Nrf2 Negatively Regulates Osteoblast Differentiation via Interfering with Runx2-dependent Transcriptional Activation*

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Nrf2 (nuclear factor E2 p45-related factor 2) is believed to be a transcription factor essential for the regulation of many detoxifying and antioxidative genes in different tissues. In the present study, we investigated the role of Nrf2 in the regulation of osteoblastic differentiation. nrf2 mRNA expression was significantly up-regulated in femur isolated from ovariectomized mice, whereas in situ hybridization analysis revealed that up-regulation of nrf2 mRNA was mainly found in osteoblasts attached on cancellous bone in femur of ovariectomized mice. Expression of Nrf2 protein was also seen in osteoblasts in neonatal mouse tibia and calvaria. In osteoblastic MC3T3-E1 cells stably transfected with nrf2 expression vector, significant inhibition was seen in the maturation-dependent increase in alkaline phosphatase activity as well as the mineralized matrix formation. Stable overexpression of nrf2 significantly impaired Runx2 (runt-related transcription factor 2)-dependent stimulation of osteocalcin promoter activity and recruitment of Runx2 on osteocalcin promoter without affecting the expression of runx2 mRNA. Coimmunoprecipitation and mammalian two-hybrid assay revealed a physical interaction between Runx2 and Nrf2, whereas cellular distribution of endogenous Runx2 was not apparently changed by nrf2 overexpression in MC3T3-E1 cells. Alternatively, Nrf2 bound to antioxidant-responsive element-like-2 sequence of osteocalcin promoter. The inhibition by nrf2 on runx2-dependent osteocalcin promoter activity was partially prevented by the introduction of reporter of deletion mutant for ARE-like-2 sequence of osteocalcin promoter. These data suggest that Nrf2 may negatively regulate cellular differentiation through inhibition of the Runx2-dependent transcriptional activity in osteoblasts.

In bone tissues, both formation and maintenance are sophisticatedly regulated by bone-forming osteoblasts and bone-resorbing osteoclasts (1–3). The osteoblast lineage is derived from primitive multipotent mesenchymal stem cells with potentiality to differentiate into bone marrow stromal cells, chondrocytes, muscles, and adipocytes (4), whereas osteoclasts are multinucleated cells derived from the fusion of mononuclear hematopoietic precursors (3). The development and differentiation of these two distinct cells are under tight regulation by a number of endogenous substances. These include growth factors, cytokines, and hormones, which are individually secreted through endocrine, paracrine/autocrine, and neuroendocrine systems essential for the del-...
stress in many tissues (23, 24). However, little attention has been paid to a role other than gene transcription in response to oxidative stress of Nrf2 in osteoblasts. In the present study, therefore, we have investigated the role of Nrf2 in mechanisms underlying the regulation of cellular proliferation, differentiation, and maturation processes using osteoblastic MC3T3-E1 cells stably transfected with nrf2 expression vector.

**EXPERIMENTAL PROCEDURES**

**Ovariectomy and Analysis of Skeletal Morphology**—The protocol employed here meets the guidelines of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Kanazawa University. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques. Eight-week-old female ddY mice were subjected to ovariectomy or a sham operation. Mice were killed by decapitation 28 days after ovariectomy, followed by dissection of femora and tibiae and subsequent removal of adhering muscles around the bone for fixation with 70% ethanol. Bone mineral density of samples obtained at 28 days after operation was measured by single energy x-ray absorptiometry using a bone mineral analyzer (DHS-600R; Aloka Co., Tokyo, Japan), whereas micro-CT scans were done using a composite x-ray analyzing system (NX-HCP; NS-ELEX Inc., Tokyo, Japan).

**RT-PCR**—Total RNA was extracted from cultured osteoblasts as described previously (25). cDNA was synthesized with the oligo(dT) primer and reverse transcriptase (Invitrogen) from extracted total RNA. PCR amplification was performed using specific primers, and PCR products were subcloned into a TA cloning vector (Promega) for determination of DNA sequences by ABI Prism 310 Genetic Analyzer (PerkinElmer Life Sciences) using a cycle sequencing kit (Amersham Biosciences). Although results obtained using RT-PCR are by definition not quantitative, apparent quantitative PCR analysis was done at around 30 cycles with relatively high linearity using primers for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase toward the possible comparative reference. PCR products were quantified by using a densitograph, followed by calculation of ratios of expression of mRNA for each gene over that for glyceraldehyde-3-phosphate dehydrogenase.

**In Situ Hybridization Analysis**—In situ hybridization was carried out as described previously (26). In brief, sections mounted were fixed with 4% paraformaldehyde, followed by prehybridization with HCl, proteinase K, and triethanolamine/acetic anhydride treatment. Sections were then incubated with digoxigenin-labeled cRNA probes at 65 °C for 16 h. The slides were treated with RNase A, blocked with 1.5% blocking buffer, incubated with anti-digoxigenin-AP-Fab fragments (Roche Applied Science) at 4 °C for 16 h and treated with nitro blue tetrazolium/formalin neutral buffer solution, followed by decalcification with 20% formic acid. The slides were then incubated with 0.3% H2O2 in methanol. After blocking with normal goat serum or bovine serum albumin and 0.1% Triton X-100, sections were incubated with primary antibodies and subsequently with biotinylated secondary antibodies, followed by incubation with VECTASTAIN Elite ABC Reagent (Vector Laboratories). Finally, immunostaining was done using 0.05% dianminobenzidine and 0.03% hydrogen peroxide.

**Cell Cultures**—MC3T3-E1 cells were purchased from RIKEN Cell Bank. MC3T3-E1 cells were cultured in α-minimal essential medium (Invitrogen) containing 10% fetal bovine serum. For differentiation induction, culture medium was replaced with α-minimal essential medium containing 50 μg/ml ascorbic acid and 5 mM β-glycerophosphate. Culture medium was changed every 2–3 days.

**Establishment of Stable Transfectants**—MC3T3-E1 cells were plated at a density of 1.5 × 10^5 cells/cm². After 24 h, they were stably transfected with PEI containing the full-length coding region of nrf2, which was kindly donated by Dr. J. Alam (University of Massachusetts Medical School). Reporter vectors were co-transfected with a TK-Renilla luciferase construct in either the presence or absence of expression vectors into MC3T3-E1 and COS7 cells. Two days after transfection, cells were lysed for determination of luciferase activity using specific substrates in a luminometer according to the manufacturer’s protocol (Promega). Transfection efficiency was normalized by determining the activity of Renilla luciferase.

**Determination of ALP Activity and Ca⁺² Accumulation and Alizarin Red Staining**—Determination of ALP activity and Ca⁺² accumulation was done as described previously (25). In brief, osteoblasts were solubilized with 0.1% Triton X-100, followed by determination of the ALP activity in lysates using p-nitrophenol phosphate as a substrate. Protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad). The lysates solubilized with 0.1% Triton X-100 were also treated with hydrochloric acid for 16–24 h, followed by centrifugation at 20,000 × g and subsequent determination of Ca⁺² content in the supernatant using the C-TEST kit (Wako). Osteoblasts were fixed with 10% formalin and stained with 2% Alizarin Red S (pH 4.0; Sigma) solution for Alizarin Red staining.

**Bromodeoxyuridine Incorporation Assay**—Cultured cells were treated with dilute bromodeoxyuridine labeling solution for 2 h at 37 °C, followed by immunohistochemical staining using a bromodeoxyuridine staining kit (Zymed Laboratories) according to the manufacturer’s directions.

**Terminal Deoxynucleotidyltransferase-mediated dUTP Nick End Labeling (TUNEL) Assay**—TUNEL staining was performed to detect apoptotic cells based on labeling of DNA strand breaks. Cultured cells were fixed with 10% formalin neutral buffer solution, followed by a TUNEL assay with the TUNEL detection kit (Roche Applied Science) according to the manufacturer’s instructions.

**Chromatin Immunoprecipitation (ChIP) Assay**—ChIP experiments were performed essentially following the protocol provided with the ChIP assay kit (Upstate Biotechnology) using MC3T3-E1 cells. MC3T3-E1 cells were treated with formaldehyde to cross-link and subsequently subjected to sonication in lysis buffer containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and...
1 μg/ml pepstatin). Immunoprecipitation was performed with the anti-Runx2 antibody generously given by Dr. G. Karsenty, followed by extraction of DNA with phenol/chloroform. PCR was performed using the primers 5'-CTTGTCTCTAGGGCGACCCA-3' and 5'-AGCATC-CAGTAGCATTTATA-3' in the promoter region of the mouse osteocalcin gene.

**Immunocytochemistry**—Cultured cells fixed with 4% paraformaldehyde were blocked with 1% bovine serum albumin containing 0.1% Triton X-100, followed by incubation with primary antibodies and subsequently with secondary antibodies conjugated with fluorescein isothiocyanate or rhodamine. Cells were then observed under a confocal laser-scanning microscope (Carl Zeiss).

**Immunoprecipitation and Immunoblotting**—MC3T3-E1 cells were solubilized in lysis buffer containing 1% Nonidet P-40, followed by incubation with an antibody for 1 h at 4 °C and subsequent immunoprecipitation with protein A-Sepharose (Amersham Biosciences). Immunoprecipitates were washed five times with lysis buffer and boiled in SDS sample buffer. Samples were then separated by SDS-polyacrylamide gel electrophoresis, followed by transfer to nitrocellulose membranes (Millipore) and subsequent immunoblotting assay as described previously (29).

**Mammalian Two-hybrid Assay**—The mammalian two-hybrid vectors (Promega) (pBIND, encoding GAL4 DNA-BD fusion protein, and pACT, encoding VP16AD fusion protein) were co-transfected with the luciferase reporter plasmid pG5luc, carrying five GAL4-binding sites upstream of the TATA box, and the TK-Renilla luciferase construct. Cells were incubated for 2 days and subjected to a luciferase reporter assay.

**Yeast Two-hybrid Assay**—Yeast cells were co-transfected with pGBKT7 vector, encoding GAL4 DNA-BD fused to the full-length nrf2 gene, and pGADT7 vector encoding GAL4-AD fused to the full-length runx2 gene. Co-transformed cells were further cultured on plates lacking leucine and tryptophan in either the presence or absence of histidine.

**Electrophoretic Mobility Shift Assay (EMSA)**—Nuclear extracts were prepared as described previously (25), and assays were carried out using 32P-labeled double-stranded oligonucleotides. The oligonucleotide sequences used were as follows: GST-Ya, 5'-CTTGTTCTCTTGGCCCTACCC-3'; ARE-like-1, 5'-CTTCACTTCCAAGTTGCATAA-3'; ARE-like-2, 5'-CCACTGACGTGGCGGCCACCC-3'; ARE-like-2 mutant, 5'-CACTGCCATGGCGGCCACCC-3'. After determining protein contents, an aliquot of nuclear extracts was incubated at a fixed amount of 3 μg of protein with a radiolabeled probe and analyzed by electrophoresis on a 6% polyacrylamide gel. Gels were fixed and dried, followed by exposure to x-ray films for different periods to obtain autoradiograms appropriate for subsequent quantification by a densitograph. Supershift analysis was carried out using the anti-Nrf2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

**Data Analysis**—Results are all expressed as the mean ± S.E., and statistical significance was determined by the two-tailed and unpaired Students’ t test or one-way analysis of variance with a Bonferroni/Dunnnett post hoc test.

**FIGURE 1.** Expression of nrf2 in bone of ovariectomized mice. Eight-week-old female ddY mice were subjected to ovariectomy (OVX) or a sham operation, followed by determination of bone mineral density of both tibia and femur by dual-energy x-ray absorptiometry at 28 days (A) and micro-CT analysis of cancellous bone at 28 days (B). Typical pictures are shown in the figure for micro-CT analysis, and similar results were invariably obtained in at least three independent determinations. C, mRNA was extracted from femora isolated from sham-operated and ovariectomized mice for subsequent RT-PCR using primers for nrf2. Typical pictures are shown on the left, whereas quantitative data are shown on the right. D, tibiae were fixed with formalin, followed by dissection of frozen sections and subsequent in situ hybridization analysis using antisense probe of nrf2. Typical micrographic pictures are shown in the figure, and similar results were invariably obtained in at least three independent determinations. Values are the mean ± S.E. from different experiments shown in the figure. *, p < 0.05; **, p < 0.01, significantly different from each control value obtained in sham-operated mice. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
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FIGURE 2. Distribution profiles of nrf2 in bone and MC3T3-E1 cells. A, tibial sections from neonatal mice (P1) at 1 day old were subjected to ALP staining, tartrate-resistant acid phosphatase staining, and immunohistochemical analysis using an antibody against Nrf2 or PPR. B, calvaria obtained from mice at postnatal 1 day were fixed with formalin, followed by dissection of frozen sections and subsequent ALP staining, tartrate-resistant acid phosphatase staining, in situ hybridization using antisense probe of type I collagen, and immunohistochemical analysis using an antibody against Nrf2. Typical micrographic pictures are shown in the figure, whereas similar results were invariably obtained in at least three independent determinations. C, MC3T3-E1 cells were cultured for 3–28 days in the presence of 50 μg/ml ascorbic acid and 5 mM β-glycerophosphate, followed by isolation of mRNA and subsequent RT-PCR using primers specific for different differentiation markers and Nrf2/Maf signaling machineries. Typical pictures are shown in the figure with similar results in three separate determinations.

RESULTS

Up-regulation of nrf2 Expression in Bone of Ovariectomized Mice—To examine whether or not Nrf2 signaling is altered in bone under pathological conditions, such as osteoporosis, we conducted ovariectomy in mice for subsequent investigation of the expression level of nrf2 mRNA in bone by semiquantitative RT-PCR and in situ hybridization analyses. Ovariectomy drastically decreased uterine weight at 28 days after operation without markedly affecting body weight at any days examined (data not shown). Ovariectomy induced a significant reduction of bone mineral density in both total tibia and total femur when determined by dual energy x-ray absorptiometry at 28 days after operation (Fig. 1A). Bone loss was evident in cancellous bone (Fig. 1B), but not in cortical bone (data not shown), by micro-CT analysis. mRNA was extracted from femur after removing bone marrows by flush out, followed by determination of nrf2 expression by semiquantitative RT-PCR. nrf2 mRNA expression was significantly up-regulated by 3-fold in femur isolated from ovariectomized mice when compared with that in sham-operated mice (Fig. 1C). In addition, in situ hybridization analysis revealed that up-regulation of nrf2 mRNA was mainly found in osteoblasts attached on cancellous bone in femur of ovariectomized mice (Fig. 1D). The use of sense probe did not result in detectable expression of nrf2 mRNA in femoral cancellous bone at 28 days after operation (data not shown).

Expression of Nrf2 in Bone and Osteoblasts—In order to evaluate the possible expression of Nrf2 protein in bone, immunohistochemical analysis was conducted on sections dissected from both tibia and calvaria of neonatal mouse at 1 day old (P1), in addition to staining for tartrate-resistant acid phosphatase, which is an osteoclastic marker, and for ALP, which is an osteoblastic marker, respectively. Both tartrate-resistant acid phosphatase and ALP staining clearly confirmed the presence of osteoclasts and osteoblasts in neonatal P1 mouse (Fig. 2A). Upon immunohistochemical analysis, expression of parathyroid hormone/parathyroid hormone-related protein receptor (PPR) protein was highly restricted to osteoblasts attaching on the cancellous bone surface, with similarly selective localization of Nrf2 protein in osteoblasts on the cancellous bone surface. The similar presence of both osteoclasts and osteoblasts was confirmed in neonatal P1 mouse calvaria, whereas in situ hybridization and immunohistochemical analyses revealed the expression of type I collagen mRNA and Nrf2 protein by osteoblasts in parietal bone, respectively (Fig. 2B). Interestingly, Nrf2 protein was also detected in osteocytes embedded in bone in both trabecular and parietal sections. In sections not treated with a primary antibody, however, no marked immunoreactivity was detected for either Nrf2 or PPR (data not shown).

The osteoblastic cell line MC3T3-E1 cells were cultured for 3–28 days in the presence of 50 μg/ml ascorbic acid and 5 mM β-glycerophosphate, followed by determination of the expression profile of each Nrf2/Maf signaling machinery by RT-PCR. Expression of mRNA was drastically increased for osteocalcin in cells cultured for 14–28 days, whereas expression of mRNA for type I collagen was gradually increased from 3 to 14 days with a plateau up to 28 days (Fig. 2C). By contrast, sustained expression was seen with runx2 mRNA for a period up to 28 days. Under these conditions, expression of nrf2 mRNA was gradually increased from 3 to 14 days with a gradual decrease thereafter up to 28 days. Although both mafF and mafG mRNA were constitutively expressed during cultivation from 3 to 28 days, no marked alteration was seen in their mRNA expression at all stages examined. Weak expression was seen with mafK mRNA throughout the culture period.

Effect of nrf2 Overexpression on Osteoblastic Differentiation—To assess the role of Nrf2 in osteoblastic differentiation toward maturation, MC3T3-E1 cells were stably transfected with pEF containing the full-length coding region of nrf2 (MC3T3-E1-nrf2) or with the empty vector (MC3T3-E1-EV). The expression level of nrf2 was examined by semiquantitative RT-PCR, immunoblotting, and reporter assay analyses. Several clones of cells transfected with nrf2 expression vector showed markedly elevated expression of nrf2 compared with cells transfected with empty vector alone. Among different clones of MC3T3-E1 cells stably transfected with nrf2 expression vector, relatively high expression was seen in the clone(1) for mRNA (Fig. 3A, a) and corresponding protein (Fig. 3A, b) in addition to promoter activity of heme oxygenase-1 (Fig. 3A, c), which is a target gene of nrf2, with relatively low expression in the clone(2).

To investigate whether Nrf2 indeed affects cell differentiation of osteoblasts, these transfected cells were cultured for 4–28 days in the

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To evaluate the effect of stable nrf2 overexpression on cell survivability, cell proliferation and apoptosis assays were individually conducted. Although cell proliferation was markedly decreased in MC3T3-E1-nrf2 clone(1) cells, as revealed by both bromodeoxyuridine incorporation (Fig. 4A, a) and cyclin D1 promoter activity (Fig. 4A, b), no significant difference was seen in the number of TUNEL-positive cells between MC3T3-E1-nrf2 clone(1) and MC3T3-E1-EV cells (Fig. 4A, c). Furthermore, mRNA expression of transcription factors essential for osteoblast differentiation was determined by semiquantitative RT-PCR in MC3T3-E1-EV and MC3T3-E1-nrf2 clone(1) cells. Expression of mRNA was not markedly changed for both osterix and atf4, in addition to runx2, between MC3T3-E1-EV and MC3T3-E1-nrf2 clone(1) cells (Fig. 4B). Accordingly, stable overexpression of nrf2 would lead to marked prevention of developmental differentiation toward maturation without affecting either cellular survivability or gene expression of several master regulators required for differentiation in cultured osteoblastic cells.

Effect of nrf2 on runx2-dependent Transcription and DNA Recruitment of runx2—An attempt was thus made to elucidate underlying mechanisms for the inhibition by nrf2 overexpression of osteoblastic differentiation by determination of reporter activity using the mouse osteocalcin promoter, OG2 (osteocalcin gene 2).

Both COS7 and MC3T3-E1 cells were transiently transfected with a reporter construct of either OG2 (Fig. 5A) or p6OSE2 (Fig. 5B), which is a chimeric gene containing six copies of OSE2 (osteocalcin-specific element 2), in either the presence or absence of nrf2 and runx2 expression vectors, followed by determination of luciferase activity. In COS7 cells transfected with OG2 reporter, luciferase activity was almost quintupled by runx2 expression, whereas no marked increase was found by transfection with nrf2 alone (Fig. 5A, a). However, nrf2 co-expression significantly impaired the runx2-dependent enhancement of OG2 reporter activity in COS7 cells when runx2 was transfected with nrf2 at a similar amount. In MC3T3-E1 cells, similarly significant inhibition was induced by co-transfection of nrf2 on runx2-dependent transactivation of OG2 promoter activity (Fig. 5A, b). In both COS7 (Fig. 5B, a)
and MC3T3-E1 (Fig. 5B, b) cells, runx2 significantly enhanced reporter activity of p6OSE2 by more than 4-fold, with nrf2 by itself being without effect. In contrast to the complete inhibition of OG2 promoter activity, however, co-introduction of nrf2 and runx2 at similar amounts significantly but partially inhibited the runx2-mediated activation of p6OSE2 reporter activity in both COS7 and MC3T3-E1 cells.

We next examined the effect of nrf2 on the association of runx2 with the osteocalcin promoter using a ChIP assay in MC3T3-E1 cells. A protein-genomic DNA complex was prepared from MC3T3-E1 cells transiently transfected with HA-tagged runx2 and/or FLAG-nrf2 expression vector, followed by immunoprecipitation with the anti-Runx2 antibody and subsequent extraction of DNA for PCR using primers to amplify a region containing OSE2 of the osteocalcin promoter or osteocalcin coding sequence. Amplified PCR products were clearly detected in lysates immunoprecipitated by the anti-Runx2 antibody and anti-HA antibody in cells transfected with runx2 alone, whereas no marked PCR products were seen in samples immunoprecipitated by the anti-Runx2 antibody and anti-HA antibody in cells transfected with both nrf2 and runx2. In control experiments, both the anti-Runx2 antibody and anti-HA antibody failed to immunoprecipitate the first exon of the osteocalcin gene (Fig. 5B, top). Quantitative analysis clearly confirmed the almost complete abolition of the association of runx2 with the osteocalcin promoter after the co-transfection of nrf2 along with runx2 (Fig. 5C, bottom). However, co-transfection of nrf2 did not affect mRNA expression of runx2 in MC3T3-E1 cells transfected with runx2 expression vector (data not shown).

Nuclear Co-localization and Physical Interaction of Nrf2 with Runx2—COS7 cells were then transfected with FLAG-tagged nrf2 and/or His-tagged runx2 expression vector, followed by immunocytochemistry with anti-His and anti-FLAG antibodies. Immunostaining analysis revealed that both Nrf2 and Runx2 efficiently underwent nuclear translocation when separately transfected or co-transfected in COS7 cells (Fig. 6A, a). Consistent with the results in COS7 cells, intro-


FIGURE 6. Cellular localization and physical interaction between Nrf2 and Runx2. A, a, COS7 cells were transiently transfected with His-tagged runx2 vector and/or FLAG-tagged nrf2 vector, followed by incubation for 48 h and subsequent immunocytochemistry using the anti-His and anti-FLAG antibodies. b, MC3T3-E1 cells were transiently transfected with nrf2 expression vector, followed by incubation for 48 h and subsequent immunocytochemistry using the anti-nrf2 antibody. Typical pictures are shown in the figure, with similar results in three separate determinations. B, a, COS7 cells were transiently transfected with HA-tagged runx2 vector and/or FLAG-tagged nrf2 vector, followed by immunoprecipitation (IP) with the anti-HA antibody and subsequent immunoblotting (IB) with the anti-FLAG antibody. b, COS7 cells were transiently transfected with pG5-luc in either the presence or absence of pACT-nrf2 vector and/or pBIND-runx2 vector, followed by incubation for 48 h and subsequent determination of luciferase activity. Values are the mean ± S.E. obtained in four independent experiments. c, yeast cells were co-transfected with pGBK7 vector fused to the full-length nrf2 and pGADT7 vector fused to the full-length runx2, followed by determination of the protein-protein interaction on plates lacking leucine and tryptophan, in either the presence or absence of histidine.

The expression pattern, DNA binding activity was increased in proportion to the culture duration from 0 to 14 days, without inducing an addition of 30-fold (Fig. 7B, a and b). To verify nuclear proteins involved in ARE binding, supershift analysis was carried out in nuclear extracts prepared from MC3T3-E1 cells using the anti-Nrf2 antibody. The anti-Nrf2 antibody was effective in inhibiting ARE binding in nuclear extracts of MC3T3-E1 cells cultured for 14 days, without inducing an upward shift of the probe-protein complex on gels when the anti-Nrf2 antibody prepared from epitope mapping at the C terminus of Nrf2 of human origin was used (Fig. 7B, a, 3), as shown in our previous study (30). Moreover, ARE binding was completely inhibited by unlabeled double-stranded oligonucleotide containing an ARE-like-2 sequence, whereas oligonucleotide with mutations on whole ARE core sequence did not markedly affect ARE binding even at a molon concentration ratio of 30-fold (Fig. 7B, a, 3).

An attempt was made to determine the possible involvement of ARE-like-2 sequence in the inhibition by Nrf2 on Runx2-dependent activation of osteocalcin promoter. Although Nrf2 almost completely impaired the Runx2-dependent enhancement of wild-type OG2 reporter activity, as shown in Fig. 5A, the introduction of nrf2 signifi-
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**DISCUSSION**

The essential importance of the present findings is that Nrf2 markedly inhibited cellular maturation through interfering with functionality of the osteoblastic master regulator Runx2 in osteoblastic cell line MC3T3-E1 cells in addition to up-regulation by ovariectomy of nrf2 mRNA in femur. To our knowledge, this paper deals with the first direct demonstration of the crucial involvement of the antioxidative transcription factor Nrf2 in osteoblastic differentiation. Although several previous studies have demonstrated the functional expression of Nrf2 in bone as a transcription factor regulating basal and inducible expression of numerous detoxifying and antioxidant genes (31, 32), no direct evidence for a role in mechanisms underlying the cellular differentiation in osteoblasts is available in the literature to date.

Our results suggest that Nrf2 would regulate osteocalcin promoter activity through at least two distinct pathways. Although Runx2-dependent osteocalcin promoter activity was almost completely blocked by overexpression of nrf2, Runx2-dependent OSE2 reporter activity was not completely inhibited by Nrf2 with complete inhibition by Nrf2 of binding of Runx2 to the osteocalcin promoter. Furthermore, our ChIP analysis gives rise to an idea that a protein-protein interaction would at least in part underlie the almost complete abolition of the association of Runx2 with the osteocalcin promoter after the introduction of nrf2 along with runx2. A physical interaction of Runx2 with Nrf2 could be responsible for the reduction of Runx2-dependent osteocalcin promoter activity by Nrf2, whereas another additional pathway could be also involved in the inhibition. Indeed, overexpression of nrf2 significantly but partially inhibited the Runx2-dependent transactivation of OG2 promoter activity when an OG2 promoter with a mutated ARE core sequence (Fig. 7C, a) was used in both COS7 and MC3T3-E1 cells (Fig. 7C, b).

**FIGURE 7. Regulation of Runx2-dependent transcriptional activity by Nrf2 DNA binding.**

A, six putative Runx2 binding sites (OSE2) and two putative Nrf2 binding sites (ARE-like-1 and ARE-like-2) are shown in the promoter region of the mouse osteocalcin gene. b, COS7 cells were transiently transfected with nrf2 expression vector, followed by incubation for 48 h and subsequent EMSA using ARE-like-1 or ARE-like-2 probe. Typical autoradiograms are shown in the figure, and similar results were invariably obtained in at least three independent determinations. b, MC3T3-E1 cells were cultured for up to 28 days, followed by cell harvest for preparation of nuclear extracts and subsequent EMSA using ARE-like-2 probe. Supershift analysis was carried out by the anti-Nrf2 antibody, whereas competition analysis was conducted using unlabeled wild type (Wt) and mutant (Mt) oligonucleotides for ARE-like-2 sequence. Typical pictures are shown in the figure with similar results in three separate determinations. b, quantitative data are shown with time course experiments on DNA binding of OG2-ARE-like-2 probe in cells cultured from 0 to 28 days. C, nucleotide sequences are shown for constructs with wild type and mutant OG2 reporters. b, COS7 and MC3T3-E1 cells were transiently transfected with wild type or mutant OG2 reporter in either the presence or absence of nrf2 and runx2 expression vectors, followed by incubation for 48 h and subsequent determination of luciferase activity. Values are the mean ± S.E. obtained in four independent experiments. **, p < 0.01, significantly different from each control value obtained in cells with empty vector alone. #, p < 0.05; ##, p < 0.01, significantly different from the value obtained in cells transfected with runx2 expression vector alone.
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Based on experiments using cancer cells with artificial expression techniques, the anti-Nrf2 antibody appropriate for immunoprecipitation is not commercially available for the evaluation of the interaction between endogenous proteins in osteoblasts so far. The final conclusion should thus await the demonstration of an interaction between Nrf2 and Runx2 in primary cultured osteoblastic cells in vitro in addition to immunoprecipitation and co-localization in vivo. In contrast to the results from immunoprecipitation and mammalian two-hybrid assay, moreover, no direct physical interaction was demonstrated between Nrf2 and Runx2 by yeast two-hybrid analysis and GST pull-down assay. Accordingly, the possibility that Nrf2 and Runx2 may be a part of large protein complex that comprises other proteins sandwiched between Nrf2 and Runx2 is not ruled out. It is also conceivable that Nrf2 and/or Runx2 may need particular posttranslational modifications specific for eukaryotic cells, such as phosphorylation and glycosylation, before the interaction.

In osteoblastic cells, at any rate, the functional interaction between Nrf2 and Runx2 could undoubtedly inhibit cellular differentiation toward maturation in a particular situation.

In a data base search, moreover, we have already found the presence of an ARE-like sequence in the mouse type I collagen promoter. Since Runx2 is absolutely required for the induction of osteoblast differentiation in a manner that regulates expression of most genes essential for the osteoblast phenotype, such as osteocalcin and type I collagen (11, 12, 14), it is likely that Nrf2 would interfere with transactivation by Runx2 of osteocalcin through DNA binding to ARE-like sequence, in addition to the protein-protein interaction, to lead to suppressed cellular differentiation in osteoblasts.

By contrast, numerous transcription factors other than Runx2 are shown to play a pivotal role in cellular maturation and functionality in osteoblasts. In the present study, for instance, expression of mRNA was not markedly changed for both osterix and atf4, in addition to runx2, by stable overexpression of nrf2 in MC3T3-E1 cells. In order to verify whether overexpression of nrf2 influences the activity of transcription factors other than Runx2, we also evaluated DNA binding activity of different transcription factors on a transcription factor array (33, 34), using nuclear extracts from MC3T3-E1-EV and MC3T3-E1-nrf2 cells. Among detectable transcription factors, however, no significant alteration was found for DNA binding activities of other transcription factors. These included activator protein-1, cAMP-responsive element binding protein, NF-κB, CCAAT/enhancer-binding proteins, Myc-Max, p53, peroxisome proliferator-activated receptor, Sp1, heat shock element, vitamin D receptor, signal transducer and activator of transcription 1, and Smad3/4 (data not shown). It is therefore conceivable that Nrf2 rather specifically interferes with the activity of the transcription factor Runx2 absolutely required for osteoblastic differentiation.

There is increasing evidence suggesting the role of free radicals in a variety of pathological situations, such as postmenopausal osteoporosis. In women, there is a close association among oxidative stress, antioxidant levels, and bone mineral density (35, 36). Hydrogen peroxide is believed to be a reactive oxygen species (ROS) responsible for the bone loss by estrogen deficiency (37). In addition to postmenopausal osteoporosis, several other risk factors, including diabetes mellitus (38), atherosclerosis (39), smoking (40), and hypertension (41), are all associated with increased oxidative stress. In bone, H2O2 is shown to directly stimulate osteoclastic formation and function through the induction of many cytokines and growth factors (8, 42), whereas this ROS is shown to up-regulate or down-regulate the expression by osteoblasts of receptor activator of NF-κB ligand or osteopro tegerin, the decoy receptor for receptor activator of NF-κB ligand (43). In osteoblasts, ROS is shown to inhibit cell differentiation (44) and to induce apoptosis (45). The administration of antioxidants, such as N-acetyl cysteine and ascorbate, increases tissue glutathione levels and abolishes ovariectomy-induced bone loss, indeed, whereas l-buthionine-(S,R)-sulfoximine, a specific inhibitor of glutathione synthesis, causes substantial bone loss (8). Nrf2 is known to regulate many phase II detoxifying enzymes and oxidative stress-inducible proteins in different tissues through binding to ARE or electrophile-responsive elements on targets genes after dimerization with small Maf proteins when cells are exposed to different insults related to oxidative stress, including ROS, ionizing radiation, and a variety of chemical entities of electrophile compounds, lipid peroxides, and antioxidants. It is thus conceivable that ovariectomy would lead to bone loss through interference with Runx2 required for cellular differentiation by Nrf2 up-regulated following generation of ROS in osteoblasts. The exact mechanism as well as functional significance of Nrf2 on bone loss in ovariectomized mice, however, remains to be elucidated in future studies on deletion of Nrf2 by germline mutation and/or localized knockdown techniques.

Keap1 (Kelch-like ECH-associating protein 1) is identified as a potential effector of Nrf2 and is composed of two distinguishable motifs: the Kelch domain and a bric-a-brac, tramtrack, broad complex/Parvovirus zinc finger domain (46, 47). The bric-a-brac, tramtrack, broad complex domain is shown to form homomeric and heteromeric oligomers, whereas the Kelch domain is thought to interact with Nrf2 for sequestration in the cytoplasm through association with the actin cytoskeleton (48, 49). Under normal physiological conditions, Nrf2 binds to the Kelch domain and is thereby retained in the cytoplasm. When cells encounter oxidative or xenobiotic stress, Nrf2 is released from Keap1 for subsequent translocation to the nucleus (50, 51). Homozygous keap1 mutant newborns are normal, but all died by the third week after birth as a result of the abnormal hyperkeratosis of the esophagus and forestomach and consequent feeding problems. In addition, the pups have severe growth retardation and a gross scaling phenotype that becomes evident by 5 days after birth. Keap1-dependent phenotypes, including severe growth retardation, are all reversed by double mutation of keap1-nrf2, indicating that the Keap1 deficiency would allow Nrf2 to constitutively accumulate in the nucleus (52). Taken together, severe growth retardation could be brought about through a direct action of Nrf2 constitutively accumulated in the nucleus of osteoblasts in keap1-null mice. Of course, growth retardation in this case may be simply not attributable to the direct action of Nrf2 in bone. In this regard, analysis on nrf2 transgenic or nrf2 knock-out mice could therefore be essential for the demonstration of the possible participation of Nrf2 in bone formation as described above.

Nrf2/Maf signaling machineries could thus be a novel target for the development of a drug useful for the treatment and therapy of a variety of bone diseases relevant to abnormal development and maturation of osteoblasts in human beings.
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