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Regulation of Adult Bone Turnover by Sex Steroids

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Abstract

Recent reports reveal increasing complexity of mechanisms underlying the bone sparing effects of sex steroids. This review focuses on mechanisms by which sex steroids attenuate endocortical and trabecular adult bone turnover, perhaps their most important property as bone mass regulators. Clearly, estrogen withdrawal increases osteoclast number and bone resorption; however, important open questions are the extent to which osteoblasts and their precursors are involved, and the relative contributions of the RANK/RANKL/OPG system, Fas ligand and Runx2. In addition to reviewing these aspects of estrogen action, we also discuss proskeletal effects of androgens on the adult male skeleton, including aromatization to estrogens and male-specific mechanisms. Detailed understanding of skeletal site- and gender-dependent mechanisms by which sex steroids protect the adult skeleton will provide the foundation for improved risk assessment, prevention and management of osteoporosis.

After decades of work on the bone sparing effects of sex steroids, discoveries are still continuously made, which provide new insight into the underlying mechanisms and offer opportunities for the development of therapeutic approaches to hypogonadism-induced osteoporosis. Here, we review and put in context recent findings regarding the bone-sparing

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effects of estrogens and androgens. We limit the review to adult trabecular and endocortical bone turnover, and do not discuss mechanisms by which sex steroids regulate longitudinal bone growth (reviewed in Vanderschueren et al., 2004) or periosteal expansion (reviewed in Vanderschueren et al., 2006). We also limit the discussion to cells of the osteoblast and osteoclast lineages, although other cell types, in particular immune cells, are also involved in the proskeletal properties of sex steroids (reviewed in Lorenzo et al., 2008).

Estrogens Attenuate Bone Turnover

Maintenance of bone tissue by estrogens has been attributed to both their anti-resorptive and anabolic effects (Riggs et al., 2002; Syed and Khosla, 2005). However, loss of bone mass in the first few years after menopause is associated not only with increased bone resorption but also with increased – not decreased – bone formation (Riggs et al., 2002; Syed and Khosla, 2005). Although estrogens have been shown to promote osteoblast commitment and survival (Khosla, 2008), multiple studies with women and animal models of postmenopausal osteoporosis documented increases in both bone resorption and bone formation based on serum and urinary markers as well as direct histological assessment of bone turnover (Gorai et al., 1997; Weitzmann et al., 2002; Eghbali-Fatourehchi et al., 2003; Usui et al., 2004). Thus, the present review focuses on the attenuation of bone turnover by estrogens and the increased bone turnover that follows menopause.

A critical question remains: which are the primary and which are the secondary effects of estrogens in keeping bone turnover in check? Do estrogens primarily restrain proliferation and differentiation of cells in the monocyte/osteoclast lineage, with osteoblastic cells following suit via coupling mechanisms? Or, do estrogens primarily restrain proliferation and differentiation of cells in the osteoblast lineage, with attenuation of monocytes/osteoclasts as a secondary effect? A related but distinct issue is the identification of the cell type(s) in which estrogens activate estrogen receptor α (ER α)—the main receptor involved in their proskeletal properties (Lindberg et al., 2002; Sims et al., 2003)—and the downstream molecular events that ultimately restrain bone turnover and preserve bone mass.

Estrogens Attenuate Bone Turnover Via Regulation of Cell Proliferation in the Osteoblast Lineage

Although estrogen withdrawal clearly results in increased osteoclast number and accelerated bone resorption, early studies by Manolagas and colleagues (Jilka et al., 1998; Di Gregorio et al., 2001) suggested that the anti-resorptive action of estrogens was rooted in cells of the osteoblast lineage. Fibroblast and osteoblast colony formation assays using bone marrow stromal cells from ovariectomized (OVX) mice and mice receiving estradiol after OVX, suggested that the anti-resorptive effect of estrogens was mediated by attenuating cell proliferation early in the osteoblast lineage (Jilka et al., 1998; Di Gregorio et al., 2001). Furthermore, the effect of estrogen withdrawal on the marrow stromal cells persisted under conditions that suppressed osteoclastogenesis (Jilka et al., 1998). Thus, although bone multicellular units (BMU) initiate the microanatomical cycle of bone turnover with osteoclastic bone resorption, the regulation of bone turnover was assigned to osteoblast progenitors, with estrogens keeping the regulatory process in check. According to this

concept, loss of estrogen unleashes the otherwise restrained proliferation and differentiation of mesenchymal progenitors, and the stimulated mesenchymal cells go on to fuel excessive bone turnover, a major contributor to bone loss under these conditions.

Estrogens Attenuate Osteoclast Differentiation and Survival Via Effects on Mesenchymal Cells

Within the osteoblast lineage, estrogens not only attenuate proliferation of early osteoblast progenitors (Jilka et al., 1998), but also influence cell-cell communications by which mesenchymal cells regulate osteoclastogenesis and bone resorption. Below we describe estrogen-responsive molecular mechanisms that regulate osteoclast differentiation and survival via osteoblasts and their precursors.

FASL

An important means by which mesenchymal cells regulate bone resorption is the production of Fas Ligand (FASL), which in turn binds to pro-apoptotic Fas receptors on osteoclasts. Treatment of osteoblasts with estradiol resulted in increased FASL expression and accelerated apoptosis of co-cultured osteoclasts (Krum et al., 2008). The enhancement of FASL expression was mediated by binding of ER α to an enhancer located 86-kb downstream of the FASL transcription start site (Krum et al., 2008). Earlier studies suggested that estrogens stimulated osteoclast apoptosis in a cell autonomous manner (Kameda et al., 1997) and that this occurred via stimulation of FASL expression in the osteoclast itself (Nakamura et al., 2007; Imai et al., 2009). Whereas a role for FASL is undisputed, the work described above (Krum et al., 2008) argues that mesenchymal cells are required, based on the inability of pure osteoclast cultures to respond to estrogens. Either way, an important mechanism underlying the proskeletal effect of estrogens has been established: they stimulate FASL expression (likely in mesenchymal cells) leading to promotion of osteoclast apoptosis, thereby limiting the number and thus the activity of osteoclasts. Indeed, estradiol did not induce osteoclast apoptosis in vitro in the presence of FASL neutralizing antibody, or when the osteoclasts were derived from mice with defective FASL signaling (Krum et al., 2008). Furthermore, such mice did not suffer bone loss after ovariectomy (Nakamura et al., 2007).

RANKL and OPG

Whereas Fas/FASL is the pathway most recently implicated in the bone-sparing effect of estrogens, previous studies provided ample evidence for the involvement of osteoblastic RANKL and its osteoclastic receptor RANK, activation of which is critical for osteoclast differentiation and function. Most notably, estradiol attenuates RANK/RANKL signaling by strongly stimulating OPG biosynthesis in osteoblasts (Hofbauer et al., 1999a). In addition, postmenopausal women have increased RANKL expression in pre-osteoblasts (as well as T- and B-cells) as compared either to premenopausal women or postmenopausal women on estrogen therapy (Eghbali-Fatourehchi et al., 2003). Inhibition of osteoclastogenesis by estrogens was earlier attributed to reduced expression of proinflammatory cytokines including IL-1, IL-6, TNF α , M-CSF and PGE2, some of which function upstream of RANK/RANKL (Pacifci, 1996; Manolagas, 2000).

Runx2

Runx2 is the master regulator of osteoblast differentiation and bone formation (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997). Besides this classical role, Runx2 also promotes osteoblast-mediated osteoclastogenesis and bone resorption through stimulation of genes such as M-CSF, RANKL and MMP-13, (Liu et al., 2001; Geoffroy et al., 2002; Enomoto et al., 2003; Maruyama et al., 2007). It appears, therefore, that Runx2 activity has to be well regulated in order to support normal bone metabolism; sufficient Runx2 activity is necessary to promote bone formation, but this activity should not exceed certain limits (Fig. 1). Indeed, the activity of Runx2 is tightly regulated by a panel of co-repressors (Westendorf, 2006). A recent addition to this panel is ER α (Khalid et al., 2008). Thus, estrogens plausibly attenuate bone turnover, at least in part, by keeping Runx2 activity in check. At menopause, unleashing of Runx2 would then contribute to excessive bone resorption and loss of bone mass (Fig. 1).

Given its classical role in osteoblast differentiation and bone formation (Komori et al., 1997; Otto et al., 1997), the initial finding of increased bone resorption in mice whose osteoblasts overexpress Runx2 was unexpected (Liu et al., 2001). Initially, the increased bone resorption was attributed to abnormal composition of the bone matrix produced by the Runx2-overexpressing osteoblasts (Liu et al., 2001). However, a similar study published soon thereafter, with similar in vivo results, was accompanied by co-culture assays, which demonstrated increased ex vivo osteoclastogenic activity by Runx2-overexpressing osteoblasts (Geoffroy et al., 2002). This was the first clear demonstration of stimulated osteoclastogenesis by osteoblastic Runx2. Runx2-mediated osteoclastogenesis in a coculture assay, was demonstrated again more recently by viral transduction of osteoblasts with Runx2 (Maruyama et al., 2007). Moreover, these authors also over-expressed a Runx2 dominant negative (DN) form in osteoblasts, which resulted in a mirror effect with respect to wild type Runx2, namely reduced osteoclastogenesis in co-culture assays and reduced osteoclast number and increased bone mass in vivo (Maruyama et al., 2007). The investigators went on to show that overexpression of the Runx2-DN form rendered mice resistant to OVX-induced high turnover bone loss (Maruyama et al., 2007). Physiologically, estrogens may fulfill a similar function by activating ER α , which then directly interacts with and inhibits Runx2 (Khalid et al., 2008).

In summary, we discussed two concepts by which cells of the osteoblast lineage mediate proskeletal effects of estrogens: (1) estrogens restrain proliferation of early osteoblast progenitors, hence estrogen withdrawal results in accelerated proliferation of these mesenchymal cells, which in turn fuels excessive bone turnover; and (2) estrogens restrain signals emanating from cells of the osteoblast lineage that promote osteoclastogenesis and increase the osteoclast lifespan; therefore, after menopause the same number of osteoblast progenitors will more efficiently promote osteoclast formation and survival. Both these mechanisms (which are not mutually exclusive) ascribe the antiresorptive property of estrogens to ER signaling within osteoblasts and their progenitors.

Proskeletal Properties of Estrogens Require ER α Signaling in Osteoclasts

Female mice with osteoclast-specific ablation of ER α have a high bone turnover and low trabecular bone mass (Nakamura et al., 2007). This seminal report introduced a paradigm shift by ascribing the proskeletal effect of estrogens, at least in part, to cells in the osteoclast lineage. The osteoclast-specific ER α knockout mice do not suffer further trabecular bone loss at least until 2 weeks after OVX (but they do later), and they do not benefit from estrogen replacement (Nakamura et al., 2007; Millan et al., 2009). Interestingly, cortical bone in female and both trabecular and cortical bone in male mice lacking osteoclastic ER α were not affected by estrogen deprivation (see below).

In osteoclasts, activated ER α attenuates cell proliferation and thus bone resorption through several possible mechanisms. As described above, it is still unclear to what extent transcriptional activation of FASL is responsible for a cell autonomous pro-apoptotic activity of estradiol in osteoclasts (Nakamura et al., 2007; Krum et al., 2008; Imai et al., 2010). Other, yet unknown target genes with ERE-containing promoters and enhancers may be involved. Alternatively, ER α may attenuate expression of genes in osteoclasts in a non-classical fashion, for example by inhibiting NF κ B (Ray et al., 1994), or by antagonizing JNK activity, thus limiting RANKL-mediated activation of AP-1 target genes (Srivastava et al., 2001). Be that as it may, the unexpected outcomes of osteoclast-specific ER α gene ablation (Nakamura et al., 2007) certainly set a novel concept in the field: ER α signaling in osteoclasts is involved in the proskeletal property of estrogens in females.

The landmark discovery of a requirement for ER α signaling in osteoclasts raised the question of whether the new paradigm should replace the old one. In other words, how complete is the evidence assigning the proskeletal role of estrogens to ER α signaling in cells of the osteoblast lineage? To date, only meeting abstracts have reported adverse effects of ER α ablation specifically in osteoblasts and their precursors (Lagerquist et al., 2008; Kondoh et al., 2009). Completion of these and other studies may lead to a unitary picture, whereby proskeletal properties of estrogens are assigned to ER α signaling in cells of both the osteoblast and the osteoclast lineages.

Androgens Protect the Skeleton Via Aromatization to Estrogens

The impact of androgens on bone turnover has been attributed to both aromatization-dependent effects mediated by ER α and aromatization-independent effects mediated by the Androgen Receptor (AR). The view ascribing bone-sparing effects of sex hormones to ER activation in females and AR activation in males was shattered with the landmark report of osteoporosis in a man with a mutation in the ER α gene (Smith et al., 1994) and the similar phenotype later described in men with mutations in the CYP19 gene (Bouillon et al., 2004; Gennari et al., 2004; Rochira et al., 2007; Lanfranco et al., 2008), whose product, aromatase, is the enzyme responsible for converting testosterone to estradiol. Men deficient in aromatase activity respond well to estrogen treatment, with suppression of bone resorption and increased bone mass (Carani et al., 1997; Bilezikian et al., 1998), whereas treatment with androgens has little to no effects (Carani et al., 1997). Similar to the clinical cases, male mice deficient in aromatase (ArKO) display a high bone turnover and low bone mass

phenotype, which can be corrected by administration of estradiol (Oz et al., 2000; Miyaura et al., 2001). Both the “experiments of nature” and those with the ArKO mice highlight the aromatase-dependent proskeletal action of androgens.

Two groups investigated the role of aromatization in male bone turnover by experimentally treating healthy men with combinations of a GnRH analog (to induce hypogonadism) along with sex steroids and an aromatase inhibitor, thereby selectively restoring AR or ER signaling (Falahati-Nini et al., 2000; Leder et al., 2003). Based on serum and urinary markers of bone resorption and formation, both studies demonstrated that bone metabolism in the hypogonadal men was corrected to the highest degree when both androgen and estrogen signaling were restored. These results suggest that androgens preserve bone mass in adult men by activation of both AR and ER α . However, there were differences between the two studies, for example, the subjects’ age, which resulted in closer-to-normal bone metabolism with restoration of *estrogen* signaling in the study employing older men (Falahati-Nini et al., 2000) versus closer-to-normal bone metabolism with restoration of *androgen* signaling in the study employing younger men (Leder et al., 2003). Therefore, androgens may restrain bone turnover in older men primarily through aromatization and activation of ER α , while activation of AR may be more important early in life (Khosla and Riggs, 2003). The importance of ER signaling is further supported by recent clinical studies, suggesting a minimal threshold level of estradiol for the maintenance of bone mass and the prevention of fractures in men (Mellstrom et al., 2008; LeBlanc et al., 2009; Vandenput and Ohlsson, 2009; Khosla, 2010).

Aromatase-Independent Proskeletal Effects of Androgens: Evidence From Animal Models

As discussed above, the increased bone turnover and decreased bone mass in aromatase deficient male mice demonstrate the importance of estrogen signaling in protecting the male skeleton (Oz et al., 2000; Miyaura et al., 2001). Interestingly, however, bone loss between 9 and 32 weeks of age is less severe in the male as compared to the female knockout mice (where aromatase is also necessary for the conversion of testosterone to estradiol) (Miyaura et al., 2001). As is the case with the human studies, this observation again suggests that androgen signaling in males provides an additional mechanism of skeletal protection, especially in young adults. Earlier studies demonstrated that testosterone could protect against orchietomy-induced bone loss even when ER α activation was not possible due to pharmacological blockade (Vandenput et al., 2002) or ER α gene ablation (Vandenput et al., 2001). Moreover, orchietomy in ArKO mice resulted in further bone resorption and further bone loss beyond the adverse effects of aromatase deficiency alone (Matsumoto et al., 2006). These studies show that at least in mice, androgens significantly attenuate bone turnover and preserve bone mass independently of aromatization.

Conditional ablation of the AR in bone was recently described, providing direct evidence for AR-mediated proskeletal effects of androgens. Interpretation of earlier mouse studies, in which AR was globally deficient, was limited because the mice had low levels of circulating testosterone (Yeh et al., 2002; Kawano et al., 2003; Vandenput et al., 2004), and thus the

observed increase in bone turnover and decreased bone mass were equally attributable to loss of ER signaling and loss of AR signaling. In contrast, sex organs and thus circulating testosterone were normal when the AR was ablated specifically in osteoblasts, allowing the investigators to attribute the observed phenotype to loss of AR signaling in bone. When AR was conditionally ablated by crossing AR-floxed with $\alpha 1(I)$ Collagen-Cre mice ($\alpha 1(I)$ -Cre;AR^{f/f}), the bone loss could be attributed to decreased AR signaling in osteoblasts (Notini et al., 2007). Based on the reduction in trabecular number, the authors concluded that the bone loss was due to increased resorption (Notini et al., 2007). Interestingly, this phenotype was age dependent at the distal femur, again pointing to an age-dependent contribution of AR signaling to the control of bone metabolism (Notini et al., 2007). Despite minor differences with respect to the $\alpha 1(I)$ -Cre;AR^{f/f} mice (Notini et al., 2007), crossing the AR-floxed mice with Osteocalcin-Cre mice also resulted in bone loss, most likely secondary to increased bone turnover (Chiang et al., 2009). This skeletal phenotype was mirrored in male mice with osteoblast-specific AR overexpression, which exhibited reduced bone turnover and increased bone mass (Wiren et al., 2004).

Molecular Mechanisms by Which AR Restrains Bone Turnover

Much like ER α in adult women and men, the bone-sparing effects of the AR in adult men are thought to be exerted primarily by keeping bone turnover in check. This is likely mediated by AR signaling in cells of the osteoblast lineage, as suggested by the conditional ablation of AR in $\alpha 1(I)$ collagen-and osteocalcin-expressing cells (Notini et al., 2007; Chiang et al., 2009). The question of whether AR signaling in osteoclasts also plays a role in the bone-sparing effect of androgens awaits results from mice with osteoclast-specific AR knockout. With regard to AR signaling in osteoblasts, and similar to the case of ER α , a key question remains. Does AR in males simply keep at bay the proliferation of osteoblastic cells, which otherwise fuel excessive bone remodeling? Or, does it also restrain osteoclastogenic signals emanating from osteoblasts and their precursors? In favor of the latter scenario, osteoclastogenesis was increased in co-cultures containing AR-deficient compared to wild type osteoblasts (Kawano et al., 2003).

In contrast to estrogens, androgens do not stimulate FASL expression, and male mice are not affected by osteoclast-specific ER α ablation (Nakamura et al., 2007), suggesting that both ER α and AR employ other molecular mechanisms to restrain bone turnover and maintain bone mass in males. Among such mechanisms, AR signaling in osteoblasts inhibits RANKL expression and IL6 production (Bellido et al., 1995; Hofbauer et al., 1999b; Kawano et al., 2003). The effect of AR activation on OPG expression remains controversial as both stimulation and repression have been reported (Hofbauer et al., 2002; Chen et al., 2004; Wiren et al., 2004). How AR inhibits the expression of these and/or other osteoblast-derived osteoclastogenic factors is not well understood. Among potential underlying mechanisms is one similar to that proposed above for ER α , namely inhibition of Runx2, a regulator of not only bone formation but also osteoblast-driven osteoclastogenesis (Liu et al., 2001; Geoffroy et al., 2002; Enomoto et al., 2003; Maruyama et al., 2007). Indeed, similar to ER α (Khalid et al., 2008), AR also directly interacts with and inhibits Runx2 (Baniwal et al., 2009). Both these inhibitory activities are tightly regulated, presumably allowing Runx2 to stimulate expression of bone anabolic genes during specific phases of osteoblast differentiation, while

keeping Runx2 in check when it promotes expression of osteoclastogenic genes during other stages of differentiation. Notably, the interaction of Runx2 with AR is stronger than that with ER α as is the resulting inhibition of Runx2 (Khalid et al., 2008; Baniwal et al., 2009). Possibly related to this, Runx2's PST domain is sufficient for interaction with ER α (Khalid et al., 2008), whereas interaction with AR occurs through simultaneous contacts with the PST and the DNA-binding domain of Runx2 (Baniwal et al., 2009; Fig. 2). Furthermore, unlike ER α , the interaction of AR with Runx2 in solution occurs in the absence of ligand. In the living cell, however, ligand is required in order to translocate the AR into the nucleus, where it then binds and inhibits Runx2 (Baniwal et al., 2009). Thus, in addition to activation of ER α after aromatization to estradiol, androgens have the capacity to restrain Runx2 activity and thus bone turnover by activation of the AR.

Conclusion

With the recent discovery of mechanisms underlying the bone-sparing effects of sex steroids, the picture appears increasingly complex. A recent breakthrough is the discovery of the central role of osteoclastic ER α in the anti-resorptive effect of estrogens in females, but interestingly not in males (Nakamura et al., 2007). Nevertheless, the old, simple concept of 'estrogens for women, androgens for men' is long shattered. Instead, aromatase-driven estradiol is now thought to be the predominant player in male bone mass regulation, with aromatization-independent AR action taking a back, yet still comfortable seat. It is important to remember the complexity and limitations of animal models from which we extract much of our knowledge. For example, the contribution of aromatase-independent androgen-mediated regulation of bone turnover is significantly more important in mice than in men (Carani et al., 1997; Oz et al., 2000; Miyaura et al., 2001; Khosla, 2007). In addition to species-dependence, the molecular mechanisms responsible for the bone-sparing effects of estrogens and androgens vary as a function of gender, age, skeletal site and the bone compartment. For instance, the dramatic role of osteoclastic ER α signaling in regulating trabecular bone turnover (Nakamura et al., 2007) is restricted to females. Sexual differences in the skeletal response to estrogens are further emphasized by preliminary results from mice with osteoblastic ER α knockout (Lagerquist et al., 2008; Kondoh et al., 2009). While estrogens affect trabecular bone primarily through *osteoclastic* ER α in females, their effect is mainly mediated by *osteoblastic* ER α in males (Nakamura et al., 2007; Kondoh et al., 2009). Bone compartment specificity is also demonstrated by the unaffected endocortical bone in osteoclastic ER α -KO mice (Nakamura et al., 2007). It appears therefore that estrogens inhibit endocortical bone resorption through osteoblastic ER signaling (Lagerquist et al., 2008). Interestingly, the endocortical bone surface is the very site that is specifically affected by over expression of Runx2 in osteoblasts (Liu et al., 2001; Geoffroy et al., 2002). This cellular and anatomical link between osteoblastic ER α signaling and Runx2-mediated bone resorption strongly supports the idea that unleashing of Runx2 activity upon estrogen loss (Khalid et al., 2008) is preferentially involved in postmenopausal endocortical bone resorption.

The complex bone phenotypes of newly developed mouse models may reflect a variety of molecular mechanisms selectively operative in different bone compartments, genders, ages and species. Understanding these complexities will offer opportunities for the development

of novel approaches to treat osteoporosis. Early investigations of mouse models with global genetic ablation of receptors and enzymes involved in sex steroid action highlighted the limitations of such models in bone research, primarily due to the indirect effects of such genetic lesions through extraskeletal tissues (Syed and Khosla, 2005; Ohlsson and Vandenput, 2009). While evaluating the results of the more sophisticated tissue-specific knockout mouse models, we must keep in mind that these models too are complex and not without their own limitations. For example, the receptors are knocked out during early developmental stages, which may confound the interpretation of the results in the context of adult bone metabolism and their implementation to the understanding and the development of therapies for adult bone metabolic disease. Thus, along with expansion of our in vivo work, we should continue expanding our knowledge of sex steroid action using cell culture models, as well as biochemical and molecular approaches to bone biology. The results of such studies would then be tested in animal models and human subjects while paying close attention to lessons learned from mouse genetics.

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Fig. 1.
Proposed model for a biphasic relationship between bone mass and Runx2 activity.

Fig. 2.

Comparison between ER α /Runx2 and AR/Runx2 interaction. Both ER α and AR interact with Runx2 via their respective DNA-binding domains (DBD). However, the interactions require different domains of Runx2 as shown by the thick horizontal lines (Khalid et al., 2008; Baniwal et al., 2009). Z1 and Z2 represent the zinc fingers at the DBDs of ER α and AR.