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Direct Transcriptional Targets of Sex Steroid Hormones in Bone

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Abstract

The sex steroid hormones, androgens and estrogens, via their respective nuclear receptors, regulate bone mineral density in humans and mice. Very little is known about the direct targets of the androgen and estrogen receptors in bone cells. First, models of hormone and receptor deficiency in mouse and human bone are discussed. This review then focuses on the direct targets of the receptors in osteoblasts and osteoclasts. A direct target of a NR is defined here as a gene that is regulated by NR binding to the DNA (either through DNA binding or association with a DNA binding protein) at an enhancer or promoter of that gene. The experimental evidence that illustrates androgen and estrogen gene regulation in osteoblasts and osteoclasts will be summarized and compared with the phenotype of the hormones *in vivo*.

Osteoporosis is a disease characterized by low bone mass due to increased osteoclast activity, and/or decreased osteoblast activity, which leads to an increased risk of fractures. The disease is multi-factorial and low calcium intake, low vitamin D levels and low sex steroid hormone levels are correlated with the disease. Over 10 million people in the United States have osteoporosis and an additional 33.6 million individuals in the US have osteopenia, or low bone mass, that could lead to osteoporosis. In 1940 Fuller Albright described postmenopausal osteoporosis and proposed that it was due to estrogen deficiency [Albright et al., 1940]. We now know that both estrogens and androgens are important in the development of bone and maintenance of bone mineral density throughout life.

The sex steroid hormones bind to their cognate steroid hormone nuclear receptors (NRs) to activate transcription of target genes. Estrogens bind to estrogen receptors (ERα and ERβ) and androgens bind to the androgen receptor (AR). Recently, 17β-estradiol (E2) has also been shown to bind to GPR30 to regulate intracellular calcium [Revankar et al., 2005]. Estrogen responsive genes are best-studied in breast cancers and androgen responsive genes are likewise best-studied in prostate cancers; however, the hormone responsive genes in the bone are relatively unknown. ERα, ERβ and AR have each been detected by several methods in both osteoblasts and osteoclasts [Bord et al., 2001; Braidman et al., 2001], but very little is known about their mechanism of action. In this review the genes directly regulated by estrogens and androgens will be described.

Genetic Models of ERα in Bone

Estrogens are important in the development of bone in both mice and humans, as evidenced by genetic mouse models and case reports of human mutations. A man with a mutation in ERα had a lack of full bone maturation with a failure of epiphyseal closure and osteoporosis at age 28 [Smith et al., 1994]. Genetic mouse models have demonstrated that both ERs are important for proper bone mineral density. Female and male ERα null mice (ERαKO) have a

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decrease in cortical bone mineral density, whereas ERβ knockout (ERβKO) mice have normal cortical and cancellous bone mineral density. Mice without either ERα or ERβ (ERαβKO) have both decreased cortical and decreased cancellous bone mineral density, suggesting that ERα and ERβ can replace each other in cancellous bone, but they also have distinct roles [Windahl et al., 2002].

Because of the interaction between the many tissues in which ERα plays a role, ERα was specifically knocked out in osteoclasts ($ER\alpha^{\Delta Oc/\Delta Oc}$) using a cathepsin K-cre recombinase [Nakamura et al., 2007]. The female $ER\alpha^{\Delta Oc/\Delta Oc}$ mice have a decrease in trabecular bone, but not in cortical bone, as do the ERαKO mice. The molecular mechanism explaining the difference in phenotype between the ERαKO and the $ER\alpha^{\Delta Oc/\Delta Oc}$ bones remains to be determined. Confirming the results of Nakamura, et al., Martin-Millan, et al., knocked out ERα in osteoclast precursors using the promoter of lysozyme M (LysM); the $ER\alpha^{LysM-/-}$ mice also have a decrease in trabecular bone mineral density [Martin-Millan et al., 2010]. An osteoblast-specific ERαKO mouse has not been generated, but would provide valuable information to determine the role of ERα (vs. ERβ) specifically in osteoblasts (vs. osteoclasts and other cell types).

To differentiate between classical and non-classical/non-genomic ERα signaling, ERαKO mice were crossed to mice with a mutant ERα allele (a two amino acid substitution (E207A/G208A) in the first zinc finger of the DNA binding domain) [Syed et al., 2005]. The mutant ERα cannot bind to an ERE and are thus named Non-classical ER knock-in (NERKI) mice. The $ER\alpha^{-/NERKI}$ mice have a decrease in cortical, but not trabecular bone, as do ERαKO mice. However, ovariectomized $ER\alpha^{-/NERKI}$ mice *gained* bone, in contrast to wildtype mice that have a decrease in bone after ovariectomy. The authors of these studies suggest that there is a balance between classical and non-classical ERα signaling in osteoblasts [Syed et al., 2005].

Protective Effects of Estrogen in Bone

Loss of estrogens results in increased bone turnover, with both increased osteoclast activity and osteoblast activity [Riggs, 2000]. The protective effects of estrogens on bone are through several mechanisms. Estrogens repress osteoclastogenic cytokine production from immune cells [Weitzmann and Pacifici, 2006], increase osteoblast proliferation, decrease osteoblast and osteocyte apoptosis [Kousteni et al., 2002] and induce osteoclast apoptosis [Kameda et al., 1997]. A decrease in estrogen levels during menopause leads to a decrease in both cortical bone mineral density and cancellous bone mineral density. Similarly, ovariectomy in mice also leads to a decrease in both cortical and cancellous bone mineral density. In 2002 the Women's Health Initiative (WHI) showed that estrogen supplementation, either with or without progesterone as part of hormone replacement therapy (HRT), prevents bone fractures. However, the routine use of HRT has diminished significantly due to other results of the WHI suggesting an increased risk of breast cancer, heart disease and stroke in women taking HRT.

The effects of estrogens on osteoblasts are confusing. E2 has been shown to increase osteoblast proliferation [Scheven et al., 1992], have no proliferative effect [Keeting et al., 1991], or to decrease osteoblast proliferation. Jilka and colleagues have demonstrated that the number of osteoblast progenitors increases after ovariectomy [Jilka et al., 1998]. Thus, estrogens may suppress the self-renewal of immature osteoblasts. The reports of the effects of E2 on osteoblast differentiation are also mixed [Robinson et al., 1997]. Ovariectomy leads to an increase in osteoblast apoptosis; therefore it is thought that estrogens protect against osteoblast cell death. Indeed, in osteoblasts and osteocytes E2 prevents DNA damage-induced apoptosis. E2 leads to rapid phosphorylation of the Src/Shc/ERK pathway

that blocks apoptosis [Kousteni et al., 2001]. The contradictory effects of estrogens on osteoblasts may depend on the source of the osteoblasts (human vs. mouse, calvarial vs. MSC-derived, young vs. old cells), amount of differentiation, amount of ERα due to over-expression, normal cells vs. osteosarcoma cells and other factors.

Estrogens have been shown to induce apoptosis in osteoclasts [Kameda et al., 1997; Kousteni et al., 2002] and in contrast, ovariectomy of mice leads to an increase in the number of osteoclasts. Kousteni, et al., suggested that apoptosis in osteoclasts was induced via a non-genomic pathway [Kousteni et al., 2001]. This study used estren (4-estren-3,17-diol), which is an estrogen receptor agonist that was thought to only have non-genomic activity. However, recent papers have shown that estren does have transcriptional effects [Hewitt et al., 2006]. Furthermore, estren is thought to activate the androgen receptor. While non-genomic signaling may contribute to apoptosis of osteoclasts, this is not the sole mechanism of action (see the regulation of FasL described below).

Estrogens also regulate bone through the immune system. Menopausal women have increased serum levels of the osteoclastogenic cytokines IL-1, IL-6, IL-7, TNFα and M-CSF. The major sources of the cytokines are monocytes, macrophages, T-cells and stromal cells, although the cells in which estrogens regulate cytokines are not conclusively identified (reviewed in [Weitzmann and Pacifici, 2006]).

Direct Targets of Steroid Hormones

Upon binding to ligand (i.e. testosterone or estradiol), NRs are translocated to the nucleus where they bind to enhancers or promoters to regulate a variety of tissue-specific and ligand-specific genes. The “classical” ERE is a 13 base pair inverted palindromic sequence—GGTCANNNTGACC and AR binds to the “classical” ARE: AGAACAnnnTGTTCT [Verrijdt et al., 2003]. In addition, ERα can indirectly activate “non-classical” transcription by binding to other DNA binding proteins such as Sp1 and AP-1 family members (e.g. c-fos or c-jun) [Castro-Rivera et al., 2001]. Finally, ERα can regulate “non-genomic” signaling in which instead of ERα entering the nucleus to activate transcription, ERα activates phosphorylation cascades such as the MAP kinase pathway [Levin, 2005]. AR has also been shown to activate non-genomic signaling [Foradori et al., 2008].

A direct target of a NR is defined here as a gene that is regulated by NR binding to the DNA (either through DNA binding or association with a DNA binding protein) at an enhancer or promoter of that gene. Therefore, experimental evidence to demonstrate DNA binding (i.e. chromatin immunoprecipitation, ChIP) and subsequent gene regulation would support the notion of a direct target. However, DNA binding is missing for the majority of the targets described for bone. Because ER and AR can bind to DNA in less than one hour after ligand addition [Metivier et al., 2003] and mRNA increases of steroid responsive genes can be detected in as little as 1 hour [Park et al., 2005], a direct target could be detectable in less than a few hours. A second category of direct targets contains those genes that are “late” direct targets. These genes are regulated directly by nuclear receptor binding at or near DNA later than the “early” targets. Therefore, for this review, to include early and late genes, direct targets of the steroid hormone receptors will be defined as genes regulated by hormones in 24 hours or less. Many studies have shown that these hormones can regulate, for example type 1 collagen in osteoblasts, after weeks of hormone treatment, but these could be indirect targets resulting from increased differentiation due to regulation of other direct hormone target genes. For this review, studies over-expressing ERs or AR in osteoblasts and studies using osteosarcoma cell lines or non-osteoblast cell lines have been omitted, although many of them provide valuable model systems.

Direct Targets of E2 in Osteoblasts

In Table 1 the genes that are possible direct targets of E2 in osteoblasts are listed. They have been shown to be upregulated by E2 in 24 hours or less.

Denger and colleagues [Denger et al., 2008] identified 46 genes that were direct targets of E2 in primary human osteoblasts. Their microarray analysis included cycloheximide and ICI treatments of E2 treated osteoblasts to identify the direct ER targets. The majority of up-regulated genes were involved in signal transduction and regulation of transcription, and many of the down-regulated genes included immune and inflammatory response genes. They focused their work on the up-regulation of IGFBP4 by ERα. ERα was shown to bind to an intron of IGFBP4. IGF-1 and –II, which bind to, and are regulated by, IGFBP4, regulate osteoblast proliferation, differentiation and activity.

Recent work from our lab has begun to elucidate the mechanism underlying E2-induced apoptosis in osteoclasts [Krum and Brown, 2008; Krum et al., 2008a]. We showed that E2, via ERα, induces transcription of Fas Ligand (FasL) in osteoblasts, resulting in a paracrine signal that induces osteoclast apoptosis. Our data contradicts the work of Nakamura et al., [Nakamura et al., 2007] where it is suggested that FasL is transcriptionally induced by E2 in *osteoclasts*. Nakamura et al., used an ERα specific knockout in osteoclasts ($ER\alpha^{\Delta OC/\Delta OC}$) and showed a lack of regulation of FasL expression in $ER\alpha^{\Delta OC/\Delta OC}$ mice. However, this work did not examine the localization of FasL in either osteoblasts or osteoclasts, as they used whole bones instead of purified cell types in their assays. In contrast, we showed that E2 induces FasL in primary calvarial osteoblasts and an osteoblast cell line (MC3T3, which is a calvarial-derived osteoblast cell line). *In vivo*, E2 induces FasL expression in osteoblasts but not in osteoclasts, as demonstrated by co-localization with RUNX2; FasL is detected at the growth plate and endosteal surface of mouse femurs, but not in osteoclasts at either location. In addition, antibody-purified osteoclasts do not undergo E2-induced apoptosis unless osteoblasts are added in a co-culture system. Finally, co-cultures of MC3T3 osteoblasts and ERαKO bone marrow-derived osteoclasts demonstrate that ERα in *osteoblasts* is necessary and sufficient for osteoclast apoptosis. Together these experiments support our model that E2 induces FasL in osteoblasts to induce apoptosis of osteoclasts [Krum and Brown, 2008].

Alkaline phosphatase is a marker of early osteoblast differentiation and is important in the mineralization of bone. For years it has been shown that alkaline phosphatase is up-regulated in the presence of E2, as compared with a vehicle control [Scheven et al., 1992]. In mouse femurs, E2 upregulates alkaline phosphatase after 4-8 days, as detected by Northern blot [Plant and Tobias, 2001]. In primary calvarial osteoblasts alkaline phosphatase mRNA can be detected in 3 hours by quantitative PCR [Krum et al., 2008b], and is thus a likely direct target of E2.

Receptor activator of nuclear factor κB ligand (RANKL) is an essential cytokine for osteoclastogenesis [Boyle et al., 2003]. RANKL is made in osteoblasts, marrow stromal cells and T-cells and binds to the RANK receptor on osteoclast progenitors [Boyle et al., 2003]. Binding of RANKL to the RANK receptor leads to activation of TNF receptor-associated factors (TRAFs) 1, 2, 3, 5 and 6 and subsequent NF-κB up-regulation of osteoclast target genes [Lee and Kim, 2003]. The RANKL pathway can be inhibited by osteoprotegerin (OPG), which acts as a decoy receptor for RANKL. Thus the RANKL:OPG ratio is critical for osteoclastogenesis. E2 has been shown to decrease the RANKL:OPG ratio in primary human osteoblast cells by increasing the amount of OPG [Bord et al., 2003] after 24 hours of hormone treatment. Longer E2 treatments have revealed both repression of OPG [Cheung et al., 2003] and an increase in OPG [Chen et al., 2003].

Withdrawal of estrogens leads to apoptosis of both osteocytes and osteoblasts. Furthermore, E2 protects against DNA damage induced by drugs such as etoposide [Kousteni et al., 2002]. Pantschenko and colleagues demonstrated that E2 induces the anti-apoptotic protein Bcl-2 in *ex-vivo* osteoblast cultures [Pantschenko et al., 2005]. Interestingly, the over-expression of Bcl-2 in osteoblasts results in loss of the sex differences between male and female bone turnover [Pantschenko et al., 2005]. Therefore, E2 induces Bcl-2 to prevent osteoblast and osteocyte apoptosis.

Not only does E2 inhibit apoptosis in osteoblasts, but it may decrease proliferation and/or increase differentiation (see above). Theses changes may be mediated through an increase in Rbbp1 (Retinoblastoma-binding protein 1) by E2 [Monroe et al., 2006]. Rbbp1 has been shown to interact with Rb to decrease E2F1-mediated proliferation. Rb is also known to interact with Runx2 to increase osteoblast differentiation [Thomas et al., 2001]. E2 also increases osteoblast differentiation by up-regulating bone morphogenic protein 2 (BMP-2) after 24 hours in murine osteoblasts [Zhou et al., 2003]. BMP-2 stimulates bone formation *in vitro* and *in vivo*. BMP-2 is a direct target of E2, as demonstrated by cycloheximide studies. This would suggest that an important role for E2 in osteoblast direct-gene regulation is the inhibition of proliferation and/or activation of differentiation-specific genes.

It has been suggested that E2 also regulates BMP-4 and BMP-6 in osteoblasts, although there is no data to suggest that these are direct targets of E2 in primary osteoblasts. BMP-4 is increased 1 week after ovariectomy [Min et al., 2007] and this data is supported by luciferase assays in the U2OS osteosarcoma cell line [van den Wijngaard et al., 2000]. In contrast, BMP-6 is up-regulated 8 days after E2 treatment of ovariectomized mice (but not after 1, 2 or 4 days) [Plant and Tobias, 2002] and in cell lines that over-express ERα [Ong et al., 2004; Rickard et al., 1998].

After ovariectomy TGF-β3 mRNA decreases in rat femora. In as little as 2 hours after E2 treatment, TGF-β3 mRNA increases in the bone. In contrast, TGF-β1 and –β2 did not increase after E2 treatment. Raloxifene, being estrogenic in the bone, also increases TGF-β3 mRNA [Yang et al., 1996]. These studies used whole bones, but it is likely that the TGF-β3 is transcribed in osteoblasts. Reporter assays confirmed regulation in an osteoblast-like cell line (MG63). Thus, one mechanism of the protective effects of E2 is to increase TGF-β3, which inhibits osteoclast differentiation and bone resorption.

ERα is known to be regulated through transcriptional, posttranscriptional and translational mechanisms. Bord et al, demonstrated that E2 treatment increased ERα mRNA after 24 hours and an increase in ERα protein after 48 hours in primary human osteoblasts [Bord et al., 2003]; earlier time points were not described. This is in contrast to a decrease in ERα mRNA and protein that is seen in the model E2-responsive cell line MCF7 cells [Pink and Jordan, 1996].

ERα vs. ERβ in Osteoblasts

Both ERα and ERβ are expressed in osteoblasts. However, we do not know if the genes described above are truly direct targets of ERα, ERβ or both because binding of a receptor to the DNA has not been shown for any of the genes. Chromatin immunoprecipitation of ERα or ERβ would answer this. A second way to demonstrate the role of ERα or ERβ in primary osteoblasts would be to use an ERα specific ligand such as PPT or an ERβ specific ligand such as DPN.

Rbbp1 is a target of E2 in primary osteoblasts (see above). Chromatin immunoprecipitation has shown that ERα and ERβ bind to intron 1 of Rbbp1 in U2OS cells over-expressing the

receptor. However, ChIP has not been performed to prove that ERβ regulates Rbbp1 in primary osteoblasts.

Direct Targets of E2 in Osteoclasts

In 1991 Oursler and colleagues treated avian osteoclasts for 30 minutes with E2 and saw increases in c-fos and c-jun mRNA (Table 2) [Oursler et al., 1991]. C-fos and c-jun are members of the AP-1 family of transcription factors. C-Fos has an important role in osteoclast differentiation; mice lacking c-fos do not have osteoclasts and are osteopetrotic. E2 signals through AP-1 family members, and E2 up-regulation of c-fos and c-jun may amplify the E2 signal.

E2 decreases the osteoclastic resorption pit in bone. This effect is mediated by E2 decreasing secretion of cathepsin B, cathepsin L, cathepsin K and TRAP [Kremer et al., 1995; Mano et al., 1996] into the acidic extracellular compartment responsible for eroding bone. Each of these enzymes is potentially a direct target of E2 as the proteins are down-regulated in less than 24 hours. Cathepsin K was confirmed to be a direct target with transcription and translation inhibitors. In the presence of cycloheximide there was still a decrease in cathepsin K mRNA, but not with the transcription inhibitor actinomycin D.

Interleukin-1 is a pro-inflammatory cytokine that is capable of inducing osteoclast formation from precursor cells Not only is IL-1 repressed by E2 in immune cells, but the IL-1 receptor (IL-1R) is repressed by E2 in osteoclasts. Furthermore, the decoy receptor IL-1RII is increased by E2. Together, the osteoclastogenic IL-1 signaling is decreased by E2 [Sunyer et al., 1999].

Nakamura et al., used an ERα specific knockout in osteoclasts ($ER\alpha^{\Delta OC/\Delta OC}$) and show a lack of regulation of FasL expression in $ER\alpha^{\Delta OC/\Delta OC}$ mice. However, this work did not examine the localization of FasL in either osteoblasts or osteoclasts, as they used whole bones instead of purified cell types in their immunoblot and microarray analyses [Nakamura et al., 2007] (see FasL regulation in osteoblasts, above). Furthermore, we and others have not detected an increase in FasL in wildtype osteoclasts [Krum et al., 2008a; Martin-Millan et al., 2010].

In summary, there are several known E2 targets in osteoclasts. Microarray analysis of wildtype osteoclasts treated with E2 for a short time period (i.e. 3 hours), or osteoclasts from ERαKO mice or osteoclast specific-ERαKO mice may reveal additional potential targets of ERα. However, any differentially regulated genes must be analyzed to be direct or indirect targets of estrogens.

Effects of Androgens in Bone

Androgens affect the skeleton in both men and women. Androgen deficiency is associated with low bone mineral density [Finkelstein et al., 1987], partly through loss of muscle mass, which decreases mechanical strain and bone mineral density. It was previously thought that testosterone effected bone largely via aromatization to estrogen [Compston, 2001]. However, we now know that androgens have a direct effect through the androgen receptor in bone. AR is expressed in both osteoblasts and osteoclasts [Vanderschueren et al., 2004]. The few known direct targets of AR in these cell types are described below and listed in Tables 3 and 4.

Androgens have been shown to increase osteoblast proliferation in mouse and human primary osteoblasts [Kasperk et al., 1989]. Furthermore, DHT, a non-aromatizable form of testosterone, has been shown to increase proliferation of MC3T3-E1 calvarial osteoblasts in

a dose dependent fashion that signals through both AR and the PI3-kisase/AKT pathway [Kang et al., 2004].

In addition to increasing osteoblast proliferation, androgens, like estrogens, inhibit osteoblast apoptosis. Osteoblast apoptosis, induced by a number of stimuli, can be prevented with DHT [Kousteni et al., 2001]. Furthermore, orchidectomy of mice increases osteoblast apoptosis *in vivo* [Kousteni et al., 2001]. These protective effects of androgens are mediated through rapid activation of both AR and the Src/Shc/ERK signaling pathways, as opposed to classical AR activation of transcription.

Like estrogens, androgens effect on bone may also be indirectly mediated by regulation of cytokines from immune cells and/or stromal cells/osteoblasts that are important for osteoclastogenesis. For example, men treated with an GNRH antagonist and an aromatase inhibitor (to prevent the enzyme aromatase from converting testosterone to estrogen) had more of the osteoclastogenic cytokine TNFα in the serum than men treated with replacement testosterone [Khosla et al., 2002].

Genetic Models of Androgens in Bone

AR knockout (AndRKO) mice have increased bone resorption leading to reduced trabecular and cortical bone mass [Kawano et al., 2003], indicating the importance of androgen action through AR and not through aromatization to estrogen. However, these results are difficult to interpret due to low levels of testosterone and estradiol in these mice.

Testicular Feminization Male (TFM) rats are unresponsive to androgens due to a single base mutation in the ligand binding domain of AR. These rats have increased estradiol levels and the bone structures are more “female-like” in shape. The rats do not have a decrease in trabecular bone, as do orchidectomized rats, but do lose bone after orchidectomy [Vanderschueren et al., 1994].

Aromatase knockout (AromKO) mice have decreased cortical and cancellous bone in both female and male mice [Miyaura et al., 2001]. When these mice were treated with E2 the bone mass was completely restored in both sexes. The AromKO mice have a similar phenotype as described in a case study of a man with a point mutation in the aromatase gene. Unlike the men with point mutations in ERα, the man with the aromatase mutation had increased bone mineral density when treated with E2 [Bilezikian et al., 1998].

Direct Targets of Androgens in Osteoblasts (Table 3)

Microarray analysis of human fetal osteoblasts (hFOB) revealed 430 genes regulated by 10 nM DHT for 12 hours [Miki et al., 2007]. Of these, MYBL2, HOXD11 and ADCYAP1R1 were validated in qPCR assays. MYBL2 regulates cell cycle and proliferation [Joaquin and Watson, 2003]. Interestingly, the DHT-mediated increase in MYBL2 mRNA was not blocked by the AR inhibitor hydroxyflutamide, suggesting that it is not a direct target of AR. HOXD11 is a homeobox gene that encodes for a protein involved in the development of the limbs and cortical bone formation [Villavicencio-Lorini et al., 2010]. ADCYAP1R1 encodes for a type I adenylate cyclase activating polypeptide receptor, and its role in bone or its activity in response to androgens are unknown.

Orchidectomy of mice reduces skeletal TGF-β by over 80 percent [Gill et al., 1998] and *in vitro* studies have shown that TGF-β is likely a direct target of androgens [Gill et al., 1998; Kasperk et al., 1990]. In as little as 10 hours, TGF-β protein is induced by androgens *in vitro* in mouse calvarial osteoblasts [Kasperk et al., 1990], suggesting a direct regulation by androgens. TGF-ß1 stimulates osteoblast proliferation of early osteoblastic precursor cells

[Janssens et al., 2005], and thus androgens stimulate osteoblast proliferation, at least partly, through up-regulation of TGF-β.

The literature suggests that androgens regulate OPG and IL6 in osteoblasts, as do estrogens. However, there is no evidence that these are direct AR targets in normal osteoblasts. Whereas E2 has been shown to up-regulate OPG, OPG mRNA and protein were shown to be repressed by androgen in an osteoblast cell line over-expressing AR [Hofbauer et al., 2002]. IL-6 induces osteoclastogenesis and may be repressed by androgens in osteoblasts, bone marrow stromal cells or another cell in the bone marrow. Orchidectomy increases IL-6 levels in the serum of mice 3 days after surgery and IL-6 in the bone marrow 28 days after surgery [Zhang et al., 1998]. IL-6 mice do not lose bone mass after orchidectomy [Bellido et al., 1995]. *In vitro*, androgens repress IL-6 secretion from bone marrow stromal cells and from hFOB/AR-6 cells (hFOB cells over-expressing the androgen receptor) [Bellido et al., 1995; Hofbauer et al., 1999]. Thus, it is likely that IL-6 is a direct target of androgens in osteoblasts, but additional experiments need to be performed.

Direct Targets of Androgens in Osteoclasts (Table 4)

While E2 inhibits osteoclast formation through induction of FasL in osteoblasts [Krum et al., 2008a], androgens decrease osteoclastogenesis by a distinct, yet unknown, mechanism than E2 [Michael et al., 2005]. Testosterone has a direct inhibitory effect on osteoclast formation. CD14+ human monocytes, differentiated to osteoclasts, do not form as many TRAP+ osteoclasts when treated with testosterone, whereas E2 has no effect in the absence of osteoblasts [Krum et al., 2008a; Michael et al., 2005].

Very few studies describe the direct targets of androgens in osteoclasts. Pederson, et al, treated avian osteoclasts with DHT and saw an increase in TGF-β and decreases in cathepsin B and TRAP protein 24 hours after treatment [Pederson et al., 1999]. TGF-β1 inhibits osteoclastogenesis under some conditions while stimulating osteoclast formation and resorption under other *in vitro* conditions [Janssens et al., 2005]. Although Cathepsin K is the major cathepsin expressed in osteoclasts, cathepsin B also is expressed and found in resorption pits [Goto et al., 1994]. Thus, the decrease in resorptive activity of osteoclasts treated with DHT can be explained by the repression of TRAP and cathepsin B. The genes involved in androgen-mediated apoptosis of osteoclasts remain to be identified.

Summary

We know relatively little about the direct targets of sex steroid hormones in bone. Microarray analysis has been under-utilized to determine the genes regulated by estrogens and androgens. Technology such as ChIP-sequencing makes it possible to analyze all of the direct genomic targets of ERs and AR in primary cells. Knowing all of the targets of sex steroid hormones in bone cells will facilitate the development of tissue-specific ligands (selective estrogen receptor modulators (SERMs) or selective androgen receptor modulators (SARMs)) for treatment or prevention of osteoporosis.

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Table 1

E2 Targets in Osteoblasts

Target	Regulation	Time	Cell type	Reference
FasL	⇑	3 hours	Mouse calvarial osteoblasts	[Krum et al., 2008a]
Alkaline phosphatase	⇑	3 hours	Mouse calvarial osteoblasts	[Krum et al., 2008b]
Bcl-2	⇑	24 hours	Mouse calvarial osteoblasts	[Pantschenko et al., 2005].
OPG	⇑	24 hours	Primary human osteoblasts	[Bord et al., 2003]
Rbbp1	⇑	4 hours	Mouse calvarial osteoblasts	[Monroe et al., 2006]
BMP-2	⇑	24 hours	Mouse MSCs differentiated to OBs	[Zhou et al., 2003]
IGFBP4	⇑	24 hours	Primary human osteoblasts	[Denger et al., 2008]
ERα	⇑	24 hours	Primary human osteoblasts	[Bord et al., 2003]
TGF-β3	⇑	2 hours	Rat femora	[Yang et al., 1996]

Table 2

E2 Targets in Osteoclasts

Target	Regulation	Time	Cell type	Reference
c-fos and c-jun	⇑	30 minutes	Avian osteoclasts	[Oursler et al., 1991]
Cathepsin K	⇓	3 hours	Rabbit osteoclasts	[Mano et al., 1996]
TRAP, Cathepsins B, L, K	⇓ (secreted proteins)	18 hours	Avian osteoclasts	[Kremer et al., 1995]
IL-1RI	⇓	4 hours	Human osteoclasts	[Sunyer et al., 1999]
IL-1RII	⇑	4 hours	Human osteoclasts	[Sunyer et al., 1999]

Table 3

Androgen Targets in Osteoblasts

Target	Regulation	Time	Cell type	Reference
Mybl2	⇑	12 hours	Human fetal osteoblasts	[Miki et al., 2007]
Hoxd11				
OSTM1				
TGF-β	⇑	10 hours	Mouse calvarial osteoblasts	[Kasperk et al., 1990]

Table 4

Androgen Targets in Osteoclasts

Target	Regulation	Time	Cell type	Reference
TGF-β	⇑	24 hours	Avian osteoclasts	[Pederson et al., 1999]
Cathepsin B and TRAP	⇓	24 hours	Avian osteoclasts	[Pederson et al., 1999]