Mock, siRNA-vector infected cells; siRNA-HDAC1, HDAC1-siRNA infected cells

Repression of Transcriptional Activity of Runx2 by HDAC1

It has been shown recently that the proteins with intrinsic HAT activity, including p300 and act as transcriptional coactivators by acetylating not only histone proteins but also transcription factors, such as p53 (32). It has been also reported that p300 associates with Runx2 and activates its transcriptional activity on the osteocalcin promoter (33). To determine whether Runx2 is directly acetylated by HATs, we cotransfected Runx2 and p300 into human embryonic kidney (HEK) 293 cells and studied its effects. Consistent with a previous report (33), p300 physically interacted with Runx2 and induced acetylation of Runx2 (Fig. 5A). In addition, the transcriptional activity of Runx2 was stimulated by coexpression of p300 (Fig. 5E).

In addition, we examined the interaction between HDAC1 and Runx2. Interestingly, Runx2 physically associated with HDAC1, implying that Runx2 might regulate its target gene expression by recruiting HDAC1 during osteogenesis (Fig. 5B). To gain the functional role of HDAC1 on Runx2 activity, HDAC1 was overexpressed with Runx2 in HEK293 cells. The transcriptional activity of Runx2 was decreased by HDAC1 in a dose-dependent manner (Fig. 5, C and D). Furthermore, HDAC1 remarkably repressed the effects of p300 on Runx2 transcriptional activity (Fig. 5E). Although it remains to be clarified whether p300 and HDAC1 modify histone proteins and/or Runx2 to regulate its transcriptional activity, these results indicate that HDAC1 is clearly able to repress the transcriptional activity of Runx2 at its target genes.

Induction of Cell Cycle Arrest Genes by HDAC Inhibitor during Osteogenesis

Cell cycle arrest is one of the early events that occur during osteoblast differentiation. It has been reported that activation of the cyclin-dependent kinase inhibitors (CKIs), such as p21 and p27, is essential for the regulation of growth and differentiation of osteoblasts (34, 35). Interestingly, the level of p21 peaked as early as 6 h after induction of osteogenic differentiation whereas the level of p27 reached a peak at 24 h in ROS17/2.8 cells (Fig. 6A). Osteopontin expression was enhanced as differentiation was induced. Similar results were obtained with C3H10T1/2 cells for the induction of p27 during osteoblast differentiation (supplemental Fig. 2A published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). Like other osteogenic marker genes (Fig. 2A), HDAC1 was recruited to the promoter region of p27 gene in preosteoblasts, and its association was decreased during osteogenesis, which might enhance hyperacetylation of histone H4 at p27 promoter region in differentiated osteoblasts (Fig. 6B).

The fact that HDAC inhibitors exhibit potent antiproliferative properties in cancer cells (25, 36) prompted us to examine the effects of NaB on the expression of cell cycle-related genes at the early stage of osteogenesis. Shown in Fig. 6C, NaB treatment augmented the expression of p27 at the early differentiation periods without significantly affecting cyclin E, suggesting that NaB might accelerate osteoblast differentiation not only by increasing osteoblast marker gene expression, but also by regulating cell cycle arrest. In support of this idea, we obtained similar results with C3H10T1/2 cells (supplemental Fig. 2B).

DISCUSSION

As coregulators, HDACs have been recently implicated in tissue differentiation. For instance, HDAC4, -5, and -7 associate with mouse embryo fibroblast 2 and act as potent inhibitors of mouse embryo fibroblast 2-dependent transcription during myogenesis (37). Also, HDAC1 directly deacetylases MyoD and

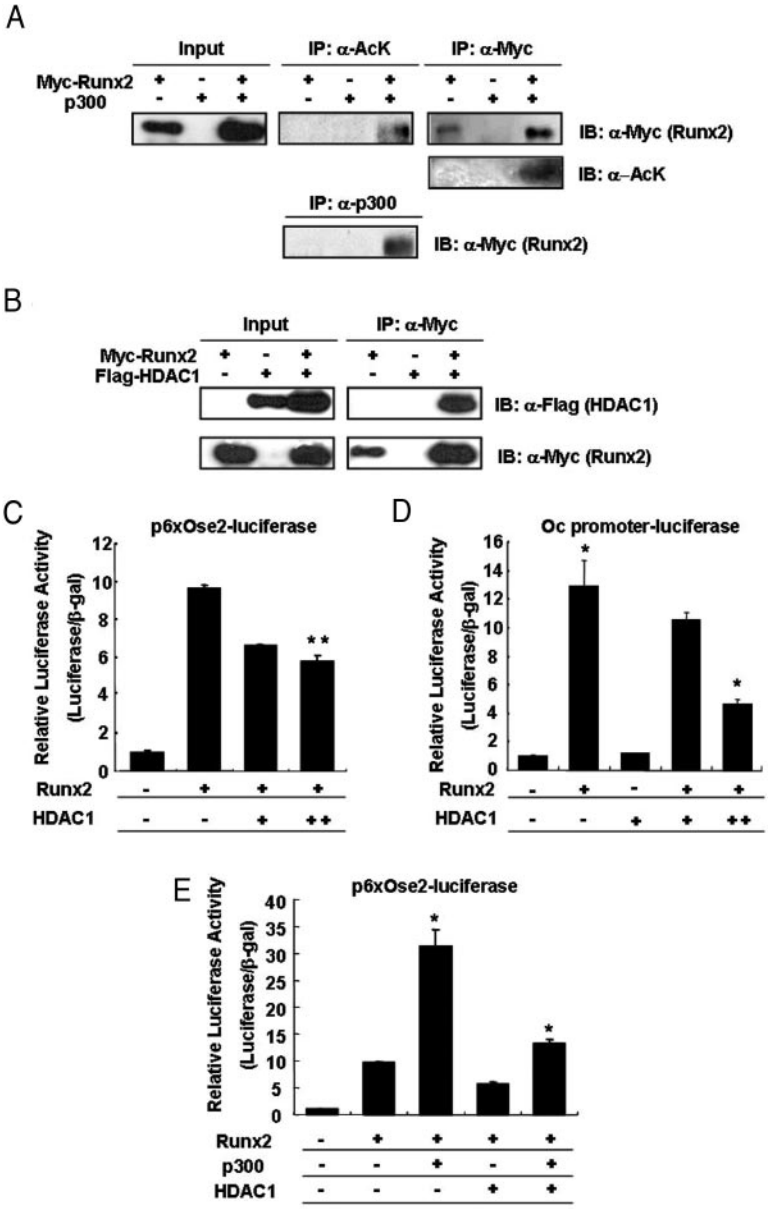

Fig. 5. Regulation of Transcriptional Activity of Runx2 by HDAC1

A, myc-Tagged Runx2 was expressed in HEK293 cells in the absence or presence of p300 overexpression. After immunoprecipitation with antiacetyl lysine antibodies (α-AcK), anti-myc antibodies (α-Myc), or anti-p300 antibodies (α-p300), the samples were resolved by SDS-PAGE, and existence of Runx2 in the immune complexes was determined with anti-myc antibodies (α-Myc) or antiacetyl lysine antibodies (α-AcK). The level of myc-Runx2 in total cell lysates was determined by Western blot analysis. Input represents 10% of total protein used in the immunoprecipitation experiment. B, myc-tagged Runx2 was expressed in HEK293 cells in the absence or presence of FLAG-tagged HDAC1 overexpression. After immunoprecipitation with anti-myc antibodies (α-Myc), the samples were resolved by SDS-PAGE and existence of HDAC1 in the immune complex was determined with anti-FLAG antibodies (α-Flag). The level of Runx2 in the immunoprecipitates was determined with anti-myc antibodies (α-Myc). The levels of myc-Runx2 and FLAG-HDAC1 in total cell lysates were determined by Western blot analyses. Input represents 10% of total protein used in the immunoprecipitation experiment. C and D, p6OSE2-luc reporter (p6XOse2-luciferase) and reporter of osteocalcin promoter (Oc promoter-luciferase) were cotransfected with HDAC1 and/or Runx2 expression vector into HEK293 cells. E, HEK293 cells were transfected with p6OSE2-luc reporter (0.1 μg), Runx2 (1 μg), p300 (1.5 μg), and HDAC1 (0.7 μg). Luciferase activities were determined 24 h after transfection. Transfection efficiency was normalized using β-galactosidase activity. Values are expressed as the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$. IB, Immunoblotting; IP, immunoprecipitation.

inhibits its transcriptional activity during muscle differentiation (29, 38). Furthermore, it has been reported that HDAC3 physically interacts with Runx2 and suppresses its transcriptional activity in osteoblast differentiation (39). In spite of these reports, it is not clearly understood whether global changes in enzymatic ac-

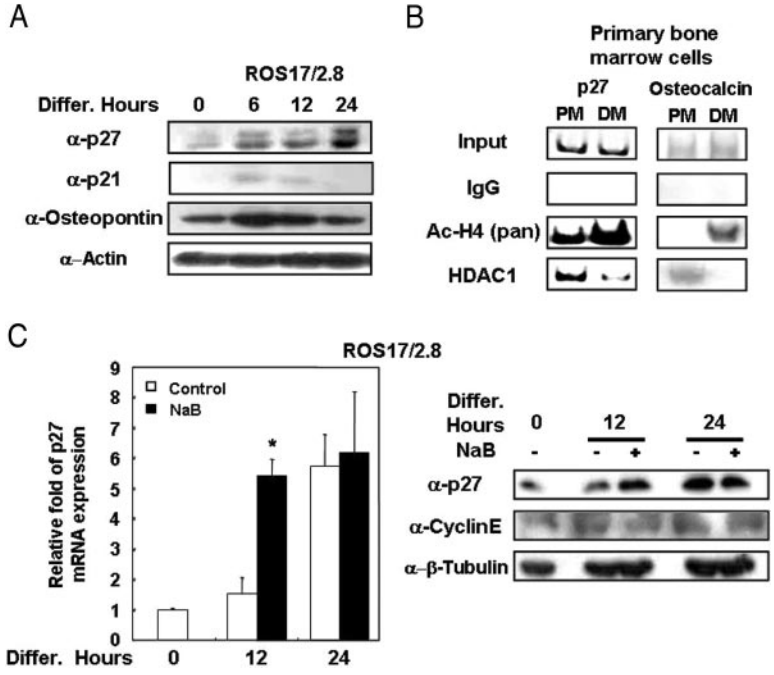

Fig. 6. Effect of NaB on Cell Cycle Arrest Genes during Osteogenesis in ROS17/2.8 Cells

A, Western blot analyses of osteopontin and cell cycle arrest proteins, including p27 and p21, during osteoblast differentiation. Antibodies against actin were used as loading control. B, ChIP assays were performed with preosteoblast or differentiated osteoblast (differentiation d 9) from primary bone marrow cells. Cells were cross-linked with formaldehyde, and isolated nuclei were immumoprecipitated with the indicated antibodies. Immunoprecipitated DNA fragments were amplified by PCR at the promoter regions of p27 and osteocalcin. Input represents 10% of the total input chromatin. Rabbit preimmune serum (IgG) was used as negative control. PM, Proliferation media; DM, differentiation media. C, ROS17/2.8 cells were differentiated into osteoblasts with or without NaB (100 μM). Cells were harvested at the indicated time points. mRNA and protein level of p27 significantly increased at early time point with NaB. Relative amounts of p27 mRNA were determined using real-time PCR. Values are normalized to the levels of GAPDH and are represented as means ± SEs (n = 3). Antibodies against β-tubulin were used as loading control. Values are expressed as the mean ± SEM. *, $P < 0.05$. DM, Differentiation media; PM, proliferation media; Differ., differentiation.

tivity and/or expression of HDACs affect certain cell type differentiation such as osteoblast differentiation.

In the present study, we revealed that regulation of total HDAC enzymatic activity is a crucial step for osteogenesis. In differentiated osteoblasts, total HDAC enzymatic activity was significantly decreased, accompanied by increase of hyperacetylation of histone H3 and H4 (Fig. 1 and supplemental Fig. 1A). Previously, it has been reported that NaB enhanced ALP activity in preosteoblast cells (40),and that several HDAC inhibitors, especially TSA, promote osteoblast maturation in MC3T3-E1 cells (41). In addition, we found that NaB enhanced osteogenesis and osteoblast marker gene expression in several osteogenic cells including ROS17/2.8, primary bone marrow, MC3T3-E1, and C3H10T1/2 cells (Fig. 3 and supplemental Fig. 1C), suggesting that modulation of endogenous HDAC enzymatic activity is important for the execution of the osteogenic program.

Until now, tissue-specific transcription factors have played a major role in understanding the molecular mechanisms of certain tissue differentiation. To bind tissue-specific transcription factors to their target genes, local chromatin structure needs to have loose status, which can be regulated by several chromatin-remodeling factors such as HATs and HDACs. In this regard, it is plausible that down-regulation of HDAC in osteogenesis might be a prerequisitive step for Runx2 or other osteogenic transcription factors to access and/or to bind to their target genes for execution of the differentiation process (Fig. 7). Very recently, we have also discovered that expression and enzymatic activity of HDACs are down-regulated during adipogenesis (42). Although we cannot rule out the possibility that regulation of HATs also contributes to osteogenesis and/or adipogenesis, it is likely that decrease of total HDAC enzymatic activity is, at least in part, a common step for execution of osteogenesis and adipogenesis, which occur in differentiation of mesenchymal stem cells.

There are several reports that HDACs act as a regulator in bone formation. HDAC4 or HDAC5 is involved in TGF-β/Sma- and Mad-related protein (Smad) 3-mediated repression of the Runx2 function in osteogenesis and mineralization (43). HDAC represses the activity of Runx2, and overexpression of HDAC4 in chondrocytes *in vivo* inhibits chondrocyte hypertrophy and differentiation, indicating that HDAC4 would act

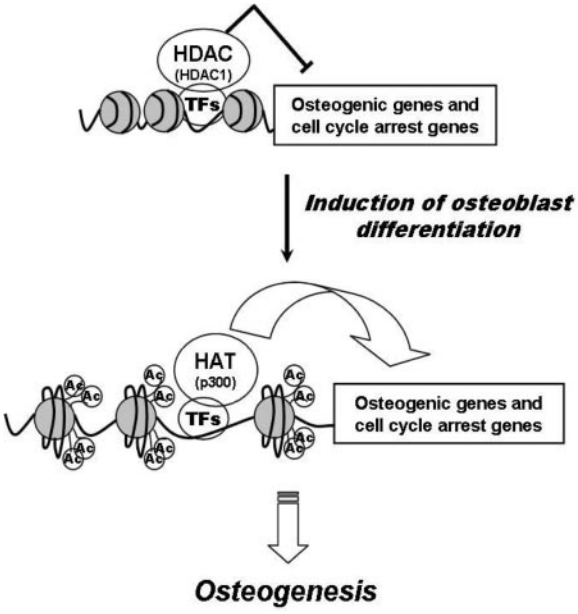

Fig. 7. Regulation of Osteogenesis by Histone-Modifying Enzymes

HDAC complexes, including HDAC1, are recruited to the promoter regions of osteogenic genes (*i.e*. Runx2, osterix, and osteocalcin) and cell cycle arrest gene (p27), which results in histone deacetylation at those promoters to maintain preosteoblasts. When osteoblast differentiation is induced, promoters of osteogenic genes, such as osterix and osteocalcin, are hyperacetylated with HAT complexes including p300. Thus, chromatin structures become more accessible to osteogenic transcription factors such as Runx2 and osterix for execution of osteoblast differentiation. TFs, Transcription factors; Ac, acetylation; HDAC, histone deacetylase; HAT, histone acetyltransferase.

as a key regulator of endochondral bone formation (44). Also, knockdown of HDAC3 in MC3T3-E1 cells promoted a late-stage event in osteoblast differentiation (39). In the present study, we provide several lines of evidence that HDAC1 is a key isoform of HDACs for osteoblast differentiation. First, expression of HDAC1 was most remarkably reduced during osteoblast differentiation in both mRNA and protein level in several osteoblasts (Fig. 1). Second, ChIP assays demonstrated that recruitment of HDAC1, but not HDAC2 and -3, to the promoters of osteoblast marker genes was decreased in differentiated osteoblasts (Fig. 2). Third, total HDAC enzymatic activity was affected by the expression level of HDAC1 in osteoblasts. Suppression of HDAC1 expression via siRNA significantly decreased total HDAC enzymatic activity in ROS17/2.8 cells (Fig. 4), whereas overexpression of HDAC1 substantially increased total HDAC enzymatic activity (supplemental Fig. 3 published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). Also, HDAC1 overexpression decreased osteoblast differentiation in C3H10T1/2 cells (supplemental Fig. 3), suggesting that HDAC1 is the major functional HDAC isoform in osteogenesis. Fourth, HDAC1 interacted with Runx2 and repressed its transcriptional activity. Previously, it has been demonstrated that the Runx family proteins (Runx1/AML1 and Runx3/AML2) are acetylated by p300 except Runx2, and these modifications are involved in control of transcriptional activity and protein stability (45–47). To our knowledge, this is the first report that Runx2 is directly acetylated by p300, which might result in increase of its transcriptional activity (Fig. 5, A and E). Furthermore, we reveal that HDAC1 could interact with Runx2 and repress its transcriptional activity, even in the presence of p300 (Fig. 5). These results also imply that Runx2 may mediate the recruitment of HDAC1 and p300 to repress certain target genes such as osterix and osteocalcin in the absence of osteogenic induction signals (Fig. 2). Although it remains to be elucidated whether p300 and HDAC1 could regulate transcriptional activity of Runx2 by acetylating and deacetylating histone proteins at Runx2 target promoters and/or Runx2 protein itself, it is feasible to postulate that Runx2 may switch its coregulator(s) from HDAC1 to p300 upon differentiation signals, which leads to hyperacetylation of histone proteins at the promoters of osteoblast marker genes as well as Runx2 during osteogenesis. Together, these data strongly support the idea that HDAC1 would function as the important regulator of osteogenic gene expression and execution of osteogenesis (Fig. 7).

During bone formation, osteoblasts undergo a series of maturation stages, including cell proliferation, matrix synthesis, and a final stage of differentiation concomitant with extracellular matrix mineralization. Proliferating osteoprogenitors must exit the cell cycle to differentiate into mature osteoblasts. For execution of cell cycles, phase transition of each cell cycle is regulated by phosphorylation of various proteins including pRb proteins (48), which are phosphorylated by cyclin-dependent kinase (CDK) and cyclin complexes. Kinase activity of CDK/cyclin complexes is negatively modulated by CKIs, resulting in the dephosphorylation of pRb (49, 50); thereby, activation of CKIs represses G_1/S phase transition by negatively regulating CDK/cyclin complexes. Recently, it has been reported that HDAC1 is implicated in the cell cycle regulation by suppressing the transcriptional activity of Tcf/Lef in response to glucocorticoid (51). However, several lines of evidence indicate that suppression of HDAC1 is essential for cell cycle arrest and osteoblast differentiation. For example, HDAC1 is essential for the proliferation of embryonic stem cells by suppressing the expression of CKIs such as p21 and p27 (52). Furthermore, HDAC6 repressed p21 expression in pre-MC3T3-E1 cells (53). Among HDAC inhibitors, butyrate, has potent effects on cell growth arrest at the G_1 phase and differentiation *in vitro* in various malignant tumor cell lines (54–57). Accordingly, we observed that NaB markedly enhanced expression of p27 at the early stage of differentiation in ROS17/2.8 cells and C3H10T1/2 (Fig. 6C and supplemental Fig. 2B). Moreover, we observed that increased total cel-

lular HDAC enzymatic activity by overexpression of HDAC1 blocked osteoblast differentiation with reduced expression of p27 (supplemental Fig. 3), and that recruitment of HDAC1 to the promoter of p27 decreased in differentiated osteoblasts (Fig. 6B). Therefore, it is likely that total HDAC enzymatic activity regulates osteoblast differentiation not only by changing osteoblast marker gene expression, but also by controlling cell cycle arrest genes, which are induced at the early stage of osteoblast differentiation.

Here we demonstrated, for the first time, that down-regulation of HDAC1 expression and total HDAC enzymatic activity is a critical step for osteoblast differentiation. Because HDAC suppression enhances osteoblast differentiation, it would be possible to develop tissue-selective HDAC inhibitors for potential remedy of bone-related diseases including osteoporosis.

MATERIALS AND METHODS

Cell Culture and Osteoblast Differentiation

Primary bone marrow cells were isolated from femur and tibia of 4- to 6-wk-old C57BL/6J mice and maintained in α-MEM with 20% horse serum and 100 nM hydrocortisone. For osteoblast differentiation, primary bone marrow cells were cultured in α-MEM with 10% fetal bovine serum (FBS), 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate. ROS17/2.8 rat osteosarcoma cells, and MC3T3-E1 calvaria cells were maintained in DMEM supplemented with 10% FBS (13). Osteoblast differentiation was induced with DMEM containing 10% FBS, 50 μM ascorbic acid, and 10 mM β-glycerophosphate. In HDAC inhibitor experiments, NaB was treated during the differentiation period.

Constructs, DNA Transfection, and Reporter Assay

For luciferase assays, HEK293 cells were cultured into 12-well plates for 2 d before transfection. pcDNA3-FLAG-HDAC1, pCMV-p300, pCS4–3myc-Runx2, p6OSE2-luc, or Oc promoter-Luc (1.3 kb of osteocalcin promoter) was transiently transfected using the calcium phosphate method (58). At 24 h posttransfection, cell lysates were analyzed for luciferase activity (58, 59). The pCMV-β-galactosidase plasmid was used as an internal control for transfection efficiency.

Immunoprecipitations, Western Blotting, and Acid Extraction of Histone Protein

HEK293 cells were transfected with pCS4–3myc-Runx2, pcDNA3-FLAG-HDAC1, and pCMV-p300 using the calcium phosphate method (58, 59). At 24 h posttransfection, cells were lysed on ice by using NETN buffer [20 mM Tris-HCl (pH 7.9), 1 mM EDTA, 100 mM NaCl, 0.5% Nonidet P-40, and protein inhibitors (Roche Applied Science, Indianapolis, IN)]. To detect Runx2 acetylation, lysates were treated with 1 μM TSA before immunoprecipitation. Lysates were then incubated with nonspecific rabbit IgG, antiacetyl-lysine (Cell Signaling Technology, Beverly, MA), or anti-myc antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as indicated for 12 h at 4 C. Immune complexes were collected with Protein A-Sepharose CL-4B (Amersham Biosciences) for 2 h at 4 C. The beads were washed three times with NETN buffer. Proteins were eluted from the beads by boiling in 5$\times$ sodium dodecyl sulfate (SDS) sample buffer for 5 min, separated by electroporesis on SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA) or nitrocellulose membranes (Schleicher & Schuell Bioscience, Inc., Keene, NH). After transfer, the membranes were blocked with skim milk and probed with primary antibodies at 1:1000 dilutions. Antibodies against-HDAC1 (H-51), HDAC2 (H-54), HDAC3 (N-19), HDAC4 (H-92), p300 (N-15), p21, p27, cyclin E, osteopontin, actin, myc, FLAG, β-tubulin (Santa Cruz Biotechnology), and acetyllysine (Upstate Biotechnology, Inc., Lake Placid, NY) were used. Western blot analyses were visualized with horseradish peroxidase-conjugated secondary antibodies (Sigma Aldrich, St. Louis, MO) and enhanced chemiluminescence.

Histones were prepared from ROS17/2.8 cells by acid extraction (Upstate Biotechnology's protocol). Western blotting was performed as mentioned above. Antibodies against-K9-acetylated histone H3, K9-methylated histone H3, and histone H3 were purchased from Upstate Biotechnology.

Northern Blotting

Total RNA was isolated with Trizol (Invitrogen, San Diego, CA) according to the manufacturer's protocol. Northern blotting was performed as previously described (59). cDNA probes were labeled by random priming kit using the Klenow fragment of DNA polymerase I (Promega Corp., Madison, WI) and [α-^{32}P]dCTP (Amersham Pharmacia Biotech, Piscataway, NJ). cDNAs for HDAC1, HDAC2, osterix, osteopontin, and ALP were used as probes. Equal loading was confirmed by 28S RNA.

Real-Time PCR

After isolation of total RNA, complementary DNA generated by M-MuLV Reverse Transcriptase (Fermentas, Inc., Hanover, MD) was analyzed by real-time PCR (Bio-Rad Laboratories, Inc., Hercules, CA) using an SYBR Green (BioWhittaker Molecular Application, Frederick, MD). All reaction products were normalized to the expression level of mRNA. Primer pairs used are as follows: Runx2 forward, 5′-GAA GGA AAG GGA GGA GGG GT; reverse, 5′-TCT GTC TCT CCT TCC CTT CC; osteocalcin forward, 5′-CGC TCT CAG GGG CAG ACA CT; reverse, 5′-GCA CCC TCC AGC ATC CAG TA; osteopontin forward, 5′-TGC CTG ACC CAT CTC AGA AGC A; reverse, 5′-TGA GAG GTG AGG TCC TCA TC; ALP forward, 5′-GAC TGG TAC TCG GAT AAC GA; reverse, 5′-TGC GGT TCC AGA CAT AGT GG; p27 forward, 5′-CGA CTT TCA GAA TCA TAA GCC C; reverse, 5′-GGG AAC CGT CTG AAA CAT TTT C.

ALP Staining and Alizarin Red Staining

Differentiated osteoblast cells were stained for ALP activity using the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium color development substrate (Promega). Alizarin red staining for mineralization was conducted as previously described (60).

Deacetylase Enzymatic Assay

Total protein extracts were isolated from cultured cells using NETN buffer. Histone deacetylase enzymatic assays were carried out using an HDAC assay kit (Upstate Biotechnology). Briefly, prepared total cell extracts (~30–40 μg) were incubated at 30 C for 30–60 min with the HDAC assay substrate, allowing deacetylation of the fluorometric substrate. After the plates had been incubated at room temperature for 10–15 min, the plates were read in a fluorescence plate reader (excitation = 350–380 nm and emission = 440–460 nm) within 60 min.

ChIP Assay

ChIP assays were performed as described previously (61). In brief, pre- and differentiated osteoblasts were cross-linked in 1% formaldehyde at 37 C for 10 min and resuspended in 200 μl of Nonidet P-40-containing buffer (5 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 8.0; 85 mM KCl, and 0.5% Nonidet P-40). Crude nuclei were precipitated and lysed in 200 μl of lysis buffer [1% SDS, 10 mM EDTA, and 50 mM Tris-HCl (pH 8.1)], and the nuclear lysates were sonicated and diluted 10-fold with immunoprecipitation buffer (16.7 mM Tris-HCl, pH 8.1; 167 mM NaCl; 1.2 mM EDTA; 0.01% SDS; and 1.1% Triton X-100). Then lysates were immunoprecipitated with nonspecific rabbit IgG, antiacetylated-H3 (K9), acetylated-H4 (pan), HDAC1, -2, -3, or p300 antibodies for 12 h at 4 C. Immune complexes were incubated with Protein A-Sepharose CL-4B (Amersham Biosciences) for 2 h at 4 C. After successive washings, immune complexes containing DNA were eluted and the precipitated DNA was amplified by PCR. Conditions for PCR were as follows: 0.25 μM concentrations of each primer, 0.1 mM concentrations of each deoxynucleotide triphosphate, 1× PCR buffer, 1 U of Ex Taq polymerase (TaKaRa), 0.06 mCi/ml [α-^{32}P]dCTP in a 20-μl reaction volume. The products were resolved on 6% polyacrylamide/1× Tris-borate/EDTA gels. Primer pairs used in this study are as follows: osteocalcin promoter forward, 5′-CGC TCT CAG GGG CAG ACA CT; reverse, 5′-GCA CCC TCC AGC ATC CAG TA; osterix promoter forward, 5′-GGA CTC CGA GTC AAG AGT AG; reverse, 5′-AGG GAG GCA GAG GGT CCA AA; peroxisome proliferator-activated receptor-γ promoter forward, 5′-CTG TAC AGT TCA CGC CCC TC; reverse, 5′-TCA CAC TGG TGT TTT GTC TAT G; p27 promoter forward, 5′-CCC TGA TAA GAG CGG TCA; reverse, 5′-GAA TCT AAG CCC GCG CCA.

siRNA

The mammalian retroviral expression vector, pSUPER-retro (OligoEngine), was used for expression of siRNA in ROS17/2.8 cells. The gene-specific insert specifies a 19-nucleotide sequence corresponding to nucleotides 1102–1121 downstream of the transcription start site (GCAGCGTCTCTTTGAGAAC) of HDAC1, separated by a nine-nucleotide noncomplementary spacer (TCTCTTGAA) from the reverse complement of the same 19-nucleotide sequence. This sequence was inserted into the pSUPER-retro vector after digestion with *Bgl*II and *Hind*III to yield pSUPER-retro-HDAC1-siRNA. This vector and the pSUPER-retro empty vector were transfected into a packaging cell line by the calcium phosphate method (59). After 48 h incubation, supernatants containing retroviral particles were collected and used to infect ROS17/2.8 cells. Retrovirally infected cells were selected with 2 μg/ml puromycin (Sigma Aldrich).

Statistics

Data were analyzed using Student's test. $P < 0.05$ and $P < 0.01$ were considered significant.

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