Background: How reduced ROS signaling regulates inflammation and remodeling in bone remains unknown. Results: Age-related switch of bone mass in p47phox-deficient mice occurs through an increased inflammatory milieu in bone. Conclusion: p47phox-Nox2-dependent physiological ROS signaling suppresses inflammation in aging. Significance: p47phox-Nox2 and Nox4 may play different roles during early development and skeletal involution because they serve unique functions on osteoblast differentiation and proliferation.

Bone remodeling is age-dependently regulated and changes dramatically during the course of development. Progressive accumulation of reactive oxygen species (ROS) has been suspected to be the leading cause of many inflammatory and degenerative diseases, as well as an important factor underlying many effects of aging. In contrast, how reduced ROS signaling regulates inflammation and remodeling in bone remains unknown. Here, we utilized a p47phox knock-out mouse model, in which an essential cytosolic co-activator of Nox2 is lost, to characterize bone metabolism at 6 weeks and 2 years of age. Compared with their age-matched wild type controls, loss of Nox2 function in p47phox−/− mice resulted in age-related switch of bone mass and strength. Differences in bone mass were associated with increased bone formation in 6-week-old p47phox−/− mice but decreased in 2-year-old p47phox−/− mice. Despite decreases in ROS generation in bone marrow cells and p47phox-Nox2 signaling in osteoblastic cells, 2-year-old p47phox−/− mice showed increased senescence-associated secretory phenotype in bone compared with their wild type controls. These in vivo findings were mechanistically recapitulated in ex vivo cell culture of primary fetal calvarial cells from p47phox−/− mice. These cells showed accelerated cell senescence pathway accompanied by increased inflammation. These data indicate that the observed age-related switch of bone mass in p47phox-deficient mice occurs through an increased inflammatory milieu in bone and that p47phox-Nox2-dependent physiological ROS signaling suppresses inflammation in aging.

Bone remodeling is a process in which osteoblasts and osteoclasts are coupled. It changes dramatically during the course of development as it undergoes age-dependent regulation. It has been suggested that bone loss during aging is largely associated with decreases in osteoblast number and bone formation rate (1), and this age-dependent loss of bone mass is also accompanied by increases in the generation or accumulation of reactive oxygen species (ROS)2 (2). Whether such age-dependent accumulation of ROS plays a direct role on impairing bone remodeling is unknown. On the other hand, appropriate concentrations of ROS have also been shown to serve as signaling molecules controlling cell differentiation and proliferation (3). Therefore, maintaining a fine balance between ROS production and elimination of excessive ROS seems to be essential for physiologic bone homeostasis at any age.

During the past several decades, investigations on the potential harmful effects of increased levels of ROS to damage DNA, lipids, and proteins leading to many pathophysiologic conditions, including degenerative diseases, have dominated over research examining the consequences of reduced ROS production (4). However, literature reports suggest that a reduction in ROS levels and signaling may also be detrimental (5, 6). For example, in the musculoskeletal system, decreased ROS concentrations have been shown to promote inflammation (6). Further investigations on the anti-inflammatory activity of NADPH-oxidases (Nox), the origins and dynamics of ROS production, and their critical biological targets in bone are required.

Although ROS can be generated by several sources, the tightly controlled and cell-specific Nox represent one of the major sources of ROS signaling molecules, including superoxide, hydrogen peroxide, and hydroxyl radicals in many cell types. Activation mechanisms, tissue distribution, and subcellular localization of different members of the Nox family are markedly different (7). Given that they are macrophage in nature, bone resorptive osteoclasts are expected to express all forms of Nox (8). We have previously identified only the expression of Nox 1, 2, and 4 in nonphagocytic osteoblastic cells, with

The abbreviations used are: ROS, reactive oxygen species; Nox, NADPH-oxidase; CGD, chronic granulomatous disease; pQCT, peripheral quantitative computerized tomography; BMD, bone mineral density; ALP, alkaline phosphatase; 2,7-DCF-DA, 2,7-dichlorodihydrofluorescein diacetate; SABG, senescence-associated β-galactosidase; DPI, diphosphorylidencyanide chloride.
isoforms 4 and 2 being the most dominant (9). One of the important roles of the catalytically active Nox complex in neutrophils and macrophages is the killing of microbes by producing ROS (8). The release of such ROS and its downstream products from phagocytic cells is also known as the respiratory burst (8). Reduced respiratory burst occurs in chronic granulomatous disease (CGD). These patients commonly inherit abnormalities of Nox2 or of the essential Nox2 co-factors p22phox, p47phox, or p67phox and display persistent inflammation in many tissues (4). Although data regarding bone inflammation in CGD patients is lacking, age-dependent increases in development of arthritis have been recently shown in a Nox2-deficient mouse model (6). Membrane-associated Nox-p22phox complex is thought to be activated by subsequently recruiting cytosolic proteins p47phox, p40phox, and p67phox. Thereafter, this complex interacts with the small GTPase Rac1 to generate superoxide by transporting electrons from intracellular NADPH to oxygen (10, 11).

It is not surprising that the majority of ROS generated in osteoclasts is through a Nox-dependent mechanism (12, 13). Recently, RANKL and TNFα have been shown to stimulate ROS generation in osteoclasts or their precursors (14, 15). It was unexpected that bone resorption in Nox2-deficient mice was apparently normal during early development (4). Although it may be possible that normal bone resorption in Nox2-deficient mice is partially due to compensatory up-regulation of other isoforms of Nox in osteoclasts (16), data from these reports led us to hypothesize that osteoblastic bone formation is disrupted in p47phox−/−-Nox2 signaling−/−mice. Moreover, animals with deletion of p47phox, one of the complex components of Nox2, showed less severe age-related joint destruction and decreased chondrocyte death (17) compared with animals that had Nox2 entirely knocked out. This suggests that Nox2 signaling may be partially preserved in its components in deficient animals. In the present study, we set to answer some of these critical questions in bone and focus on osteoblasts using p47phox knock-out young and old mice.

Experimental Procedures

Animals—Time-impregnated pregnant female C57BL/6-Ncf1M1J/J (p47phox−/−) mice and their wild type controls were obtained from The Jackson Laboratory (Bar Harbor, ME). Dams and litters were housed in polycarbonate cages in an Association of Laboratory Animal Care-approved animal facility in an environmentally controlled room at 22 °C with a 12-h light/dark cycle and fed standard rodent chow ad libitum throughout experimental period including pregnancy and lactation. Male wild type control and p47phox−/− pups (n = 10 per group) were fed chow ad libitum as previously described (18), for 6 weeks and 2 years. We have recorded body weights on a weekly basis and food intake daily for 7 days after 1, 4, and 12 weeks on the diet. After the mice were sacrificed, serum, legs, and vertebrae were collected and stored at −80 °C until use.

Bone Analyses—Peripheral quantitative computerized tomography (pQCT) was performed on formalin-fixed left tibia for bone mineral density (BMD) measurement using a method established in our laboratory (19). A STRATEC XCT 960 M unit (XCT Research SA, Norland Medical Systems, Fort Atkins, WI) specifically configured for small bone specimens was utilized. Software version 5.4 was used with thresholds of 570 mg/cm² to distinguish cortical bone and 214 mg/cm² to distinguish trabecular from cortical and subcortical bone. Tibial BMD and bone mineral content were calculated. The position for pQCT scanning was defined at a distance from proximal tibia 1 mm below growth plate corresponding to 7% of the total length of the tibia. The distance between each scanning was 1 mm, and total of five scans (five slices) were carried out. The data are expressed as the means of three contiguous slices with the greatest trabecular bone density.

Sequential dehydration of right rear tibial was carried out using different concentrations of alcohol. Proximal tibial bone samples were embedded, cut, and Masson and TRAP (tartrate-resistant acid phosphatase) stained by standard histology special procedures (20, 21). For histomorphometric analysis, sections were read in a blinded fashion. Parameters of cancellous bones in the proximal tibia were measured with a digitizing morphometric system, which consists of an epifluorescent microscope (model BH-2; Olympus), a color video camera, and a digitizing pad (Numonics 2206) coupled to a computer (Sony) and a morphometry program from OsteoMetrics, Inc. Total bone area, total bone surface, osteoblast surface, osteoclast surface, and eroded surface were obtained by manual tracing. Ver- tebrae (L4) histology and immunostaining including above unstained tibia sections using TNFα antibody (Cell Signaling) were carried out using standard protocol from VectaStain ABC kit (Vector Laboratory, Burlingame, CA).

Microcomputed tomography measurements of trabecular of the tibia bone after the above pQCT process were evaluated by using Scanco microcomputerized tomography scanner (μCT-40; Scanco Medical AG, Bassersdorf, Switzerland) at 6-μm isotropic voxel size with x-ray source power of 55 kV and 145 μA and integration time of 300 ms. The gray scale images were processed using a low pass Gaussian filter (σ = 0.8, support = 1) to remove noise, and a fixed threshold of 220 was used to extract the mineralized bone from the soft tissue and marrow phase. Cancellous bone was separated from the cortical regions by semiautomatically drawn contours. A total of 120 slices starting from about 1 mm distal to growth plate, constituting 0.70-mm length, was evaluated for trabecular bone structure by using software provided by Scanco, as described in detail previously (22).

Three-point bending of the left femur was performed at room temperature using a miniature bending apparatus with the posterior femoral surface lying on lower supports (7 mm apart) and the left support immediately proximal to the distal condyles. Load was applied to the anterior femoral surface by an actuator midway between the two supports moving at a constant rate of 3 mm/min to produce a physiological in vivo strain rate of 1% for the average mouse femur. The mechanical properties including ultimate strength/stress and stiffness were recorded with a digital caliper.

Serum Bone Turnover Markers—The serum bone formation marker alkaline phophatase (ALP) and the serum bone resorption marker C-terminal telopeptides of type I collagen (CTX-1) RatLaps were measured by Rat-MID™ ALP ELISA and RatLaps 1-3 ELISA, respectively, from Nordic Biosciences Diagnostic (Herlev, Denmark). Serum total osteocalcin levels were mea-
sured by an ELISA-based kit from TAKARA (TAKARA Bio Inc.). ELISA for mouse mTRAP5b levels was performed using enzyme immunoassay kits from TSZ ELISA (TSZ Scientific).

**Real Time RT-PCR Analysis—** Mouse femur bone RNA and osteoblastic cell RNA were extracted using TRI reagent (MRC Inc., Cincinnati, OH) according to the manufacturer’s recommendation followed by DNase digestion and column cleanup using Qiagen mini columns. Briefly, for RNA isolation from bone tissue, at the time of sacrifice, the right femur was taken, and bone marrow cells were flushed with Eagle’s minimum essential medium + Hanks’ salts after cleaning the surrounding connective tissue. Femoral bones were then frozen in liquid nitrogen and smashed. Femoral bone was placed in 1000 μl of TRI reagent and homogenized using a Polytron aggregate (Kinematica, Luzern, Switzerland). 100 μl of 1-bromo-3-chloropropano was added, and the mixture was centrifuged for 15 min at speed of 16,000 rpm, 4 °C. 450 μl of supernatant was taken, and an equal volume of isopropanol was added and centrifuged for an additional 15 min (16,000 rpm, 4 °C). After washing the RNA pellet with 75% ethanol, isolated RNA was resuspended in RNase-free water. For RNA isolation from cultured cells, treated cells from 6-well plates were washed twice with PBS, and 1000 μl of TRI reagent was added into each well. Cells were scraped into a 1.5-ml Eppendorf tube. RNA preparation was identical to that of isolation of RNA from bone tissue. Reverse transcription was carried out using an iScript cDNA synthesis kit from Bio-Rad. Real time RT-PCR was carried out using SYBR Green and an ABI 7500 fast sequence detection system (Applied Biosystems, Foster City, CA) (23) (Table 1).

**Western Blotting and Antibody Array—** Femoral bone tissue proteins were extracted using a cell lysate buffer as described previously (24). Collagen 1, Runx2, RANKL, TNFα, and MMP9 protein expression in bone tissue was assessed by standard Western immunoblotting using antibodies recognizing these proteins (Santa Cruz Biotechnology, Inc.) followed by incubation with secondary antibodies conjugated with horseradish peroxidase (Santa Cruz). β-Actin protein in bone tissue was analyzed by immunoblotting, using mouse monoclonal antibody recognizing β-actin (Sigma) followed by incubation with a secondary anti-mouse antibody conjugated with horseradish peroxidase (Santa Cruz). Immunoblots were visualized using SuperSignal West Pico chemiluminescent (Pierce). Quantitation of the intensity of the bands in the autoradiograms was performed using a VersaDoc™ imaging system (Bio-Rad). Mouse inflammation antibody arrays were carried out using proteins isolated either from vertebræ or cultured cells according to the procedure provided by manufacture in detail (RayBiotech, Inc., catalog no. AAM-INF-1-8).

**Flow Cytometric Measurement of ROS—** The cell-permeable dye 2,7-dichlorodihydrofluorescein diacetate (2,7-DCF-DA) (Sigma-Aldrich) becomes fluorescent upon reaction with ROS. 2,7-DCC-DA was dissolved in dimethyl sulfoxide and stored as 50 μM stock. Bone marrow cells or calvarial cells were loaded with 10 μM 2,7-DCC-DA for 30 min and then washed three times with PBS before they were harvested. Washed cells were resuspended in 500 μl of PBS and kept on ice until flow cytometric measurement was started. ROS measurement was immediately carried out by flow cytometry using FACSor (Becton-Dickinson, Rutherford, NJ) with a 488-nm excitation beam. The signals were obtained using a 530-nm band pass filter for DCF. Each determination was based on the mean fluorescence intensity of 5000 cells. Basal and phorbol 12-myristate 13-ace-tate-stimulated hydrogen peroxide production was measured in neonatal calvarial cells using the Amplex Red hydrogen peroxide/peroxidase assay (Invitrogen, Molecular Probes) as per the manufacturer’s instructions and our previous description (26).

**Cell Proliferation Assay—** Nonradioactive cell proliferation assay was performed following the protocol provided by manufacturer (Promega Corporation, part no. TB169). According to the manufacturer’s instructions, this assay measures absorbance at 490 nm, and there is a linear correlation (the correlation coefficient of the line is 0.997) between cell number and absorbance at 490 nm; absorbance was converted to cell number.

| Gene   | Forward primer | Reverser primer |
|--------|----------------|-----------------|
| Alp    | TAA CGG CTA CCC GGA TCC TA | TGT CTT GGA CAG AGG CAT GTG |
| Trif   | GAC GTC GAA CTT GCA GGA GAG | GCC ACA AGC AGG AAG AGA |
| Mmp9   | TCT TCT GCC GTC GCA GTA GTG GCC A | TGC ACT GCA GCG TGG AAG CAA A |
| Osteocalcin | TCG TGC TGG AGT GCT TCT TAT GAC | CAC CCT TCT CCA ACA CTG TAC A |
| Runx2  | GTG GGT GAA CGA TCC TGC A | GCG TTC TCG CAG TAC TAC |
| p16    | GTC GCA CGC TCC CGA TGA T | TGG TCC TCG AAG TCA TCT |
| p53    | GGA GAC ATT TTC AGG CTT ATG GA | GCC TCC AAA AAA CTC TCC AAC ATC |
| Cyclin A | ACT CTG TCT GCC TCT TTT GC | TCA AGCT CCC AAG TCC A |
| Cyclin B | GCC AAG CAG TCT GGC CCC AATA | GCT GAA CAA GAG CCA AAG |
| Gapdh  | GTA TGA CAC TCA CAC TCA CGG CAA A | GOT CTC GCT CCT GGA AGA TG |

**Table 1**

| Gene   | Forward primer | Reverser primer |
|--------|----------------|-----------------|
| Alp    | TAA CGG CTA CCC GGA TCC TA | TGT CTT GGA CAG AGG CAT GTG |
| Trif   | GAC GTC GAA CTT GCA GGA GAG | GCC ACA AGC AGG AAG AGA |
| Mmp9   | TCT TCT GCC GTC GCA GTA GTG GCC A | TGC ACT GCA GCG TGG AAG CAA A |
| Osteocalcin | TCG TGC TGG AGT GCT TCT TAT GAC | CAC CCT TCT CCA ACA CTG TAC A |
| Runx2  | GTG GGT GAA CGA TCC TGC A | GCG TTC TCG CAG TAC TAC |
| p16    | GTC GCA CGC TCC CGA TGA T | TGG TCC TCG AAG TCA TCT |
| p53    | GGA GAC ATT TTC AGG CTT ATG GA | GCC TCC AAA AAA CTC TCC AAC ATC |
| Cyclin A | ACT CTG TCT GCC TCT TTT GC | TCA AGCT CCC AAG TCC A |
| Cyclin B | GCC AAG CAG TCT GGC CCC AATA | GCT GAA CAA GAG CCA AAG |
| Gapdh  | GTA TGA CAC TCA CAC TCA CGG CAA A | GOT CTC GCT CCT GGA AGA TG |
Senescence-associated β-Galactosidase (SABG) Staining and Activity Assay—SABG activity assay was performed by β-galactosidase enzyme assay kit (Promega) measured the absorbance at 420 nm according to the manufacturer’s instructions. Cell β-galactosidase staining was also performed according to a method published previously (23). Senescent cells were identified as blue-stained cells by standard light microscopy.

Statistical Analyses—The data are expressed as means ± S.E. t test or one-way or two-way analysis of variance followed by Student-Newman-Keuls post hoc analysis was used to compare the treatment groups. Values were considered statistically significant at p < 0.05.

Results

Increased Bone Formation, Mass, and Strength in Young p47phox−/− Mice, Is Reversed in Old p47phox−/− Mice Compared with Their Age-matched Wild Type Controls—To determine p47phox−/− mouse bone phenotypes at both age of 6 weeks and 2 years, we first analyzed tibial bone by pQCT (peripheral quantitative computed tomography scan). At 6 weeks of age, BMD (with exception of trabecular BMD) and bone mineral content were both significantly higher in p47phox−/− mice compared with those from wild type animals (Fig. 1A). To our surprise, at 2 years of age, all pQCT parameters measured in the p47phox−/− mice were significantly lower relative to their wild type counterparts (Fig. 1A). To determine whether the observed lower bone quantity, i.e., bone mass, is associated with poor bone quality, three-point bone bending test were performed to evaluate bone strength in femurs. As expected, and consistent with our pQCT analysis, both stiffness and peak load were significantly higher in 6-week-old p47phox−/− mice and lower in 2-year-old p47phox−/− mice compared with their respective wild type controls (Fig. 1B). The observed differences in bone quantity and quality between p47phox−/− and wild type mice at 6 weeks and 2 years were reflected by the bone metabolite levels in the serum. Levels of bone formation markers osteocalcin and ALP were significantly higher in serum of 6 weeks p47phox−/− mice, whereas they were lower in 2-year-old p47phox−/− mice compared with their respective age-matched wild type controls (Fig. 1C). On the other hand, bone resorption marker CTX-1, reflecting osteoclast activity, was only significantly different between p47phox−/− and wild type mice at 6 weeks (Fig. 1C). TRAP5b,
believed to be a marker of osteoclast number, was significantly different between \textit{p47}\textit{phox}\textsubscript{-/-} and wild type mice at age 2 years, but not at 6 weeks (Fig. 1C). Bone histomorphometric analytical data further supported these results on bone mass and bone metabolic markers. In 6-week-old mice, with the exception of osteoblast surfaces, increased osteoblast indices including osteoblast number and bone volume were found in \textit{p47}\textit{phox}\textsubscript{-/-} mice compared with their respective controls (Fig. 2A). Decreased osteoclast surface accompanied by erosion surface was also observed in \textit{p47}\textit{phox}\textsubscript{-/-} mice compared with their control at 6 weeks of age (Fig. 2A). In contrast to 6-week-old \textit{p47}\textit{phox}\textsubscript{-/-} mice, 2-year-old \textit{p47}\textit{phox}\textsubscript{-/-} mice had significantly decreased bone volume and osteoblast number and increased osteoclast number but no differences in osteoclast surface and erosion surface compared with their wild type controls (Fig. 2B). Despite changes in osteoclast indices in both young and aged \textit{p47}\textit{phox}\textsubscript{-/-} mice, we could not quantify obvious differences between control and \textit{p47}\textit{phox}\textsubscript{-/-} on bone sections using TRAPase staining (data not shown). These data suggest that osteoblast bone formation was largely affected in both rapidly growing and aged \textit{p47}\textit{phox}\textsubscript{-/-} mice, although there were some osteoclast bone resorption changes. Moreover, microcomputed tomography analysis confirmed the increased bone volume in 6-week-old \textit{p47}\textit{phox}\textsubscript{-/-} mice and decreased bone volume in 2-year-old \textit{p47}\textit{phox}\textsubscript{-/-} mice compared with their respective wild type controls (Fig. 2C). The changes of an index of bone strength, polar moment of inertia (\textit{J}; mm\textsuperscript{4}), was especially consistent with the femur bending test in \textit{p47}\textit{phox}\textsubscript{-/-} mice at 6 weeks and 2 years compared with their controls.

\textbf{Reduced ROS Generation and \textit{p47}\textit{phox}-Nox2 Signaling Is Associated with Increased Osteoblast Differentiation in Early Life but Enhanced Inflammation in Aged \textit{p47}\textit{phox}\textsubscript{-/-} Mice—}To explore mechanistic underpinnings of observed bone mass and quality changes with age in \textit{p47}\textit{phox}\textsubscript{-/-} mice compared with their wild type controls, we isolated primary bone marrow cells
and neonatal osteoblastic calvarial cells. Flow cytometric analysis for total ROS production in bone marrow cells using DCF fluorescence revealed that ROS levels in mice increased with age ($p < 0.001$, between wild type), but that ROS production in p47phox$^{-/-}$ mice was significantly lower at 6 weeks and 2 years than their corresponding wild type controls ($p < 0.001$) (Fig. 3A). Interestingly, according to this measurement, ROS levels in 2-year-old p47phox$^{-/-}$ mice were similar to those from 6-week-old wild type mice. Next, isolated neonatal calvarial cells were utilized to determine whether p47phox$^{-/-}$-Nox2-dependent ROS production and Nox2 signaling were abolished in osteoblastic cells from p47phox$^{-/-}$ mice. Calvarial cells from neonatal p47phox$^{-/-}$ mice had lower basal production of hydrogen peroxide resulting from the action of superoxide dismutase on Nox-generated superoxide, as measured using Amplex red, compared with cells isolated from wild type mice (Fig. 3B) ($p < 0.05$). In addition, hydrogen peroxide production in response to treatment with the Nox2 activator phorbol 12-myristate 13-acetate was significantly lower in osteoblastic calvarial cells from p47phox$^{-/-}$ mice compared with cells from wild type controls (Fig. 3C). These data indicate that p47phox$^{-/-}$-Nox2-dependent ROS generating signaling in osteoblastic cells from p47phox$^{-/-}$ mice is indeed impaired. To determine osteoblastic cell differentiation potential of calvarial cells in the absence of functioning Nox2 signaling from p47phox$^{-/-}$ mice, we cultured these cells in the presence of osteoblastogenic medium for 12 days. Evaluated by ALP staining, we found that cells from p47phox$^{-/-}$ mice have increased differentiation potential compared with cells isolated from wild type neonates (Fig. 3D) ($p < 0.05$). Expression of Nox4, another major isoform of Nox in nonphagocytic osteoblasts, was increased in cells from p47phox$^{-/-}$ mice (Fig. 3E); however, knocking down Nox4 gene expression using Nox4 shRNA (Fig. 3E) was associated with decreased ALP gene expression compared with cells from p47phox$^{-/-}$ mice with control shRNA (Fig. 3F). Interestingly, ALP gene expression was also significantly lower in cells from wild type control animals with Nox4 shRNA compared with without Nox4 shRNA (Fig. 3F). These data suggest that p47phox$^{-/-}$-Nox2-dependent ROS suppresses osteoblast differentiation, but Nox4 is at least required for increased osteoblast differentiation in p47phox$^{-/-}$ mice.

The above observed results may explain bone phenotype and metabolism in p47phox$^{-/-}$ mice during early development (age 6 weeks) compared with their wild type controls. However, it is

**FIGURE 3. Nox2 signaling is not required for osteoblast differentiation.** A, ROS measurements in bone marrow cells from p47phox$^{-/-}$ and wild type control mice using flow cytometry. Three animal samples per group were presented, bone marrow cells were loaded with 10 μM 2,7-DCF-DA for 30 min, living cells were collected, and ROS were monitored (2,7-DCF-DA fluorescence) by flow cytometry. The numbers in the upper right corners indicate cell number in this panel with ROS positive. B and C, basal (B) and phorbol 12-myristate 13-acetate (PMA)-stimulated (C) H$_2$O$_2$ generation was measured in wild type and p47phox$^{-/-}$ mouse neonatal calvarial cell cultures using the Amplex Red hydrogen peroxide/peroxidase assay kit. D, neonatal calvarial cells from wild type or p47phox$^{-/-}$ mice were cultured in 6-well plates for 12 days, and ALP staining was performed. E, Nox4 shRNA was transfected into calvarial cells from either wild type or p47phox$^{-/-}$ mice. Nox4 expression in these transfected cells were determined using Western blots. F, after 24 h, ALP gene expression was examined in calvarial cells from either wild type or p47phox$^{-/-}$ mice with or without Nox4 shRNA using real time PCR. Significant differences ($p < 0.05$) are indicated by * and # compared with either wild type mice or control.
still not clear why ROS production is reduced but both bone quantity and quality are reversed in p47phox−/− mice at age 2 years compared with their wild type control. Although p47phox and Nox2 themselves are different genes, deletion of p47phox or Nox2 in mice are animal models for CGD, and they all produce less ROS. Age-dependent increases in inflammation, such as rheumatoid arthritis, have been reported in Nox2-deficient mice (6). We therefore next explored bone inflammation in p47phox−/− mice to determine whether this was associated with their lower bone mass compared with wild type controls at age 2 years. Proteins were isolated from vertebrae, and Western blots showed a decrease in RANKL expression with no obvious differences in the other two inflammatory markers, TNFα and MMP9, between 6-week-old p47phox−/− and wild type animals (Fig. 4A). Col-1 (type 1 collagen) and Runx2 expression were higher in bone from p47phox−/− mice compared with wild type controls at age 6 weeks (Fig. 4A). In 2-year-old mice, however, RANKL, TNFα, and MMP9 were all expressed at significantly higher levels in bone from p47phox−/− mice compared with wild type controls (Fig. 4A). Runx2 expression was lower in bone from p47phox−/− mice (Fig. 4A). Although specific cell types were not defined, the increased TNFα expression in bone from 2-year-old p47phox−/− mice was further confirmed using antibody immunostaining of decalcified vertebrae bone sections, and TNFα was intensively stained red (Fig. 4B). In accordance with changes of protein levels, mRNA expression of these pro-inflammatory cytokines were also greater in bone of p47phox−/− mice at 2 years compared with their age-matched controls (Fig. 4C) (p < 0.05). However, there were no differences in mRNA expression of these pro-inflammatory cytokines between wild type and p47phox−/− mice at age 6 weeks (Fig. 4C). Gene expression of osteoblast differentiation and activity markers revealed significantly higher mRNA expression of ALP, osteocalcin, and Runx2 in bone of p47phox−/− mice at 6 weeks compared with their wild type controls (Fig. 4C). Among these markers, only osteocalcin was found to be significantly decreased in bone from p47phox−/− mice at 2 years compared with their controls (Fig. 4C). Proteins isolated from vertebrae were further analyzed using inflammation antibody array (Fig. 4D). Although
the signature of inflammation was slightly different between p47<sup>phox</sup>-/- and control mice at 2 weeks of age, significant amounts of inflammatory factors were found increased in their expression from p47<sup>phox</sup>-/- compared with their control mice at 2 years of age.

**Bone and Osteoblastic Cell Senescence Is Accelerated in Old p47<sup>phox</sup>-/- Mice**—Progressive accumulation of excess ROS has been suspected to be the leading cause of many tissue inflammation and degenerative diseases and underlies the effects of aging (2). This is somehow paradoxical to our current findings showing that reduced ROS production was associated with increased inflammation in bone from aged p47<sup>phox</sup>-/- mice, and this was further associated with decreased bone mass. We therefore asked whether physiological levels of p47<sup>phox</sup>-Nox2-dependent ROS signaling protects against the skeletal aging process. SABG activity is the most widely used assay and marker to detect aging or senescent cells both in vivo and in vitro, and the SABG activity in bone was measured in p47<sup>phox</sup>-/- and wild type mice at 6 weeks and 2 years. Although there was no significant difference in SABG activity in p47<sup>phox</sup>-/- and wild type mice at 6 weeks, SABG activity was significantly higher in p47<sup>phox</sup>-/- mice at age 2 years (p < 0.05) (Fig. 5A). It needs to be mentioned that this assay was consistent and generated very small standard deviation (n = 9 was used for in vivo samples), including in ex vivo assay below; error bars are difficult to determine. Each individual sample from p47<sup>phox</sup>-/- mice at age 2 years was measured for SABG showed higher than any samples from control mice. In accordance with these results, p16 and p53 gene expression were also significantly higher in bone from 2-year-old p47<sup>phox</sup>-/- mice compared with wild type controls (Fig. 5B). There was no difference in expression of these genes between p47<sup>phox</sup>-/- and wild type mice at 6 weeks (Fig. 5B). Cell division progression regulator anaphase-promoting complex substrates cyclin A and B were also examined. We found that both cyclin A and B expression in bone were significantly higher in p47<sup>phox</sup>-/- mice at 6 weeks but lower in p47<sup>phox</sup>-/- mice at 2 years compared with their respective wild type controls (Fig. 5C). This is interesting, because the results implicated that without sufficient p47<sup>phox</sup>-Nox2-dependent ROS production, skeletal aging may be accelerated.

Finally, the in vivo results demonstrating accelerated cell senescence were recapitulated using isolated calvarial cell cultures. We tried to establish replicative senescent osteoblastic cells in vitro; we usually need to passage calvarial cell cultures at least up to 20 times as described in detail under “Experimental Procedures” and in our previous study (23). Cells isolated from p47<sup>phox</sup>-/- mice showed significantly decreased proliferation and differentiation capacities after only eight passages compared with cells isolated from wild type mice passaged the same number of times (Fig. 6A). Gene expression of ALP, Runx2, p16, and p53 from passage eight calvarial cells from p47<sup>phox</sup>-/- mice were further compared with passage 8 cells from wild type controls. Gene expression of ALP and Runx2 gene was significantly
lower, whereas that of p16 and p53 genes was significantly higher in passage 8 cells from p47phox<sup>+/−</sup> mice compared with those from wild type mice (Fig. 6B). Consistent with cell senescence-associated gene expression, SABG activity was also significantly higher in passage 8 calvarial cells from p47phox<sup>+/−</sup> mice compared with their control cells as evaluated by SABG ELISA measurement and SABG blue staining (Fig. 6, C and D). Furthermore, substantial increases in TNFα expression were also found in passage 20 calvarial cells from p47phox<sup>+/−</sup> mice compared with wild type control cells (Fig. 6E). As we published previously, to generate replicative senescent primary cells, cells need to be passaged at least 20 times. In current experiment, we only passaged cells from p47phox<sup>+/−</sup> mice eight times, and they were quicker to enter senescence program than cells from wild type mice, suggesting that the p47phox gene exerts roles on anti-osteoblastic cell senescence or skeletal aging. Passage 20 cells from both p47phox<sup>+/−</sup> and wild type control mice were further analyzed using inflammation antibody array (Fig. 6F). Expre-
sion of large amounts of inflammatory factors was significantly increased in passage 20 cells from p47phox−/− mice compared with those cells from wild type controls.

Different effects of transient versus sustained inhibition of Nox activity on osteoblastic cell senescence signaling and inflammation from control mice were examined. Fig. 7A shows increased expression of cyclin A and B in passage 2 calvarial cells from p47phox−/− mice but decreased expression of both cyclin A and B in passage 8 and 20 cells from p47phox−/− compared with cells from control. B, calvarial cells from control mice were treated with Nox pan inhibitor DPI for 4, 24, and 72 h with three different doses. Proteins were collected for SABG activity measurement. C, calvarial cells from control mice were treated with Nox pan inhibitor DPI 50 nm for 4, 24, and 72 h, and RNA were collected for TNFα mRNA expression using real time PCR. Significant differences (p < 0.05) are indicated by * compared with respective cells from wild type control mice or vehicle-treated cells.

**Discussion**

In the present study, we investigated bone metabolism in p47phox−/− knock-out mice. We have shown that Nox2 activity and signaling in the bone was markedly reduced in p47phox−/− mice, as indicated by decreased ROS generation in both bone marrow cells and primary osteoblastic cells, and osteoblastic cells from p47phox−/− mice failed to respond Nox2 activator phorbol 12-myristate 13-acetate. We observed increased bone mass and strength during early development of p47phox−/− mice that was reversed in 2-year-old p47phox−/− mice compared with their age-matched respective wild type controls. Therefore, p47phox−Nox2-mediated reduction of ROS generation may be only temporally beneficial for early bone development. The age-related switch of bone mass in p47phox−/− mice appears to occur through an increased inflammatory milieu in bone accompanied by accelerated senescent signaling pathway in osteoblasts and that p47phox−/−-dependent physiological ROS signaling suppresses inflammation in aging. Our data in aged p47phox−/− mice may conflict with a previous expectation that age-associated bone loss and inflammation are associated with increased ROS production, but they are consistent with recent evidence that showed age-dependent increases in development of inflammatory arthritis in an entirely Nox2-deficient mouse model (6). Our data are also consistent with clinical observations where data indicated increased inflammatory diseases in CGD patients (27). Although the use of a global knock-
Bone Mass in p47<sup>phox</sup>-deficient Mice

out for p47<sup>phox</sup> may have limitations to discriminate a specific bone effect from a generalized endocrine effect, there is no evidence for such a generalized endocrine effect on bone.

ROS have previously been thought to be toxic by-products of cellular function, but specific Nox-dependent ROS production is not recognized as a by-product. More recent evidence suggests that Nox-dependent ROS signaling may play an important role on cell cycle progression and proliferation (28, 29). This may be true, particularly in bone cells from 2-year-old p47<sup>phox</sup>/−/− mice, which showed less activity to secrete osteocalcin. Similarly, passage 8 osteoblastic cells from neonatal calvarias of p47<sup>phox</sup>/−/− mice showed less potential to proliferate compared with cells from wild type animals. We could not definitively address whether p47<sup>phox</sup>-Nox2-dependent ROS generation plays an age-dependent role to promote osteoblastic cell proliferation in vivo; however, increased cell senescent signaling pathway in aged p47<sup>phox</sup>/−/− mice may be pivotal in suppression of cell proliferative capacity.

The p47<sup>phox</sup> deficiency in mouse is one of the animal models for the human disease CGD in which patients commonly inherit its abnormalities of Nox2, p22<sup>phox</sup>, or p67<sup>phox</sup> and display persistent inflammation in many tissues. Patients usually die early in life because of a variety of infections; bone metabolism in these patients has never been documented. However, according to Nox2-deficient data (16), bone resorption was not suspected to be significantly changed in CGD patients. This prompted us to focus on examining bone formation in p47<sup>phox</sup>-deficient mice. We have previously demonstrated that although Nox2 was highly expressed in osteoblastic cells, Nox4 expression was also abundant. In contrast, very low expression of Nox1 and no expression of Nox3 were observed in these cells (9). These results were confirmed in RNA from the bone tissue after aspiration of bone marrow cells in the current study. Our results suggest that without fully functioning p47<sup>phox</sup>-Nox2, osteoblast differentiation is increased during early life bone development. We therefore suspect that before inflammatory factors have a significant impact, Nox4 is up-regulated in p47<sup>phox</sup>/−/− mice to compensate for a decrease in Nox2 activity, and this up-regulation of Nox4 may be essential for increasing osteoblast differentiation in young mice (Fig. 4). Although there are discrepancies from a recent study using global Nox4 knock-out animal (30), cell-type-specific conditional knock-out animal models may be used to untangle this possibility. However, in the current study, we used an ex vivo cell culture model and determined that knocking down Nox4 in cells from p47<sup>phox</sup>/−/− mice blocked temporal osteoblastic cell differentiation signaling. These data are consistent with recent findings demonstrating that Nox4 is required for cardiomyocyte and adipocyte differentiation in vitro via redox activation (31), and ROS-dependent signaling pathways are also known to play an important role in osteoblast differentiation (32). Our data therefore suggest that Nox2 and Nox4 function differently during early development and skeletal involution to preserve different roles on osteoblast differentiation and proliferation, respectively.

Early life bone development and skeletal remodeling during aging are believed to be two completely different processes. The current understanding on bone development throughout life is that increases in bone formation relative to bone resorption in rapidly growing rodents result in bone accrual. On the other hand, decreases in bone formation and osteoblast function in addition to increases in bone resorption are considered features of the aging skeleton (1, 2). Although osteocytes were suspected to be involved in bone remodeling (33), bone loss during aging is still recognized largely as a result of decreased osteoblast number and bone formation rate (1). Decreased osteoblast number in 2-year-old p47<sup>phox</sup>/−/− mice could explain diminished osteoblast proliferation. Moreover, it is possible that increased TNFα in 2-year-old p47<sup>phox</sup>/−/− mice suppresses Runx2 expression. Therefore, we believe that, in contrast to the redundant role of p47<sup>phox</sup>-Nox2 in osteoblast differentiation in early development, p47<sup>phox</sup>-dependent Nox2 signaling or constitutive p47<sup>phox</sup>-dependent Nox2-derived ROS production is indispensable for osteoblast proliferation in aged animals. To promote cell proliferation, a certain level of p47<sup>phox</sup>-Nox-associated ROS production may be required. A recent study in a primary acute myeloid leukemia cell line and primary acute myeloid leukemia blasts showed that Nox-derived ROS promoted cell proliferation and was associated with defective oxidative stress signaling (34). In our hands, ROS levels in bone marrow cells of 2-year-old p47<sup>phox</sup>/−/− mice were similar to 6-week-old wild type mice. This ROS levels in 2-year-old p47<sup>phox</sup>/−/− mice may be far lower to promote osteoblast proliferation.

The root cause of increased inflammation in aged p47<sup>phox</sup>/−/− mice characterized by elevation of numbers of inflammatory factors in bone is still not clear, but it appears to be associated with accelerated activation of senescence pathway in osteoblasts. Based on our previous in vitro and in ex vivo cell culture experiments (23), osteoblastic cells (including primary cells and cell line) need to be passaged 20–30 times to generate replicative senescent and aging osteoblasts. However, neonatal calvarial cells isolated from p47<sup>phox</sup>/−/− mice showed rapidly increased cell senescent pathway after only eight passages. We therefore speculate that physiological p47<sup>phox</sup>-dependent Nox2-derived ROS levels may suppress not only inflammation but also osteoblast aging. Our speculation may be contradictory to the accepted idea of the universal role of ROS accumulation plays in the aging process; however, it agrees with evidence provided recently in which yeast cells were grown anaerobically without ROS. The lifespan of these cells was shorter than that of cells grown aerobically and was further shortened by caloric restriction (35). Therefore, the current understanding of the free radical theory in aging process has been challenged (36). An increasing number of studies contradicting the free radical theory of aging have been published recently and have been very useful in defining the contribution of oxidative damage to the aging process (37–39). Moreover, evidence has shown that increased antioxidant protection may even lead to shortened lifespan, and decreased antioxidant function may extend it (40). It is not known whether increased inflammation up-regulates tissue senescence pathway or whether accelerated cell senescence signaling causes tissue inflammation; nonetheless, they seem to be always accompanied by each other. Most of the time, increased cell senescent signaling is also associated with increased tissue inflammation (41). It has been known that cellular senescence is a stable form of cell cycle arrest that may
limit the proliferative potential of a cell and promotes aging (42). Our understanding of how ROS and cellular senescence contributes to noncancer tissue pathophysiology is extremely limited. However, as we previously hypothesized, increased osteoblastic cell senescence may play a central role in bone pathology or osteoblastic cellular senescence may be a fundamental factor contributing to bone loss associated with, not only aged p47^{phox−/−} mice, but also high fat Western diet-induced obesity, sex steroid deficiency, and other conditions, such as systemic insulin resistance and chronic alcohol abuse (43).

In conclusion, based on our data, we believe that greater bone mass and strength in young p47^{phox−/−} mice are due to increased osteoblastic cell differentiation leading to increased bone formation. Reversal of bone mass and strength in older p47^{phox−/−} mice is due to decreased bone forming cell proliferation and accelerated cell senescence accompanied by increased inflammation. Our data indicate that p47^{phox−}·Nox2 and Nox4 may play different roles during early development and skeletal involution because they serve unique functions on osteoblast differentiation and proliferation.

References

1. Kassem, M., and Marie, P. J. (2011) Senescence-associated intrinsic mechanisms of osteoblast dysfunctions. Aging Cell 10, 191–197
2. Almeida, M. (2012) Aging mechanisms in bone. Bonekey Rep. 1, 102
3. Tsukagoshi, H., Busch, W., and Benfey, P. N. (2010) Transcriptional regulation of ROS controls transition from proliferation to differentiation in the root. Cell 143, 606–616
4. Bedard, K., and Krause, K. H. (2007) The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol. Rev. 87, 245–313
5. Hagenow, K., Gelderman, K. A., Hultqvist, M., Merky, P., Bäcklund, J., Frey, O., Kamradt, T., and Holmdahl, R. (2009) Ncf1-associated reduced oxidative burst promotes IL-33R+ T cell-mediated adjuvant-free arthritis in mice. J. Immunol. 183, 874–881
6. Lee, K., Won, H. Y., Bae, M. A., Hong, J. H., and Hwang, E. S. (2011) Spontaneous and aging-dependent development of arthritis in NADPH oxidase 2 deficiency through altered differentiation of CD11b+ and Th1/ Treg cells. Proc. Natl. Acad. Sci. U.S.A. 108, 9548–9553
7. Maghazl, G. I., Krause, K. H., Stocker, R., and Jaquet, V. (2012) Detection of reactive oxygen species derived from the family of NOX NADPH oxidases. Free Radiol. Biomed. 53, 1903–1918
8. Lambeth, J. D., Kawahara, T., and Diebold, B. (2007) Regulation of Nox and Duox enzymatic activity and expression. Free Radiol. Biomed. 43, 319–331
9. Chen, J. R., Lazarenko, O. P., Shankar, K., Blackburn, M. L., Lumphkin, C. K., Badger, T. M., and Ronis, M. J. (2011) Inhibition of NADPH oxidases prevents chronic ethanol-induced bone loss in female rats. J. Pharmacol. Exp. Ther. 336, 734–742
10. Groemping, Y., Lapouge, K., Smerdon, S. J., and Rittinger, K. (2003) Molecular basis of phosphorylation-induced activation of the NADPH oxidase. Cell 113, 343–355
11. Schrenzel, J., Serrander, L., Båndi, B., Nüsse, O., Foyouzzi, R., Lew, D. P., Demaurex, N., and Krause, K. H. (1998) Electron currents generated by the human phagocyte NADPH oxidase. Nature 392, 734–737
12. Geisz, M., and Leto, T. L. (2004) The Nox family of NAD(P)H oxidases: host defense and beyond. J. Biol. Chem. 279, 51715–51718
13. Key, L. L., Jr., Ries, W. L., Taylor, R. G., Hays, B. D., and Pitzer, B. L. (1990) Oxygen derived free radicals in osteoclasts: the specificity and location of the nitroblue tetrazolium reaction. Bone 11, 115–119
14. Lee, N. K., Choi, Y. G., Baik, J. Y., Han, S. Y., Jeong, D. W., Bae, Y. S., Kim, N., and Lee, S. Y. (2005) A crucial role for reactive oxygen species in RANKL-induced osteoclast differentiation. Blood 106, 852–859
15. Yang, S., Madyzastha, P., Ries, W., and Key, L. L. (2002) Characterization of interferon gamma receptors on osteoclasts: effect of interferon gamma on osteoclastic superoxide generation. J. Cell. Biochem. 84, 645–654
16. Yang, S., Madyszata, P., Bingel, S., Ries, W., and Key, L. (2001) A new superoxide-generating oxidase in murine osteoclasts. J. Biol. Chem. 276, 5452–5458
17. Sareith, O., Kellkta, T., Pizzolla, A., Hultqvist, M., and Holmdahl, R. (2011) NOX2 complex-derived ROS as immune regulators. Antioxid. Redox Signal. 15, 2197–2208
18. Reeves, P. G., Nielsen, F. H., and Fahey, G. C., Jr. (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J. Nutr. 123, 1939–1951
19. Chen, J. R., Lazarenko, O. P., Blackburn, M. L., Badeaux, J. V., Badger, T. M., and Ronis, M. J. (2009) Infant formula promotes bone growth in neonatal piglets by enhancing osteoblastogenesis through bone morphogenic protein signaling. J. Nutr. 139, 1839–1847
20. Chen, J. R., Lazarenko, O. P., Zhang, J., Blackburn, M. L., Ronis, M. J., and Badger, T. M. (2014) Diet-derived phenolic acids regulate osteoblast and adipocyte lineage commitment and differentiation in young mice. J. Bone Miner. Res. 29, 1043–1053
21. Zhang, J., Lazarenko, O. P., Kang, J., Blackburn, M. L., Ronis, M. J., Badger, T. M., and Chen, J. R. (2013) Feeding blueberry diets to young rats dose-dependently inhibits bone resorption through suppression of RANKL in stromal cells. PLoS One 8, e70438
22. Cao, J. J., Gregoire, B. R., and Gao, H. (2009) High-fat diet decreases cancellous bone mass but has no effect on cortical bone mass in the tibia in mice. Bone 44, 1097–1104
23. Zhang, J., Lazarenko, O. P., Blackburn, M. L., Badger, T. M., Ronis, M. J., and Chen, J. R. (2014) Soy protein isolate down-regulates calcitonin-1 expression to suppress osteoblastic cell senescence pathways. FASEB J. 28, 3134–3145
24. Chen, J. R., Zhang, J., Lazarenko, O. P., Cao, J. J., Blackburn, M. L., Badger, T. M., and Ronis, M. J. (2013) Soy protein isolates prevent loss of bone quantity associated with obesity in rats through regulation of insulin signaling in osteoblasts. FASEB J. 27, 3514–3523
25. Wong, G. L., and Cohn, D. V. (1975) Target cells in bone for parathormone and calcitonin are different: enrichment for each cell type by sequential digestion of mouse calvaria and selective adhesion to polymeric surfaces. Proc. Natl. Acad. Sci. U.S.A. 72, 3167–3171
26. Chen, J. R., Shankar, K., Nagarajan, S., Badger, T. M., and Ronis, M. J. (2008) Protective effects of estradiol on ethanol-induced bone loss involve inhibition of reactive oxygen species generation in osteoblasts and down-stream activation of the extracellular signal-regulated kinase/signal transducer and activator of transcription 3/receptor activator of nuclear factor-kappaB ligand signaling cascade. J. Pharmacol. Exp. Ther. 324, 50–59
27. Marks, D. J., Miyagi, K., Rahman, F. Z., Novelli, M., Bloom, S. L., and Segal, A. W. (2009) Inflammatory bowel disease in CGD reproduces the clinicopathological features of Crohn’s disease. Am. J. Gastroenterol. 104, 117–124
28. Tan, C., Day, R., Bao, S., Turk, J., and Zhao, Q. D. (2014) Group VIA phospholipase A2 mediates enhanced macrophage migration in diabetes mellitus by increasing expression of NADPH oxidase 4. Arterioscler. Thromb. Vasc. Biol. 34, 768–778
29. Lambeth, J. D. (2004) NOX enzymes and the biology of reactive oxygen. Nat. Rev. Immunol. 4, 181–189
30. Goettsch, C., Babelowa, A., Trummer, O., Erben, R. G., Ram metall, S., Weismann, N., Weinberger, V., Benkhoff, S., Kampschulte, M., Obermayer-Pietsch, B., Hofbauer, L. C., Brandes, R. P., and Schröder, K. (2013) NADPH oxidase 4 limits bone mass by promoting osteoclastogenesis. J. Clin. Invest. 123, 4731–4738
31. Murray, T. V., Smyrnias, I., Shah, A. M., and Brewer, A. C. (2013) NADPH oxidase 4 regulates cardiomyocyte differentiation via redox activation of c-Jun protein and the cis-regulation of GATA-4 gene transcription. J. Biol. Chem. 288, 15745–15759
32. Arakaki, N., Yamashita, A., Niimi, S., and Yamazaki, T. (2013) Involvement of reactive oxygen species in osteoblastic differentiation of MC3T3-E1 cells accompanied by mitochondrial morphological dynam-
Bone Mass in p47phox-deficient Mice

33. Xiong, J., Onal, M., Jilka, R. L., Weinstein, R. S., Manolagas, S. C., and O’Brien, C. A. (2011) Matrix-embedded cells control osteoclast formation. Nat. Med. 17, 1235–1241
34. Hole, P. S., Zabkiewicz, J., Munje, C., Newton, Z., Pearn, L., White, P., Marquez, N., Hills, R. K., Burnett, A. K., Tonks, A., and Darley, R. L. (2013) Overproduction of NOX-derived ROS in AML promotes proliferation and is associated with defective oxidative stress signaling. Blood 122, 3322–3330
35. Koc, A., Gasch, A. P., Rutherford, J. C., Kim, H. Y., and Gladyshev, V. N. (2004) Methionine sulfoxide reductase regulation of yeast lifespan reveals reactive oxygen species-dependent and -independent components of aging. Proc. Natl. Acad. Sci. U.S.A. 101, 7999–8004
36. Gladyshev, V. N. (2014) The free radical theory of aging is dead: long live the damage theory! Antioxid. Redox Signal. 20, 727–731
37. Gems, D., and Doonan, R. (2009) Antioxidant defense and aging in C. elegans: is the oxidative damage theory of aging wrong? Cell Cycle 8, 1681–1687
38. Ristow, M., and Schmeisser, S. (2011) Extending life span by increasing oxidative stress. Free Radic. Biol. Med. 51, 327–336
39. Van Raamsdonk, J. M., and Hekimi, S. (2012) Superoxide dismutase is dispensable for normal animal lifespan. Proc. Natl. Acad. Sci. U.S.A. 109, 5785–5790
40. Van Raamsdonk, J. M., and Hekimi, S. (2009) Deletion of the mitochondrial superoxide dismutase sod-2 extends lifespan in Caenorhabditis elegans. PLoS Genet. 5, e1000361
41. Howcroft, T. K., Campisi, J., Louis, G. B., Smith, M. T., Wise, B., Wyss-Coray, T., Augustine, A. D., McElhaney, J. E., Kohanski, R., and Sierra, F. (2013) The role of inflammation in age-related disease. Aging 5, 84–93
42. Campisi, J. (2011) Cellular senescence: putting the paradoxes in perspective. Curr. Opin. Genet. Dev. 21, 107–112
43. Chen, J. R., Lazarenko, O. P., Haley, R. L., Blackburn, M. L., Badger, T. M., and Ronis, M. J. (2009) Ethanol impairs estrogen receptor signaling resulting in accelerated activation of senescence pathways, whereas estradiol attenuates the effects of ethanol in osteoblasts. J. Bone Miner. Res. 24, 221–230