

## ARTICLE

## Nrf2 is required for normal postnatal bone acquisition in mice

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A large body of literature suggests that bone metabolism is susceptible to the ill effects of reactive species that accumulate in the body and cause cellular dysfunction. One of the body's front lines in defense against such damage is the transcription factor, Nrf2. This transcription factor regulates a plethora of antioxidant and cellular defense pathways to protect cells from such damage. Despite the breadth of knowledge of both the function of Nrf2 and the effects of reactive species in bone metabolism, the direct role of Nrf2 in skeletal biology has yet to be thoroughly examined. Thus, in the current study, we have examined the role of Nrf2 in postnatal bone metabolism in mice. Mice lacking Nrf2 (Nrf2<sup>-/-</sup>) exhibited a marked deficit in postnatal bone acquisition, which was most severe at 3 weeks of age when osteoblast numbers were 12-fold less than observed in control animals. While primary osteoblasts from Nrf2<sup>-/-</sup> mice functioned normally *in vitro*, the colony forming capacity of bone marrow stromal cells (BMSCs) from these mice was significantly reduced compared to controls. This defect could be rescued through treatment with the radical scavenger *N*-acetyl cysteine (NAC), suggesting that increased reactive species stress might impair early osteoblastogenesis in BMSCs and lead to the failure of bone acquisition observed in Nrf2<sup>-/-</sup> animals. Taken together, these studies suggest Nrf2 represents a key pathway in regulating bone metabolism, which may provide future therapeutic targets to treat osteoporosis.

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## INTRODUCTION

Postnatal bone growth in mammals involves the concerted action of three principal cell types: osteoblasts, osteoclasts and osteocytes.<sup>1</sup> Osteoblasts, derived of a mesenchymal origin, produce the organic bone matrix that is composed largely of type I collagen and facilitate its mineralization.<sup>2</sup> During this process, a subset of osteoblasts become entombed in the newly produced matrix and further differentiates into osteocytes. These osteocytes form a vast interconnected network of cells within the bone that continue to promote mineralization of the newly deposited matrix, as well as perform additional functions of mechanosensing, regulating local bone turnover and participating in endocrine signaling.<sup>3–4</sup> Osteoclasts, derived of hematopoietic origin, are responsible for the resorption of mineralized bone matrix to facilitate bone remodeling and maintain serum calcium levels.<sup>5</sup> A considerable body of literature exists that implicates the accumulation of reactive oxygen species (ROS) and other free radicals as a major contributor to the functional decline observed in numerous tissues and

cellular processes<sup>6–7</sup> and it is highly unlikely bone would be an exception. Indeed, recent studies in both animals and humans<sup>8</sup> suggest roles—both physiological and pathological—for ROS in the regulation of bone mass.

ROS are formed predominantly in mitochondria as electrons escape the electron transport chain during the aerobic metabolism of glucose and fats to generate ATP.<sup>9</sup> Until recently, these ROS were believed to be an unavoidable, deleterious by-product of cellular metabolism in an oxygen-rich environment. However, extensive data are emerging that indicates that ROS are purposely produced by cells to trigger physiological signaling cascades (ERK, p38, JNK, PI3K/Akt, AP-1, p53, Wnt/β-catenin), as well as activate or modify the activity of tyrosine phosphatases, proteases, chaperones and transcription factors (NF-κB, glucocorticoid receptor, estrogen receptor, HIF-1α, etc.).<sup>10</sup> In this regard, ROS and other free radicals seem to behave with a split personality in the cell, reminiscent of Dr Jekyll and Mr Hyde. At low levels, ROS contribute positively to cellular processes, often acting as mitogens and stimulating differentiation. At high levels, we observe

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the cellular damage (protein adducts, lipid oxidation, DNA damage, *etc.*) for which ROS and other radical species first gained notoriety. Thus, cells contain elaborate antioxidant and detoxifying enzyme systems that are responsible for precisely regulating the levels of free radical species (glutathione peroxidase, GSH, glutathione S-transferase, superoxide dismutase, NADPH quinone oxidoreductase-1, heme oxygenase-1, ferritin H, *etc.*).<sup>11</sup> While some of these antioxidant and detoxification pathways function basally in cells, many more are upregulated in response to endogenous or exogenous stressors. One such 'master regulatory' antioxidant response pathway is controlled by the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2).<sup>12</sup>

Nrf2 is a prolific transcription factor, activated in response to oxidative stress caused by wide variety of environmental or endogenous stressors. In an unstressed condition, Nrf2 is sequestered in the cytoplasm by Kelch ECH associating protein 1 (Keap1) and targeted for proteosomal degradation via cullin 3 ubiquitin ligase. Keap1 serves as the primary sensor of various free radical stresses, and upon experiencing a stress signal, Keap1 undergoes a conformational change resulting in loss of Keap1-cullin 3 ligase activity and stabilization of Nrf2. Free to translocate to the nucleus, Nrf2 regulates the transcription of broad range of genes (antioxidant enzymes, glutathione homeostasis, proteasome pathway, detoxification enzymes, *etc.*) whose promoters contain an antioxidant response element.<sup>12</sup> Our studies and others have shown that in mice, disruption of Nrf2 significantly impairs the induction of these downstream cellular defense pathways and exaggerates the effects of oxidative stress, nitrosative stress, inflammation and endoplasmic reticulum stress after exposure to environmental agents, such as cigarette smoke,<sup>13–17</sup> bacteria<sup>18–20</sup> or viruses.<sup>21–22</sup> Conversely, enhancing the Nrf2 signaling pathway by genetic disruption of its inhibitor partner, Keap1, increases antioxidant defenses and diminishes tissue injury caused by smoking.<sup>19,23</sup> This pathway is highly conserved,<sup>24</sup> and we have recently demonstrated that a defect in Nrf2 signaling pathways occurs in COPD patients that renders them susceptible to pathological drivers of COPD such as oxidative stress and inflammation.<sup>14–15,25</sup> Additionally, human DNA polymorphisms leading to reduced Nrf2 abundance<sup>26–27</sup> have been associated with skin vitiligo,<sup>28</sup> chronic gastritis,<sup>29</sup> peptic ulcer,<sup>30</sup> ulcerative colitis<sup>31</sup> and adult respiratory distress syndrome.<sup>27</sup>

Despite ample evidence implicating oxidative stress in age-related bone loss in humans,<sup>32–39</sup> and the prominent role of Nrf2 in responding to oxidative stress in other tissues, the involvement of Nrf2 in skeletal maintenance has not been thoroughly examined. In this study, we have examined the effect of loss of Nrf2 throughout the postnatal

skeletal development of mice. We have found that Nrf2 is critical for normal postnatal bone development, as mice lacking Nrf2 demonstrated significantly impaired bone acquisition and reduced bone volume. These changes appear to be primarily due to defects in osteoblastogenesis that result from increased oxidative stress in the absence of Nrf2.

## MATERIALS AND METHODS

### Materials

Cell culture media,  $\alpha$ -minimal essential medium ( $\alpha$ -MEM), was obtained from Cellgro-Mediatech (Herndon, VA, USA) and fetal bovine serum (FBS) was from Gibco (Gaithersburg, MD, USA). Recombinant human RANKL and M-CSF were purchased from PeproTech (Rocky Hill, NJ, USA). ELISA Kits were purchased from R&D Systems (Minneapolis, MN, USA). Assay kits for flow cytometric analysis of cell proliferation were purchased from BD Pharmingen (San Jose, CA, USA). All other reagents not specified here were purchased from Sigma (St. Louis, MO, USA).

### Animal studies

Female wild-type (Nrf2<sup>+/+</sup>) and Nrf2-deficient (Nrf2<sup>-/-</sup>) on a C57Bl6J background<sup>40</sup> were used for all experiments. Female Nrf2<sup>+/+</sup> (control mice) or Nrf2<sup>-/-</sup> mice were killed at 3, 6, 8, 12 and 24 weeks of age and bone volume was assessed using a desktop micro-tomographic imaging system (Skyscan 1172; Skyscan, Kontich, Belgium) at the Center for Musculoskeletal Research at Johns Hopkins University. Histological analyses, using a semi-automatic method (Osteoplan II, Kontron) were carried out in the Department of Nephrology, Bone and Mineral Metabolism at the University of Kentucky on 8 week old mice that had received intraperitoneal injections of 1% calcein (w/v) (to assess dynamic histomorphometric parameters) 5 and 3 days prior to killing. All analyses of bone structure were completed in accordance with the recommendations of the American Society for Bone and Mineral Research.<sup>41–42</sup> Serum C-terminal telopeptide (CTX) (Immunodiagnostik, Bensheim, Germany) and Serum OPG & RANKL (R&D Systems) levels were determined by ELISA.

### Osteoblast isolation and culture

Osteoblasts were isolated from calvaria of newborn control and Nrf2<sup>-/-</sup> mice by serial digestion in 1.8 mg·mL<sup>-1</sup> collagenase type I (Worthington, Lakewood, NJ, USA) solution. Calvaria were digested in 10 mL of digestion solution for 15 min at 37 °C with constant agitation. The digestion solution was collected, and digestion was repeated with fresh digestion solution an additional four times. Digestions 3–5 (containing the osteoblasts) were pooled together, centrifuged, washed with  $\alpha$ -MEM containing 10% FBS, 1% pen/strep and plated overnight at 37 °C in a humidified

incubator supplied with 5% CO<sub>2</sub>. For differentiation assays, osteoblasts were grown to confluence and then switched to differentiation media supplemented with  $\beta$ -glycerophosphate and ascorbic acid for 14 days prior to alkaline phosphatase (ALP) staining or 21 days prior to alizarin red S (ARS) staining by standard methods. ALP and ARS staining was quantified by image density analysis of plate images using ImageJ software (NIH, Bethesda, MD, USA).

#### Osteoblast proliferation assays

Osteoblasts were plated in six-well plates at low cell density ( $9 \times 10^4$  cells per well) and cultured in  $\alpha$ -MEM containing 0.5% FBS for 24 h to arrest the cells in G0 phase. For proliferation analysis of the cells,  $10 \mu\text{mol}\cdot\text{L}^{-1}$  BrdU was added to the medium at the time of mitogenic stimulation. The cells were then fixed and stained with anti-BrdU-APC and 7-AAD and analyzed by FACS Calibur (Becton-Dickson, Franklin Lakes, NJ, USA). 20 000 events were collected for each sample and results were analyzed with WinMDI version 2.8.

#### Colony-forming unit (CFU) assays

CFU-osteoblast (CFU-OB) assay using stromal flushes were performed on femurs and tibiae of 8-week-old female control and Nrf2<sup>-/-</sup> mice, and these cells were plated at low density in triplicate. Non-adherent cells were removed after four days, and the adherent stromal population was allowed to grow in osteogenic differentiation media for 18 days. The number of osteoblast colonies was assessed by both ALP and ARS staining and followed by methylene blue staining to assess total number of colonies.

#### Osteoclastogenesis assays

Whole bone marrow cells flushed from femurs and tibiae of 8-week-old control and Nrf2<sup>-/-</sup> mice were cultured in  $\alpha$ -MEM containing 10% FBS for 1 day. Suspended cells were harvested and further cultured with  $30 \text{ ng}\cdot\text{mL}^{-1}$  M-CSF in  $\alpha$ -MEM containing 10% FBS. After 3 days, adherent cells (bone marrow-derived macrophages) were used for osteoclastogenesis assays. Bone marrow-derived macrophages were cultured in medium containing  $30 \text{ ng}\cdot\text{mL}^{-1}$  M-CSF and  $200 \text{ ng}\cdot\text{mL}^{-1}$  RANKL and were seeded on 96-well plates ( $1 \times 10^4$  cells per well) for 7–8 days, with culture media changed every other day. Tartrate-resistant acid phosphatase-positive multinucleated cells with more than three nuclei were counted as osteoclast cells.

#### Gene expression studies

Total RNA was extracted using TRIzol (Life Technologies, Grand Island, NY, USA), reverse transcribed using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) and amplified by real-time PCR using primers specific for heme oxygenase 1 and NADPH quinone oxidoreductase-1 primers. Reactions were normalized to actin levels.

#### Statistical analysis

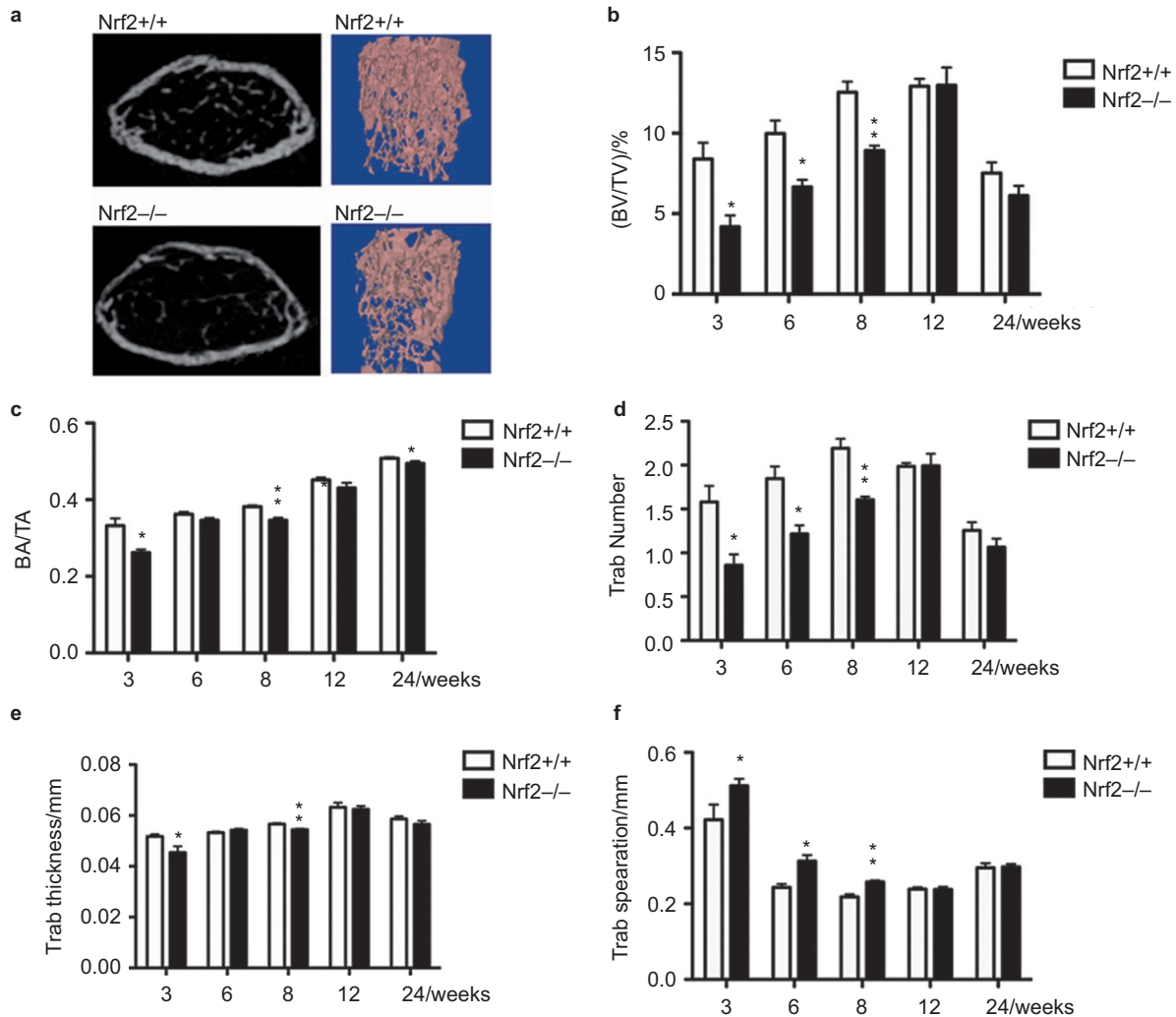
All values are expressed as a mean  $\pm$  standard error of the mean. All statistical analyses were performed using the Microsoft Excel data analysis program for ANOVA or Student's *t*-test analysis with an assigned significance level of 0.05 ( $\alpha$ ).

## RESULTS

Nrf2 is required for normal postnatal bone acquisition

Nrf2<sup>-/-</sup> mice breed normally and have previously been reported to demonstrate normal growth patterns as compared to age-matched Nrf2<sup>+/+</sup> mice. However, after 1 year of age, Nrf2<sup>-/-</sup> mice begin to acquire autoimmune diseases not seen in their wild-type counterparts.<sup>43</sup> In the course of investigating these autoimmune changes, we noted a difference in the bone strength of Nrf2<sup>-/-</sup> mice while harvesting long bones to perform marrow flushes. The long bones of Nrf2<sup>-/-</sup> mice seemed to be more fragile than those of their wild-type counterparts. Thus, to investigate the possibility of Nrf2 playing a previously unappreciated role in bone maintenance, we analyzed the long bones of Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> mice at 3, 6, 8, 12 and 24 weeks of age by micro-CT. As expected for C57BL/6 mice, Nrf2<sup>+/+</sup> mice attained peak bone mass by 8 weeks of age. By contrast, Nrf2<sup>-/-</sup> mice demonstrated severely reduced bone mass at 3 weeks of age, which persisted through 12 weeks of age (Figure 1). Micro-CT analysis of the distal femur revealed approximately 45%, 30% and 20% reductions in bone volume fraction (BV/TV) in Nrf2<sup>-/-</sup> mice compared to controls at 3, 6 and 8 weeks of age, respectively (Figure 1a and 1b). Trabecular number and thickness were decreased in Nrf2<sup>-/-</sup> mice compared to controls, while trabecular separation was increased (Figure 1d–1f). However, by 12 weeks, all parameters were similar between Nrf2<sup>+/+</sup> and Nrf2<sup>-/-</sup> mice (Figure 1). Taken together, the result suggests that loss of Nrf2 impairs early postnatal bone acquisition.

To better understand the cellular mechanisms responsible for the poor bone acquisition in Nrf2<sup>-/-</sup> mice, we performed histomorphometric analysis on trabecular bone of the distal femur of control and Nrf2<sup>-/-</sup> mice at 3, 6, 8, 12 and 24 weeks of age (Figure 2 and Table 1). At 3 weeks of age, osteoblast numbers were 12-fold lower in Nrf2<sup>-/-</sup> mice as compared to control mice (Figure 2a). However, the osteoblast number per bone length rapidly compensated and was similar between the genotypes by 6 weeks of age, remaining so throughout all remaining time points. There were no significant differences in osteoclast number per bone length or resorption-related parameters between control and Nrf2<sup>-/-</sup> mice at any of the indicated ages (Figure 2b), suggesting that an osteoblast defect was primarily responsible for the reduced bone phenotype observed *in vivo*. While there were some transient



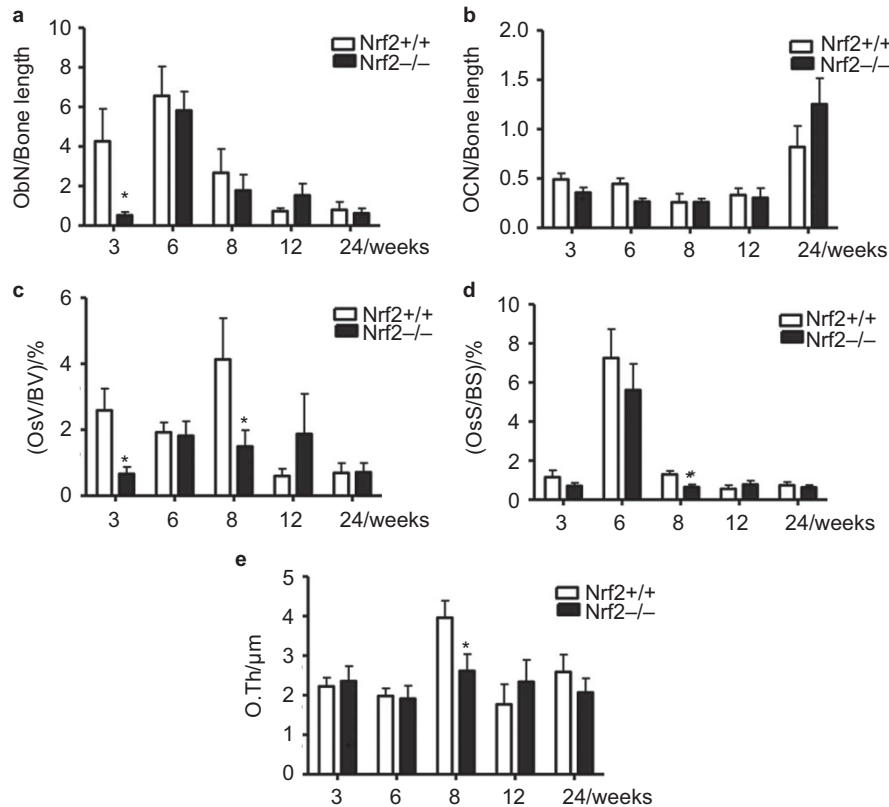
**Figure 1.** Nrf2 is required for normal postnatal bone acquisition. (a) Representative micro-CT images of trabecular bone structure in the distal femur of control and Nrf2<sup>-/-</sup> mice at 8 weeks of age. (b–f) MicroCT analysis of trabecular bone structure at the distal femur in control and Nrf2<sup>-/-</sup> mice at 3, 6, 8, 12 and 24 weeks of age (*n*=4–6 mice/group/age). Bone volume/tissue volume, BV/TV (%); bone area/tissue area, BA/TA (%); trabecular number, Tb. N (no./mm); trabecular thickness, Tb. Th (mm); Trabecular separation, Tb. Sp (mm). Values shown are mean ± s.e.m.

reductions in formation-related parameters at 8 weeks of age in the Nrf2<sup>-/-</sup> mice compared to controls—osteoid surface per bone surface (OsS/BS, %), osteoid volume per bone volume (OsV/BV, %) and osteoid thickness (O.Th,  $\mu$ m) were significantly less (Figure 2c–2e)—these changes do not appear to be significant enough, nor temporally positioned to have been the primary cause of reduced bone volume in Nrf2<sup>-/-</sup> mice. Thus, it appears more likely that the early failure in bone acquisition in Nrf2<sup>-/-</sup> mice would result from the early and severe reduction in osteoblast numbers as compared to control animals.

Nrf2 does not significantly affect primary osteoblast proliferation or differentiation *in vitro*

To further probe the cellular mechanisms responsible for the reduced bone volume observed in Nrf2<sup>-/-</sup> mice, we

next investigated whether the reduction in osteoblast numbers observed in Nrf2<sup>-/-</sup> mice was the result of defective osteoblast proliferation. Primary calvarial osteoblasts were isolated from control and Nrf2<sup>-/-</sup> newborn mice and their proliferation analyzed by BrdU incorporation and flow cytometric analysis. Surprisingly, we observed no difference in osteoblast proliferation following serum starvation and mitogenic stimulation with 10% FBS (Figure 3a). We also examined primary osteoblast differentiation in control and Nrf2<sup>-/-</sup> primary calvarial osteoblasts, given the transient decrease in formation-related histomorphometric parameters observed in 8-week-old Nrf2<sup>-/-</sup> mice *in vivo*. Again, in contrast to our expectation, there were no significant differences in ALP or ARS staining following 14 or 21 days of culture in osteogenic media, respectively (Figure 3b and 3c).



**Figure 2.** Osteoblast numbers are significantly reduced in the early life of *Nrf2*<sup>-/-</sup> mice. Quantitative histomorphometry of trabecular bone at the distal femur of control and *Nrf2*<sup>-/-</sup> mice at 3, 6, 8, 12 and 24 weeks of age (*n*=4–6 mice/group/age). (a) Osteoblast number/bone length—number per 100  $\mu$ m; (b) osteoclast number/bone length—number per 100  $\mu$ m; (c) osteoid volume/bone volume; (d) osteoid surface/bone surface; (e) osteoid thickness. Values shown are mean $\pm$ s.e.m.

Deletion of *Nrf2* impairs colony-forming capacity of bone marrow stromal cells

Given that we did not observe any functional deficit in osteoblasts *in vitro*, we next sought to determine whether the reduction in osteoblast numbers observed *in vivo* might

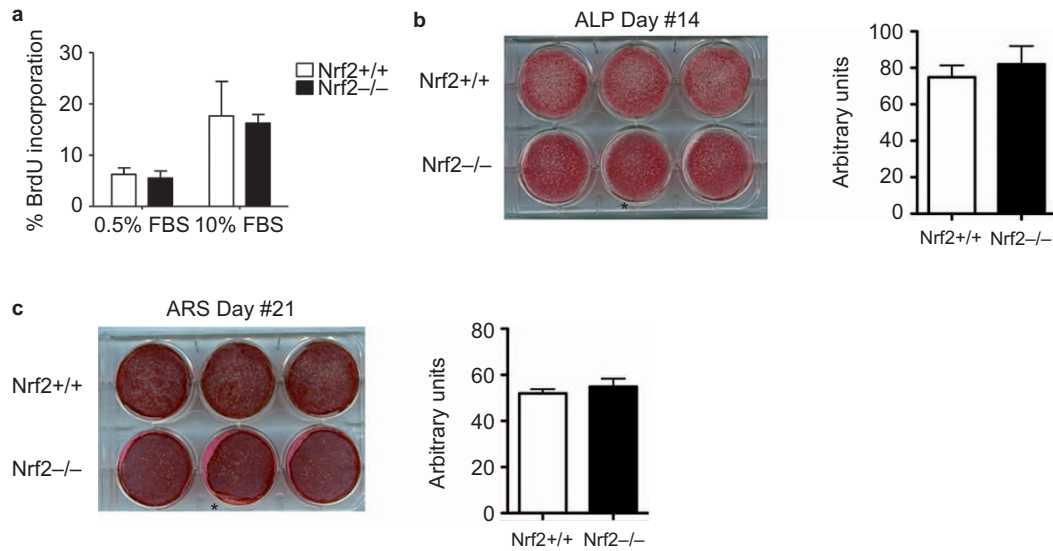
have been due to impairment of bone marrow stromal cell (BMSC) function. We have previously reported that deletion of *Nrf2* decreases antioxidant defenses and survival signals, which attenuate the proliferative capacity of hematopoietic stem progenitor cells *in vitro* and *in vivo*.<sup>44</sup> In accord

**Table 1.** Complete quantitative histomorphometry in trabecular bone in the distal femur at 8 weeks of age<sup>a</sup>

Quantitative histomorphometry parameters	<i>Nrf2</i> <sup>+/+</sup>	<i>Nrf2</i> <sup>-/-</sup>
Dynamic parameters		
Mineralizing surface/bone surface (MS/BS; %)	6.36 $\pm$ 0.69	5.96 $\pm$ 2.28
Mineral apposition rate (MAR; $\mu$ m $\cdot$ d <sup>-1</sup> )	1.81 $\pm$ 0.28	1.07 $\pm$ 0.22*
Bone formation rate/bone surface (BFR/BS; mm <sup>3</sup> $\cdot$ cm <sup>-2</sup> per year)	26.55 $\pm$ 13.4	15.03 $\pm$ 11.5
Mineralization lag time (Mlt; day)	2.25 $\pm$ 1.06	1.97 $\pm$ 0.6
Formation parameters		
Osteoid volume/bone volume (OV/BV; %)	2.96 $\pm$ 0.69	1.16 $\pm$ 0.32*
Osteoid surface/bone surface (OS/BS; %)	9.00 $\pm$ 1.91	4.60 $\pm$ 1.07*
Osteoid thickness (O.Th; $\mu$ m)	3.87 $\pm$ 0.22	2.69 $\pm$ 0.34*
Osteoblast surface/bone surface (Ob.S/BS; %)	2.52 $\pm$ 0.84	2.07 $\pm$ 0.58
Osteoblast number/bone length (Ob.N/BLength; no. per 100 $\mu$ m)	1.96 $\pm$ 0.61	1.72 $\pm$ 0.47
Osteoblast number/tissue area (Ob.N/TA; no. per mm <sup>2</sup> )	9.05 $\pm$ 2.91	7.47 $\pm$ 2.19
Resorption parameters		
Erosion surface/bone surface (ES/BS; %)	2.00 $\pm$ 0.46	2.47 $\pm$ 0.79
Osteoclast surface/bone surface (OC.S/BS; %)	2.00 $\pm$ 0.39	2.17 $\pm$ 0.66
Osteoclast number/bone length (Oc.N/BLength; no. per 100 $\mu$ m)	0.63 $\pm$ 0.11	0.78 $\pm$ 0.23
Osteoclast number/tissue area (Oc.N/TA; no. per mm <sup>2</sup> )	3.03 $\pm$ 0.62	3.47 $\pm$ 1.19

<sup>a</sup> Values are shown as mean $\pm$ s.e.

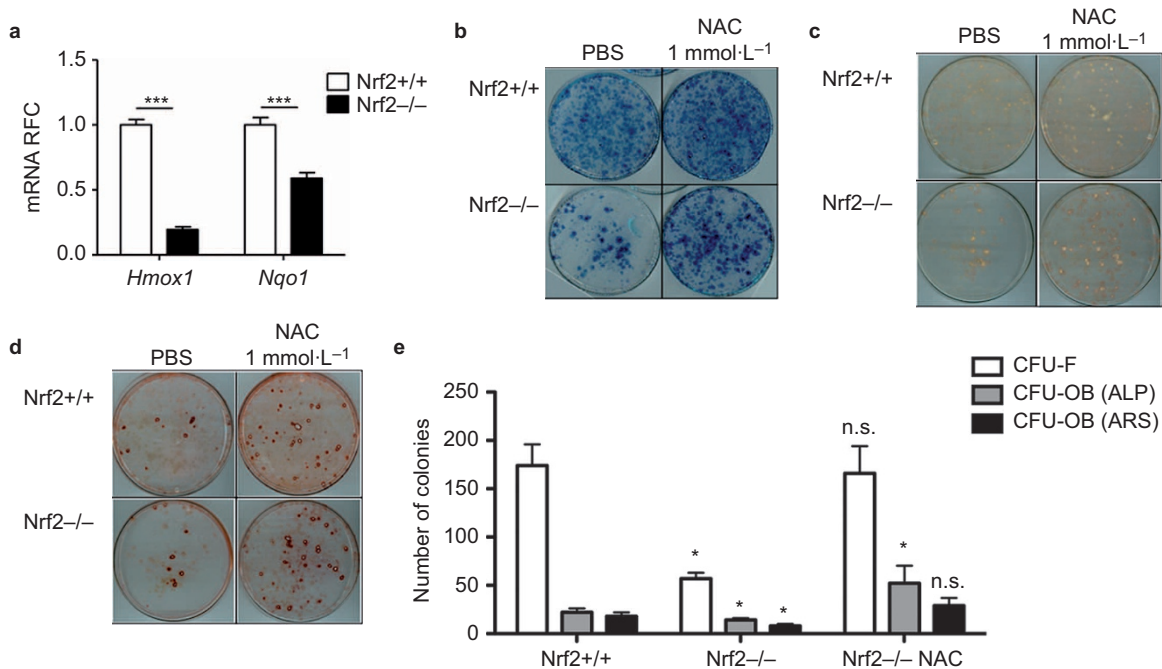
\* Statistically significant difference from control, *P*<0.05.



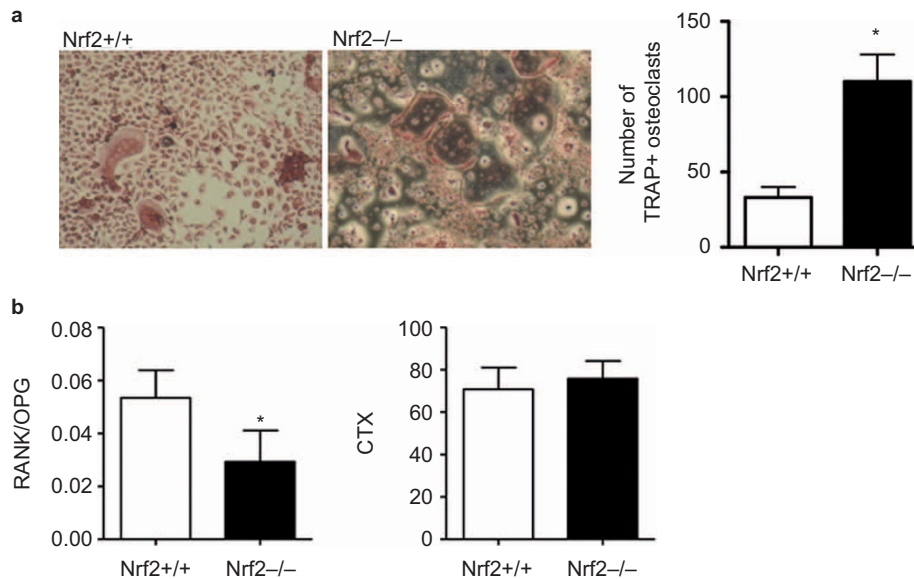
**Figure 3.** Nrf2 does not affect primary osteoblast proliferation or differentiation. (a) Quantification of primary calvarial osteoblast proliferation by BrdU incorporation and flow cytometric analysis in control and Nrf2<sup>-/-</sup> osteoblasts. (b) ALP staining after 14 days and (c) ARS staining after 21 days of primary calvarial osteoblast differentiation under osteogenic conditions. Values shown are mean  $\pm$  s.e.m.

with this notion, BMSCs derived from Nrf2<sup>-/-</sup> mice demonstrated significantly reduced expression of Nrf2-regulated antioxidants, heme oxygenase-1 and NADPH quinone oxidoreductase-1 as compared to BMSCs from control mice (Figure 4a). Moreover, we also observed a reduction in total colony forming units (Figure 4b and 4e), as well as alkaline

phosphatase and alizarin red positive colony forming units (Figure 4c–4e). To determine if the decreased colony forming capacity of Nrf2<sup>-/-</sup> BMSCs was due to increased levels of oxidative stress resulting from poor antioxidant defense, we also cultured the BMSCs in presence or absence of N-acetyl cysteine (NAC), a free-radical scavenger. NAC



**Figure 4.** The colony forming capacity of BMSCs is impaired in Nrf2<sup>-/-</sup> mice. (a) Real-time qPCR analysis of Hmox and Nqo1 in bone marrow stromal cells harvested from 8-week-old control and Nrf2 KO mice. (b–d) CFUs assessed in control and Nrf2<sup>-/-</sup> BMSCs after PBS or 1 mmol·L<sup>-1</sup> NAC treatment and growth under osteogenic conditions for 18 days. Images represent at least 3 experiments. (b) Methylene blue staining – CFU-F; (c) ALP staining – CFU-OB (ALP); (d) ARS staining – CFU-OB (ARS). (e) Quantitative representation of experiments depicted in b–d. Values shown are mean  $\pm$  s.e.m. Hmox, heme oxygenase.



**Figure 5.** Osteoclastogenesis is enhanced in the absence of Nrf2 *in vitro*, but not *in vivo*. (a) Bone marrow macrophages from control and Nrf2<sup>-/-</sup> mice were stimulated with MCS-F and RANKL to induce osteoclastogenesis for 7 days prior to TRAP staining to identify osteoclasts. (b) Serum was harvested from 8-week-old control and Nrf2<sup>-/-</sup> mice and RANKL, OPG and CTX levels were assessed by ELISA. TRAP, tartrate-resistant acid phosphatase.

supplementation restored the number of colonies formed in Nrf2<sup>-/-</sup> BMSCs to levels equal to, or greater in the case of CFU-OB (ALP), than that of control BMSCs (Figure 4b–4e). Taken together, these data suggest that poor postnatal bone acquisition in Nrf2<sup>-/-</sup> mice might result from a failure in the proliferative/survival capacity of the osteoprogenitor population, which results in the low osteoblast numbers observed early in the life of Nrf2<sup>-/-</sup> mice.

Osteoclastogenesis is dramatically enhanced in the absence of Nrf2 *in vitro*, but not *in vivo*

Our data, so far, have indicated that the reduced postnatal bone acquisition observed in Nrf2<sup>-/-</sup> mice is primarily the result of dramatically reduced osteoblast numbers in early life, which likely results from impaired BMSC proliferation or survival and a subsequent lack of osteoprogenitors. However, our previous studies indicated that loss of Nrf2 has deleterious effects on hematopoietic populations as well, from which osteoclasts are derived. Thus, our final question was whether loss of Nrf2 had any impact on osteoclastogenesis. Quite to our surprise since Nrf2<sup>-/-</sup> mice displayed no abnormality in osteoclast-related parameters by histomorphometry, we noticed a dramatic enhancement of RANKL induced osteoclastogenesis of Nrf2<sup>-/-</sup> bone marrow macrophages as indicated by tartrate-resistant acid phosphatase-positive multinucleated cells, compared to controls (Figure 5). Using serum samples from 8-week-old control and Nrf2<sup>-/-</sup> mice, we next examined both the RANKL/OPG ratio and serum CTX by ELISA. Interestingly, the RANKL/OPG ratio was

significantly lower in the serum of Nrf2<sup>-/-</sup> mice. Further, there was no difference in CTX levels, a marker of osteoclast-mediated resorption, between control and Nrf2<sup>-/-</sup> mice. Thus, while Nrf2 appears to be inhibitory for osteoclastogenesis *in vitro*, there appear to be compensatory mechanisms *in vivo* that nullify these effects.

## DISCUSSION

The deleterious effects of reactive species in bone have been extensively studied. Likewise, the function of the master regulatory transcription factor, Nrf2, has been thoroughly detailed in response to various cellular stresses. However, the role of Nrf2, itself, in bone metabolism has not been adequately examined to date. In the present studies, we examined the effect of loss of Nrf2 on bone metabolism throughout postnatal life in mice. Interestingly, loss of Nrf2 resulted in a failure of normal bone acquisition in mice, with the deficit appearing most severe at a young age and correcting by adulthood. This is in contrast to the traditional view of how reactive species impact bone metabolism—slowly accumulating throughout life and ultimately leading to cellular dysfunction<sup>6</sup>—and may ultimately highlight physiological roles of reactive species that are critical in early life, particularly in progenitor populations.

Histomorphometric analysis suggested that the poor acquisition of bone mass in Nrf2<sup>-/-</sup> mice was the result of dramatically reduced osteoblast numbers early in life (3 weeks of age). While osteoblast numbers recovered during pubertal growth and remained comparable to

control animals after this point, the bone volume of Nrf2<sup>-/-</sup> mice remained less than controls until adulthood (12 weeks), after bone volume in control animals had plateaued. Our *in vitro* studies would suggest that this is not, in fact, due to any defect in osteoblasts themselves. Primary calvarial osteoblasts from mice lacking Nrf2 proliferated and differentiated normally in our studies, if even in a slightly enhanced fashion, when compared to control osteoblasts. Recent reports suggest that Nrf2 may, in fact, be inhibitory to osteoblastogenesis. Hinoi and colleagues<sup>45</sup> suggested that Nrf2 inhibited osteoblastogenesis in MC3T3-E1 cells by inhibiting the activity of Runx2. While we did not observe such dramatic effects in our primary osteoblast cultures, we did note a dramatic effect of Nrf2 at earlier stages of cell differentiation, namely, in bone marrow stromal cells. Similar to our previous findings in hematopoietic stem cells,<sup>44</sup> loss of Nrf2 had a dramatic impact on colony forming capacity of BMSCs, significantly inhibiting both total colony formation (CFU-F), as well as osteoblast colonies (CFU-OB). This inhibition of colony forming capacity in BMSCs appeared to be due to increased reactive species present, as treatment with the reactive species scavenger, NAC, was able to rescue the defect in BMSCs and restore colony counts of Nrf2<sup>-/-</sup> BMSCs to levels comparable to control BMSCs. We believe this to be the cause of the early defect in osteoblast number observed in Nrf2<sup>-/-</sup> mice, as we were unable to detect any primary defect in osteoblast function. It is likely that, in the whole animal, additional mechanisms to activate antioxidant pathways independent of Nrf2 signaling, such as FoxO,<sup>46</sup> could compensate for the loss of Nrf2 and are responsible for the recovery of osteoblast numbers observed during pubertal growth. This hypothesis will be tested in future studies, in which we will attempt to rescue this early phenotype through administration of NAC to young animals.

Interestingly, despite any evidence for osteoclastic involvement in the phenotype observed *in vivo*, this population of cells is strikingly affected by loss of Nrf2 *in vitro*. Similar results have also been observed in a recent report by Park *et al.*,<sup>47</sup> in which they also observed no osteoclastic phenotype in Nrf2<sup>-/-</sup> mice, but dramatic increases in osteoclastogenesis *in vitro*, owing to Nrf2 inhibiting activation of NF- $\kappa$ B and induction of c-fos and NFATc1.<sup>47</sup> Also similar to the findings of Park and colleagues, we observed a reduction in the RANKL/OPG ratio in Nrf2<sup>-/-</sup> mice, which may explain the discrepancy between this osteoclastic phenotype *in vitro* and the lack of any osteoclast disturbance *in vivo*.

Finally, despite many similarities in our findings and those of the recent report by Park and colleagues, they reached the conclusion that Nrf2 is solely an inhibitor of bone acquisition and showed increased bone volume in

Nrf2<sup>-/-</sup> mice by micro-CT analysis at 9 weeks of age. This is, obviously, in stark contrast to our present findings, although we are inclined to believe that both pieces of work are accurate. First, we used female animals for the experiments described, whereas the sex of animals used in their studies was not stated. It is possible that there may be sexual dimorphism in the phenotype. Moreover, Park and colleagues examined only a single time point—9 weeks of age. In our examination of bone volume in the Nrf2<sup>-/-</sup> mice, we observed an initial failure in bone acquisition at 3 weeks, which then caught up to control animals by 12 weeks of age. We observed 45%, 30% and 20% reductions in bone volume fraction (BV/TV) in Nrf2<sup>-/-</sup> mice compared to controls at 3, 6 and 8 weeks of age, respectively (Figure 1a and 1b), so it is entirely possible that in the time between 8 and 12 weeks of age, the Nrf2<sup>-/-</sup> mice may overshoot control animals in attaining their own peak bone mass. Consistent with our current findings in global Nrf2<sup>-/-</sup> mice, previous studies have demonstrated that osteoblast-targeted deletion of family member Nrf1 resulted in reduced trabecular bone volume.<sup>48</sup> Differences in the diet or genetic background (i.e., variable compensatory responses from Nrf family members Nrf1 and Nrf3) of animals could also contribute to the contrasting *in vivo* results with the Park *et al.*'s study. Regardless, both studies indicate Nrf2 is a key player in skeletal development, which is worthy of future study and may ultimately provide additional therapeutic targets for age-related bone loss.

### Competing interests

The authors declare no conflict of interest.

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