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N-Acetylcysteine Stimulates Osteoblastic Differentiation of Mouse Calvarial Cells

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Abstract Estrogen deficiency causes osteoporosis via increased generation of reactive oxygen species (ROS), and thus, antioxidants may prove to be the effective therapeutic candidates. We examined the effects of the antioxidant *N*-acetylcysteine (NAC) on osteoblastic differentiation in mouse calvarial cells. NAC (10–30 mM) enhanced alkaline phosphatase activity, mRNA expression of osteoblast differentiation-associated genes and mineralized nodule formation. It also increased expression of bone morphogenetic proteins-2, -4, and -7. The osteogenic activity of NAC was partially reduced by inhibition of glutathione synthesis. Since caffeic acid phenethyl ester did not stimulate osteoblast differentiation, it is unlikely that ROS scavenging activity of NAC is sufficient for osteogenic activity. We observed that NAC suppressed small GTPase RhoA activity and activation of RhoA by *Pasteurella multocida* toxin suppressed the osteogenic activity of NAC. These results suggest that NAC might exert its osteogenic activity via increased glutathione synthesis and inhibition of RhoA activation. J. Cell. Biochem. 103: 1246–1255, 2008.

Key words: *N*-acetylcysteine; osteoblast differentiation; glutathione; inhibition of RhoA activity

Reactive oxygen species (ROS) are produced in cells as a by-product of normal metabolism. High levels of oxidants perturb the normal redox balance and shift cells into a state of oxidative stress [Finkel, 2003]. Oxidative stress has been implicated in a large number of human diseases, including atherosclerosis, pulmonary fibrosis, cancer, neurodegenerative diseases, and aging [Halliwell et al., 1992].

Postmenopausal osteoporosis is caused by estrogen deficiency, which leads to bone loss through increased osteoclastic activity, combined with a relative deficiency of osteoblast function [Riggs et al., 2002]. Pharmacologic treatment of patients with osteoporosis includes calcium and vitamin D_3, bisphosphonates, calcitonin, estrogens, and selective estrogen receptor modulators [Lin and Lane, 2004]. These drugs retard further bone loss but their effect on recovery of lost bone is not obvious. Recent reports have demonstrated that: (i) estrogen deficiency induces bone loss through increased ROS production [Lean et al., 2003, 2005; Jagger et al., 2005]; (ii) ROS stimulates osteoclastogenesis while antioxidants suppress osteoclast differentiation and activity [Aitken et al., 2004; Ha et al., 2004]; and (iii) oxidative stress inhibits osteoblast differentiation while metallothionein overexpression protects bone marrow stromal cells against hydrogen peroxide-induced inhibition of osteoblastic differentiation [Mody et al., 2001; Bai et al., 2004; Liu et al., 2004]. These findings suggest that ROS

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may represent a critical target for the treatment and/or prevention of osteoporosis.

Although several reports have demonstrated that osteoblast differentiation can be inhibited by oxidative stress, induced by exogenous stimuli such as hydrogen peroxide or xanthine/xanthine oxidase [Mody et al., 2001; Bai et al., 2004], we found no reports concerning the role of spontaneously produced ROS and redox regulation in osteoblasts. Therefore, we examined the effects of the antioxidant *N*-acetylcysteine (NAC) on osteoblastic differentiation of mouse calvarial cells. NAC is a precursor of glutathione (GSH) and functions as a source of sulfhydryl groups and as an ROS scavenger [Aruoma et al., 1989]. In this study, we demonstrated that NAC stimulates osteoblastic differentiation and mineralized nodule formation in mouse calvarial cells.

MATERIALS AND METHODS

Reagents

Cultural reagents were obtained from Invitrogen Life Technologies (Carlsbad, CA). NAC, GSH, L-buthionine-[*S,R*]-sulfoxide (BSO), *N*-acetylalanine (NAA), *Pasteurella multocida* toxin (PMT), Rho kinase (ROCK) inhibitor Y27632, hydrogen peroxide, and 2′7′-dichlorofluorescin diacetate (DCFH-DA) were obtained from Sigma-Aldrich (St. Louis, MO). Caffeic acid phenethyl ester (CAPE) was purchased from BIOMOL Research Labs (Plymouth Meeting, PA). BCA protein assay reagent and mouse anti-Cdc42 antibody were purchased from Pierce (Rockford, IL). Goat anti-bone morphogenetic protein (BMP)-2 and -7 antibodies, ImmunoPure goat anti-mouse IgG horseradish peroxidase (HRP)-conjugated and bovine anti-goat IgG HRP-conjugated antibodies were purchased from Santa Cruz (Santa Cruz, CA). Mouse anti-RhoA and anti-Rac1 antibodies, as well as the Rho and Rac/cdc42 assay Reagent kits were from Upstate (Lake Placid, NY). Alexa Fluor 546 phalloidin was from Molecular Probes, Inc. (Eugene, OR).

Isolation of Mouse Calvarial Cells

Mouse calvarial cells were obtained as described previously [Kim et al., 2002]. In brief, the frontal and parietal bones of neonatal ICR mice (Samtako BIO KOREA, Osan, Korea) were dissected aseptically and digested consecutively for 10, 10, 10, 20, 20, and 20 min each, using an enzyme mixture containing 0.1% collagenase, 0.05% trypsin, and 0.5 mM EDTA. Calvarial cells released at the later three periods were pooled and cultured in Dulbecco's modified eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 95% humidified air with 5% CO_2. Second passage cells were used for experiments.

Measurement of Intracellular ROS

Intracellular ROS was measured using DCFH-DA which diffuses into cells where it is hydrolyzed to DCFH. DCFH is then converted by ROS-mediated oxidation to the highly fluorescent derivative 2′,7′-dichlorofluorescein (DCF) [Bass et al., 1983]. Cells (5×10^4 cells/well) were seeded into 96-well culture plates, incubated overnight and serum-starved for 16–18 h. Cells were then pre-incubated for 30 min in serum-free DMEM containing 50 μM DCFH-DA, transferred to fresh serum-free DMEM and incubated for times indicated in the presence of the reagents indicated. DCF fluorescence was measured using a FLUOstar OPTIMA (BMG Lab-Technologies, Offenburg, Germany).

Alkaline Phosphatase (ALP) Activity

Cells (1.3×10^5 cells/well) were seeded into 24-well culture plates and incubated for 2 days in DMEM supplemented with 10% FBS. Cells were harvested and homogenized, then aliquots were incubated with 15 mM *p*-nitrophenyl phosphate in 0.1 M glycine-NaOH buffer (pH 10.3) at 37°C for 30 min. Reactions were stopped by the addition of 0.25 N NaOH. The optical density of the reaction product *p*-nitrophenol was read at 410 nm using a microplate reader. Total protein in the cell homogenates was determined using the BCA protein assay.

Mineralized Nodule Formation

Cells (2×10^5 cells/well) were seeded into 4-well culture plates and incubated for 14 days in osteoblast differentiation medium containing α-minimum essential medium (α-MEM) supplemented with 10% FBS, 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate. The medium was changed every other day. At the end of culture, cells were fixed with 70% ethanol and mineralized deposits detected by in situ Alizarin red staining. Mineralized nodules were counted at 40× magnification using a light microscope.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Expression levels of osteoblast differentiation-associated genes were examined by semiquantitative RT-PCR. Cells were cultured in osteoblast differentiation medium for 3 and 8 days. At the end of culture, total RNA was isolated using easy-BLUE reagent (iNtRON, Seoul, Korea). Complementary DNA (cDNA) was synthesized from 1 μg total RNA using the AccuPower RT PreMix (BIONEER, Daejeon, Korea) and PCR was performed using *i-star* Taq DNA polymerase (iNtRON) on 10% of the cDNA. The following mouse genes and their primer sequences were used for PCR: tissue nonspecific (liver–bone–kidney type) ALP-forward (f) 5′-AGGCAGGATTGACCACGG-3′, ALP-reverse (r) 5′-TGTAGTTCTGCTCATGGA-3′; type I collagen (Col1(α)1)-f 5′-GAAGTCAG-CTGCATACAC-3′, Col1(α)1-r 5′-AGGAAGTC-CAGGCTGTCC-3′; osteopontin (OPN)-f 5′-3′, OPN-r 5′-CTGGGCAACTGGGATGACCTT-3′; bone sialoprotein (BSP)-f 5′-GTCAACGGCA-CCAGCACCAA-3′, BSP-r 5′-GTAGCTGTATT-CGTCCTCAT-3′; osteocalcin (OCN)-f 5′-ATG-AGGACCCTCTCTCTGCT-3′, OCN-r 5′-CCGT-AGATGCGTTTGTAGGC-3′; osterix-f 5′-CTG-GGGAAAGGAGGCACAAAGAAG-3′, osterix-r 5′-GGGTTAAGGGGAGCAAAGTCAGAT-3′; Runx2-f 5′-CCGCACGACAACCGCACCAT-3′, Runx2-r 5′-CGCTCCGGCC CACAAATCTC-3′; BMP-2-f 5′-CGGGAACAGATACAGGAA-3′, BMP-2-r 5′-GCTGTTTGTGTTTGGCTTGA-3′; BMP-4-f 5′-CGTTACCTCAAGGGAGTG-GA-3′, BMP-4-r 5′-CACACCCCTCTACCAC-CATC-3′; BMP-7-f 5′-TACGTCAGCTTCCGA-GACCT-3′, BMP-7-r 5′-GCTCAGGAGAGGTT-GGTCTG-3′; transforming growth factor-β_1 (TGF-β_1)-f 5′-ATACGCCTGAGTGGCTGTCT-3′, TGF-β_1-r 5′-TGGTTGTAGAGGGCAAG-GAC-3′; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-f 5′-ACCACAGTCCATG-CCATCAC-3′, GAPDH-r 5′-TCCACCACCCT-GTTGCTGTA-3′. PCR products were separated by electrophoresis on 1.2% agarose gels, stained with ethidium bromide and visualized under UV light.

Measurement of Intracellular Glutathione

Cells (5×10^5) were seeded into 60 mm culture dishes and incubated for 2 days in differentiation medium. Then they were cultured in the presence or absence of NAC for 6 or 12 h and harvested. Total glutathione and the ratio of its reduced to oxidized forms were determined using a BIOXYTECH GSH/GSSG-412 kit (OXIS International, Portland, OR).

Western Blot Analysis

We performed Western blot analyses in order to observe the effects of NAC on expression of BMPs. Cells were cultured with osteoblast differentiation medium for 1 or 6 days. Then the culture medium was changed to α-MEM supplemented with 1% bovine serum albumin (BSA), 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate. Cells were cultured for an additional 2 days, and the medium was collected, concentrated, subjected to SDS–PAGE and transferred to PVDF membranes. Blots were probed with anti-BMP-2 or anti-BMP-7 antibodies, and then incubated with HRP-conjugated secondary IgG. Immunoreactivity was detected with a Supersignal chemiluminescent substrate (Pierce) using a LAS plus (Fuji Photo Film Co. Ltd., Tokyo, Japan).

The levels of Runx2 and small GTPases in cell lysates were examined by Western blot analyses. Cells were cultured in osteoblast differentiation medium for the times indicated and cell lysates were prepared using RIPA buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.2% SDS, 1% sodium deoxycholate, 50 mM NaF, 0.2 mM Na_3VO_4, 1 mM PMSF and protease inhibitor cocktail). Protein samples (50 μg) were separated by SDS–PAGE and immunoblotting was performed as described above.

F-Actin Staining

Cells (2×10^4 cells/well) were plated in chamber slides and treated with NAC for 24 h, then fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. After blocking with 1% BSA, cells were labeled with Alexa Fluor 546 phalloidin (1:40) and visualized under confocal microscopy (Olympus FluoView FV300; Tokyo, Japan).

Small GTPase Activity Assays

Small GTPase activity assays were performed using the Rho and Rac/cdc42 Assay Reagent kits, according to the manufacturer's protocols. Cell lysates were prepared using lysis buffer containing 25 mM HEPES (pH7.5), 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 25 mM NaF, and 1 mM Na_3VO_4.

Pull-down of activated, GTP-bound small GTPases from whole cell lysate was performed using glutathione-sepharose beads linked to either a GST-PBD fusion protein containing the Rac1- and Cdc42-binding regions of PAK-1B or a GST-RBD fusion protein containing the Rho-binding region of rhotekin. Activated small GTPases were eluted from the beads by boiling for 5 min in Laemmli buffer. SDS–PAGE and immunoblot analyses were performed as described above.

Statistical Analyses

All results were expressed as means ± SEM. Statistical differences were analyzed using the Student's *t*-test. P values ≤ 0.01 were considered to indicate a statistically significant difference.

RESULTS

NAC Stimulates Osteoblastic Differentiation

To examine the effect of NAC on osteoblastic differentiation, we observed mineralized nodule formation in mouse calvarial cells. These cells formed a few mineralized nodules in the presence of ascorbic acid and β-glycerophosphate. However, mineralized nodule formation increased greatly in the presence of 10–30 mM NAC, with a peak effect at 20 mM (Fig. 1A). Under our culture conditions, mineralized nodule formation in mouse calvarial cells can be subdivided into three stages: proliferation and early differentiation (0–4 days); matrix deposition and early nodule formation (4–8 days); and nodule growth and matrix mineralization (9–14 days). To determine the critical period for NAC-induction of mineralized nodule formation, we added NAC from days 1 to 4, 8 or 14, as well as from days 9 to 14. The stimulatory effect was evident in cells treated from day 1, even when NAC was present for only the first 4 days (Fig. 1B). However, NAC did not exhibit any stimulatory effects if treatment was started at day 9, a point at which the cellular nodules had started to form but were not mineralized. These results suggest that NAC stimulates an early differentiation event, rather than the later mineralization process. In vivo exposure of calvarial bone to NAC resulted in a marked local increase in calvarial bone thickness and secondary bone marrows were observed inside the newly formed bone, which indicates that NAC exerts a stimulatory influence on bone formation, as well as an inhibitory effect on osteoclastic bone resorption (Supplementary Fig. 1).

Since stimulation by NAC occurs during early osteoblast differentiation, we examined its effect on ALP activity, which is an early marker of differentiation. In agreement with the mineralized nodule data, ALP activity was enhanced significantly (ca. twofold) by NAC (Fig. 1C). We used RT-PCR to examine mRNA expression levels of osteoblast differentiation-associated genes such as ALP, Col1(α)1, BSP, OCN, osterix, and Runx2 (Fig. 1D). With the exception of OPN expression, which was unaffected by the presence of NAC, mRNA levels of the differentiation-associated genes increased following NAC stimulation. Stimulatory effect of NAC was also observed in mouse bone marrow stromal cells (Supplementary Fig. 2). In these cells, NAC increased significantly ALP activity in low concentration range (0.5–1 mM) and its effect was higher than that of BMP-2 (50 ng/ml).

Western blot analysis was used to confirm increased levels of Runx2 in the whole cell lysate and nuclear fraction (Fig. 1E). Runx2 is a master regulatory switch for bone formation that mediates temporal activation and/or repression of cell growth and phenotypic genes expression during the various stages of osteoblast differentiation [Lian et al., 2004]. Therefore, our results suggest that NAC might up-regulate osteoblast differentiation-associated genes via up-regulation of Runx2.

Since BMPs are known to induce and/or augment osteoblastic differentiation [Wozney et al., 1988; Katagiri et al., 1994], we examined whether or not NAC induced their expression. In the presence of NAC, mRNA encoding BMP-2, BMP-4, and BMP-7 increased but no obvious effect on TGF-β_1 expression was observed (Fig. 1F). The enhanced production of BMP-2 and -7 was confirmed by Western blotting (Fig. 1G). These results suggest that NAC stimulates production of BMPs, which leads to stimulation of osteoblastic differentiation.

Stimulatory Effect of NAC Depends Partially Upon Enhanced Synthesis of GSH

To verify whether or not stimulation of osteoblast differentiation is a common property among antioxidants, we compared the effects of NAC, GSH, and CAPE. NAA was used as a negative control. Under serum-starved conditions, calvarial cells produced ROS

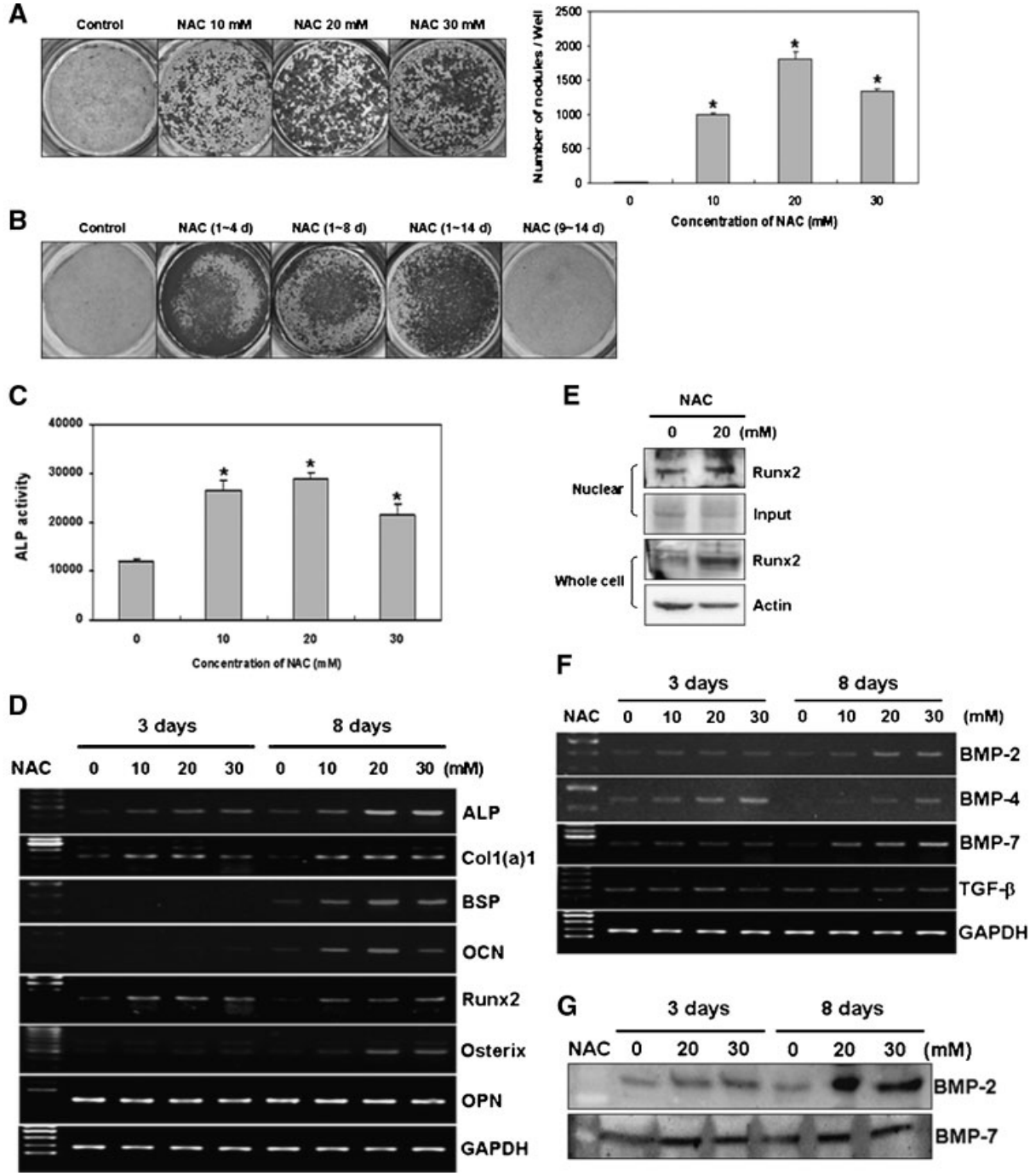

Fig. 1. NAC stimulates osteoblastic differentiation and mineralized nodule formation in mouse calvarial cells. **A**,**B**: Cells were cultured for 14 days in osteoblast differentiation medium with varying concentrations of NAC (A) or treated with 20 mM NAC for the periods indicated (B). At the end of culture, cells were stained with Alizarin red and nodules were counted (A, **right panel**). Data represent means ± SEM (N = 4). * Significant difference from the control ($P < 0.01$). **C**: Cells were cultured for 2 days and then ALP activity (nmole *p*-nitrophenol liberated/h/mg protein) was measured. Data represent means ± SEM (N = 4). * Significant difference from the control ($P < 0.01$). **D**,**F**: Cells were cultured in osteoblast differentiation medium for 3 and 8 days, and gene expression levels were examined by semi-quantitative RT-PCR. **E**: The nuclear fraction and whole cell lysates were prepared from cells cultured in differentiation medium for 2 days. Proteins were separated by SDS–PAGE, then immunoblot analysis was performed using anti-Runx2 antibody. Input represents Ponceau S staining of nuclear proteins. **G**: Cells were cultured in differentiation medium for 1 or 6 days in the presence or absence of NAC. The medium was changed to α-MEM supplemented with 1% BSA, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate, then cells were cultured for an additional 2 days. Culture medium was collected and concentrated, then separated by SDS–PAGE. BMP-2 and -7 were detected by immunoblot analysis.

spontaneously (Fig. 2A). NAC (20 mM) and GSH (20 mM) decreased ROS levels and stimulated mineralized nodule formation of osteoblasts significantly, whereas the cells were unaffected by NAA (20 mM; Fig. 2A,B, respectively). GSH increased mineralized nodule formation to a lesser extent than NAC. The phenolic antioxidant CAPE (5 μg/ml) exhibited ROS scavenging activity that was comparable to 20 mM NAC, but it did not stimulate mineralized nodule formation (Fig. 2B). At concentrations > 5 μg/ml, CAPE induced cell death in long-term culture (data not shown). These results suggest that NAC effect is not due to nonspecific osmolarity but depends upon the presence of both a thiol group and antioxidant activity.

Since NAC functions as a precursor in GSH synthesis, we examined whether or not enhanced GSH synthesis was responsible for NAC-induced stimulation of mineralized nodule formation. Within 12 h of NAC treatment, cellular levels of total GSH increased (Fig. 2C), as did the ratio of reduced to oxidized GSH (data not shown). BSO is a specific inhibitor of GSH synthesis and its presence suppressed the stimulatory effect of NAC significantly (Fig. 2D). These results indicate that NAC stimulation requires increased synthesis of GSH, as well as the resulting change in intracellular redox state.

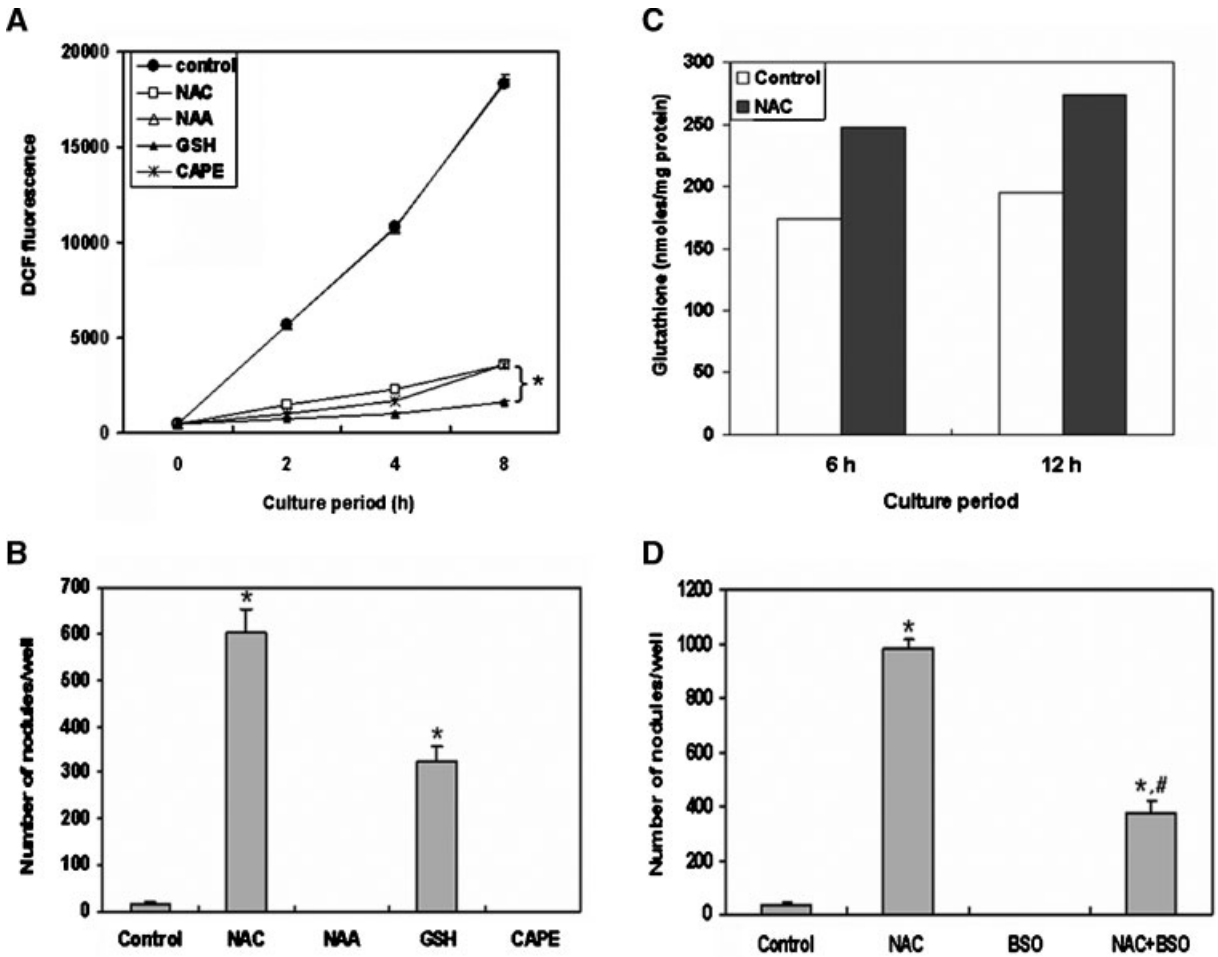

Fig. 2. Stimulatory effect of NAC requires enhanced GSH synthesis. **A**: Intracellular ROS levels were measured by detecting ROS-mediated conversion of DCFH-DA to DCF. Data represent means ± SEM (N = 6). * Significant difference from the control ($P < 0.01$). **B**,**D**: Cells were cultured for 14 days in osteoblast differentiation medium in the presence of the reagents indicated. At the end of culture, cells were stained with Alizarin Red and nodules were counted. Data represent means ± SEM (N = 4). * Significant difference from the control ($P < 0.01$), # significant difference from NAC alone ($P < 0.01$). **C**: Total GSH was determined, as described in the Materials and Methods Section. Reagent concentrations: 20 mM NAC; 20 mM NAA; 20 mM GSH; 5 μg/ml CAPE; and 100 μM BSO.

NAC Suppresses RhoA Activity and Inhibition of RhoA-ROCK Stimulates Osteoblastic Differentiation

Since NAC treatment induced a morphological change in calvarial cells, we observed the actin cytoskeleton by staining with Alexa fluor phalloidin. Calvarial cells treated with NAC exhibited reduced stress fiber formation, as well as induction of a prominent cortical actin cytoskeleton (Fig. 3). Since the actin cytoskeleton is regulated by Rho family small GTPases, we examined the effects of NAC on the activity of Rac1, Cdc42, and RhoA. NAC induced activation of Rac1 and Cdc42, but down-regulated RhoA activity (Fig. 3C). Since RhoA activation leads to the assembly of stress fibers, NAC-induced rearrangement of the actin cytoskeleton coincides with its regulatory effects on small Rho GTPase activity.

Previous reports have shown that the osteoblastic differentiation of mouse calvarial cells is suppressed and stimulated by activation and inhibition of RhoA, respectively [Harmey et al., 2004]. Therefore, we examined whether or not activation of RhoA inhibited NAC stimulation of osteoblastic differentiation. As demonstrated previously [Essler et al., 1998], PMT activated RhoA, but did not affect the activities of Rac1 or Cdc42 in calvarial cells (Fig. 4A). Y27632 is a specific inhibitor of the Rho downstream effector ROCK [Harmey et al., 2004]. Although RhoA activity was unaffected by Y27632, activation of both Rac1 and Cdc42 was enhanced (Fig. 4A). These results suggest that the activation of Rac1 and Cdc42 in NAC- or Y27632-treated cells might represent a compensatory response to inhibition of Rho-ROCK signaling. Consistent with a previous report [Harmey et al., 2004], osteoblastic differentiation was inhibited by PMT and stimulated by Y27632 (Fig. 4B,C). Furthermore, PMT suppressed NAC stimulation of ALP activity and expression of osteoblast differentiation-associated genes (Fig. 4B,C). Although Y27632 did not enhance significantly the stimulatory effect of NAC, alone it did stimulate expression of osteoblast differentiation-associated genes and mineralized nodule formation, although to a lesser extent than NAC (Fig. 4B,C, Supplementary Fig. 3). Similarly, induction of BMP-7 was observed only in NAC treated cells (Fig. 4D). In addition to inhibition of the Rho-ROCK pathway, these results suggest that other mechanisms might be involved in the stimulatory effect of NAC.

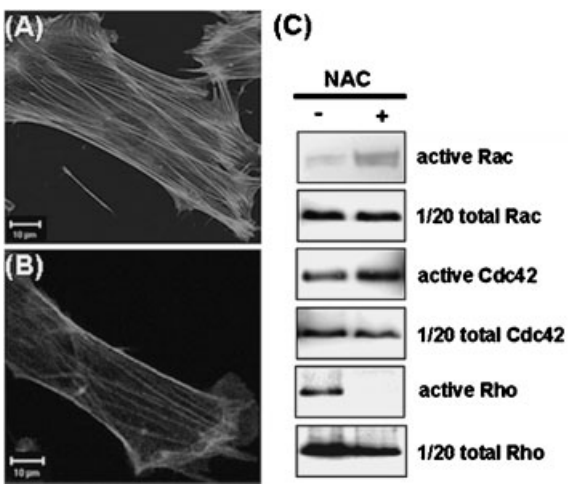

Fig. 3. NAC reduced stress fiber formation via down-regulation of RhoA activity in calvarial cells. **A**,**B**: Cells were cultured for 24 h in the absence (A) or presence (B) of 20 mM NAC, and stained with Alexa fluor-labeled phalloidin. Bars: 10 μm (A,B). **C**: Cells were treated with 20 mM NAC for 48 h, then cell lysates were subjected to pull-down using either the GST-PAK-CD fusion protein (Rac1 and Cdc42) or GST-C21 (RhoA). Activated Rac1 (Rac1-GTP), Cdc42 (Cdc42-GTP) and RhoA (RhoA-GTP) were detected in the pull-down fractions by Western blotting using antibodies specific to Rac1, Cdc42, and RhoA, respectively. Similarly, total Rac1, Cdc42, and RhoA levels were detected by immunoblotting a 1/20 volume of cell lysate with the same antibodies.

Rac is known to be involved in the control of ROS production in both phagocytic and non-phagocytic cells [Roberts et al., 1999; Bokoch and Knaus, 2003]. Therefore, we examined whether or not regulation of Rho family GTPases might alter intracellular ROS levels in calvarial cells. We observed that neither PMT nor Y27632 affected cellular ROS levels (Supplementary Fig. 4). Since the oxidative stress caused by hydrogen peroxide is known to inhibit osteoblastic differentiation [Mody et al., 2001; Bai et al., 2004], we examined whether or not it exerted any effect on Rho family GTPases. Hydrogen peroxide alone enhanced the activity of both Rac1 and Cdc42, but did not stimulate or inhibit RhoA (Fig. 4E). These results indicate that the inhibition of RhoA activity by NAC is independent of its hydrogen peroxide scavenging activity.

DISCUSSION

We found that primary calvarial cells spontaneously produced significant amounts of ROS, even in the absence of extracellular stimuli

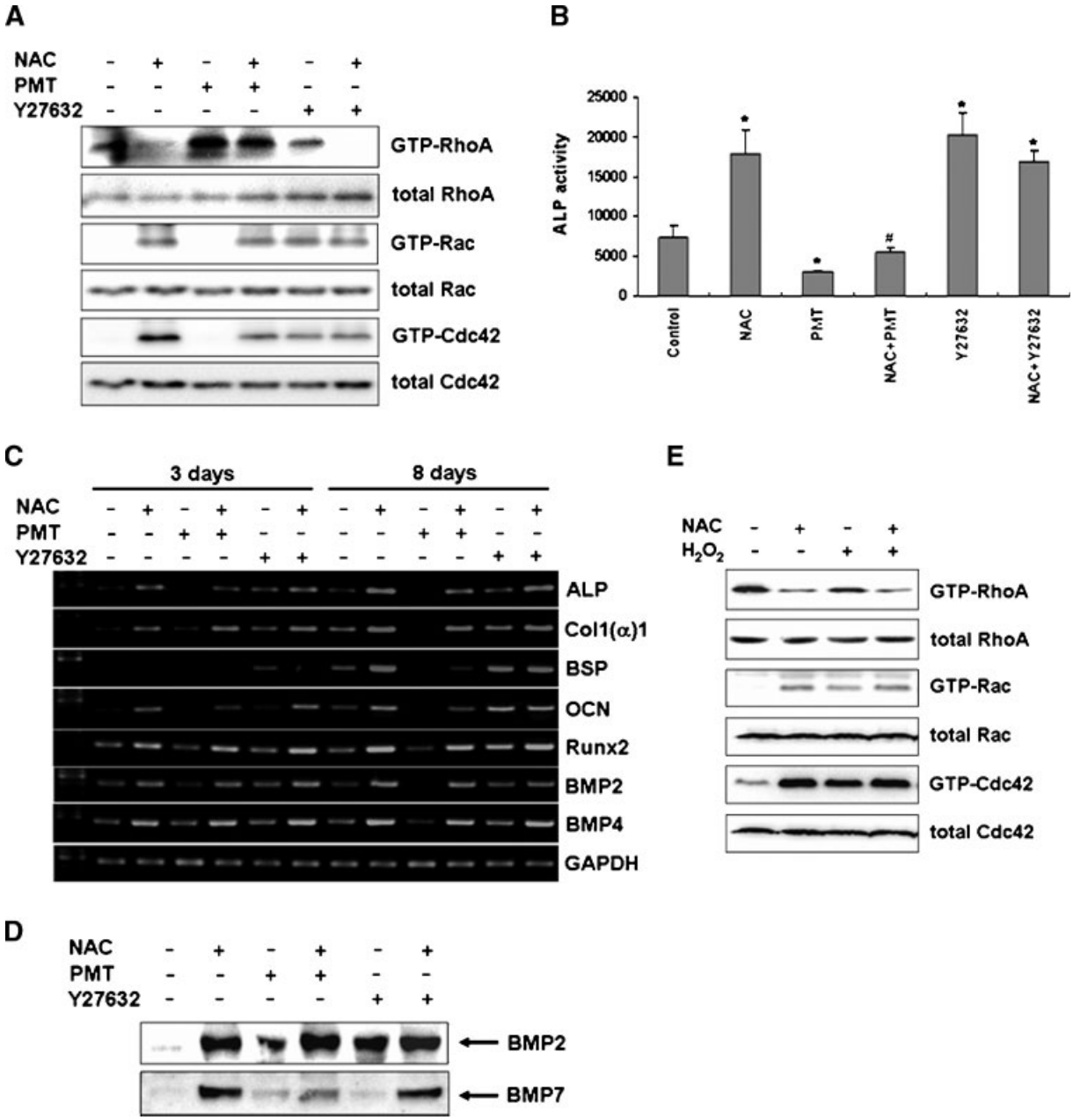

Fig. 4. The stimulatory effect of NAC involves inhibition of RhoA/ROCK signaling but inhibition of RhoA is independent of its H_2O_2 scavenging activity. **A**,**E**: Cells were cultured for 2 days in differentiation medium containing the reagents indicated. Activity of Rac1, Cdc42, and RhoA were detected using a pull-down assay and immunoblot analysis. **B**: ALP activity was measured from cells cultured for 2 days in differentiation medium containing the reagents indicated. Data represent means ± SEM (N = 4). * Significant difference from the control ($P < 0.01$), # significant difference from NAC alone ($P < 0.01$). **C**: Osteoblast differentiation-associated gene expression levels were examined by semiquantitative RT-PCR. **D**: Expression of BMP-2 and -7 were detected by Western blot analysis of collected culture medium, as described in the Materials and Methods Section. Reagent concentrations: 20 mM NAC; 10 ng/ml PMT; 10 μM Y27632; and 100 μM H_2O_2.

such as serum. Osteoblastic differentiation and mineralized nodule formation were stimulated significantly by the reduction of intracellular ROS and increased thiol levels, resulting from treatment with NAC or GSH. The stimulatory effect of NAC was suppressed partially by BSO which blocks GSH synthesis. Although CAPE has been shown to partially prevent hydrogen peroxide-induced inhibition of ALP activity [Bai et al., 2004], alone it did not induce osteoblastic differentiation. Therefore, although these antioxidants share common

activities such as ROS scavenging, they may exert different cellular effects depending upon chemical structure and/or mechanisms of action.

In this study, we showed for the first time that NAC exerts a regulatory effect on Rho family small GTPases and in particular, it inhibits RhoA activation. Previous studies have demonstrated that PMT stimulates DNA synthesis and cell proliferation, while interfering with differentiation in quiescent osteoblasts [Mullan and Lax, 1996; Harmey et al., 2004]. PMT also stimulates the Rho-ROCK pathway, which inhibits mineralized nodule formation in mouse calvarial cell culture [Harmey et al., 2004]. Consistent with these reports, the activation of RhoA by PMT suppressed the stimulatory effect of NAC on osteoblastic differentiation. Although Y27632 stimulated ALP activity and expression of osteoblast differentiation-associated genes, its effect on mineralized nodule formation and expression of BMPs was less pronounced than that of NAC. Differences between the effects of NAC and Y27632 might be caused by the absence of the down-regulation of RhoA downstream effector functions other than ROCK and/or by the lack of antioxidant activity in Y27632 treated cells. In a previous report, the inhibitory effect of PMT on bone nodule formation was reversed completely by the Rho inhibitor C3 transferase but only partly reversed by ROCK inhibitors, which suggests that other downstream effectors of RhoA might play roles in the regulation of osteoblastic differentiation [Harmey et al., 2004]. In contrast to the data obtained in mouse calvarial cells, activation of Rho-ROCK signaling has been shown to cause osteogenic differentiation of human mesenchymal stem cells [McBeath et al., 2004]. In addition, RhoA inhibition and cytoskeletal disruption reduce osteoblastogenesis of human mesenchymal stem cells in a microgravity model [Meyers et al., 2005]. Although the reasons for these discrepancies are unclear, they might indicate that the role played by RhoA differs depending upon species and/or differentiation stage. On the other hand, statins have also been shown to induce osteoblast differentiation and stimulate bone formation [Mundy et al., 1999; Song et al., 2003]. Statins inhibit HMG-CoA reductase and result in inhibition of small GTPases activation via prevention of prenylation. In addition, our data have shown that NAC also enhances osteoblast differentiation of mouse bone marrow stromal cells. Taken together, these reports and our results suggest that inhibition of RhoA GTPase may induce osteoblast differentiation in osteoblastic or bone marrow stromal cells.

Although it is not clear how NAC regulates the activity of Rho family GTPases, previous report has shown that cold-induced superoxide production stimulates RhoA/ROCK signaling and NAC abolishes cold-induced RhoA activation in arteriolar smooth muscle cells [Bailey et al., 2005]. Taken together with our observation that hydrogen peroxide did not regulate RhoA activity, it is likely that superoxide scavenging is responsible for the regulatory effect of NAC on RhoA activity. However, further investigation will be needed to explain the mechanism of NAC regulation of RhoA activity in calvarial cells.

Differentiation of osteoblasts is regulated by a variety of extracellular growth factors and transcription factors: BMPs are the growth factors that induce bone formation [Urist, 1965; Wozney et al., 1988] and stimulate osteoblast differentiation of mesenchymal cells [Katagiri et al., 1994]; and Runx2 [Ducy et al., 1997] and osterix [Nakashima et al., 2002] are bone-associated transcription factors that are essential for osteoblast differentiation. Runx2 is required for the early commitment of mesenchymal cells to chondro/osteoblastic lineages, as well as for the expression of phenotypic markers in differentiated cells [Franceschi, 2003]. Osterix functions downstream of Runx2 and is necessary for Runx2 transcriptional activation of Runx2 target genes such as OCN, BSP, and Col1(α)1 [Nakashima et al., 2002; Franceschi, 2003]. Although over-expression of BMPs (BMP-2, -4, or -7) or Runx2 alone only stimulates modest osteoblast differentiation, over-expression of BMP and Runx2 together increased differentiation synergistically [Franceschi et al., 2003]. In this study, NAC enhanced expression of Runx2, osterix and BMPs, which indicates that the stimulatory effect of NAC on osteoblastic differentiation might be mediated by enhanced expression of central transcription factors and BMPs.

Taken together, our results demonstrate that NAC stimulates the osteoblastic differentiation and mineralized nodule formation of mouse calvarial cells and that this stimulatory effect depends upon enhanced synthesis of GSH and down-regulation of RhoA activity.

REFERENCES

Aitken CJ, Hodge JM, Nishinaka Y, Vaughan T, Yodoi J, Day CJ, Morrison NA, Nicholson GC. 2004. Regulation of human osteoclast differentiation by thioredoxin binding protein-2 and redox-sensitive signaling. J Bone Miner Res 19:2057–2064.

Aruoma OI, Halliwell B, Hoey BM, Butler J. 1989. The antioxidant action of N-acetylcysteine: Its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. Free Radic Biol Med 6:593–597.

Bai XC, Lu D, Bai J, Zheng H, Ke ZY, Li XM, Luo SQ. 2004. Oxidative stress inhibits osteoblastic differentiation of bone cells by ERK and NF-κB. Biochem Biophys Res Commun 314:197–207.

Bailey SR, Mitra S, Flavahan S, Flavahan NA. 2005. Reactive oxygen species from smooth muscle mitochondria initiate cold-induced constriction of cutaneous arteries. Am J Physiol Heart Circ Physiol 289:H243–H250.

Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC, Thomas M. 1983. Flow cytometric studies of oxidative product formation by neutrophils: A graded response to membrane stimulation. J Immunol 130:1910–1917.

Bokoch GM, Knaus UG. 2003. NADPH oxidases: Not just for leukocytes anymore. Trends Biochem Sci 28:502–508.

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. 1997. Osf2/Cbfa1: A transcriptional activator of osteoblast differentiation. Cell 89:747–754.

Essler M, Hermann K, Amano M, Kaibuchi K, Heesemann J, Weber PC, Aepfelbacher M. 1998. *Pasteurella multocida* toxin increases endothelial permeability via Rho kinase and myosin light chain phosphatase J Immunol 161:5640–5646.

Finkel T. 2003. Oxidant signals and oxidative stress. Curr Opin Cell Biol 15:247–254.

Franceschi RT. 2003. Functional cooperativity between osteoblast transcription factors: Evidence for the importance of subnuclear macromolecular complexes? Calcif Tissue Int 72:638–642.

Franceschi RT, Xiao G, Jiang D, Gopalakrishnan R, Yang S, Reith E. 2003. Multiple signaling pathways converge on the Cbfa1/Runx2 transcription factor to regulate osteoblast differentiation. Connect Tissue Res 44:109–116.

Ha H, Kwak HB, Lee SW, Jin HM, Kim HM, Kim HH, Lee ZH. 2004. Reactive oxygen species mediate RANK signaling in osteoclasts. Exp Cell Res 301:119–127.

Halliwell B, Gutteridge JM, Cross CE. 1992. Free radicals, antioxidants, and human disease: Where are we now? J Lab Clin Med 119:598–620.

Harmey D, Stenbeck G, Nobes CD, Lax AJ, Grigoriadis AE. 2004. Regulation of osteoblast differentiation by *Pasteurella multocida* toxin (PMT): A role of GTPase in bone formation. J Bone Miner Res 19:661–670.

Jagger CJ, Lean JM, Davies JT, Chambers TJ. 2005. Tumor necrosis factor-α mediates osteopenia caused by depletion of antioxidants. Endocrinology 146:113–118.

Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, Rosen V, Wozney JM, Fujisawa-Sehara A, Suda T. 1994. Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol 127:1755–1766.

Kim YH, Kim GS, Baek JH. 2002. Inhibitory action of bisphosphonates on bone resorption does not involve the regulation of RANKL and OPG expression. Exp Mol Med 34:145–151.

Lean JM, Davies JT, Fuller K, Jagger CJ, Kirstein B, Partington GA, Urry ZL, Chambers TJ. 2003. A crucial role for thiol antioxidants in estrogen-deficiency bone loss. J Clin Invest 112:915–923.

Lean JM, Jagger CJ, Kirstein B, Fuller K, Chambers TJ. 2005. Hydrogen peroxide is essential for estrogen-deficiency bone loss and osteoclast formation. Endocrinology 146:728–735.

Lian JB, Javed A, Zaidi SK, Lengner C, Montecino M, van Wijnen AJ, Stein JL, Stein GS. 2004. Regulatory controls for osteoblast growth and differentiation: Role of Runx/Cbfa/AML factors. Crit Rev Eukaryot Gene Expr 14:1–41.

Lin JT, Lane JM. 2004. Osteoporosis: A review. Clin Orthop Relat Res 425:126–134.

Liu AL, Zhang ZM, Zhu BF, Liao ZH, Liu Z. 2004. Metallothionein protects bone marrow stromal cells against hydrogen peroxide-induced inhibition of osteoblastic differentiation. Cell Biol Int 28:905–911.

McBeath R, Prione DM, Nelson CM, Bhadriraju K, Chen CS. 2004. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Dev Cell 6:483–495.

Meyers VE, Zayzafoon M, Douglas JT, McDonald JM. 2005. RhoA and cytoskeletal disruption mediate reduced osteoblastogenesis and enhanced adipogenesis of human mesenchymal stem cells in modeled microgravity. J Bone Miner Res 20:1858–1866.

Mody N, Parhami F, Sarafian TA, Demer LL. 2001. Oxidative stress modulates osteoblastic differentiation of vascular and bone cells. Free Radic Biol Med 31:509–519.

Mullan PB, Lax AJ. 1996. *Pasteurella multocida* toxin is a mitogen for bone cells in primary culture. Infect Immun 64:959–965.

Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossini G, Boyce B, Zhao M, Gutierrez G. 1999. Stimulation of bone formation in vitro and in rodents by statins. Science 286:1946–1949.

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B. 2002. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108:17–29.

Riggs BL, Khosla S, Melton LJ III. 2002. Sex steroids and the construction and conservation of the adult skeleton. Endocr Rev 23:279–302.

Roberts AW, Kim C, Zhen L, Lowe JB, Kapur R, Petryniak B, Spaetti A, Pollock JD, Borneo JB, Bradford GB, Atkinson SJ, Dinauer MC, Williams DA. 1999. Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense. Immunity 10:183–196.

Song C, Guo Z, Ma Q, Chen Z, Liu Z, Jia H, Dang G. 2003. Simvastatin induces osteoblastic differentiation and inhibits adipocytic differentiation in mouse bone marrow stromal cells. Biochem Biophys Res Commun 308:458–462.

Urist MR. 1965. Bone: Formation by autoinduction. Science 150:893–899.

Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA. 1988. Novel regulators of bone formation: Molecular clones and activities. Science 242:1528–1534.