PERSPECTIVES

Aging and Oxidative Stress: A New Look at Old Bone

Maria Almeida

Division of Endocrinology and Metabolism, Center for Osteoporosis and Metabolic Bone Diseases, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA

Abstract

Advancing age is a major contributor to a decline of bone mass and strength and, consequently, a rise in the incidence of bone fractures. Skeletal aging in a murine model closely recapitulates age-related skeletal changes in humans, including decreased osteoblast number as well as decreased bone mass and strength and is associated with increased oxidative stress. This *Perspective* reviews what is known about the effects of the aging process on bone and its cellular constituents. Specifically, it discusses how pathways activated by oxidative stress can contribute to the decrease in osteoblast number and bone formation that are the hallmarks of involutional osteoporosis. Activation of the p53/p66^{shc} signaling cascade in the skeleton by reactive oxygen species increases osteoblast/osteocyte apoptosis. On the other hand, reactive oxygen species stimulate the FoxO family of transcription factors that defend cells against oxidative stress. In line with this, loss of FoxO function in the skeleton increases oxidative stress and decreases bone mass. Conversely, gain of FoxO function specifically in osteoblasts decreases oxidative stress and increases bone mass. This *Perspective* also reviews the potential contribution of loss of sex steroids, glucocorticoids, as well as oxidized lipids to oxidative stress and skeletal involution. *IBMS BoneKEy*. 2010 October;7(10):340-352.
©2010 International Bone & Mineral Society

Keywords: Aging; Oxidative stress; p66^{Shc}; FoxO; Wnt signaling; Sex steroids; Lipid oxidation; Glucocorticoids

The Pathophysiology of Skeletal Aging in Humans and Mice

The primary manifestation of aging is an overall decline in the capacity of various organs, including the skeleton, to maintain homeostasis. Age is a critical risk factor for the development of cardiovascular and metabolic diseases like atherosclerosis, insulin resistance, and metabolic syndrome. Importantly, advancing age is also a major risk factor for a decline in bone mass and strength and, consequently, a rise in the incidence of bone fractures. Loss of cancellous bone mass in humans starts as early as the 3rd decade, well before any change in sex steroid production (1). Nonetheless, in women the loss of trabecular bone in the spine accelerates substantially after menopause, attesting to the adverse role of estrogen deficiency on skeletal homeostasis and its contribution to the acceleration of age-associated bone loss. Cortical bone loss begins to decline after the age of 50 in both sexes, albeit at a faster rate in women than in men, suggesting that this bone compartment is more sensitive to sex steroids. Recently, Zebaze and colleagues have shown that bone loss at peripheral sites in the first 15 years after menopause makes only a small contribution to total bone loss throughout life (2). The majority of cortical bone loss occurs after the age of 65 years, and is not driven solely by the fall in estrogen levels.

The key pathogenetic mechanism leading to age-related skeletal fragility is impaired bone formation. A gradual decrease in bone formation begins shortly after peak bone mass (3;4). Age-related bone loss is due primarily to an insufficient number of osteoblasts resulting from the exhaustion of multipotential mesenchymal stem cell progenitors (3;4) and the diversion of these

progenitors toward the adipocyte lineage (5-11).

Studies by our group and others have demonstrated that mice and rats also lose bone mass and strength with age (5-8). Loss of bone mass and strength in rodents with advancing age is associated with an increase in the prevalence of apoptotic osteoblasts and a corresponding decrease in osteoblast number and bone formation rate. The loss of bone mass with age in C57BL/6 female or male mice is associated with increased oxidative stress in bone and diminished canonical Wnt signaling, a critical regulator of bone formation (5;9;10).

Conserved Pathways That Regulate Skeletal Aging: The Role of Reactive Oxygen Species

Progressive oxidative damage has long been considered as the fundamental mechanism of age-associated physiological function decline (11;12). Modest levels of cellular reactive oxygen species (ROS) – the radical forms of oxygen – are generated by the mitochondrial electron transport chain during normal metabolism. Progressive mitochondrial damage with age, however, may result in excessive ROS production that damages proteins, lipids, and DNA, leading to cell death (13;14). It is now known that generation of H_2O_2 is a controlled process, involved in complex intracellular signal transduction cascades that regulate cell proliferation, differentiation, and migration in response to growth factors (15). NADPH oxidase (Nox) is largely responsible for receptor-dependent H_2O_2 production. Activated Nox complexes assemble within discrete subcellular compartments and, along with localized inactivation of antioxidant enzymes like peroxiredoxin I, allow restricted H_2O_2 accumulation for cell signaling (16;17).

In support of the contention that oxidative stress plays an important role in the skeleton, we and others have demonstrated that the loss of bone caused by gonadectomy in mice is prevented by antioxidants (5;18;19). Moreover, overexpression of thioredoxin-1, a major intracellular antioxidant, prevented the increase in oxidative stress and attenuated the suppression of bone formation following streptozotocin-induced diabetes in mice (20). Both osteoblast number and bone formation are decreased in mice treated with the pro-oxidant buthionine sulfoximine (19), and murine models of premature aging associated with oxidative damage exhibit osteoporosis (21;22). Importantly, the increased osteoblast apoptosis in the bone of aged mice is abrogated by administration of the antioxidant N-acetyl-cysteine (NAC) or catalase (10). Thus, oxidative stress appears to be a critical contributor to the adverse effects of aging on bone mass and strength, as is the case in other tissues (23).

The p53-p66^{Shc} signaling pathway controls intracellular redox status and attenuates osteoblastic cell differentiation and survival

Mammalian cells respond to oxidative stress using either long-lasting protective responses or apoptosis as a clearance mechanism for damaged cells. Increased ROS and osteoblast apoptosis caused by aging in the murine skeleton are associated with an increase in the phosphorylation of p53 and p66^{Shc} in bone (5). Conversely, apoptosis and p66^{Shc} phosphorylation in aged mice decrease with antioxidant administration (10). p66^{Shc}, an isoform of Shc, plays an important role as a redox enzyme implicated in mitochondrial ROS generation and translation of oxidative signals into apoptosis (24). p66^{Shc} localizes predominantly in the cytoplasm, with a small fraction (10-40%) in the mitochondria. Oxygen-derived free radicals activate the protein kinase C-β isoform to induce Ser^{36} phosphorylation of p66^{Shc}, promoting the translocation of the protein from the cytosol to mitochondria. In the mitochondria, p66^{Shc} acts as a redox enzyme to increase the production of H_2O_2 and apoptosis (25). Importantly, global p66^{Shc} deficiency decreases ROS production in murine bone (Almeida M, Han L, and Manolagas S, unpublished data) and increases osteoblast resistance to oxidative stress in a cell-autonomous fashion (26).

In cell cultures, p53 activation by oxidative stress leads to an increase in p66^{Shc} protein abundance, and phosphorylation of Ser36 of p66^{Shc} is a prerequisite for oxidant stress-induced apoptosis (27). Either p53-null or p66^{Shc}-null cells are relatively resistant to oxidant-induced apoptosis and exhibit lower endogenous ROS production and reduced oxidative damage to DNA as compared to wild-type cells. In line with this, mouse genetic studies have revealed that p53 negatively regulates osteoblastogenesis and bone formation (21;28;29). However, it remains unknown whether ROS are involved in the actions of p53 on bone.

One possible consequence of reduced ROS production is less damage to macromolecules, including DNA and protein, and thus a slower rate of aging, as well as protection against age-related diseases. In support of this theory, genetic deletion of p66^{Shc} in the mouse leads to a 30% longer lifespan (30). Remarkably, p66$^{Shc-/-}$ mice are more resistant to diabetes and exhibit a reduced risk of atherosclerosis and cardiovascular damage when submitted to a high-fat diet [reviewed in (24)]. p66$^{Shc-/-}$ mice are also protected against acute tissue damage after hind limb ischemia. The possibility that p66$^{Shc-/-}$ deletion also confers resistance to age-related bone loss is under current investigation by our group.

FoxOs and the skeleton

Activation of FoxOs and their transcriptional targets, in particular the peroxide-scavenging enzymes MnSOD and catalase, constitute part of cellular antioxidant defense mechanisms (31). Recent work by our group and others has established that FoxOs play an important role in skeletal homeostasis. Specifically, combined somatic deletion of FoxO1, 3, and 4 at 3 months, for just 5 weeks, resulted in increased oxidative stress in bone, and bone loss at both cancellous and cortical sites (32). The decreased bone mass was due to deficient bone formation and resulted from decreased osteoblast number and increased osteoblast apoptosis. In contrast, bone resorption was not affected. *Ex vivo* studies performed using cells from FoxO-deleted mice demonstrated that FoxOs have cell-autonomous effects on osteoblastic cells, including an increase in the basal rate of apoptosis of calvaria- and bone marrow-derived osteoblasts due to oxidative stress. Interestingly, bone marrow-derived stromal cells from FoxO-deleted mice also exhibited increased expression of PPARγ and, consequently, increased adipogenic capacity (32), as well as a decreased number of colony forming units of osteoblasts (CFU-OB) (Ambrogini E, Almeida M, and Manolagas S, unpublished data). These results suggest that FoxOs might contribute to the cell lineage specification of early multipotent mesenchymal stem cells. Mice with a gain of function of FoxO3 in osteoblasts exhibited decreased oxidative stress and osteoblast apoptosis, as well as increased osteoblast number, bone formation rate, and bone mass. This evidence favors the idea that FoxOs promote osteoblast survival via antioxidant actions. In agreement with our findings, Rached *et al*. reported that deletion of FoxO1 in osteoblasts expressing col1a1 leads to a decrease in osteoblast number and bone mass as a consequence of increased oxidative stress (33). However, in contrast to our studies, deletion of FoxO1 decreased osteoblastic cell proliferation but did not affect apoptosis. FoxO1 deletion also decreased protein synthesis in osteoblasts, leading to diminished levels of glutathione and collagen. Nonetheless, the same mice exhibited a striking increase in the production of osteocalcin (34). The increase in osteocalcin along with a decrease in Esp, which encodes a thyrosine phosphatase called OST-PTP, resulted in increased pancreatic β-cell proliferation, insulin secretion and insulin sensitivity, suggesting that FoxO1 may be a key modulator of the ability of the skeleton to function as an endocrine organ regulating glucose metabolism. Nonetheless, combined somatic deletion of FoxO1, 3, and 4 or combined deletion of FoxOs in osteoblast progenitor cells and their progeny has no effect on glucose homeostasis (Ambrogini E, Almeida M, and Manolagas S, unpublished data). Overall, these lines of evidence clearly show that FoxOs have critical functions in skeletal homeostasis (Fig. 1).
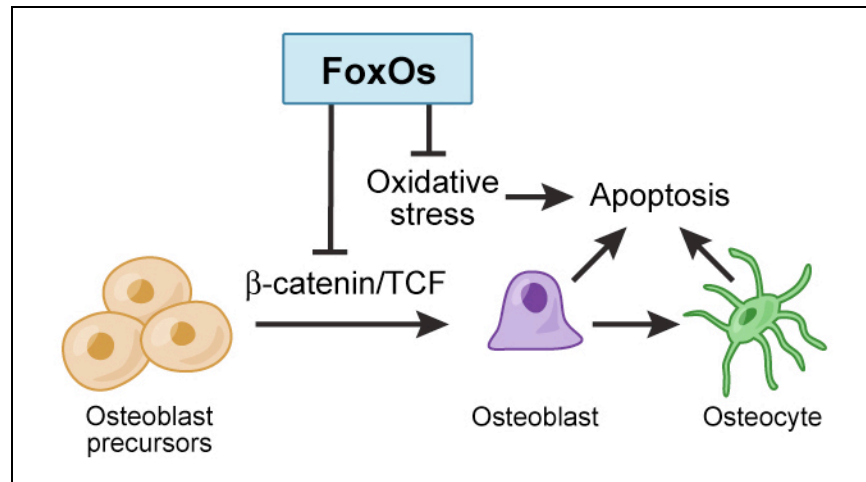
Fig. 1. The role of FoxOs in cells of the osteoblast lineage. Activation of FoxOs diverts β–catenin from TCF-to FoxO-mediated transcription. In osteoblast precursor cells, this mechanism leads to a decrease in Wnt-induced osteoblastogenesis. On the other hand, FoxOs prevent oxidative stress and, consequently, apoptosis of osteoblasts and osteocytes.

Oxidative Stress and Wnt Signaling

Studies from our group have also revealed that the expression of β-catenin/TCF target genes decreases and that of FoxO target genes increases with age in murine bone, along with an increase in markers of oxidative stress and decreased bone formation (9). In osteoblastic cell models, oxidative stress induces the association of FoxOs with β-catenin, and β-catenin is required for the stimulation of FoxO target genes. Notably, oxidative stress induced by H_2O_2 promotes FoxO-mediated transcription at the expense of Wnt/TCF-mediated transcription and osteoblast differentiation. The effect of H_2O_2 on TCF transcription is attenuated by increasing levels of β-catenin, strongly suggesting that a limited pool of active β-catenin is diverted from TCF to FoxO transcription under stress conditions. Studies in colon carcinoma cells confirmed that the same mechanism operates in these models (35). Taken together, these observations suggest that age-related bone loss might result, at least in part, from the suppressive effect of ROS on Wnt/β-catenin signaling via FoxO activation (Fig. 1). In line with this idea, mice with targeted expression of Wnt10b in the bone marrow had increased bone mass and no evidence of age-related loss of bone mass (36). Activation of FoxOs might also decrease β-catenin/TCF-mediated transcription via

alternative mechanisms. Indeed, in neural stem cells FoxOs promote the transcription of the Wnt signaling inhibitors Sost, sFRP1, and sFRP2 (37). Oxidative stress might also decrease Wnt signaling via dephosphorylation of GSK-3β (38;39) and activation of Dkk1 transcription (40;41). Interestingly, however, Wnt signaling can also be activated by oxidative stress (42;43) and administration of antioxidants to cultured osteoblastic cells or mice decreases Wnt signaling and osteoblastogenesis, respectively (10).

While this evidence clearly indicates that oxidative stress modulates Wnt signaling, there is also data indicating that β-catenin promotes the expression of several antioxidant genes in different cell types and prevents oxidative stress in the liver (44-46). It remains unknown whether this function of β-catenin occurs in cells of the osteoblast lineage or whether it contributes to its osteoblastogenic properties.

Systemic Hormones Like Sex Steroids and Glucocorticoids, as Well as Oxidized Lipids, Exert Their Influence on Skeletal Homeostasis, at Least in Part, via Regulation of Oxidative Stress

Age-associated oxidative stress is potentiated by loss of sex steroids

343

Similar to aging, gonadectomy promotes an increase in ROS and the phosphorylation of p53 and p66^{Shc} in bone (5;18;47). Estradiol, DHT, or several antioxidants completely reverse the effects of gonadectomy on these markers of oxidative stress. More strikingly, administration of antioxidants is as effective as sex steroid replacement in preventing the increased osteoclastogenesis, osteoblastogenesis, and increased osteoblast and osteocyte apoptosis, as well as the loss of BMD in gonadectomized female or male mice. *In vitro* studies in osteoblastic cells have elucidated that

p66^{Shc} is an essential mediator of the effects of oxidative stress, not only on apoptosis but also on NF-κb activation and the expression of cytokines like TNFα and IL6 (26). Sex steroids antagonize all these effects of oxidative stress by preventing PKCβ-induced p66^{Shc} phosphorylation via a mechanism that requires ERKs (5) (Fig. 2). Importantly, binding of the estrogen receptor (ER) α to DNA is not required for the ability of this receptor to mediate the anti-oxidant effects of estrogens on bone (48).
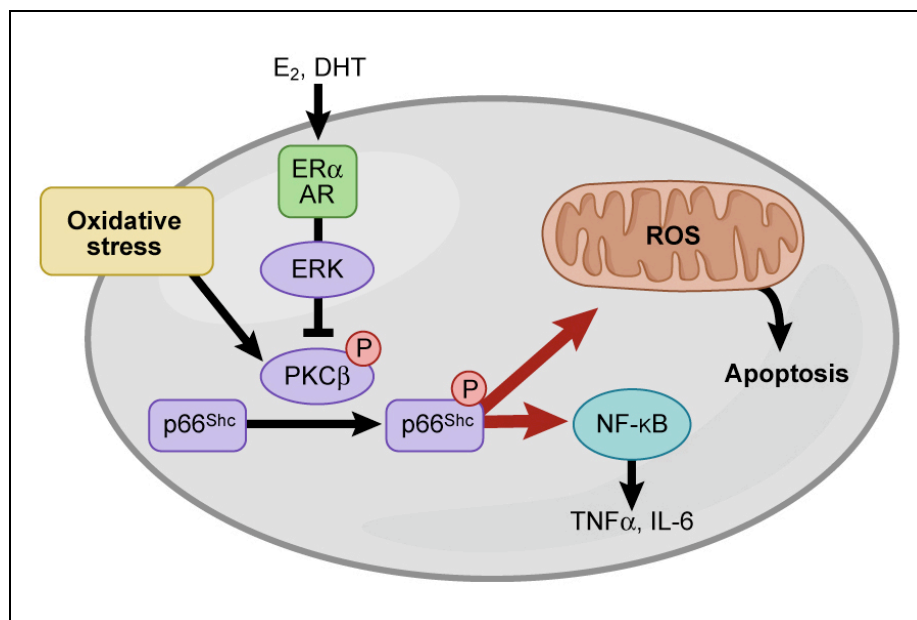

Fig. 2. p66^{Shc} is an essential mediator of the effects of oxidative stress on apoptosis, NF-κB activation and cytokine production by osteoblastic cells. Estrogens or androgens attenuate all these effects by suppressing PKCβ/p66^{Shc} phosphorylation.

Studies by several groups have shown that ROS are required for the formation and activation of osteoclasts [reviewed in (23)]. Consistent with the requirement of ROS in osteoclast generation and the *in vivo* evidence that sex steroids prevent bone loss via anti-oxidant effects, estradiol or DHT attenuates osteoclastogenesis and stimulates osteoclast apoptosis by a mechanism that involves activation of glutathione and thioredoxin reductases and inhibition of NF-κB (5;18). Similar to the case with osteoblasts, the anti-osteoclastogenic and pro-apoptotic effects of estradiol on osteoclasts are independent

of the DNA-binding function of the ER (49). Overall, these studies suggest that the loss of sex steroids contributes to age-related bone loss, at least in part by increasing oxidative stress.

Lipid oxidation amplifies oxidative stress and diminishes osteogenic Wnt signaling in the aged skeleton

Lipoxygenase-dependent formation of oxidized lipids plays an essential role in the development of atherosclerosis and epidemiologic evidence from humans as well as studies in mice point to a link

between osteoporosis and cardiovascular disease [reviewed in (23)]. The expression of the lipoxygenases Alox12, Alox12e, and Alox15, as well as the lipid oxidation product 4-HNE, and the adipogenic PPARγ, increases in the bone of C57BL/6 mice with advancing age (50). Moreover, lipoxygenases oxidize polyunsaturated fatty acids to form products that bind to and activate PPARγ. In the process, they generate pro-oxidants like 4-HNE (51). Both PPARγ and Alox15 influence skeletal homeostasis as demonstrated by evidence that BMD is increased in mice lacking Alox15 or in mice that are haploinsufficient for PPARγ (52;53). On the other hand, mice expressing high levels of Alox15 have low BMD (52), and activation of PPARγ with synthetic thiazolidinedione ligands decreases bone mass both in humans and in mice [reviewed in (54)].

The expression of Alox15 is stimulated by oxidative stress in cultured cells (55). In line with this, NAC or catalase completely reversed the age-related increase in Alox15 expression to levels found in younger mice (10). Importantly, other conditions leading to oxidative stress in the skeleton, like loss of estrogens (5;18) and deletion of FoxOs (32), also increase Alox15 expression in bone (Jilka R and Almeida M, unpublished data). Similar to aged mice, young mice expressing high levels of Alox15 exhibit increased oxidative stress, lipid peroxidation and PPARγ expression, as well as reduced Wnt signaling in the skeleton. 4-HNE activates FoxOs that in turn attenuate β-catenin/TCF-mediated transcription, as described above. Because TCF-mediated transcription suppresses PPARγ expression (56;57), inhibition of β-catenin/TCF transcriptional activity leads to an increase in PPARγ levels. Oxidized PUFAs promote PPARγ association with β-catenin and induce β-catenin degradation (50;58), thereby further decreasing β-catenin/TCF-mediated transcription (Fig. 3). Oxidized lipids also stimulate apoptosis of osteoblastic cells and inhibit BMP-2-induced osteoblast differentiation (59-61). Via these mechanisms, lipid oxidation contributes to the decline in osteoblast number and bone formation that occurs with aging.

Endogenous glucocorticoids contribute to the age-related loss of bone mass and strength

An increase in the production of endogenous glucocorticoids with age, as well as enhanced sensitivity of bone cells to glucocorticoids, represents another age-associated pathogenetic mechanism of involutional osteoporosis (62). Aging also decreased the volume of the bone vasculature and solute transport from the peripheral circulation to the lacunar-canalicular system. Furthermore, mice with osteoblast/osteocyte-specific transgenic expression of 11β-HSD2, the enzyme that inactivates glucocorticoids, are protected from the adverse effects of aging on osteoblast and osteocyte apoptosis, bone formation rate and microarchitecture, crystallinity, vasculature volume, interstitial fluid, and strength. Moreover, exposure to glucocorticoids suppressed angiogenesis in fetal metatarsals, as well as hypoxia-inducible factor 1α transcription and VEGF production in osteoblast and osteocyte cultured cells. This, and the evidence that dehydration of bone decreases strength, strongly suggests that endogenous glucocorticoids increase skeletal fragility in old age as a result of cell-autonomous effects on osteoblasts and osteocytes, leading to a decrease in bone angiogenesis, vasculature volume, and osteocyte lacunar-canalicular fluid.

Recently, we and others have found that glucocorticoids stimulate ROS production and FoxO activity (63-65). Indeed, suppression of oxidative stress reduces glucocorticoid-induced osteoblast apoptosis and FoxO activation *in vitro*. Moreover, in osteoblast progenitor cells, FoxOs mediate the inhibitory actions of glucocorticoids on Wnt signaling (Almeida M, Han L, and Manolagas S, unpublished data), suggesting that at least some of the deleterious actions of glucocorticoids on bone might be mediated by ROS (Fig. 3).

Outlook

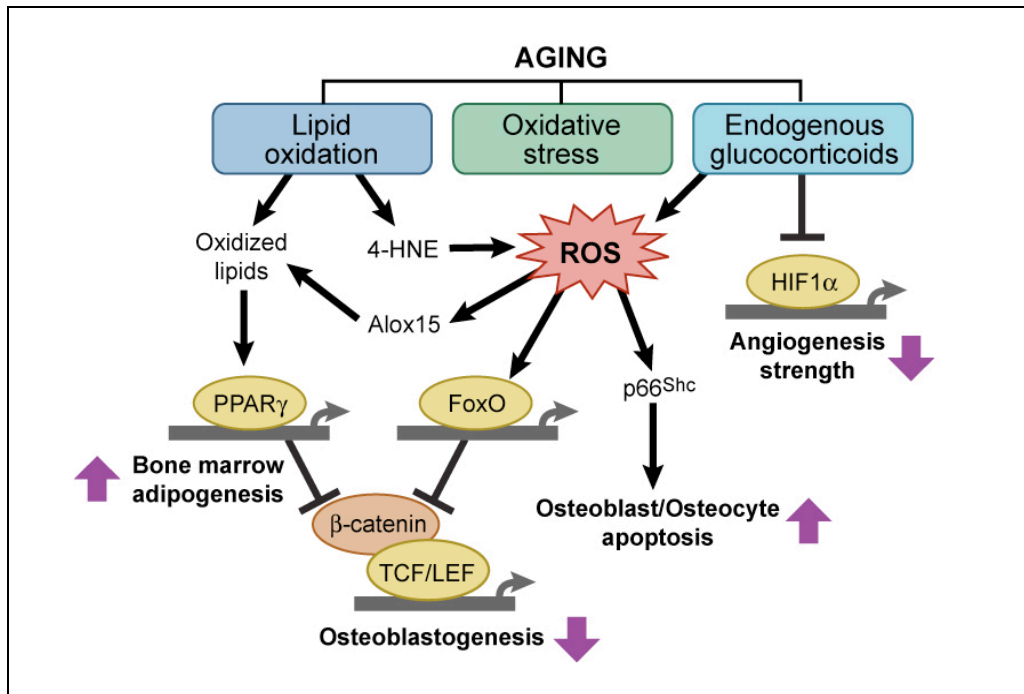Mitochondrial function and ROS have been consistently implicated in many diseases of

Fig. 3. Mechanisms that contribute to the development of involutional osteoporosis. Skeletal aging in mice is associated with increased oxidative stress, lipid oxidation, and sensitivity to endogenous glucocorticoids. Increased reactive oxygen species (ROS) induce osteoblast/osteocyte apoptosis via activation of p66[Shc] and also stimulate FoxO transcriptional activity. Activation of FoxOs decreases β-catenin/TCF-mediated transcription and osteoblastogenesis. Lipid oxidation via 4-HNE contributes to ROS generation. The expression of the lipoxygenase Alox15 is increased by ROS and feed forward on lipid oxidation. The oxidized lipids generated by this process bind to and activate PPARγ, further contributing to a decrease in β-catenin/TCF-induced osteoblastogenesis. Activation of PPARγ also leads to increased bone marrow adiposity. The increased glucocorticoid sensitivity of osteoblast/osteocytes with age leads to decreased bone angiogenesis, vasculature volume, and osteocyte lacunar-canalicular fluid, as well as decreased strength. Glucocorticoids also increase ROS levels in bone.

aging. However, conventional untargeted antioxidants have not demonstrated a clear benefit in human studies. It is now well-established that upregulation of the cellular antioxidant system protects cells from oxidative damage, but it also alters the redox signaling required for signal transduction initiated by many growth factors. Consistent with this, studies in *C. elegans* indicate that fine-tuning redox signaling to meet an optimal cellular response to certain ROS levels is of crucial importance to longevity (66). In mice, administration of NAC and/or catalase for 4 weeks did not increase bone mass or strength in aged mice, despite the ability of these agents to suppress oxidative stress and osteoblast apoptosis (10). Consistent with the evidence that the propagation of Wnt signaling is rapidly and transiently

stimulated by ROS (42), antioxidants exerted negative effects on osteoblastogenesis *in vivo*, and inhibited Wnt signaling *in vitro* (10;18). This mechanism might override the positive effect of antioxidants on Wnt/β-catenin signaling resulting from abrogation of ROS-induced FoxO activation (9;50). Perhaps more importantly, antioxidants inhibit the generation and activity of osteoclasts, leading to a reduction in bone remodeling (18;67). Thus, while effective in preventing the loss of bone mass in situations of high remodeling like in estrogen deficiency, conventional antioxidants do not restore bone mass in aged mice. Nevertheless, an emerging class of small-molecule antioxidants targeted to the mitochondria, which can mimic the activity of endogenous antioxidant enzyme systems and

subsequent physiological effects on pathways mediated by ROS, hold great promise for future therapeutic use in age-related diseases (68). In the meantime, the evidence demonstrating that ROS can decrease β-catenin/TCF signaling via FoxO activation suggests that the deleterious actions of oxidative stress in the skeleton are determined by specific signaling functions of ROS. Future studies targeting the pathways affected by ROS in bone cells should elucidate the molecular mechanisms that are relevant to age-associated skeletal involution, and may also identify novel drug targets for the prevention/treatment of age-related bone loss.

Acknowledgments

Conflict of Interest: None reported.

Peer Review: This article has been peer-reviewed.

References

1. Riggs BL, Melton LJ, Robb RA, Camp JJ, Atkinson EJ, McDaniel L, Amin S, Rouleau PA, Khosla S. A population-based assessment of rates of bone loss at multiple skeletal sites: evidence for substantial trabecular bone loss in young adult women and men. *J Bone Miner Res*. 2008 Feb;23(2):205-14.

2. Zebaze RM, Ghasem-Zadeh A, Bohte A, Iuliano-Burns S, Mirams M, Price RI, Mackie EJ, Seeman E. Intracortical remodelling and porosity in the distal radius and post-mortem femurs of women: a cross-sectional study. *Lancet*. 2010 May 15;375(9727):1729-36.

3. Lips P, Courpron P, Meunier PJ. Mean wall thickness of trabecular bone packets in the human iliac crest: changes with age. *Calcif Tissue Res.* 1978 Nov 10;26(1):13-7.

4. Parfitt AM. Bone-forming cells in clinical conditions. In: Hall BK, ed. *Bone. Volume 1. The Osteoblast and Osteocyte*. Boca Raton, FL: Telford Press and CRC Press; 1990:351-429.

5. Almeida M, Han L, Martin-Millan M, Plotkin LI, Stewart SA, Roberson PK, Kousteni S, O'Brien CA, Bellido T, Parfitt AM, Weinstein RS, Jilka RL, Manolagas SC. Skeletal involution by age-associated oxidative stress and its acceleration by loss of sex steroids. *J Biol Chem.* 2007 Sep 14;282(37):27285-97.

6. Syed FA, Mödder UI, Roforth M, Hensen I, Fraser DG, Peterson JM, Oursler MJ, Khosla S. Effects of chronic estrogen treatment on modulating age-related bone loss in female mice. *J Bone Miner Res*. 2010 May 17. [Epub ahead of print]

7. Halloran BP, Ferguson VL, Simske SJ, Burghardt A, Venton LL, Majumdar S. Changes in bone structure and mass with advancing age in the male C57BL/6J mouse. *J Bone Miner Res*. 2002 Jun;17(6):1044-50.

8. Iida H, Fukuda S. Age-related changes in bone mineral density, cross-sectional area and strength at different skeletal sites in male rats. *J Vet Med Sci*. 2002 Jan;64(1):29-34.

9. Almeida M, Han L, Martin-Millan M, O'Brien CA, Manolagas SC. Oxidative stress antagonizes Wnt signaling in osteoblast precursors by diverting beta-catenin from T cell factor- to forkhead box O-mediated transcription. *J Biol Chem*. 2007 Sep 14;282(37):27298-305.

10. Jilka RL, Almeida M, Ambrogini E, Han L, Roberson PK, Weinstein RS, Manolagas SC. Decreased oxidative stress and greater bone anabolism in the aged, as compared to the young, murine skeleton by parathyroid hormone. *Aging Cell*. 2010 Aug 4. [Epub ahead of print]

11. Harman D. Aging: a theory based on free radical and radiation chemistry. *J Gerontol.* 1956 Jul;11(3):298-300.

12. Muller FL, Lustgarten MS, Jang Y, Richardson A, Van Remmen H. Trends in oxidative aging theories. *Free Radic Biol Med.* 2007 Aug 15;43(4):477-503.

13. Balaban RS, Nemoto S, Finkel T. Mitochondria, oxidants, and aging. *Cell.* 2005 Feb 25;120(4):483-95.

14. Dröge W. Free radicals in the physiological control of cell function. *Physiol Rev.* 2002 Jan;82(1):47-95.

15. D'Autréaux B, Toledano MB. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat Rev Mol Cell Biol.* 2007 Oct;8(10):813-24.

16. Lambeth JD. NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol.* 2004 Mar;4(3):181-9.

17. Woo HA, Yim SH, Shin DH, Kang D, Yu DY, Rhee SG. Inactivation of peroxiredoxin I by phosphorylation allows localized H(2)O(2) accumulation for cell signaling. *Cell.* 2010 Feb 19; 140(4):517-28.

18. Lean JM, Davies JT, Fuller K, Jagger CJ, Kirstein B, Partington GA, Urry ZL, Chambers TJ. A crucial role for thiol antioxidants in estrogen-deficiency bone loss. *J Clin Invest.* 2003 Sep; 112(6):915-23.

19. Jagger CJ, Lean JM, Davies JT, Chambers TJ. Tumor necrosis factor-alpha mediates osteopenia caused by depletion of antioxidants. *Endocrinology.* 2005 Jan;146(1):113-8.

20. Hamada Y, Fujii H, Kitazawa R, Yodoi J, Kitazawa S, Fukagawa M. Thioredoxin-1 overexpression in transgenic mice attenuates streptozotocin-induced diabetic osteopenia: a novel role of oxidative stress and therapeutic implications. *Bone.* 2009 May;44(5):936-41.

21. Tyner SD, Venkatachalam S, Choi J, Jones S, Ghebranious N, Igelmann H, Lu X, Soron G, Cooper B, Brayton C, Hee Park S, Thompson T, Karsenty G, Bradley A, Donehower LA. p53 mutant mice that display early ageing-associated phenotypes. *Nature.* 2002 Jan 3;415(6867):45-53.

22. De Boer J, Andressoo JO, de Wit J, Huijmans J, Beems RB, van Steeg H, Weeda G, van der Horst GT, van Leeuwen W, Themmen AP, Meradji M, Hoeijmakers JH. Premature aging in mice deficient in DNA repair and transcription. *Science.* 2002 May 17; 296(5571):1276-9.

23. Manolagas SC. From estrogen-centric to aging and oxidative stress: a revised perspective of the pathogenesis of osteoporosis. *Endocr Rev.* 2010 Jun;31(3):266-300.

24. Trinei M, Berniakovich I, Beltrami E, Migliaccio E, Fassina A, Pelicci P, Giorgio M. P66Shc signals to age. *Aging (Albany NY).* 2009 Jun 5;1(6):503-10.

25. Giorgio M, Migliaccio E, Orsini F, Paolucci D, Moroni M, Contursi C, Pelliccia G, Luzi L, Minucci S, Marcaccio M, Pinton P, Rizzuto R, Bernardi P, Paolucci F, Pelicci PG. Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. *Cell.* 2005 Jul 29;122(2):221-33.

26. Almeida M, Han L, Ambrogini E, Bartell SM, Manolagas SC. Oxidative stress stimulates apoptosis and activates NF-kappaB in osteoblastic cells via a PKCbeta/p66shc signaling cascade: counter regulation by estrogens or androgens. *Mol Endocrinol.* 2010 Aug 4. [Epub ahead of print]

27. Trinei M, Giorgio M, Cicalese A, Barozzi S, Ventura A, Migliaccio E, Milia E, Padura IM, Raker VA, Maccarana M,

Petronilli V, Minucci S, Bernardi P, Lanfrancone L, Pelicci PG. A p53-p66Shc signalling pathway controls intracellular redox status, levels of oxidation-damaged DNA and oxidative stress-induced apoptosis. *Oncogene*. 2002 May 30;21(24):3872-8.

28. Lengner CJ, Steinman HA, Gagnon J, Smith TW, Henderson JE, Kream BE, Stein GS, Lian JB, Jones SN. Osteoblast differentiation and skeletal development are regulated by Mdm2-p53 signaling. *J Cell Biol.* 2006 Mar 13;172(6):909-21.

29. Wang X, Kua HY, Hu Y, Guo K, Zeng Q, Wu Q, Ng HH, Karsenty G, de Crombrugghe B, Yeh J, Li B. p53 functions as a negative regulator of osteoblastogenesis, osteoblast-dependent osteoclastogenesis, and bone remodeling. *J Cell Biol.* 2006 Jan 2;172(1):115-25.

30. Migliaccio E, Giorgio M, Mele S, Pelicci G, Reboldi P, Pandolfi PP, Lanfrancone L, Pelicci PG. The p66shc adaptor protein controls oxidative stress response and life span in mammals. *Nature*. 1999 Nov 18;402(6759):309-13.

31. de Keizer P, Burgering B, Dansen TB. FOXO as a sensor, mediator and regulator of redox signaling. *Antioxid Redox Signal*. 2010 Jul 14. [Epub ahead of print]

32. Ambrogini E, Almeida M, Martin-Millan M, Paik JH, Depinho RA, Han L, Goellner J, Weinstein RS, Jilka RL, O'Brien CA, Manolagas SC. FoxO-mediated defense against oxidative stress in osteoblasts is indispensable for skeletal homeostasis in mice. *Cell Metab*. 2010 Feb 3;11(2):136-46.

33. Rached MT, Kode A, Xu L, Yoshikawa Y, Paik JH, DePinho RA, Kousteni S. FoxO1 is a positive regulator of bone formation by favoring protein synthesis and resistance to oxidative stress in osteoblasts. *Cell Metab*. 2010 Feb 3;11(2):147-60.

34. Rached MT, Kode A, Silva BC, Jung DY, Gray S, Ong H, Paik JH, DePinho RA, Kim JK, Karsenty G, Kousteni S. FoxO1 expression in osteoblasts regulates glucose homeostasis through regulation of osteocalcin in mice. *J Clin Invest*. 2010 Jan 4;120(1):357-68.

35. Hoogeboom D, Essers MA, Polderman PE, Voets E, Smits LM, Burgering BM. Interaction of FOXO with beta-catenin inhibits beta-catenin/T cell factor activity. *J Biol Chem.* 2008 Apr 4;283(14):9224-30.

36. Bennett CN, Longo KA, Wright WS, Suva LJ, Lane TF, Hankenson KD, MacDougald OA. Regulation of osteoblastogenesis and bone mass by Wnt10b. *Proc Natl Acad Sci U S A*. 2005 Mar 1;102(9):3324-9.

37. Paik JH, Ding Z, Narurkar R, Ramkissoon S, Muller F, Kamoun WS, Chae SS, Zheng H, Ying H, Mahoney J, Hiller D, Jiang S, Protopopov A, Wong WH, Chin L, Ligon KL, DePinho RA. FoxOs cooperatively regulate diverse pathways governing neural stem cell homeostasis. *Cell Stem Cell*. 2009 Nov 6;5(5):540-53.

38. Shin SY, Chin BR, Lee YH, Kim JH. Involvement of glycogen synthase kinase-3beta in hydrogen peroxide-induced suppression of Tcf/Lef-dependent transcriptional activity. *Cell Signal.* 2006 May;18(5):601-7.

39. Lin CL, Wang JY, Ko JY, Surendran K, Huang YT, Kuo YH, Wang FS. Superoxide destabilization of beta-catenin augments apoptosis of high-glucose-stressed mesangial cells. *Endocrinology*. 2008 Jun;149(6):2934-42.

40. Shou J, Ali-Osman F, Multani AS, Pathak S, Fedi P, Srivenugopal KS. Human Dkk-1, a gene encoding a Wnt antagonist, responds to DNA damage and its overexpression sensitizes brain tumor cells to apoptosis following

alkylation damage of DNA. *Oncogene*. 2002 Jan 31;21(6):878-89.

41. Colla S, Zhan F, Xiong W, Wu X, Xu H, Stephens O, Yaccoby S, Epstein J, Barlogie B, Shaughnessy JD Jr. The oxidative stress response regulates DKK1 expression through the JNK signaling cascade in multiple myeloma plasma cells. *Blood*. 2007 May 15;109(10):4470-7.

42. Funato Y, Michiue T, Asashima M, Miki H. The thioredoxin-related redox-regulating protein nucleoredoxin inhibits Wnt-beta-catenin signalling through dishevelled. *Nat Cell Biol*. 2006 May;8(5):501-8.

43. Cheng SL, Shao JS, Halstead LR, Distelhorst K, Sierra O, Towler DA. Activation of vascular smooth muscle parathyroid hormone receptor inhibits Wnt/beta-catenin signaling and aortic fibrosis in diabetic arteriosclerosis. *Circ Res*. 2010 Jul 23;107(2):271-82.

44. Essers MA, de Vries-Smits LM, Barker N, Polderman PE, Burgering BM, Korswagen HC. Functional interaction between beta-catenin and FOXO in oxidative stress signaling. *Science*. 2005 May 20;308(5725):1181-4.

45. Zhang XF, Tan X, Zeng G, Misse A, Singh S, Kim Y, Klaunig JE, Monga SP. Conditional beta-catenin loss in mice promotes chemical hepatocarcinogenesis: Role of oxidative stress and platelet-derived growth factor receptor alpha/phosphoinositide 3-kinase signaling. *Hepatology*. 2010 Jun 15. [Epub ahead of print]

46. Seike M, Kondo T, Mori Y, Gemma A, Kudoh S, Sakamoto M, Yamada T, Hirohashi S. Proteomic analysis of intestinal epithelial cells expressing stabilized beta-catenin. *Cancer Res*. 2003 Aug 1;63(15):4641-7.

47. Muthusami S, Ramachandran I, Muthusamy B, Vasudevan G, Prabhu V, Subramaniam V, Jagadeesan A, Narasimhan S. Ovariectomy induces oxidative stress and impairs bone antioxidant system in adult rats. *Clin Chim Acta*. 2005 Oct;360(1-2):81-6.

48. Almeida M, Martin-Millan M, Ambrogini E, Bradsher R 3rd, Han L, Chen XD, Roberson PK, Weinstein RS, O'Brien CA, Jilka RL, Manolagas SC. Estrogens attenuate oxidative stress and the differentiation and apoptosis of osteoblasts by DNA-binding-independent actions of the ERalpha. *J Bone Miner Res*. 2010 Apr;25(4):769-81.

49. Martin-Millan M, Almeida M, Ambrogini E, Han L, Zhao H, Weinstein RS, Jilka RL, O'Brien CA, Manolagas SC. The estrogen receptor-alpha in osteoclasts mediates the protective effects of estrogens on cancellous but not cortical bone. *Mol Endocrinol*. 2010 Feb;24(2):323-34.

50. Almeida M, Ambrogini E, Han L, Manolagas SC, Jilka RL. Increased lipid oxidation causes oxidative stress, increased peroxisome proliferator-activated receptor-gamma expression, and diminished pro-osteogenic Wnt signaling in the skeleton. *J Biol Chem*. 2009 Oct 2;284(40):27438-48.

51. Schneider C, Porter NA, Brash AR. Routes to 4-hydroxynonenal: fundamental issues in the mechanisms of lipid peroxidation. *J Biol Chem*. 2008 Jun 6;283(23):15539-43.

52. Klein RF, Allard J, Avnur Z, Nikolcheva T, Rotstein D, Carlos AS, Shea M, Waters RV, Belknap JK, Peltz G, Orwoll ES. Regulation of bone mass in mice by the lipoxygenase gene Alox15. *Science*. 2004 Jan 9;303(5655):229-32.

53. Akune T, Ohba S, Kamekura S, Yamaguchi M, Chung UI, Kubota N, Terauchi Y, Harada Y, Azuma Y, Nakamura K, Kadowaki T, Kawaguchi H. PPARgamma insufficiency enhances osteogenesis through osteoblast formation from bone marrow

progenitors. *J Clin Invest*. 2004 Mar;113(6):846-55.

54. Grey A. Skeletal consequences of thiazolidinedione therapy. *Osteoporos Int*. 2008 Feb;19(2):129-37.

55. Seiler A, Schneider M, Förster H, Roth S, Wirth EK, Culmsee C, Plesnila N, Kremmer E, Rådmark O, Wurst W, Bornkamm GW, Schweizer U, Conrad M. Glutathione peroxidase 4 senses and translates oxidative stress into 12/15-lipoxygenase dependent- and AIF-mediated cell death. *Cell Metab*. 2008 Sep;8(3):237-48.

56. Kang S, Bennett CN, Gerin I, Rapp LA, Hankenson KD, MacDougald OA. Wnt signaling stimulates osteoblastogenesis of mesenchymal precursors by suppressing CCAAT/enhancer-binding protein alpha and peroxisome proliferator-activated receptor gamma. *J Biol Chem*. 2007 May 11;282(19):14515-24.

57. Okamura M, Kudo H, Wakabayashi K, Tanaka T, Nonaka A, Uchida A, Tsutsumi S, Sakakibara I, Naito M, Osborne TF, Hamakubo T, Ito S, Aburatani H, Yanagisawa M, Kodama T, Sakai J. COUP-TFII acts downstream of Wnt/beta-catenin signal to silence PPARgamma gene expression and repress adipogenesis. *Proc Natl Acad Sci U S A*. 2009 Apr 7;106(14):5819-24.

58. Sharma C, Pradeep A, Wong L, Rana A, Rana B. Peroxisome proliferator-activated receptor gamma activation can regulate beta-catenin levels via a proteasome-mediated and adenomatous polyposis coli-independent pathway. *J Biol Chem*. 2004 Aug 20;279(34):35583-94.

59. Brodeur MR, Brissette L, Falstrault L, Ouellet P, Moreau R. Influence of oxidized low-density lipoproteins (LDL) on the viability of osteoblastic cells. *Free Radic Biol Med*. 2008 Feb 15;44(4):506-17.

60. Klein BY, Rojansky N, Ben-Yehuda A, Abou-Atta I, Abedat S, Friedman G. Cell death in cultured human Saos2 osteoblasts exposed to low-density lipoprotein. *J Cell Biochem*. 2003 Sep 1;90(1):42-58.

61. Huang MS, Morony S, Lu J, Zhang Z, Bezouglaia O, Tseng W, Tetradis S, Demer LL, Tintut Y. Atherogenic phospholipids attenuate osteogenic signaling by BMP-2 and parathyroid hormone in osteoblasts. *J Biol Chem*. 2007 Jul 20;282(29):21237-43.

62. Weinstein RS, Wan C, Liu Q, Wang Y, Almeida M, O'Brien CA, Thostenson J, Roberson PK, Boskey AL, Clemens TL, Manolagas SC. Endogenous glucocorticoids decrease skeletal angiogenesis, vascularity, hydration, and strength in aged mice. *Aging Cell*. 2010 Apr;9(2):147-61.

63. Almeida M, Ambrogini E, Martin-Millan M, Han L, Warren A, Shelton RS, Plotkin L, Bellido T, O'Brien CA, Jilka RL, Weinstein RS, Manolagas SC. Induction of oxidative stress and diversion of beta-catenin from TCF- to FOXO-mediated transcription by glucocorticoids or TNFalpha in osteoblastic cells. *J Bone Miner Res*. 2008 Sep;23(Suppl 1):S170.

64. Houstis N, Rosen ED, Lander ES. Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature*. 2006 Apr 13;440(7086):944-8.

65. Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, Walsh K, Schiaffino S, Lecker SH, Goldberg AL. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell*. 2004 Apr 30;117(3):399-412.

66. Honda Y, Tanaka M, Honda S. Modulation of longevity and diapause by redox regulation mechanisms under the insulin-like signaling control in

Caenorhabditis elegans. *Exp Gerontol*. 2008 Jun;43(6):520-9.

67. Lean JM, Jagger CJ, Kirstein B, Fuller K, Chambers TJ. Hydrogen peroxide is essential for estrogen-deficiency bone loss and osteoclast formation. *Endocrinology*. 2005 Feb;146(2):728-35.

68. Wanagat J, Dai DF, Rabinovitch P. Mitochondrial oxidative stress and mammalian healthspan. *Mech Ageing Dev*. 2010 Jun 8. [Epub ahead of print]