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## FoxO-mediated defense against oxidative stress in osteoblasts is indispensable for skeletal homeostasis in mice

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### Summary

Aging increases oxidative stress and osteoblast apoptosis and decreases bone mass, whereas forkhead box O (FoxO) transcription factors defend against oxidative stress by activating genes involved in free radical scavenging and apoptosis. Conditional deletion of FoxO1, 3 and 4 in three month-old mice resulted in an increase in oxidative stress in bone and osteoblast apoptosis and a decrease in the number of osteoblasts, the rate of bone formation, and bone mass at cancellous and cortical sites. The effect of the deletion on osteoblast apoptosis was cell autonomous and resulted from oxidative stress. Conversely, overexpression of a FoxO3 transgene in mature osteoblasts decreased oxidative stress and osteoblast apoptosis, and increased osteoblast number, bone formation rate and vertebral bone mass. We conclude that FoxO-dependent oxidative defense provides a mechanism to handle the oxygen free radicals constantly generated by the aerobic metabolism of osteoblasts and is thereby indispensable for bone mass homeostasis.

### Introduction

Reactive oxygen species (ROS) are continuously generated as normal by-products of aerobic metabolism and can cause lipid peroxidation, protein damage, and DNA lesions leading to cell death, but also serve as signaling molecules at lower concentrations to control cell proliferation and differentiation (Balaban et al., 2005; Giorgio et al., 2007). Age- or gonadectomy-related bone loss in mice is associated with increased oxidative stress (OS) (Lean et al., 2003; Almeida et al., 2007b; Almeida et al., 2009a; Almeida et al., 2009b; Manolagas, 2009). Likewise, murine models of premature aging and signs of oxidative damage exhibit osteoporotic features (Tyner et al., 2002; De Boer et al., 2002). Moreover, antioxidants prevent the increased osteoclastogenesis and increased osteoblast and osteocyte apoptosis as well as the loss of bone caused by gonadectomy, supporting the view that OS plays an important role in skeletal homeostasis (Lean et al., 2003; Almeida et al., 2007b).

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To counteract the adverse consequences of OS, cells possess several oxidative defense mechanisms including those governed by the FoxO family of transcription factors. In mammals, this family comprises four members: FoxO1/FKHR, FoxO3/FKHRL1, FoxO4/AFX and FoxO6 (Greer and Brunet, 2005). FoxO1, 3, and 4 show broad, overlapping patterns of expression in developing and adult tissues, whereas FoxO6 is restricted to specific structures of the developing brain. In the setting of growth factor stimulation, FoxO1, 3 and 4 are subject to Akt-mediated phosphorylation which results in their nuclear export and inhibition of FoxO-mediated transcription (Brunet et al., 1999; Kops et al., 1999; Biggs, III et al., 1999; Tang et al., 1999; Takaishi et al., 1999). OS, on the other hand, causes the retention of FoxOs in the nucleus and activation of transcription, by promoting FoxO post-translational modifications, including phosphorylation (on amino-acid residues distinct from those phosphorylated by Akt), ubiquitylation and acetylation (Calnan and Brunet, 2008; van der Horst and Burgering, 2007). Stress stimuli override the sequestration of FoxOs in the cytoplasm by growth factors, both in mammalian cells and in *Drosophila* (Brunet et al., 2004; Wang et al., 2005) suggesting that protection of cells from stress is one of the crucial roles of these evolutionarily conserved proteins. FoxOs mediate OS responses by regulating the transcription of antioxidant enzymes such as MnSOD and catalase as well as genes involved in cell cycle, DNA repair, and lifespan (Salih and Brunet, 2008; van der Horst and Burgering, 2007). The importance of the antioxidant properties of FoxOs in mammalian biology has been recently highlighted by the profound effects of the deletion of FoxO3 alone or the combined deletion of FoxO1, 3, and, 4 in mice. Indeed, these studies have revealed that FoxOs are indispensable for normal erythropoiesis and hematopoietic stem cell quiescence and survival, because of their ability to prevent excessive levels of ROS (Marinkovic et al., 2007; Tothova et al., 2007).

Beta-catenin – a factor required for osteoblast differentiation (Glass et al., 2005) – is essential for ROS-induced FoxO activation (Essers et al., 2005). In osteoblastic cell models, OS induces the association of FoxOs with  $\beta$ -catenin and promotes FoxO-mediated transcription at the expense of  $\beta$ -catenin/T-cell specific transcription factor (TCF)-mediated transcription and osteoblast differentiation (Almeida et al., 2007a; Almeida et al., 2009a). The negative effect of ROS on TCF transcription is abrogated by raising the level of  $\beta$ -catenin, suggesting that a limited pool of active  $\beta$ -catenin is diverted from TCF to FoxO transcription under stress conditions (Manolagas and Almeida, 2007; Hooigeboom and Burgering, 2009). FoxOs are also known to suppress the expression and transcriptional activity of PPAR $\gamma$ , which is a potent repressor of osteoblastogenesis (Dowell et al., 2003; Armoni et al., 2006; Akune et al., 2004). Together, these observations prompt speculation that FoxOs play an important role in bone biology by enabling the maintenance of a physiologically appropriate lifespan of mature osteoblasts through their oxidative defense activities. In addition, FoxOs may control the generation of new osteoblasts from their mesenchymal stem cell progenitors by modulating their proliferation and/or differentiation through their antioxidant properties or via modulating the activity of other transcription factors such as  $\beta$ -catenin or PPAR $\gamma$ .

## Results

### FoxO3 is the predominant FoxO in bone and bone cells

Quantitative (q)RT-PCR analysis of whole tissue RNA revealed that all three *FoxO* genes were expressed in calvaria and vertebrae, with *FoxO3* showing the most abundant expression (2-3 fold higher than *FoxO1* and *FoxO4*) (Figure S1A). *FoxO1*, 3 and 4 expression remained constant between 4 and 26 month of age, as determined in two independent experiments (Figure S1B). Consistent with the whole bone findings, *FoxO3* expression was 2 fold higher than FoxO1 and 4 in bone marrow-derived osteoclasts (Figure S1C), or in calvaria- or bone marrow-derived osteoblastic cells (Figure S1D). Thus, all three *FoxOs* are expressed in bone cells and *FoxO3* is the predominant FoxO family member.

## Loss of FoxO signaling causes loss of bone mass

To elucidate the role of FoxOs in bone, we characterized the skeletal phenotype of mice in which the three main FoxO genes *FoxO1*, *3*, and *4* were conditionally deleted. For this purpose, we used 12 wk-old *Mx-Cre<sup>+</sup>;FoxO1,3,4<sup>LL</sup>* mice harboring the interferon-inducible transgene *Mx1-Cre* and conditional alleles for each of the *FoxO* genes and *Mx-Cre<sup>-</sup>;FoxO1,3,4<sup>LL</sup>* littermate controls, hereafter designated *Mx-Cre<sup>+</sup>* and *Mx-Cre<sup>-</sup>*, respectively (Paik et al., 2007). Five wk after injection of polyinosine-polycytidylic acid (pI-pC), used to activate the transgene and consequently the deletion of the three *FoxO* genes, the levels of expression of *FoxO1*, *3*, and *4* were decreased by 60-75% in calvaria and vertebrae of *Mx-Cre<sup>+</sup>* mice (Figure 1A). The efficiency of deletion of *FoxOs* in bone was comparable to that of spleen but less than in liver (Figure 1A). Body weight was not affected by FoxO deletion in *Mx-Cre<sup>+</sup>* females or males (Figure S2A). Five wk after FoxO deletion, however, male and female *Mx-Cre<sup>+</sup>* mice exhibited a significantly lower bone mass, as documented by a decrease in total and femoral bone mineral density (BMD) by dual energy X-ray absorptiometry (DEXA) (Figure 1B and Table S1). Spinal BMD also decreased in male animals. Deletion of FoxOs led to a decrease in femoral cortical width in female and male *Mx-Cre<sup>+</sup>* mice as determined by compartment-specific analysis by micro-CT (Figure 1C). Femoral cancellous bone volume was also decreased but to a lesser extent and consistent with this *Mx-Cre<sup>+</sup>* mice exhibited decreased trabecular number and increased trabecular separation (Figure S2B). Histomorphometric analysis of the vertebrae of male mice confirmed the effect of the deletion on bone mass by revealing a significant reduction in cancellous bone area as well as a decrease in trabecular width and number and increased trabecular separation (Figure 1D). In agreement with the decreased cancellous bone mass, osteoblast number, osteoid perimeter as well as bone formation rate (BFR) were also reduced (Figure 2A). In contrast, the number of osteoclasts in cancellous bone was not affected by deletion of FoxOs (Figure 2A).

## OS and osteoblast apoptosis are increased in FoxO-deficient mice

Because of the well known anti-oxidant role of FoxOs in other tissues (Marinkovic et al., 2007; Tothova et al., 2007), we examined the levels of OS in the bone of the FoxO deleted mice. FoxO deletion resulted in increased phosphorylation of p66<sup>shc</sup> – a robust marker of OS (Migliaccio et al., 1999; Giorgio et al., 2005) – in vertebral lysates from male and female *Mx-Cre<sup>+</sup>* mice (Figure 2B). In agreement with our earlier findings that increased OS in bone promotes osteoblast and osteocyte apoptosis (Almeida et al., 2007b), the prevalence of apoptosis of both these cell types was increased in vertebral cancellous bone from *Mx-Cre<sup>+</sup>* mice as a consequence of the loss of FoxO function (Figure 2C). This result suggests that decreased osteoblast survival is responsible, at least in part, for the decreased osteoblast number, bone formation, and bone mass.

To investigate whether the protective actions of FoxOs against OS and osteoblast apoptosis in bone were cell autonomous, we cultured calvaria- or bone marrow-derived osteoblastic cells from *Mx-Cre<sup>+</sup>* or *Mx-Cre<sup>-</sup>* mice. The efficiency of deletion of FoxOs in calvaria- or bone marrow-derived cells from *Mx-Cre<sup>+</sup>* mice was about 50% and 80%, respectively, as measured by qRT-PCR. The decrease of the three FoxOs was confirmed by Western blotting (Figure 3A and S3A). Similar to the *in vivo* data, apoptosis of either calvaria- or bone marrow-derived osteoblastic cells from *Mx-Cre<sup>+</sup>* mice was increased as compared to cells from control mice (Figure 3B and S3B). The higher prevalence of apoptosis of FoxO-deleted cells was due to increased OS, as the anti-oxidant N-acetyl-cysteine (NAC) abrogated the pro-apoptotic effect of FoxO deletion in calvaria-derived cells (Figure 3B). In agreement with this finding, OS was increased in calvaria-derived osteoblasts from *Mx-Cre<sup>+</sup>* mice as evidenced by a 2-fold higher basal p66<sup>shc</sup> phosphorylation (Figure 3C). As with apoptosis, addition of NAC abrogated the increased p66<sup>shc</sup> phosphorylation, a finding consistent with the view that FoxOs attenuate osteoblastic cell apoptosis through a cell autonomous mechanism that minimizes OS. There

was no difference between the *Mx-Cre*<sup>+</sup> and *Mx-Cre*<sup>-</sup> mice in the rate of the proliferation of cells in these cultures (data not shown).

The number of osteoblasts present in bone is determined by their rate of generation and their lifespan. To examine whether the lower osteoblast number in *Mx-Cre*<sup>+</sup> mice could result in part from a decrease in osteoblastogenesis, we examined the expression of *Runx2* and *Osterix* – two transcription factors required for the commitment and differentiation of mesenchymal progenitors to cells of the osteoblastic lineage (Karsenty, 2008). *Runx2* and *Osterix* mRNA levels were decreased as a result of FoxO deletion in bone marrow-derived stromal cells, as well as the levels of the osteoblast marker *alkaline phosphatase* (Figure 3D). In addition, the same cells displayed increased expression of *PPAR* $\gamma$ 2, a nuclear receptor that downregulates osteoblastogenesis (Akune et al., 2004) (Figure 3D). Consistent with the increased *PPAR* $\gamma$ 2 expression in the *Mx-Cre*<sup>+</sup> mice, the pro-adipogenic ligand rosiglitazone (RGL) caused a greater increase in the number of adipocytes in bone marrow-derived stromal cell cultures from these mice as compared to the controls (Figure 3E).

Deletion of FoxOs alters the number of hematopoietic lineage cells (Tothova et al., 2007). Prompted by this evidence, we quantified the number of osteoclast progenitors present in the bone marrow of *Mx-Cre*<sup>+</sup> mice. As shown in figure 4A, the mRNA and protein levels of the three FoxOs were dramatically diminished in cultured osteoclasts from *Mx-Cre*<sup>+</sup> mice, similar to the case of hematopoietic stem cells (Tothova et al., 2007). Osteoclasts from *Mx-Cre*<sup>+</sup> mice exhibited an increase in the rate of apoptosis (Figure 4B). This was confirmed by the lower expression of the osteoclast-specific markers like the *calcitonin receptor*, *tartrate-resistant acid phosphatase (TRAP)*, and *cathepsin K* in parallel cultures (Figure 4C). In line with evidence that FoxO3 binds to and suppresses the *FasL* promoter (Jonsson et al., 2005), the increased osteoclast apoptosis was associated with a 4-fold increase in *FasL* mRNA. Osteoclast progenitor number was increased as a result of *FoxO* deletion (Figure 4D)

### Gain of FoxO3 function in osteoblasts causes an increase in bone mass

To seek independent confirmation of the role of FoxOs in bone formation and homeostasis, we generated mice that conditionally overexpress *FoxO3* in cells of the osteoblast lineage (Figure 5A). This was accomplished by crossing mice with a conditionally activated WT *FoxO3* transgene (*FoxO3*<sup>C</sup>) with mice expressing Cre recombinase under the control of the osteocalcin (*OCN*) promoter. This promoter targets the expression of Cre to osteoblast-lineage cells at later stages of differentiation. Consistent with this, the *FoxO3* transgene was highly expressed in calvaria or vertebra from 1 month-old *OCN-Cre;FoxO3*<sup>C</sup> mice but undetectable in liver or spleen. As expected, the transgene was also undetectable in all tissues from littermate controls harboring only the *OCN-Cre* transgene (Figure 5B). The expression of FoxO-target genes, like *Gadd45* and *catalase*, was increased in calvaria of *OCN-Cre;FoxO3*<sup>C</sup> mice confirming the functionality of the transgene (Figure 5C). To further examine the functionality of the transgene, we used adenovirus (Ad)-Cre infection to activate it in calvaria cells from *FoxO3*<sup>C</sup> mice. Transfection of Ad-Cre infected cells with a FoxO-responsive luciferase (luc) reporter construct led to a 2-fold enhancement of FoxO directed transcriptional activity (Figure 5D).

Overexpression of *FoxO3* in osteoblasts led to a significant increase in bone mass in the vertebrae, as determined by BMD at 16 wk of age in the female, but not in the male, mice (Figure 6A). Nonetheless, an increase in cancellous bone mass (as determined by the measurement of bone volume over total volume) could be detected by micro-CT in both sexes, most likely reflecting the three dimensional advantage of the micro-CT measurement (Figure 6B). Female or male *OCN-Cre;FoxO3*<sup>C</sup> mice also exhibited increased trabecular number, as compared to littermate controls, while trabecular separation was decreased but this latter change was significant only in the male (Figure 6C). Vertebral BMD in female mice harboring

the *FoxO3<sup>C</sup>* transgene without Cre activation was similar to wild-type littermates, demonstrating that the insertion site of the *FoxO3<sup>C</sup>* transgene has no effect on bone mass (Figure S4A). In contrast to the lumbar spine BMD, there was no difference in femoral bone mass between the experimental and the control mice by BMD (data not shown) or micro-CT (Figure S4B). Histomorphometric analysis of vertebral bone independently confirmed that *FoxO3* gain-of-function in osteoblasts led to an increase in bone mass as demonstrated by an increase in cancellous bone area (BA/TA) and trabecular number, as well as a decrease in trabecular separation (Figure 6D).

Consistent with the gain in bone mass, the transgenic mice exhibited higher number of osteoblasts and bone formation rate in cancellous bone (Figure 7A). Moreover, in a diametrically opposite manner to the deletion model, FoxO3 overexpression in mature osteoblasts resulted in decreased phosphorylation of p66<sup>shc</sup> in vertebral lysates from the OCN-Cre;FoxO3<sup>C</sup> mice (Figure 7B). In line with the evidence for the decrease in OS in the bones of the OCN-Cre;FoxO3<sup>C</sup> mice, the prevalence of osteoblast apoptosis was diminished; whereas osteocyte apoptosis was not affected (Figure 7C).

To demonstrate that the overexpression of FoxO3 in osteoblasts did actually enhance defense against OS and thereby protected osteoblasts from OS-induced apoptosis we cultured bone marrow-derived osteoblastic cells from the control and the OCN-Cre;FoxO3<sup>C</sup> mice for 18 days in ascorbic acid (Figure 7D-E). As expected, progression of osteoblast differentiation under these culture conditions (and thereby an increase in the number of osteocalcin expressing cells) led to the expression of the FoxO3 transgene in cells from the OCN-Cre;FoxO3<sup>C</sup>, but not in cells from control mice as shown in Figure 7D by qRT-PCR and Western Blot for HA and FoxO3. For the Western blot of Figure 7D we have utilized an anti-FoxO3 antibody that recognizes both the human protein produced by the transgene as well as the endogenous mouse protein. Thus, the increased signal intensity observed in the transgene lane reflects the sum of both proteins and thereby an increase in overall FoxO3 expression in the cells from the transgenic mice. As shown in Figure 7E, osteoblasts from the OCN-Cre;FoxO3<sup>C</sup> mice, exhibited resistance to apoptosis induced by a range of H<sub>2</sub>O<sub>2</sub> concentrations.

Finally, based on our previous observations that FoxOs can divert  $\beta$ -catenin from TCF- to FoxO-mediated transcription and by this mechanism decrease the expression of the anti-osteoclastogenic factor osteoprotegerin (OPG) and consequently increase osteoclast numbers (Glass et al., 2005), we examined the number of osteoclast progenitors in the bone marrow and mature osteoclasts in vertebral bone sections of the *FoxO3* overexpressing mice. Unexpectedly, these mice displayed lower progenitors and lower osteoclast numbers (Figure 7F). This decrease could not be explained by changes in the production of osteoclastogenic cytokines by osteoblastic cells because we observed no changes in the mRNA levels of *RANKL*, *OPG* or *M-CSF* in vertebral bone from *OCN-Cre;FoxO3<sup>C</sup>* mice (data not shown).

## Discussion

FoxOs translate environmental stimuli, such as OS and hormonal changes, into dynamic gene expression programs involved in many physiological as well as pathological processes (Salih and Brunet, 2008; Dansen and Burgering, 2008; van der Horst and Burgering, 2007). Based on this and evidence that decreased defense against OS is responsible, at least in part, for the adverse effects of aging and sex steroid deficiency on the murine skeleton, we have genetically manipulated FoxOs in mice and investigated the impact of such manipulation on skeletal homeostasis. Specifically, we hypothesized, that if one were to remove an important defense mechanism against OS from bone cells, one may recapitulate, at least some of, the adverse effects of aging on bone in young mice. Our results demonstrate that conditional deletion of the three broadly expressed genes, *FoxO1*, 3, and 4, in young adult mice or targeted

overexpression of *FoxO3* in osteoblasts leads to significant changes in bone mass. Specifically, loss of FoxO function in 3 mo old mice for a period of 5 wk results in increased OS in bone as well as increased osteoblast and osteocyte apoptosis and an osteoporotic phenotype characterized by decreased bone mass at both cancellous and cortical sites. We also show that the increased osteoblast apoptosis following the triple FoxO deletion is cell autonomous and the result of increased OS, as the rate of osteoblast apoptosis of the FoxO-deficient osteoblasts in ex-vivo cultures can be reverted to normal by the addition of the antioxidant NAC.

In sharp contrast to the increased OS and osteoblast apoptosis, decreased osteoblast number and bone formation, and the loss of bone mass following the loss of FoxO function, overexpression of *FoxO3* in mature osteoblasts led to opposite effects: decreased OS and osteoblast apoptosis, increased osteoblast number and bone formation and increased bone mass. Albeit, increased bone mass was detectable in the vertebrae but not in the femur, perhaps because of the high variability of the measurement at the latter site during growth. An alternative explanation for the manifestation of the gain of FoxO3 function only in vertebrae may be the dramatically higher rate of turnover and the basal rate of osteoblast apoptosis in cancellous versus the periosteal compartment (Jilka et al., 2009) and the fact that cancellous bone makes larger contribution to BMD in the vertebrae than in the femur.

Collectively, the findings of this paper suggest that FoxOs are constantly defending against increased OS in osteoblasts, thereby assuring a normal healthy lifespan. In support of this conclusion, earlier work from our group and others over the years has established that apoptosis is the fate of the majority (60-80%) of mature osteoblasts (Jilka et al., 2007); and that this process is regulated by all major factors that influence skeletal homeostasis including systemic hormones, glucocorticoid administration, locally produced factors like IL-6 type cytokines, IGF-I, Wnts and their antagonists, parathyroid related peptide, mechanical forces, as well as OS (Jilka et al., 2008). Consistent with the contention that loss of FoxOs increases OS and thereby decreases the lifespan of osteoblasts, the number of mature osteoblasts in bone sections from the *FoxO*-deficient mice was decreased, and as a consequence their work output (i.e. the synthesis and mineralization of the bone matrix) was compromised.

Similar to our findings with osteoblasts, FoxOs promote mammalian cell survival by attenuating OS in other cell types (Tothova and Gilliland, 2007). Nonetheless, this effect is highly dependent on the cell type and even the tissue in which a particular cell type resides. For example, loss of FoxO function promotes murine hematopoietic stem cell apoptosis but decreases the apoptosis of thymocytes in the same animals (Paik et al., 2007; Tothova et al., 2007). More strikingly, loss of FoxOs decreases the apoptosis of endothelial cells from the liver but has no effect on endothelial cells residing in the lungs (Paik et al., 2007).

Osteoblast generation (determined by the expression of several lineage specific markers) in ex-vivo cultures of osteoblastic progenitors derived from the bone marrow of *FoxO*-deficient mice was attenuated as compared to the control mice. Specifically, *Runx2* and *Osterix* expression as well as *alkaline phosphatase*, markers of osteoblast differentiation, were decreased. This could result from either an increase in the apoptosis of these progenitors or attenuation of their differentiation. In support of the latter explanation, deletion of FoxOs caused an increase of *PPAR $\gamma$* — a nuclear receptor that stimulates adipogenesis (Tontonoz and Spiegelman, 2008) while represses osteoblastogenesis (Akune et al., 2004) and is tonically suppressed by FoxOs (Dowell et al., 2003; Armoni et al., 2006). Moreover cells from the FoxO deficient mice exhibited increased adipogenic potential in response to the PPAR $\gamma$  ligand rosiglitazone. Hence, increased adipocyte differentiation at the expense of osteoblast differentiation from their common mesenchymal progenitors, may have contributed to the decreased osteoblast number and bone formation in these mice.

We and others have demonstrated that both old age and acute loss of sex steroids in mice leads to increased OS and an increase in the prevalence of osteoblast and osteocyte apoptosis; and that antioxidants prevent the increased osteoblast and osteocyte apoptosis, as well as the loss of bone caused by gonadectomy (Almeida et al., 2007b; Lean et al., 2003). In addition, FoxO transcriptional activity, as measured by the expression of the *Gadd45* gene expression, was increased in the aged mice of our earlier studies (Almeida et al., 2007a). The most likely explanation for the inability of FoxOs to prevent osteoblast apoptosis in old age is that the defense provided by these factors is overwhelmed by the high level of OS or that OS activates other pathways which interfere with the activity of FoxOs, or both. Extensive evidence indicates that ROS-induced p66<sup>shc</sup> activation leads to FoxO inactivation via Akt-mediated FoxO phosphorylation (Nemoto and Finkel, 2002; Smith et al., 2005). Moreover, ROS-induced stimulation of NF- $\kappa$ B may lead to IKK-mediated phosphorylation and inhibition of Foxo3 activity, at least in part by targeting it to ubiquitin-dependent degradation (Hu et al., 2004). Increased phosphorylation of p66<sup>shc</sup> as well as increased activity of NF- $\kappa$ B are common features of old age and sex steroid deficiency (Almeida et al., 2007b; Biswas et al., 2005; Adler et al., 2007; De Bosscher et al., 2006). Hence, several counteracting mechanisms can account for the overwhelming of FoxOs ability to thwart osteoblast apoptosis in old age.

Deletion of FoxOs also caused an increase in mature osteoclast apoptosis, perhaps secondary to the observed increase in FasL production. This latter finding is in agreement with the observation that FoxO3 can bind and suppress the *FasL* promoter in neutrophils; whereas deletion of FoxO3 results in elevated expression of *FasL* and increased neutrophil apoptosis (Jonsson et al., 2005). In support of this contention, FasL is an important pro-apoptotic signal for osteoclasts, and mice lacking *Fas* have increased osteoclast numbers and decreased bone mass (Wu et al., 2003). Moreover, up-regulation of *FasL* expression by estrogens may represent an important mechanism of the pro-apoptotic effect of these hormones on osteoclasts (Manolagas, 2000; Nakamura et al., 2007). The stimulatory effect of the FoxO deletion on osteoclast apoptosis was accompanied by an increase in osteoclast progenitors, most likely due to the expansion of myeloid progenitors, described earlier by Tothova et al. (Tothova et al., 2007). In spite of these changes the number of osteoclasts in cancellous bone of the *Mx-Cre*<sup>+</sup> mice was unaltered, suggesting that the two changes may have cancelled each other. Genetic manipulation of FoxOs exclusively in cells of the osteoclast lineage in future work should help to explain these phenomena and their biologic relevance.

Lastly, overexpression of *FoxO3* in osteoblasts led to a decrease in osteoclast numbers, which was unexpected, considering the inhibitory effect of FoxOs on canonical Wnt signaling and evidence that  $\beta$ -catenin reduces the levels of OPG (a direct target of  $\beta$ -catenin/TCF transcriptional activity), and increases RANKL and consequently osteoclast numbers in bone (Glass et al., 2005; Holmen et al., 2005). Yet, *OPG* or *RANKL* expression was not altered in the bone of our *OCN-Cre;FoxO3<sup>C</sup>* mice. This latter finding suggests that increased FoxO3 transcriptional activity in mature osteoblasts controls osteoclastogenesis independently of the canonical Wnt signaling. Instead, FoxO3 overexpression may decrease osteoclast number by decreasing ROS in the bone microenvironment. This suggestion is supported by mounting evidence that mitochondria biogenesis and ROS are indeed required for osteoclast generation (Garrett et al., 1990; Levasseur et al., 2003; Bai et al., 2005; Lean et al., 2005; Lee et al., 2005).

In conclusion, the results reported in this paper strongly support the working hypothesis that ROS are seminal signals for the fate of osteoblasts and that inappropriate increase of ROS adversely affects bone formation. The FoxO family of transcription factors defends against such an increase by constantly up-regulating free radical scavenging and DNA-repair enzymes, thereby representing an indispensable homeostatic mechanism for skeletal health.

## Experimental Procedures

### Generation of FoxO3 transgenic mice

We generated mice harboring a transgene consisting of a transcriptional/translational stop cassette, flanked by loxP sites, inserted between the broadly-expressed hybrid *CMV-chicken  $\beta$ -actin promoter* (Matsuda and Cepko, 2007) and a cDNA encoding an HA-tagged wild-type human *FoxO3* (Fig. 5A), designated *FoxO3<sup>C</sup>* mice. HA-FoxO3 (Brunet et al., 1999) was provided by M. Greenberg, Harvard Medical School (Addgene plasmid 1787). When crossed with mice expressing the Cre recombinase in a specific cell type, the loxP-flanked stop cassette is removed only in the cells expressing Cre resulting in FoxO3 expression in that cell type (Fig. 5A). We generated 10 independent *FoxO3<sup>C</sup>* lines using this construct in a C57BL/6 background and tested the ability of the transgene to be activated in a cell type-specific manner by crossing each line with transgenic mice expressing Cre under the control of the human osteocalcin promoter (*OCN-Cre*) (Zhang et al., 2002). A single *FoxO3<sup>C</sup>* line demonstrating bone-specific expression (Fig. 5A) was utilized for all the studies described herein. Experimental mice were generated by crossing homozygous *OCN-Cre* mice, which are in a FVB/N background, with hemizygous *FoxO3<sup>C</sup>* mice to generate offspring that were all hemizygous for the *OCN-Cre* transgene and 50% of these were also hemizygous for the *FoxO3<sup>C</sup>* transgene. Thus all experimental mice were littermates in an F1 C57BL/6-FVB/N genetic background. Transgenic offspring were identified by PCR using the following primer sequences: forward (101bp) 5'-GAG TCG CTC GGT ACG ATT TA-3' reverse (101bp) 5'-CGG GAG GCT AGC ATA ATC AG-3'.

### Animal experimentation

Twelve week-old *Mx-Cre<sup>+</sup>;FoxO1,3,4<sup>LL</sup>* and *Mx-Cre<sup>-</sup>;FoxO1,3,4<sup>LL</sup>* littermate control mice, generated as previously described (Paik et al., 2007), received a total of three intraperitoneal injections of 300  $\mu$ g of pI-pC (Sigma) administered every other day to induce Cre activation. Five wk later the animals were sacrificed and the various tissues and cells collected. BMD and body weight measurements were performed before the first pI-pC injection and before sacrifice, as previously described (O'Brien et al., 2005). Mice were injected with tetracycline 6 and 2 days before harvesting. Protocols involving genetically modified mice and their wild-type littermates were approved by the Institutional Animal Care and Use Committees of the University of Arkansas for Medical Sciences and the Central Arkansas Veterans Healthcare System.

### Bone analysis

Femur and vertebra micro-CT scans were performed on isolated bones with a  $\mu$ CT40 scanning system (Scanco). Histology was performed in undecalcified vertebral bone sections as previously described (Weinstein et al., 1998; Weinstein et al., 2002). Details of the bone analysis can be found in Supplemental Experimental Procedures.

### Cell culture

Osteoblastic cells derived from mouse calvaria were obtained and cultured as described previously (Jilka et al., 1998) for 8 days. Adherent bone marrow cells were obtained from long bones as described previously (DiGregorio et al., 2001) and cultured in  $\alpha$ -MEM supplemented with 10% FBS and 1% PSG. Bone marrow-derived cells cultured in the absence of ascorbic acid are designated stromal cells (Fig. 3D and 3E). Cells cultured in the presence of 1% ascorbic acid to promote osteoblast differentiation are designated bone marrow osteoblastic cells (Fig. 7D, 7E, S1D and S4). To quantify adipogenesis, bone marrow-derived stromal cells were cultured to 70% confluence, and the media supplemented with rosiglitazone (5 nM/ml) or with 3.3% BSA in PBS as vehicle control. Medium was changed every 2 d for 11 d. Oil Red O



staining and quantification were performed as described previously (Almeida et al., 2009a). Osteoclast progenitors were derived from bone marrow cells cultured in  $\alpha$ -MEM supplemented with 10% FBS, 1% PSG, 30 ng/ml M-CSF and 30 ng/ml soluble RANKL (R&D Systems). Osteoclasts were enumerated after staining for TRAP; only multinucleated cells (more than three nuclei) were counted. Mature osteoclasts were derived from non-adherent bone marrow cells maintained for 5 d in same medium as for osteoclast progenitors. Apoptotic cells were quantified by measuring caspase 3 activity as described previously (Plotkin et al., 1999).

### Western blot and qRT-PCR analysis

Phosphorylated p66<sup>shc</sup> was assayed by immunoblotting in fifth lumbar vertebra or cultured cell lysates, as previously described (Kousteni et al., 2003) using a mouse monoclonal antibody recognizing Ser36 phosphorylated p66<sup>shc</sup> (Calbiochem). FoxO1, 3 and 4 levels in cultured cell lysates were determined using a rabbit monoclonal antibody for Foxo1 (Cell Signaling), a rabbit polyclonal antibody for FoxO3 (Santa Cruz Biotechnology; GenWay Biotech, Inc), and a rabbit polyclonal antibody for Foxo4 (Cell Signaling). Protein levels of HA-FoxO3 or  $\beta$ -actin were analyzed using mouse monoclonal antibodies (Cell Signaling and Sigma, respectively). Quantification of the intensity of the bands in the autoradiograms was performed using a VersaDoc<sup>TM</sup> imaging system (Bio-Rad). Total RNA was extracted from tissues or cultured cells and reverse-transcribed as previously described (Almeida et al., 2007a). Primers and probes for the different genes were manufactured by the TaqMan<sup>®</sup> Gene Expression Assays service (Applied Biosystems). Primers and probe for HA-FoxO3 were manufactured by Assays-on-demand service (Applied Biosystems): forward primer sequence 5'-CTCTGACTGACCGCGTTACTC-3'; reverse primer sequence 5'-GCCAAAATGATGAGACAGCACAATA-3'. The mRNA levels in Figure 4C, 5B, 5C, 7D and S1 were calculated by normalizing to the house-keeping gene ribosomal protein S2 using the  $\Delta$ Ct method (Livak and Schmittgen, 2001). The relative mRNA levels in Figure 1A, as well as in Figures 3A, 3D, 4A, 4C, S4A were calculated by the same method with the additional step of assigning an arbitrary value of 100 to the control sample.

### Adenovirus infection and luciferase assay

Newborn calvaria cells from Foxo3<sup>C</sup> or wild-type mice were infected twice (with 24 h interval) with adenovirus encoding Cre recombinase (Ad-Cre) (Vector Biolabs) at a titer of 100 multiplicity of infection. Twenty four h after the last infection cells were plated on a 48-well plate and 16 h later transfected with a reporter plasmid containing 6 copies of daf-16 family protein binding element (FoxO-luc) provided by B. Burgering (University Medical Center, Utrecht, Netherlands) using Lipofectamine Plus (Invitrogen). Luciferase activity was determined as previously described (Kousteni et al., 2003).

### Statistical analysis

All data are reported as the mean  $\pm$  standard deviation. Group mean values were compared, as appropriate, by Student's two-tailed *t* test or one-way ANOVA with Bonferroni's multiple comparison test. A *p* value  $\leq 0.05$  was considered significant.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

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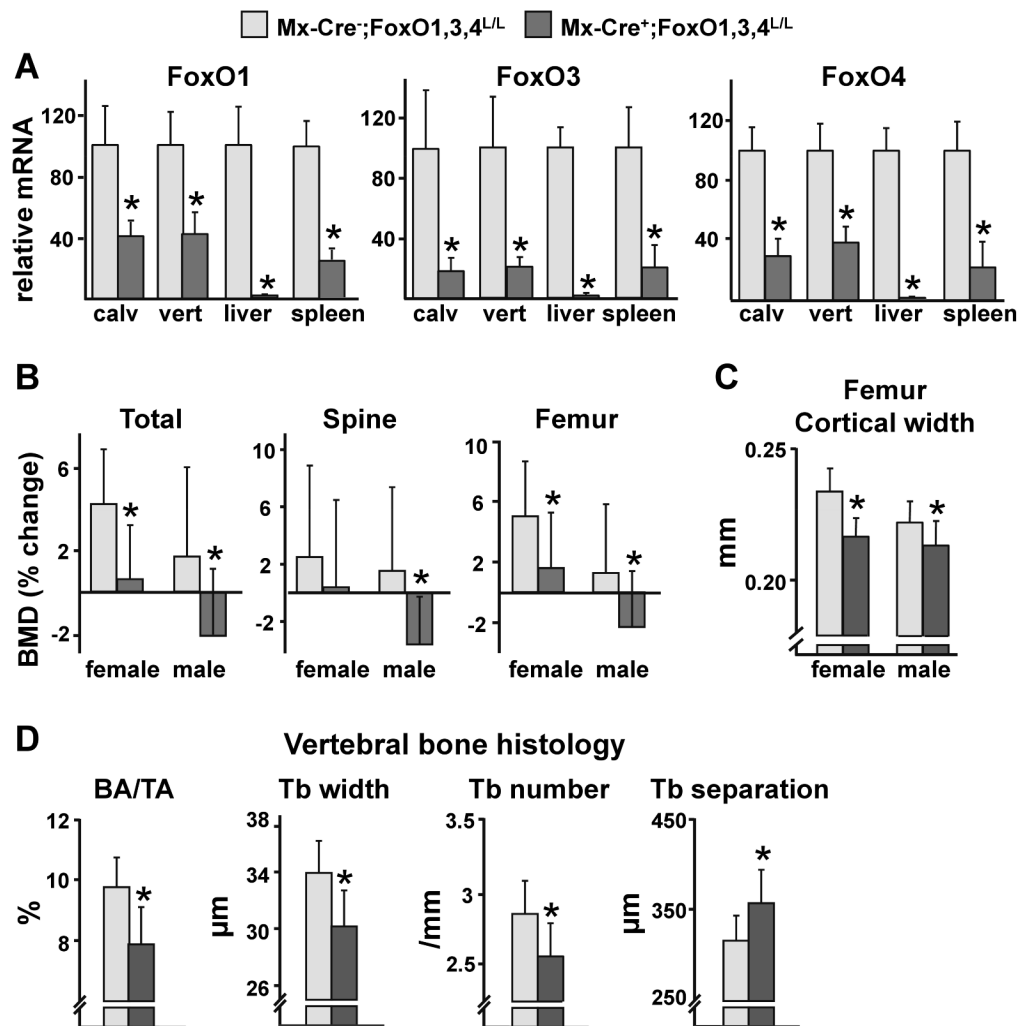
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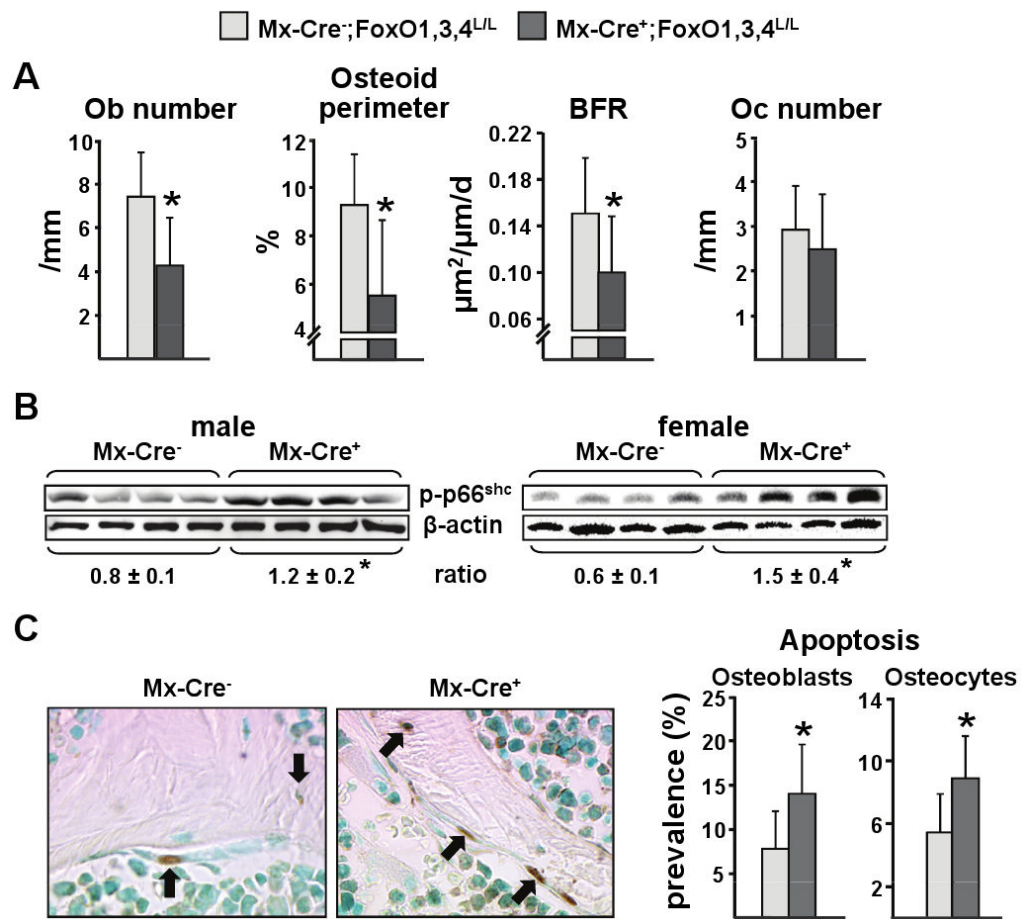
### Figure 1. Bone Mass is Decreased Following FoxOs Deletion

(A-D) 12 wk-old female and male mice were injected with pI-pC and sacrificed 5 wk later. (A) mRNA levels of FoxOs in calvaria (calv), vertebra (vert), liver or spleen from male mice determined by qRT-PCR (n=8 mice/group).

(B) BMDs determined by DEXA 1 d before and 5 wk following pI-pC injections (n=10-17 mice/group). \*p<0.05 vs respective Mx-Cre<sup>-</sup> by Student's t-test

(C) Cortical width measured by micro-CT in femurs (n=10-17 mice/group). \*p<0.05 vs respective Mx-Cre<sup>-</sup> by Student's t-test

(D) Histomorphometric analysis of longitudinal undecalcified sections of L1-L3 vertebrae of male mice (n=6-7 mice/group). BA/TA=bone area per tissue area; Tb=trabecular. \*p<0.05 by Student's t-test.



### Figure 2. Deletion of FoxOs Increases Oxidative Stress and Decreases Bone Formation

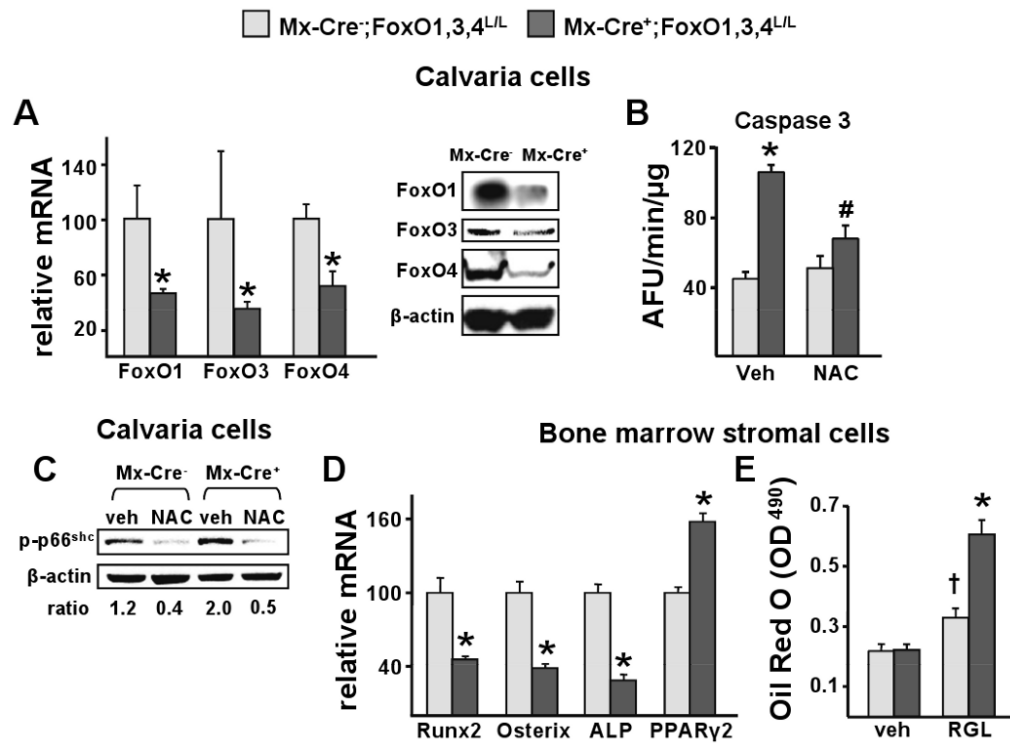
(A-C) Measurements were made using bone from the animals described in Figure 2.

(A) Histomorphometric analysis of longitudinal undecalcified sections of L1-L3 vertebrae of male mice (n=6-7 mice/group). Ob=osteoblast; Oc=osteoclast.

(B) p66<sup>shc</sup> phosphorylation determined by Western blot in vertebral lysates; each lane represents one animal. The mean ratio ± SD of phosphorylated p66<sup>shc</sup> to β-actin is depicted numerically at the bottom of the corresponding blots.

(C) Osteoblasts and osteocyte apoptosis in sections of the same mice as in A. In the photomicrographs apoptotic osteocytes and osteoblasts (stained brown by ISEL, original magnification ×630) are indicated by the arrows (n=7-8 mice/group).

\*p<0.05 by Student's t-test.



**Figure 3. ROS-Induced Apoptosis is Increased in Osteoblastic Cells from FoxO-Deleted Mice**

(A-D) Cells were obtained from the mice described in figure 2.

(A) mRNA levels of FoxOs by qRT-PCR and protein levels of FoxOs by Western Blot in calvaria-derived osteoblastic cells cultured for 8 d. \*p<0.05 by Student's t-test.

(B) Caspase 3 activity in cells described in A, cultured in the presence of vehicle or NAC (1 mM) for 24 h. \*p<0.05 vs veh in *Mx-Cre<sup>-/-</sup>*; # p<0.05 vs veh in *Mx-Cre<sup>+/+</sup>* by ANOVA.

(C) p66<sup>shc</sup> phosphorylation determined by western blot in lysates from calvaria cells described in B. The ratio of phosphorylated p66<sup>shc</sup> to  $\beta$ -actin is depicted numerically at the bottom of the corresponding blots.

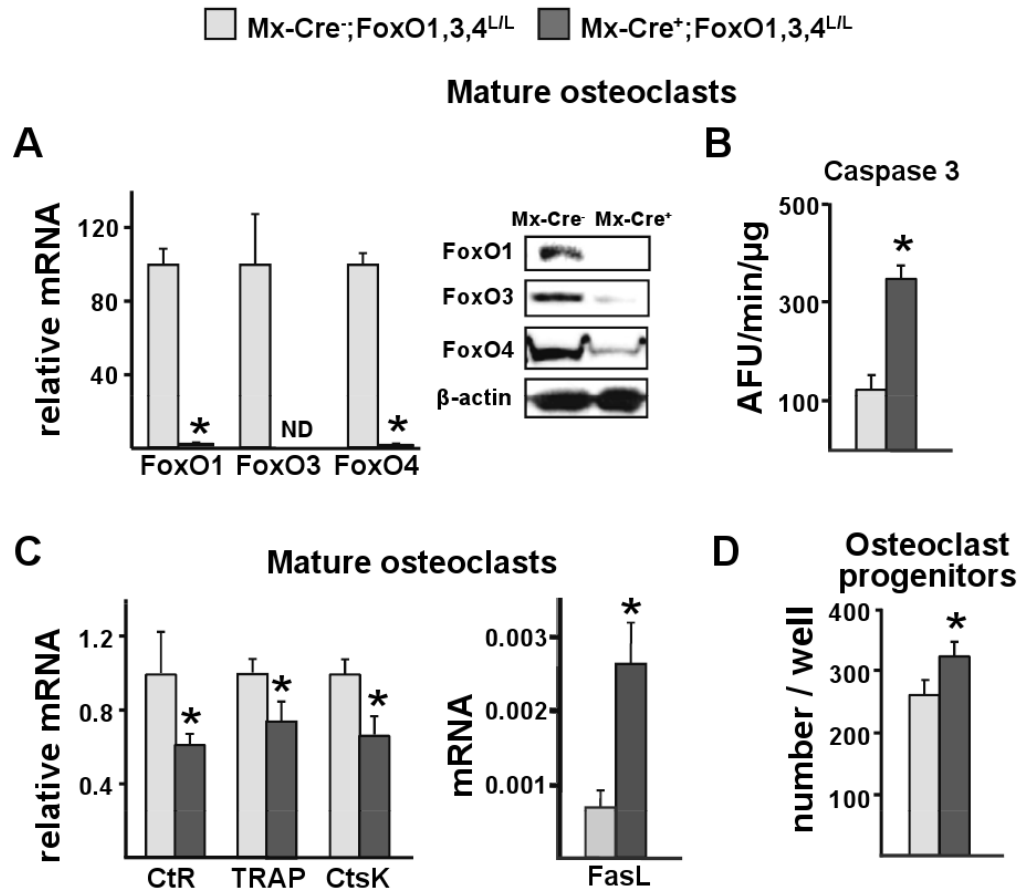
(D) mRNA levels of the indicated genes in bone marrow-derived stromal cells cultured for 8 d.

\*p<0.05 by Student's t-test

(E) Bone marrow-derived stromal cells were treated with vehicle or rosiglitazone (RGL; 5 nM/ml) for 11 days. Lipid was visualized with Oil Red O and quantified as described in Methods.

\*p<0.05 vs RGL in *Mx-Cre<sup>-/-</sup>*; †p<0.05 vs vehicle in *Mx-Cre<sup>-/-</sup>* by ANOVA.





**Figure 4. Osteoclast Progenitor Number and Mature Osteoclast Apoptosis are Increased Following FoxOs Deletion**

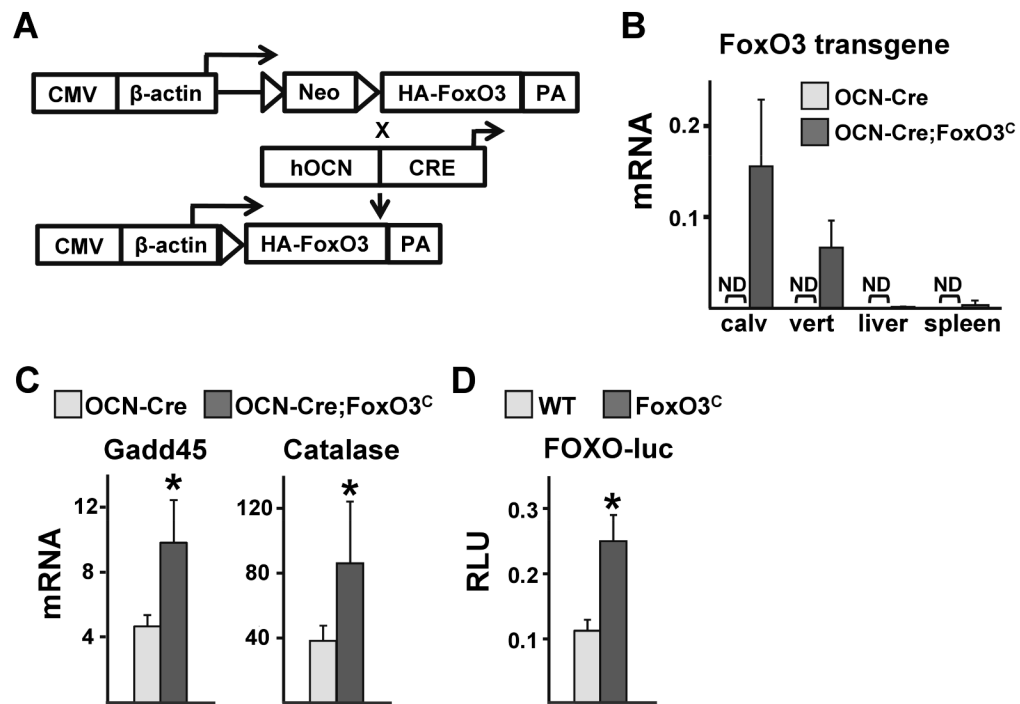
(A-D) Cells were obtained from the mice described in figure 2.

(A) mRNA levels of FoxOs by qRT-PCR and protein levels of FoxOs by Western Blot in mature osteoclasts generated from non-adherent bone marrow cells cultured with M-CSF and RANKL for 5 d; ND=not detected.

(B) Caspase 3 activity and (C) mRNA levels of the indicated genes in cells described in A. CtR=calcitonin receptor; CtsK=cathepsin K.

(D) Number of TRAP positive cells generated from bone marrow cells cultured with M-CSF and RANKL for 6 d. TRAP=tartrate-resistant acid phosphatase.

\*p<0.05 by Student's t-test.



**Figure 5. Generation of FoxO3<sup>C</sup> Transgenic Mice**

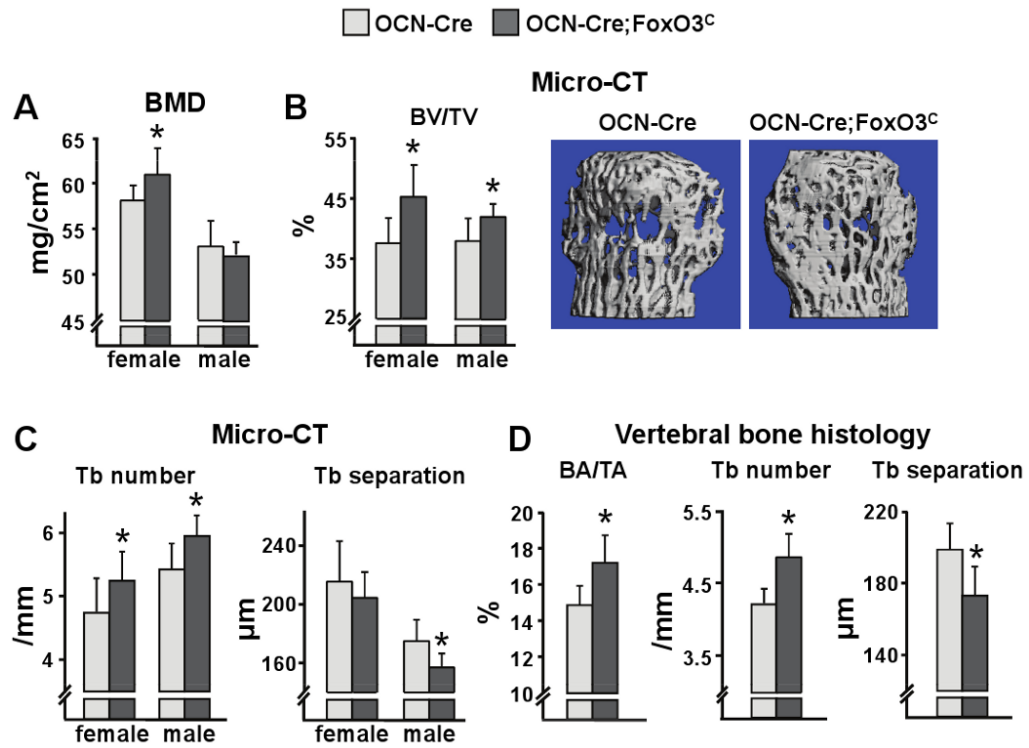
(A) Mice harboring a transgene consisting of a transcriptional/translational stop cassette, flanked by loxP sites, inserted between the broadly-expressed hybrid CMV-chicken  $\beta$ -actin promoter and a cDNA encoding an HA-tagged human FoxO3 WT, designated FoxO3<sup>C</sup> mice were crossed with mice expressing the Cre recombinase under the control of the OCN promoter. The loxP-flanked stop cassette is removed only in cells expressing Cre resulting in FoxO3 expression in that cell type. PA=polyadenylation site.

(B) mRNA levels of the FoxO3 transgene in calvaria, vertebra (L5), liver and spleen of 4 wk-old mice determined by qRT-PCR (n=3 mice/group); ND=not detected.

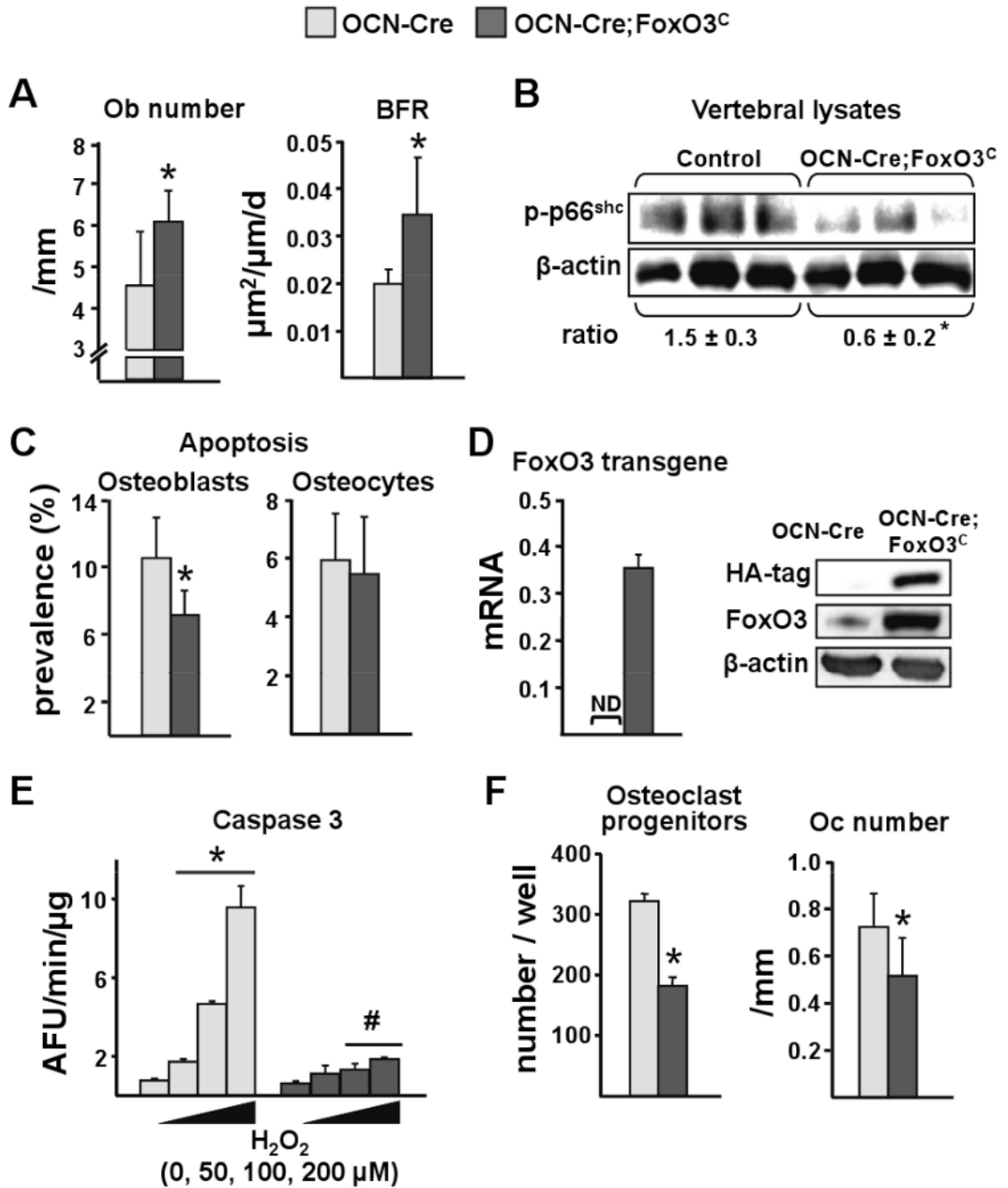
(C) mRNA levels of the indicated FoxO-target genes in calvaria from 4 wk-old mice (n=3 mice/group).

(D) Calvaria-derived osteoblastic cells isolated from newborn FoxO3<sup>C</sup> and WT littermates infected with Ad-Cre for FoxO3 overexpression and transfected with a FoxO-luc reporter construct. Luc activity was measured 24 h later.

\*p<0.05 by Student's t-test.



**Figure 6. Overexpression of FoxO3 in Osteoblasts Increases Vertebral Bone Mass**  
 (A) Spine BMD by DEXA and (B-C) micro-CT measurements in the vertebra (L5) of 16 wk-old mice (n=7-14 mice/group). BV/TV=bone volume per total volume; Tb=trabecular. Representative images of vertebral cancellous bone are shown. \*p<0.05 vs respective OCN-Cre by Student's t-test  
 (D) Histomorphometric analysis of longitudinal undecalcified sections of L1-L3 vertebrae of 16 wk-old male mice (n=6 mice/group). \*p<0.05 by Student's t-test. BA/TA=bone area per tissue area.



**Figure 7. Overexpression of FoxO3 in Osteoblasts Protects Against Oxidative Stress-Induced Apoptosis**

(A-C) Measurements were made using bones from the animals described in Figure 6.

(A) Histomorphometric analysis of longitudinal undecalcified sections of L1-L3 vertebrae of male mice (n=7 mice/group). Ob=osteoblasts; BFR=bone formation rate.

(B) p66<sup>shc</sup> phosphorylation determined by Western blot in vertebral lysates; each lane represents one animal. The mean ratio  $\pm$  SD of phosphorylated p66<sup>shc</sup> to  $\beta$ -actin is depicted numerically at the bottom of the corresponding blots.

(C) Osteoblasts and osteocyte apoptosis in sections of the same mice as in A.

(D) mRNA levels of FoxO3 transgene by qRT-PCR and protein levels of HA-FoxO3 and Foxo3 by Western blot in bone marrow-derived osteoblastic cells cultured for 18 d with ascorbic acid. (E) Caspase 3 activity in cells described in D, cultured in the presence of vehicle or H<sub>2</sub>O<sub>2</sub> at the indicated concentrations for 16 h. \*p<0.05 vs no H<sub>2</sub>O<sub>2</sub> by ANOVA. #p<0.05 vs respective H<sub>2</sub>O<sub>2</sub> dose in OCN-Cre by Student's t-test.

(F) Left panel: number of TRAP positive cells generated from bone marrow cells cultured with M-CSF and RANKL for 6 d. Right panel: osteoclast number was determined in the same sections as in A. TRAP=tartrate-resistant acid phosphatase; Oc=osteoclast. \*p<0.05 by Student's t-test.