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Estrogen Receptor α Mediates Proliferation of Osteoblastic Cells Stimulated by Estrogen and Mechanical Strain, but Their Acute Down-regulation of the Wnt Antagonist *Sost* Is Mediated by Estrogen Receptor β*[S]

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Background: Strain and estrogens down-regulate *Sost*/sclerostin and stimulate osteoblastic proliferation.
Results: ERα inhibition prevents proliferation. ERβ inhibition prevents *Sost* down-regulation by strain or estradiol. Sclerostin prevents proliferation following strain and not estradiol.
Conclusion: ERα promotes proliferation, and ERβ mediates *Sost* down-regulation following estradiol ligand stimulation and ligand independently following strain.
Significance: Selective ER modulators could promote osteogenesis through differential regulation of *Sost* and proliferation.

Mechanical strain and estrogens both stimulate osteoblast proliferation through estrogen receptor (ER)-mediated effects, and both down-regulate the Wnt antagonist *Sost*/sclerostin. Here, we investigate the differential effects of ERα and -β in these processes in mouse long bone-derived osteoblastic cells and human Saos-2 cells. Recruitment to the cell cycle following strain or 17β-estradiol occurs within 30 min, as determined by Ki-67 staining, and is prevented by the ERα antagonist 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1*H*-pyrazole dihydrochloride. ERβ inhibition with 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-β]pyrimidin-3-yl] phenol (PTHPP) increases basal proliferation similarly to strain or estradiol. Both strain and estradiol down-regulate *Sost* expression, as does *in vitro* inhibition or *in vivo* deletion of ERα. The ERβ agonists 2,3-bis(4-hydroxyphenyl)-propionitrile and ERB041 also down-regulated *Sost* expression *in vitro*, whereas the ERα agonist 4,4′,4″-[4-propyl-(1*H*)-pyrazol-1,3,5-triyl]tris-phenol or the ERβ antagonist PTHPP has no effect. Tamoxifen, a nongenomic ERβ agonist, down-regulates *Sost* expression *in vitro* and in bones *in vivo*. Inhibition of both ERs with fulvestrant or selective antagonism of ERβ, but not ERα, prevents *Sost* down-regulation by strain or estradiol. *Sost* down-regulation by strain or ERβ activation is prevented by MEK/ERK blockade. Exogenous sclerostin has no effect on estradiol-induced proliferation but prevents that following strain. Thus, in osteoblastic cells the acute proliferative effects of both estradiol and strain are ERα-mediated. Basal *Sost* down-regulation follows decreased activity of ERα and increased activity of ERβ. *Sost* down-regulation by strain or increased estrogens is mediated by ERβ, not ERα. ER-targeting therapy may facilitate structurally appropriate bone formation by enhancing the distinct ligand-independent, strain-related contributions to proliferation of both ERα and ERβ.

Bone architecture is adjusted to be functionally appropriate for load-bearing through processes in which the strains engendered by loading initiate cascades of responses in resident bone cells that in turn influence the activity of cells responsible for bone formation and resorption. The activity of these cells is also influenced by estrogens. Loss of estrogens following menopause is associated with the development of osteoporosis, a widespread condition of skeletal inadequacy that has been hypothesized to reflect a failure of the homeostatic mechanisms by which bone adapts to its functional load-bearing environment, commonly referred to as the mechanostat (1). The cellular mechanisms of the mechanostat are locally influenced by the estrogen receptors ERα[7] and ERβ acting ligand-indepen-

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[7] The abbreviations used are: ER, estrogen receptor; MPP, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1*H*-pyrazole dihydrochloride; PTHPP, 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-β]pyrimidin-3-yl]phenol; PPT, 4,4′,4″-[4-propyl-(1*H*)-pyrazol-1,3,5-triyl]tris-phenol; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile; E2, 17β-estradiol; β2-MG, β_2-microglobulin; LRP, low density lipoprotein receptor-related protein; CLBObs, cortical long bone osteoblastic cells; rhSOST, recombinant human sclerostin.

dently (2–4). This implies that compounds that target the ERs should be able to enhance the sensitivity of the mechanostat and so provide effective, mechanically appropriate, treatment for osteoporosis. The action of the selective estrogen receptor modulator tamoxifen illustrates this; it reduces fracture risk in human patients (5), and in mice it synergistically enhances the effects of loading on bone gain (6).

Loading-induced increases in bone formation involve osteoblastic cell proliferation (7, 8) and down-regulation of *Sost*/sclerostin (9–11), a glycoprotein secreted primarily by osteocytes. Although a direct effect of sclerostin on strain-induced osteoblast proliferation has never been shown, sclerostin is presumed to exert its potent anti-osteogenic effect through inhibition of the Wnt pathway in neighboring osteoblasts by antagonizing Wnts binding to their low density lipoprotein receptor-related (LRP)-5 and -6 co-receptors (12). Neutralizing antibodies against sclerostin appear to have substantial and sustained osteogenic effects in humans and are now in advanced stages of clinical trials for the treatment of osteoporosis (13). A reduction in sclerostin production is also achieved by treatment with estrogens (14, 15), which also increase osteoblast proliferation (16). However, the mechanisms by which estrogens and loading converge to achieve similar outcomes remain largely unknown.

To investigate the potential mechanisms involved, we have established a model in which human female osteoblastic Saos-2 cells are subjected to mechanical strain by four-point bending of their substrate *in vitro* (17). These cells have been reported by ourselves and others to express *Sost* and sclerostin protein (17, 18). In this model, exposure to strain causes down-regulation of *Sost* expression over a time course consistent with that observed following loading of rodent bones *in vivo* (19, 20), through mechanisms requiring extracellular signal-regulated kinase (ERK) signaling (17). ERK is activated in bones subjected to loading *in vivo* (21) and in osteoblastic cells subjected to strain *in vitro* (22–24). This involves ERα and ERβ acting ligand-independently (24).

Other effects of strain on ligand-independent ER activity include activation of genomic estrogen-response elements (25), ERα-mediated nongenomic activation of Wnt/β-catenin (26, 27), and AKT (27) signaling. Osteoblastic cells from wild type (WT) mice proliferate in response to strain in the absence of estrogenic ligands, whereas similarly derived cells from ER$\alpha^{-/-}$ mice do not (28, 29). Consistent with this observation, cells overexpressing ERα are more proliferative in response to strain than cells only expressing endogenous ERα (25). The role of ERα in bones' local adaptive responses to loading has also been demonstrated *in vivo* in a number of studies, each of which show a diminished response to loading in female mice when ERα activity is reduced (2, 30–32).

In contrast, the role of ERβ in regulating bones' adaptation to loading remains controversial. The first *in vivo* study of ER's involvement in loading-related adaptation in bone reported a lower osteogenic response to axial loading of the ulna in female ER$\beta^{-/-}$ mice compared with WT littermates (32). However, later studies using knock-outs regarded as being more "complete," showed enhanced responses to axial loading (2, 33).

ERβ has been suggested to be the dominant regulator of estrogen receptor signaling, in part due to its ability to form heterodimers with ERα (34). However, the outcomes of ERβ signaling depend on the cellular context in which it operates; whereas ERβ largely inhibits transcriptomic changes caused by estrogen treatment when ERα is present, it promotes expression of a subset of genes when ERα is deleted (35). In osteoblastic cells, ERα activation increases ERβ expression (36) and has been shown to directly bind the ERβ promoter in other cell types (37). In contrast, ERβ can repress ERα expression (38), and mice lacking ERβ have increased ERα in their bones (39). The outcomes of ERα and ERβ signaling are therefore closely linked in what has been described as a "ying yang" relationship determined by a subtle balance between them (35, 40). Compensation for the absence of ERα activity by ERβ, and vice versa, is demonstrated by the mild effect of loss of either receptor alone compared with deletion of both ERs in bone and other tissues (41–43).

Having originally reported the involvement of the ERs in bones' adaptive response to loading (30, 32), and more recently ERK's involvement in *Sost* down-regulation by mechanical strain *in vitro* (17), we hypothesized that these commonalities between estrogen and strain signaling meant that ERα and ERβ could both contribute to the ligand-independent mechanisms by which loading down-regulates *Sost* expression and in turn regulates proliferation of osteoblasts in response to strain. The studies reported here used subtype-selective receptor agonists and antagonists against the ERs to establish the contributions of ERα and ERβ to the regulation of *Sost* and proliferation by both estradiol and strain in osteoblasts.

MATERIALS AND METHODS

Reagents and Cell Culture—17β-Estradiol (E2) was from Sigma and dissolved in molecular grade ethanol (EOH). Selective estrogen receptor modulators used were the ERα-selective agonist 4,4′,4″-[4-propyl-(1*H*)-pyrazol-1,3,5-triyl]tris-phenol (PPT, 0.1 μM) (44) or antagonist 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1*H*-pyrazole dihydrochloride (MPP, 0.1 μM) (45), the ERβ agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN, 0.1 μM) (46) or antagonist 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-β]pyrimidin-3-yl] phenol (PTHPP, 0.1 μM) (47), the context-dependent agonist/antagonist tamoxifen (0.1 μM), and the nonselective ERα/ERβ antagonist fulvestrant (0.1 μM, ICI 182780). The mitogen-activated protein kinase (MAPK)/ERK inhibitor PD98059 was used at a final concentration of 10 μM. All were from Tocris Bioscience (Bristol, UK). Fulvestrant was dissolved in EOH, and all other compounds were dissolved in dimethyl sulfoxide (DMSO). Cells were pretreated with the selective antagonists MPP, PTHPP, and PD98059 30 min before strain or E2 treatment, whereas fulvestrant was added 16 h before as described previously (27).

Wnt3a and recombinant human sclerostin were from R&D Systems (Abingdon, UK). Sclerostin was dissolved in phosphate-buffered saline (PBS) and added 1 h before strain or E2 treatment. The diluents never reached concentrations greater than 0.1% in the culture medium.

Cell Culture—All cells were maintained in phenol red-free DMEM containing 10% heat-inactivated FCS (PAA, Somerset, UK), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 IU/ml streptomycin (Invitrogen) (complete medium) in a 37 °C incubator at 5% CO_2, 95% humidity as described previously (17).

Saos-2 cells were a kind gift of Dr. S. Allen (Royal Veterinary College, London, UK). Mouse cortical long bone osteoblastic cells (cLBObs) were derived from explants of young adult female C57BL/6 mice as described previously (26, 28, 30). In brief, cLBObs were explanted by harvesting the diaphyses of long bones under sterile conditions in PBS containing 1× solution of antibiotic/antimycotic (PAA, Somerset, UK). All surface tissues were removed, and marrow contamination was eliminated by repeated flushing with PBS. The bones were subsequently chipped into fragments and cultured in complete medium until cell outgrowth was observed. Like similarly derived cells from rat bone (48), mouse cLBObs express markers of osteoblastic differentiation (Runx2, collagen 1 A1, and osteocalcin) (4) and form mineralized nodules (supplemental Fig. 1 supplemental Methods), however, they do not express *Sost* under the conditions required for *in vitro* strain experiments (4).

Cell Culture for Proliferation Studies—Cells were seeded at an initial density of 5000 cells/cm^2 (Saos-2) or 10,000 cells/cm^2 (cLBObs) on sterile custom-made plastic strips and allowed to adhere and grow for 24 h. Cells were then serum-depleted overnight in 2% charcoal/dextran-stripped serum to reduce the presence of steroids and their basal proliferation, before being subjected to strain or other treatments the next morning (*i.e.* cells were cultured for 2 full days before treatment). Cells were cultured in the same medium they were strained in for the duration of each experiment.

Cell Culture for Studies of Sost Regulation—Markers of osteoblastic differentiation, including *Sost*, increase in Saos-2 cells with time in culture (18, 49). Therefore, for studies of *Sost* regulation, we used over-confluent cultures that express *Sost* at a significantly higher level than subconfluent, proliferative cultures (supplemental Fig. 2). Sclerostin protein is readily detectable in confluent Saos-2 cells by Western blotting, and sclerostin levels in the cell culture supernatant are comparable with previously reported levels in human serum (supplemental Fig. 3).

Using a previously described protocol (17), cells were seeded at an initial density of 40,000 cells/cm^2, allowed to grow for 72 h before being serum-depleted in 2% charcoal/dextran serum overnight, and then subjected to strain or other treatment(s) (4 full days in culture before treatment). As was the case for proliferation studies, cells were always cultured in the same medium they were strained in until they were harvested at the appropriate time point for quantitative RT-PCR studies.

Straining Cells in Vitro—Strain was applied to the plastic strips on which cells were adherent using a well established protocol (17, 26, 27). This involves a brief period (~17 min) of 600 cycles of four-point bending engendering a peak strain on the surface of the strip of 3400 micro-strain (unless otherwise stated). A testing machine was used (Zwick Testing Machines Ltd., Leominster, UK) to achieve peak strain rates on and off of ~24,000 micro-strain/s, dwell times on and off of 0.7 s, and a frequency of 0.6 Hz.

Ki-67 Staining to Assess Proliferation—Anti-Ki-67 antibodies were from Santa Cruz Biotechnology (mouse anti-human, sc-23900; goat anti-mouse, sc-7846). Ki-67 staining in human Saos-2 cells was performed essentially as described previously (50). However, for anti-mouse Ki-67 staining, the antigen was retrieved by heating in PBS with 0.5% v/v Triton™ X-100 (Sigma), blocked in 1% BSA solution for 30 min, 10% rabbit serum for 1 h and then 10% horse serum for 1 h at room temperature. In both cases the primary antibody was used at a 1:100 dilution overnight at 4 °C. NorthernLight™-conjugated donkey secondary antibodies were from R&D Systems (Abingdon, UK) and used at a concentration of 1:100. Slides were mounted in Fluoroshield™ containing DAPI counter-stain (Sigma) and imaged on a Leica DMRB microscope. All slides in each experiment were imaged under identical conditions.

To assess proliferation by Ki-67 staining, the percentage of cells stained positive was analyzed under ×20 magnification in four randomly chosen fields per slide. In each case, representative proliferation results are shown as the proportion of all cells in each field stained positive for Ki-67.

The nuclear distribution of Ki-67 antigen is cell cycle stage-specific, as documented previously (50–53). Cell cycle stages were analyzed under ×40 magnification using the pattern of Ki-67 nuclear distribution in individual cells. In each case, the proportion of actively replicating (Ki-67 positive) cells in G_1, G_1/S, S, G_2, or M phase are shown. For this, 213 ± 12 Ki-67-positive nuclei were analyzed in 10 randomly chosen high power fields per slide. Key results were independently confirmed by the author G. L. Galea and L. B. Meakin.

Quantitative RT-PCR—RNeasy™ Plus mini kits (Qiagen, Sussex, UK) were used to eliminate DNA and extract RNA. First strand cDNA synthesis was performed using SuperScriptII™ (Invitrogen). Product copy numbers quantified against standard curves were normalized relative to β_2-microglobulin (β2-MG). PCR primers were designed using Primer3 Plus (54). Human *Sost* and β2-MG primers were as described previously (17). ERβ primers were also described previously (55). Other primers were as follows: mouse *Sost* sense TGCCGCGAGCTGCACTACAC and antisense CACCACTTCACGCGCCCGAT; mouse β2-MG sense ATGGCTCGCTCGGTGACCCT and antisense TTCTCCGGTGGGTGCGTGA; mouse OPG sense TGTGTGTCCCTTGCCCTGACCA and antisense ACACTCGGTTGTGGGTGCGG; and mouse CCND1 sense AAGTGGAGACCATCCGCCGC and antisense GCTCCTCACAGACCTCCAGCATC.

Quantitative RT-PCR data are presented as pooled results from two to four independent experiments with $n = 4–6$ in each experiment. In each case, the control group was set at 100%.

In Vivo Studies—Adult female mice (7 months) were bred from a previously described ER$\alpha^{-/-}$ colony (26, 42). Mice were housed up to five per cage and provided standard mouse chow and water *ad libitum* throughout the study. For RNA extraction from bone, the surrounding muscle was dissected, the epiphyses were removed, and the marrow was flushed with sterile PBS. Bones were pulverized in QIAzol™ using a TissueLyser LT™

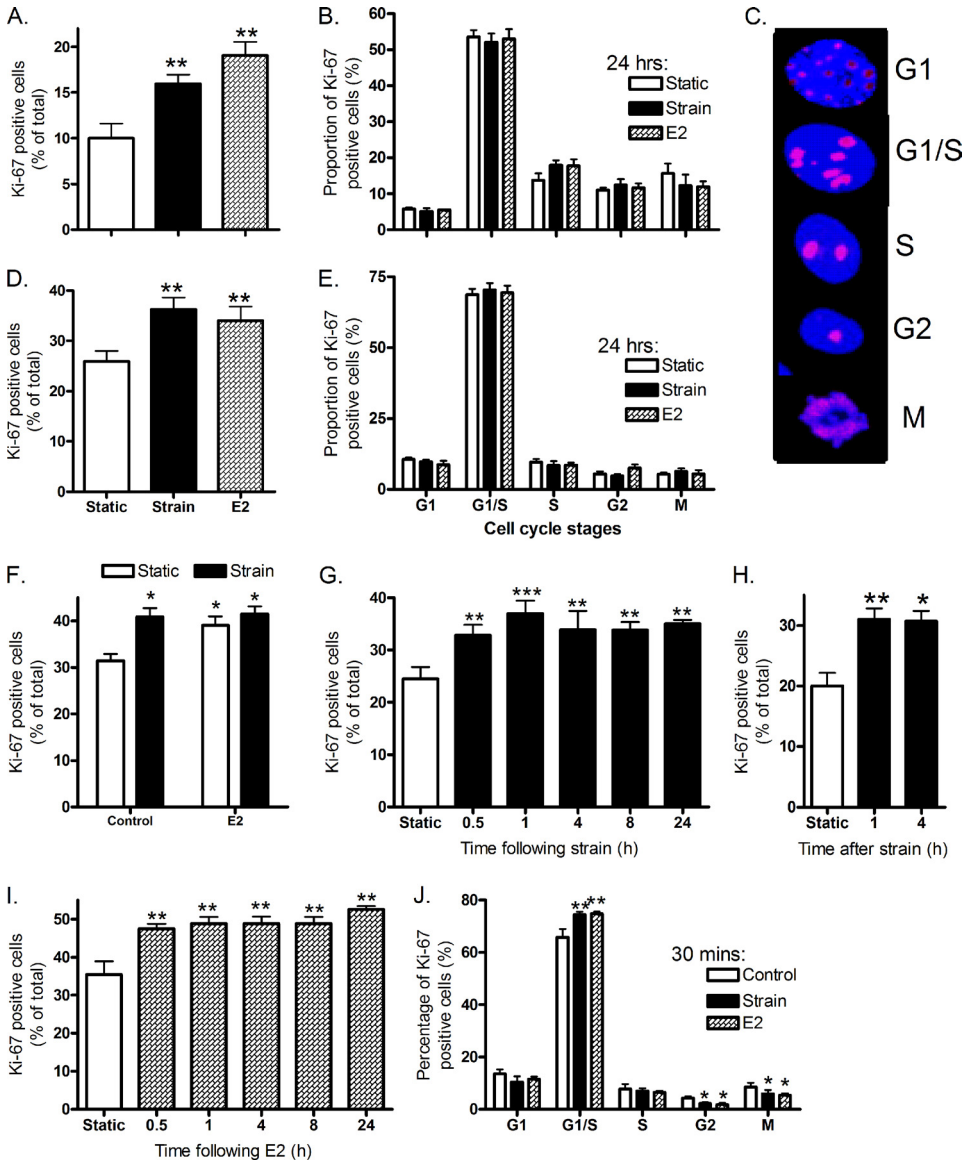

FIGURE 1. **Strain and E2 recruit osteoblastic cells to the cell cycle.** Primary mouse long bone-derived osteoblasts (*A* and *B*) or Saos-2 cells (*D* and *E*) were subjected to strain or treated with 1 μM E2 and fixed 24 h later. *C,* representative images showing the distribution of Ki-67 antigen in different stages of the cell cycle as described previously (50–53). *A* and *D,* proportion of cells staining positive for Ki-67; *B* and *E,* distribution of Ki-67-positive cells in different stages of the cell cycle was determined. Saos-2 cells were treated with 1 μM E2 before exposure to strain and fixed 8 h later (*F*). Saos-2 cells were fixed at the indicated time points following exposure to strain (*G*) or treatment with 1 μM E2 (*I*), and the percentage of cells staining positive for Ki-67 was determined. Primary mouse long bone-derived osteoblasts were fixed at the indicated time points following exposure to strain (*H*). Proportion of Ki-67-positive Saos-2 cells in different stages of the cell cycle was determined on static vehicle-treated slides or ones harvested 30 min following exposure to strain or treatment with E2 using the pattern of nuclear Ki-67 stain (*J*). *Bars* represent means ± S.E., $n = 4$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* static controls.

(Qiagen, Sussex, UK); RNA was extracted, and genomic DNA was eliminated using RNeasy™ Plus Universal kits (Qiagen, Sussex, UK).

To evaluate the effect of tamoxifen (2 mg/kg/day), mice were treated using a regimen that we have previously shown synergistically enhanced loading-related bone gain (6). At 16 weeks of age (day 1), virgin female C57BL/6 mice were sham-ovariectomized (Sham, $n = 8$) or ovariectomized ($n = 16$). Ten days after the operation (day 11), the ovariectomized mice were randomly subdivided into two groups ($n = 8$) and received either vehicle (peanut oil, 5 ml/kg; Sigma) or tamoxifen citrate (Tocris Cookson Inc., Ellisville, MO) by s.c. injection on days 11, 13, 15, 18, and 21 and were then sacrificed on day 25. All procedures were in accordance with the Institutional Animal Care and Home Office, UK, guidelines and approved by the ethics committee of the University of Bristol or of the Royal Veterinary College, London, UK.

Statistical Analysis—Statistical analysis was carried out on SPSS version 17 for Windows. Comparisons of two groups were by independent sample *t* tests, and more than two groups were compared by analysis of variance with Bonferroni or Games Howell post hoc adjustments. Data are presented as the mean ± S.E. $p < 0.05$ was considered significant.

RESULTS

Both Estrogens and Strain Rapidly Stimulate Osteoblastic Proliferation—Exposure to 1 μM E2, or a short period of mechanical strain, increased the proportion of cLBObs staining

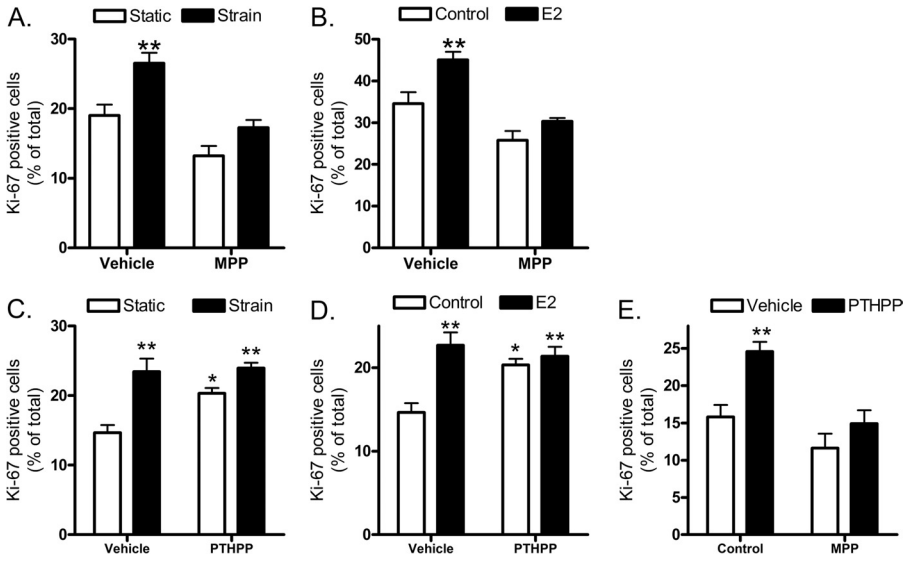

FIGURE 2. **Blockade of ERα prevents increases in osteoblast-like cell proliferation stimulated by strain and estradiol, whereas blockade of ERβ increases basal cell proliferation.** Saos-2 cells were subjected to strain (*A*) or treatment with 1 μM E2 (*B*) with or without pretreatment with 0.1 μM of the ERα inhibitor MPP and fixed 8 h later. Cells were subjected to strain (*C*) or treated with 1 μM E2 (*D*) with or without pretreatment with 0.1 μM PTHPP and fixed 8 h later. Cells were treated with 0.1 μM MPP 30 min before treatment with 0.1 μM PTHPP and harvested 8 h later (*E*). *Bars* represent means ± S.E., $n = 4$. *, $p < 0.05$; **, $p < 0.01$ relative to vehicle or static controls.

positive for the proliferating cell marker Ki-67 24 h later (Fig. 1*A*). No significant differences were detected between the proportions of Ki-67-positive cells in different stages of the cell cycle following either treatment (Fig. 1*B*). A similar proliferative response to both E2 and strain was observed in Saos-2 cells (Fig. 1*D*), with no change observed in the proportion of replicating cells in different stages of the cell cycle 24 h following treatment (Fig. 1*E*). This indicates that both estradiol and strain recruit otherwise Ki-67-negative quiescent cells to the cell cycle. Pretreatment with E2 for 30 min before exposure to strain did not significantly change the proportion of cells staining positive in these asynchronous cultures relative to treatment with strain or E2 alone (Fig. 1*F*). Thus, strain and estradiol similarly recruit a cohort of Ki-67-negative cLBObs or Saos-2 cells to the cell cycle without altering their progression through it.

Because it is not currently known when osteoblast-like cells are first stimulated to proliferate following a brief episode of strain, a time course of proliferation was undertaken. This showed a significant increase in the proportion of Saos-2 cells staining positive for Ki-67 within 30 min following strain (Fig. 1*G*). A similarly rapid response was observed in cLBObs (Fig. 1*H*). E2 also initiated Saos-2 proliferation within 30 min (Fig. 1*I*). This increase in Ki-67-positive cells was associated with a transient increase in the proportion of cells in the G_1/S phase of the cell cycle (Fig. 1*J*). Taken together, these data show that strain and estradiol both recruit a cohort of osteoblast-like cells to the cell cycle within 30 min of stimulation.

Strain and E2-induced Proliferation Requires ERα—Osteoblast proliferation following strain (28) or estradiol treatment (56, 57) has previously been reported to involve ERα. Blockade of ERα with methyl-piperidino-pyrazole (MPP) prevented the increase in Ki-67-positive cells 8 h following either strain (Fig. 2*A*) or 1 μM E2 (Fig. 2*B*) and was associated with a significant reduction in basal proliferation after 24 h (49 ± 4% decrease, $p < 0.01$ *versus* vehicle-treated controls). In contrast, blockade of ERβ with PTHPP was associated with a significant increase in basal proliferation with no significant further increase observed following strain or E2 treatment (Fig. 2, *C* and *D*). The increase in basal proliferation following ERβ blockade was prevented by pretreatment with the ERα antagonist (Fig. 2*E*). Thus, strain and estradiol involve ERα to stimulate proliferation of osteoblast-like cells, although basal proliferation of these cells is inhibited by ERβ.

Proliferation Following Strain and Wnt3a, but not E2, Is Inhibited by Exogenous Sclerostin—Osteocyte-derived sclerostin is presumed to exert its potent anti-osteogenic effect through inhibition of the Wnt pathway in neighboring osteoblasts (12). Consistent with this, pretreatment with 10 ng/ml recombinant human sclerostin (rhSOST), while not significantly changing the proportion of Saos-2 cells stained positive for Ki-67, prevented the increase in proliferation observed following treatment with 10 ng/ml Wnt3a (Fig. 3*A*). Similarly, pretreatment with rhSOST prevented the increase in Ki-67-positive cells 8 h following strain (Fig. 3*B*). However, rhSOST pretreatment did not prevent the increase in Ki-67-positive cells 8 h following treatment with 1 μM E2 (Fig. 3*C*). Thus, although both strain and estradiol stimulate rapid proliferation in osteoblastic cells, they do so by different mechanisms. Only proliferation caused by strain is prevented by the inhibitor of Wnt signaling, sclerostin.

Down-regulation of Sost Expression by E2, Activation of ERβ, and Inhibition of ERα—Both strain and estradiol trigger ER-dependent regulation of transcription in osteoblastic cells (25) and both down-regulate *Sost.* E2, at doses equal to or greater than 10 nM, down-regulated *Sost* expression within 8 h (Fig. 4*A*). Selective activation of ERα with 0.1 μM propyl pyrazole triol (PPT) had no effect on *Sost* expression after 8 h (Fig. 4*B*), whereas activation of ERβ with the agonists diarylpropionitrile

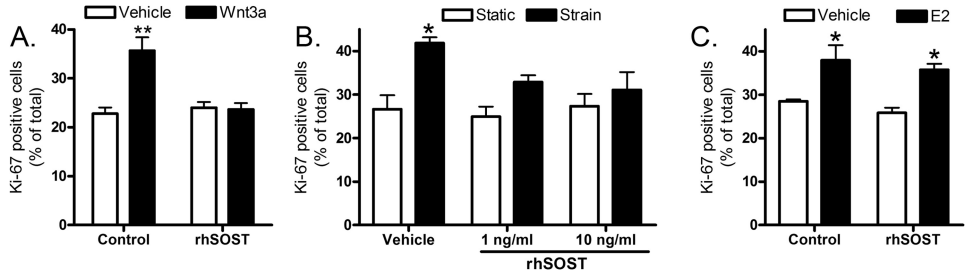

FIGURE 3. **Proliferation triggered by strain or Wnt3a, but not by estradiol, is prevented by exogenous sclerostin.** Saos-2 cells were treated with 10 ng/ml Wnt3a (*A*), subjected to strain (*B*), or treated with 1 μM E2 (*C*) with or without 1 h pretreatment with 10 ng/ml or the indicated concentration of recombinant human sclerostin (*rhSOST*) and fixed 8 h later. The percentage of cells staining positive for Ki-67 was determined. *Bars* represent means ± S.E., $n = 4$. *, $p < 0.05$; **, $p < 0.01$ relative to vehicle or static controls.

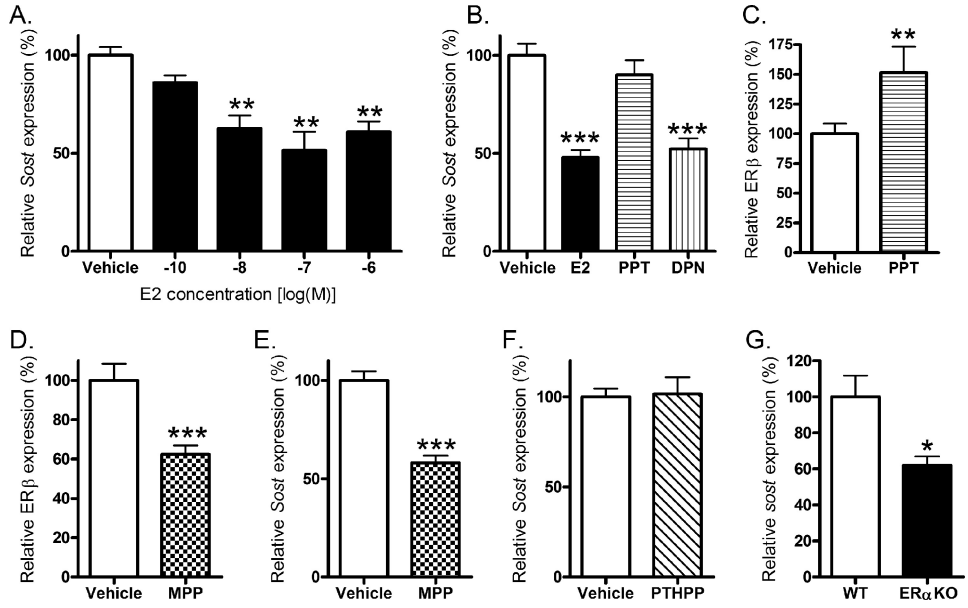

FIGURE 4. **Estradiol down-regulates *Sost* expression, as does activation of ERβ or inhibition of ERα.** Saos-2 cells were treated with the indicated doses of E2 and harvested 8 h later (*A*). Cells were treated with 0.1 μM E2, the ERα agonist PPT, or the ERβ agonist DPN and harvested 8 h later, and *Sost* levels were quantified (*B*). Cells were treated with 0.1 μM of the ERα agonist PPT (*C*) or the ERα antagonist MPP (*D*) and harvested 8 h later to quantify ERβ expression. To evaluate the effect of ER antagonists on *Sost* levels, cells were treated with the ERα antagonist MPP (*E*) or the ERβ antagonist PTHPP (*F*) and harvested 8 h later. Long bones were harvested from $ER\alpha^{-/-}$ and wild type (*WT*) mice, and *Sost* levels were quantified (*G*). *Bars* represent the mean ± S.E. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* the relevant controls.

(DPN, Fig. 4*B*) or ERB041 (ERB, supplemental Fig. 4) down-regulated *Sost* levels within 8 h.

Because ERα activation with PPT has previously been reported to up-regulate ERβ expression in osteoblastic cells (36), we quantified ERβ expression as a positive control of PPT action and found it to be elevated at this time point (Fig. 4*C*). Conversely, inhibition of ERα with 0.1 μM MPP significantly down-regulated ERβ (Fig. 4*D*).

ERα blockade with MPP also down-regulated *Sost* expression 8 h following treatment (Fig. 4*E*), whereas antagonizing ERβ with PTHPP had no effect on basal *Sost* expression (Fig. 4*F*). This suggests that, in cells not exposed to strain or estradiol, ERα ligand-independently maintains *Sost* expression. Loss of ERα function also resulted in lower *Sost* levels in bones from female $ER\alpha^{-/-}$ mice compared with WT controls (Fig. 4*G*).

ERβ Not ERα Mediates Sost Down-regulation by Strain or E2—Having established that ER signaling regulates basal *Sost* expression, we next investigated whether this is relevant to the regulation of *Sost* by strain. As reported previously (17), *Sost* expression was down-regulated in Saos-2 cells within 8 h following exposure to strains equal to or greater than 2000 micro-strain (Fig. 5*A*). Nonselective blockade of both ERα and ERβ with fulvestrant had no effect on basal expression of *Sost*, but prevented its down-regulation 8 h following strain (Fig. 5*B*) or estradiol (supplemental Fig. 5). Blockade of ERα with the selective antagonist MPP did not prevent significant *Sost* down-regulation by strain (Fig. 5*C*) or E2 (Fig. 5*D*), irrespective of its reduction in basal levels. In contrast, selective blockade of ERβ with PTHPP prevented *Sost* down-regulation following exposure to strain (Fig. 5*E*) or E2 (Fig. 5*F*).

ERK Mediates Sost Down-regulation by Strain or E2—Both ERα and ERβ mediate rapid activation of ERK signaling in osteoblastic cells subjected to strain (24). In Saos-2 cells ERK activation is required for *Sost* down-regulation by strain (17). Treatment of Saos-2 cells for 24 h with 10 μM of the ERK inhibitor PD98059 did not significantly change cell number or viability (supplemental Fig. 6, *a* and *b*), but significantly reduced ERK phosphorylation (supplemental Fig. 6, *c* and *d*). Inhibition of ERK activation also prevented *Sost* down-regulation by strain

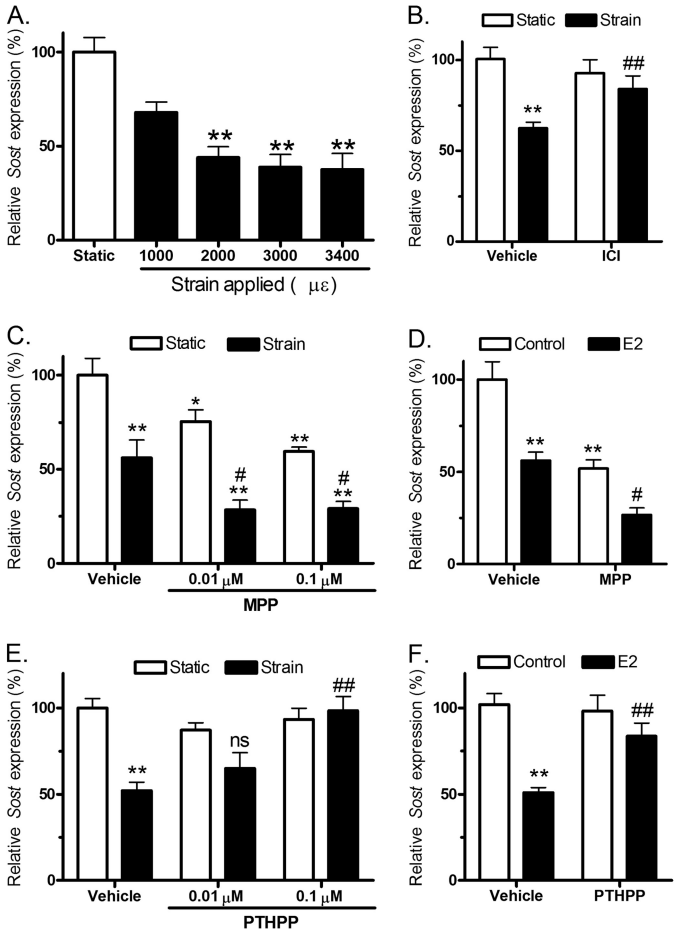

FIGURE 5. **Blockade of ERβ prevents *Sost* down-regulation by strain or estradiol.** Saos-2 cells were subjected to the indicated peak strain magnitudes and harvested 8 h later (*A*). Cells were subjected to strain with or without 16 h of pretreatment with 1 μM ICI 182,780 (*ICI*) (fulvestrant) and harvested 8 h later (*B*). Cells were subjected to strain with the indicated concentrations of MPP (*C*) or treated with E2 with or without pretreatment with 0.1 μM MPP and harvested 24 h later (*D*). Cells were subjected to strain with the indicated concentrations of PTHPP (*E*) or treated with E2 with or without pretreatment with 0.1 μM PTHPP (*F*). *Sost* levels were quantified relative to β2-MG. *Bars* represent the mean ± S.E. *ns* means not significant; *, $p < 0.05$; **, $p < 0.01$ *versus* the relevant control; #, $p < 0.05$; ##, $p < 0.01$ *versus* strained or E2-treated vehicle controls.

(Fig. 6*A*), E2 (Fig. 6*B*), and the ERβ agonist DPN (which has previously been reported to activate the nongenomic ERK pathway (Fig. 6*C*) (58)).

Tamoxifen used clinically as an ERα inhibitor in the treatment of breast cancer, also acts as a nongenomic ERβ agonist (59, 60), and in Saos 2 cells it also down-regulated *Sost* expression through a mechanism dependent on ERK activation (Fig. 6*D*).

Tamoxifen Down-regulates Sost in Vivo—We next sought to substantiate the *in vitro* finding that tamoxifen down-regulates *Sost,* using an *in vivo* approach in mice in which we had previously reported that tamoxifen synergistically enhances bones' adaptive response to mechanical loading (6). Down-regulation of *Sost* by tamoxifen was confirmed in bones *in vivo* (Fig. 7*A*) and was associated with increased expression of cyclin D1 (*CCND1*, Fig. 7*B*) and osteoprotegerin (*OPG*, Fig. 7*C*), known Wnt target genes (61, 62).

DISCUSSION

The ability of a bone to withstand loading without fracture critically depends upon the ongoing (re)modeling within its constituent tissue. The amount of bone formed as a result of the various stimuli responsible for (re)modeling is dependent upon the strength of the stimuli themselves and the responsiveness of the cells they influence. Two major regulators of bone (re)modeling are mechanical strain and estrogens. The experiments reported here demonstrate that exposure to either estradiol or a short period of dynamic strain stimulate proliferation, as indicated by an increased proportion of Ki-67-positive cells, in osteoblast-like cells derived from the weight-bearing cortical bones of female mice, and in the female human osteoblastic Saos-2 cell line. Increases in both estradiol and strain initiate this effect within 30 min, far earlier than we had assumed. Other early strain-related events in osteoblastic cells include; increased ligand-independent ERα phosphorylation within 5 min (23), ERK activation, also within 5 min (23), and an increase in β-catenin translocation to the nucleus within 30 min (26).

One potential pathway by which both strain and estrogen could exert their effects on osteoblast proliferation, and thus bone formation, is via the Wnt pathway. There are now numerous *in vivo* studies demonstrating a role for the Wnt pathway in mediating bones' response to mechanical loading (63–65). Deletion of the LRP-5 co-receptor reduces the osteogenic effects of loading (63). Conversely, mice harboring mutations in LRP-5, which make it insensitive to the antagonistic effects of sclerostin (66), show enhanced osteogenic responses to mechanical loading (64, 65). We have previously shown that Wnt activation in osteoblastic cells subjected to strain is facilitated by ERα and that activation of β-catenin and its translocation to the nucleus in response to mechanical strain is abrogated in osteoblastic cells lacking ERα (26, 27).

The finding that exogenous sclerostin prevents proliferation of osteoblastic cells stimulated by strain suggests that, in the natural situation, sclerostin down-regulation following mechanical loading relieves its inhibition of proliferation stimulated by Wnt proteins present in the local microenvironment. The parallel finding that exogenous sclerostin has no effect on estrogen-related proliferation suggests that the pathway from estrogen to osteoblast proliferation is by a different route in which *Sost* down-regulation is either not a rate-limiting step or is not involved at all. Possible *Sost*-independent mechanisms by which estrogens have their effect include ER-mediated effects on AP-1 transcription (67) or the physical association of ERα with TCF-4 independently of β-catenin (68). We have no evidence from this study to suggest a specific role for *Sost* down-regulation in the multiple responses of bone cells to estrogens, except that lower levels of extracellular sclerostin would be expected to increase the sensitivity to Wnt of any cells sharing this extracellular environment, given that sclerostin also inhibits proliferation following Wnt3a. The observation that down-regulation of *Sost* can be associated with different biological outcomes is not novel; both intermittent and continuous PTH down-regulate *Sost in vivo*, however one is anabolic and the other is catabolic (69, 70).

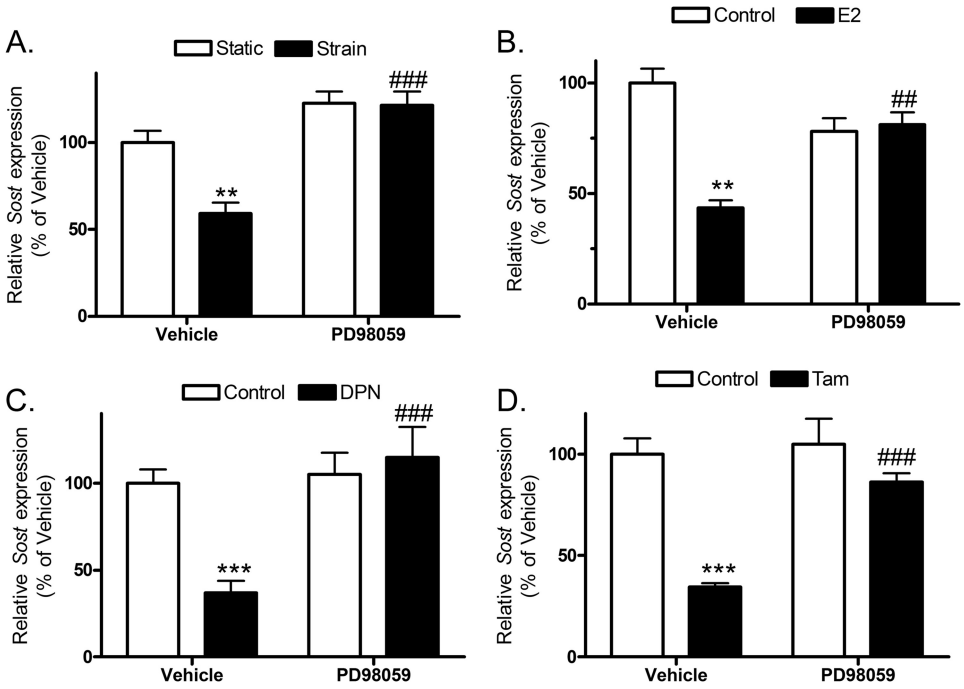

FIGURE 6. **MEK/ERK blockade prevents ER-mediated *Sost* down-regulation.** Saos-2 cells were subjected to strain (*A*) or treated with 0.1 μM E2 (*B*), DPN (*C*), or Tamoxifen (Tam) (*D*) with or without 30 min of pretreatment with 10 μM PD98059 and harvested 24 h later. *Sost* levels were quantified relative to β2-MG. *Bars* represent the mean ± S.E., $n = 12$. **, $p < 0.01$; ***, $p < 0.001$ *versus* vehicle control; ##, $p < 0.01$; ###, $p < 0.001$ *versus* the strained or agonist-treated vehicle group.

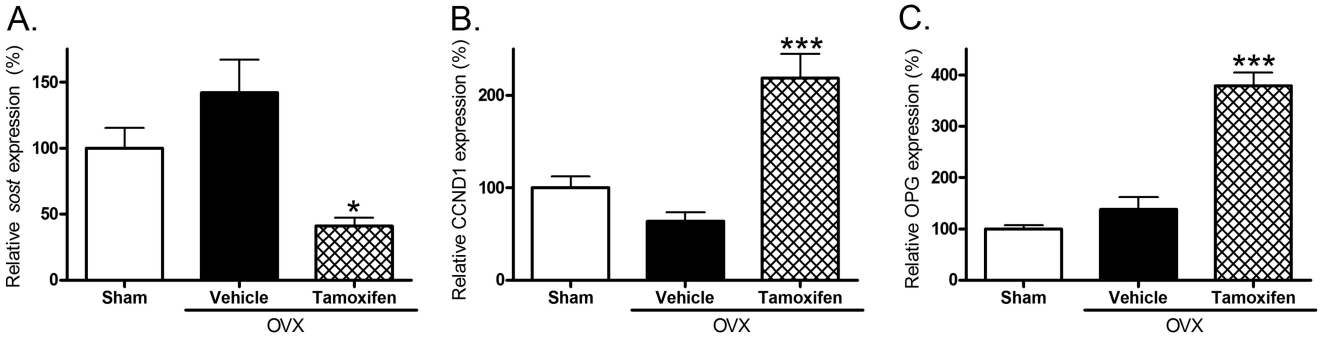

FIGURE 7. **Down-regulation of *Sost* expression by tamoxifen occurs *in vivo* as well as *in vitro*.** Adult female mice were ovariectomized (*OVX*) and treated with tamoxifen. *Sost* (*A*), cyclin D1 (*CCND1*) (*B*), and osteoprotegerin (*OPG*) (*C*) levels were quantified relative to β2-MG. *Bars* represent the mean ± S.E. *, $p < 0.05$; ***, $p < 0.001$ *versus* Sham and ovariectomized-vehicle in each case.

The finding that the increase in proliferation stimulated by strain and estradiol is mediated by ERα, is consistent with a previous report that nonselective ER blockade with fulvestrant (ICI 182780) prevents proliferation in rat osteoblasts in response to the same stimuli (71). In contrast, selective inhibition of ERβ increases proliferation, an increase that can be prevented by selectively blocking ERα. Thus, ERα and ERβ have opposite effects on basal osteoblastic cell proliferation, a situation that is well established in various other cell models (72–75).

The two ER subtypes also have different effects on *Sost*/sclerostin expression. Sclerostin is naturally produced primarily by osteocytes, and ideally, we would have wished to investigate its regulation in primary osteocyte cultures, but obtaining cultures of sufficient purity for large scale *in vitro* loading experiments is not currently possible. Unfortunately, the well established MLO-Y4 and MLO-Y5 osteocyte-like cell lines have been found to express very low to undetectable levels of *Sost* (76, 77). The recently reported IDG-SW3 cell line that replicates osteoblast to osteocyte differentiation does synthesize *Sost*, however, this requires at least 14 days of treatment with osteogenic differentiation medium that promotes mineralization (77) and mineralized cultures cannot be used for experiments involving strain. We therefore used the human osteoblastic Saos-2 cell line for our experiments because these cells secrete sclerostin when highly confluent (16, 17), and in this model exposure to strain causes down-regulation of *Sost* expression over a time course consistent with that observed *in vivo* (18).

In cultures of these cells, both estradiol and strain down-regulate *Sost*/sclerostin expression through either ERα and/or ERβ as evidenced by its blockade with fulvestrant. However, whereas the ERα agonist PPT has no effect on basal *Sost* levels, the ERα antagonist MPP causes down-regulation. Thus, although increased ERα activity does not increase basal levels of *Sost*, decreased ERα activity causes *Sost* down-regulation. Consistent with this, loss of ERα also results in lower *Sost* levels in bones from female ER$\alpha^{-/-}$ mice compared with WT controls.

In contrast, the ERβ agonist DPN and the partial ERβ agonist tamoxifen both cause down-regulation of *Sost*, whereas the ERβ antagonist PTHPP has no effect. This shows that although increased ERβ activity down-regulates basal *Sost,* the decreased ERβ activity does not increase it. Selective antagonism of ERβ rather than ERα also prevents the down-regulation of *Sost* by acute increases in either estradiol or strain.

The differences we report on the effects of ERα and ERβ on *Sost* expression were unexpected; we had anticipated that ERα would mediate strain-related down-regulation of *Sost* because the absence of ERα in female (but not male) mice has been repeatedly associated with a lower adaptive response to applied loading than in their WT background (30–32). This has been assumed to be the result of ERα's ligand-independent involvement in a number of the early stages of bones' osteogenic/anti-resorptive response to loading. ERα's strain-related functions include its association with ERK in the signalosome (24), with the IGF receptor's response to IGF (27), and its role in the translocation of β-catenin from cytoplasm to nucleus (26). Instead, these findings notwithstanding, our present data suggest that, in human female osteoblastic cells at least, the effects of acute changes in strain or estradiol are mediated primarily by ERβ. This inference is based on a number of elements in our present study as follows. (i) Selective activation of ERβ imitates the ERK-mediated down-regulation of *Sost* by strain or estradiol. (ii) Tamoxifen, which acts as a nongenomic ERβ agonist while, at least in other cell types, antagonizing ERα (59, 60), also imitates *Sost* down-regulation by strain or estradiol. (iii) Prevention of *Sost* down-regulation by nonselective inhibition of ERs with fulvestrant is not achieved by selective blockade of ERα, whereas blockade of ERβ prevents *Sost* down-regulation by both strain or estradiol.

The finding that MAPK/ERK blockade prevents ERβ-mediated *Sost* down-regulation is consistent with the report that ERβ, like ERα, is involved in the rapid strain-related activation of ERK signaling in osteoblastic cells (24). The potential involvement of ERK signaling suggests a nongenomic mode of action of ERβ, at least in the context of strain, although the involvement of this signaling pathway in a wide range of cellular processes limits interpretation of this result. Nongenomic activation of ERK signaling by either ERβ or ERα in a variety of cell types is increasingly being associated with diverse biological outcomes (78, 79). That ERβ activation may regulate *Sost* expression and activate ERK signaling has recently been suggested by the report that feeding rats soy isoflavones, which act as potent and relatively selective ERβ agonists (80, 81), increases ERK phosphorylation in bone and down-regulates *Sost*/sclerostin levels similarly to E2 treatment *in vivo* (15). The potential use of soy isoflavones for the treatment of osteoporosis is currently being investigated (82). We also demonstrate that tamoxifen reduces *Sost* expression *in vivo*, a finding consistent with an effect through ERβ. The potential for ERβ to mediate the therapeutic effects of tamoxifen treatment has been proposed elsewhere in the context of breast cancer (60), and tamoxifen administration has a profound osteogenic effect in cancellous regions of mouse bones, where ERβ is intensely expressed in osteocytes (83). A role for ERβ in mediating bone's response to loading is consistent with tamoxifen synergistically enhancing bone gain in the tibiae of female mice subjected to mechanical loading (6). In this regard, the effects of tamoxifen are similar to those of parathyroid hormone and EP4 targeting compounds, both of which have been reported to down-regulate *Sost* (17, 76) and synergistically enhance bone gain following loading (84–86).

The inference that *Sost* regulation by strain is mediated by ERβ is also consistent with the original report by Lee *et al.* (32) that mice with incomplete ablation of ERβ activity show an impaired increase in cortical bone formation following loading of the ulna. However, subsequent reports in mice with more complete ERβ ablation (32) have shown a greater cortical osteogenic response to loading (2, 33). It is only possible to speculate on the inconsistencies between these studies because of potentially compensatory up-regulation of ERα (39) and opposite global transcriptomic influences of ERβ with or without ERα (35). What is clear is that the role of ERβ in the bone's adaptation to loading remains controversial and, compared with ERα, under-studied. It is also becoming increasingly apparent that the functions of ERα and ERβ in determining the osteoregulatory effects of loading are dependent not only on the systemic biochemical/hormonal systemic context but also on the region of the bone involved (2).

Although this study investigates the role of the ERs in differentiated osteoblasts, ERs also play an active role during osteoblast lineage progression. Both ERα and ERβ are expressed in stromal pre-osteoblasts (83, 87, 88), which either proliferate to maintain the progenitor pool or differentiate into osteoblasts (as schematically represented in Fig. 8). Mechanical loading and E2 both increase osteoblast differentiation (89–91), and the mechanism by which strain promotes osteoblast differentiation involves LRP-independent activation of β-catenin (89, 92, 93). However, the role of the ERs in these processes is not clearly understood. Both ERs may mediate osteoblast differentiation following E2 treatment (94), although ERα inhibits mineralization (95) and bone morphogenetic protein-induced differentiation (96). Marrow stromal cells from individuals with a hypomorphic ERα have lower estradiol responsiveness but enhanced intrinsic differentiation (97). ERα also inhibits the transcriptional activity of the master regulator of the osteoblast lineage, Runx2 (98).

Loading and estrogen both promote proliferation of pre-osteoblasts (99–102), more differentiated osteoblastic cells (as used in the present study), and bone-lining cells (7, 8, 16). The proliferative effects of strain on osteoblastic cells have repeatedly been found to require ligand-independent activation of ERα (28, 29, 71). Similarly, estrogen promotes proliferation through a nongenomic function of ERα (56). In this study, ERα promotes proliferation following strain or estradiol treatment, whereas ERβ suppresses basal proliferation. Intriguingly, in MG63 cells, estrogen acting through ERβ has also been reported to suppress proliferation (103).

In mature osteoblasts *in vivo,* the osteogenic effects of estradiol requires a fully functional ERα (104), whereas the osteogenic effects of loading do not require the ligand-binding AF2 domain of this receptor (4). Ligand-independent functions of ERα activated in osteoblastic cells by strain include its potentiation of the IGF receptor (27) and the translocation of

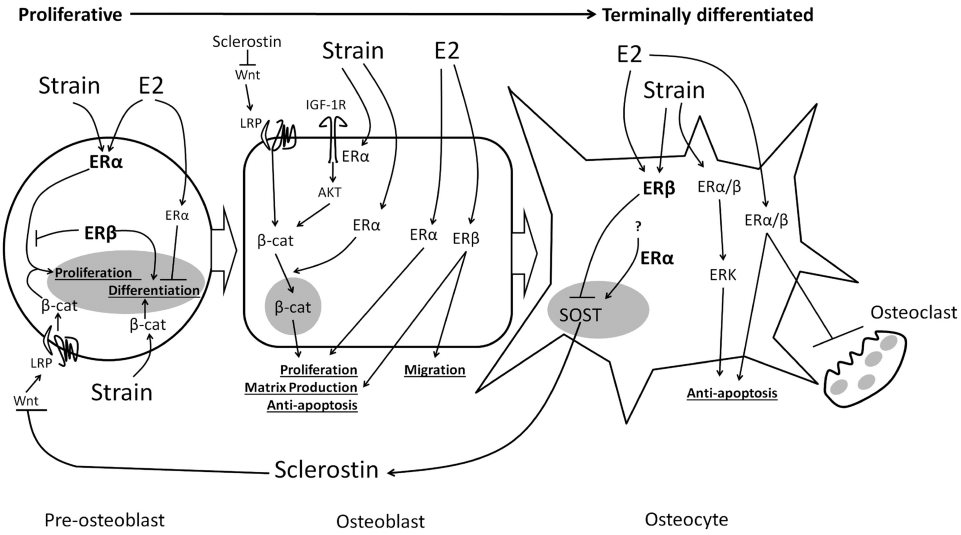

FIGURE 8. **Schematic illustrating the roles of ERα and ERβ at different stages of the osteoblastic lineage.** Early osteoblasts can proliferate or differentiate, and although ERα promotes their proliferation (28, 56, 114) and suppresses differentiation (94–96), there is evidence that ERβ promotes differentiation (94, 103) while inhibiting proliferation (103). In more mature osteoblasts, ERα promotes proliferation and ERβ inhibits it, as we have shown in this study. ERβ and ERα both contribute to matrix production (103, 115, 116), and ERβ also selectively regulates genes associated with cell migration (105). In osteoblastic cells exposed to mechanical strain, ERα facilitates other osteogenic signaling pathways, specifically IGF (27) and Wnt/β-catenin signaling (26). ERα and ERβ also contribute to anti-apoptotic signaling (55, 107, 110), and in osteocyte-like cells subjected to strain this involves ERK activation (24). Both ERs may also influence osteoclastogenic cytokine expression by osteoblastic cells (55, 95, 117–119). Both receptors regulate *Sost* expression, as described here; ERβ mediates its acute down-regulation by strain and estradiol, and ERα maintains its basal expression. However, understanding the physiological context in which this complex interaction operates requires further investigation. *β-cat*, β-catenin.

β-catenin to the nucleus (26). Although roles for ERβ in the responses of these cells to strain are largely unknown, in MG63 cells ERβ is required for estradiol to up-regulate expression of matrix components (103). ERβ also selectively regulates the expression of genes related to migration (105), a process that is likely to be involved in matrix secretion.

Following secretion of matrix, osteoblasts become quiescent, terminally differentiate into osteocytes, or become apoptotic. Loading and estrogen both reduce apoptosis (24, 106–109), and estradiol has been shown to contribute to anti-apoptotic processes by activation of either ERα or ERβ through nongenomic mechanisms in a variety of cell models (55, 107, 110). In osteocytic MLO-Y4 cells, mechanical strain exerts anti-apoptotic effects through ERK activation that requires nongenomic signaling through both ERs (24). Apoptosis, especially of osteocytes, promotes osteoclast recruitment (108, 111), and ERα expression in osteoclasts is required for maintaining trabecular bone mass in female mice (113). ERα also suppresses the expression of osteoclastogenic cytokines in osteoblastic cells (117–119).

Together, these data suggest that ERα plays key roles early on in the osteoblast lineage through its enhancement of proliferation, which is consistent with the recent finding that in mice selective deletion of ERα in pre-osteoblast mesenchymal cells using Prx-cre or Osterix1-cre is associated with reduced cortical thickness due to reduced bone formation. In contrast, its deletion later in the lineage using Col1a1-cre has no such effect (112). Conversely, as has previously been suggested by Cao *et al.* (103), the functions of ERβ may relate to the formation of post-proliferative matrix-secreting cells.

Although it is difficult to reconcile *in vitro* data with those from studies in mice *in vivo*, the additional step of relating the data to human patients is even more problematic. However, with age there is a reduction in circulating estrogens in both men and women that is accompanied by an increase in serum sclerostin (113). Reduced Wnt signaling due to elevated sclerostin levels, impairing the proliferative context in which loading acts, could partially explain the reduced ability of bone to adapt to its mechanical loading environment post-menopausally and in later life. Any attempt to explain this in terms of the activity of either ERα or ERβ would at this stage be speculation.

In conclusion, ERα and ERβ differentially regulate the responses of osteoblastic cells to acute changes in their ligand (estrogens) and to mechanical strain, thus influencing the context in which these proliferative stimuli act (schematically represented in Fig. 8). Exposure of osteoblastic cells *in vitro* to either a short period of mechanical strain or to an acute increase in the estradiol concentration in their environment stimulates proliferation mediated at least in part through ERα. Such exposure to changes in estradiol concentration and to short exposure to dynamic strain also down-regulates the expression of the Wnt antagonist *Sost*/sclerostin. Whereas ERα maintains basal expression of *Sost*, ERβ activity inhibits basal proliferation. However, it is ERβ and not ERα that mediates acute reduction in *Sost* in response to either changes in estrogens or strain. The (re)modeling response of bones to either strain or estrogens involves control of targeted formation and resorption. The extent of this osteogenic/anti-resorptive response will depend *inter alia* upon both the "proliferative context" in which it operates and the strength/duration of the stimulus to which the responsive cells are subjected. The data presented here suggest that the contribution of ERα is primarily to the proliferative context, although the contribution of ERβ is to the acute response of the resident bone cells to their mechanical and hormonal environment. In the case of exposure to

strain, this response involves reduced expression of the Wnt antagonist *Sost*.

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REFERENCES

1. Lanyon, L., and Skerry, T. (2001) Postmenopausal osteoporosis as a failure of bone's adaptation to functional loading: a hypothesis. *J. Bone Miner. Res.* **16,** 1937–1947
2. Saxon, L. K., Galea, G., Meakin, L., Price, J., and Lanyon, L. E. (2012) Estrogen receptors α and β have different gender-dependent effects on the adaptive responses to load bearing in cancellous and cortical bone. *Endocrinology* **153,** 2254–2266
3. Lanyon, L., Armstrong, V., Saxon, L., Sunters, A., Sugiyama, T., Zaman, G., and Price, J. (2007) Estrogen receptors critically regulate bones' adaptive responses to loading. *Clin. Rev. Bone Miner. Metab.* **5,** 234–248
4. Windahl, S. H., Saxon, L., Börjesson, A. E., Lagerquist, M. K., Frenkel, B., Henning, P., Lerner, U. H., Galea, G. L., Meakin, L. B., Engdahl, C., Sjögren, K., Antal, M. C., Krust, A., Chambon, P., Lanyon, L. E., Price, J. S., and Ohlsson, C. (2013) Estrogen receptor-α is required for the osteogenic response to mechanical loading in a ligand-independent manner involving its activation function 1 but not 2. *J. Bone Miner. Res.* **28,** 291–301
5. Cooke, A. L., Metge, C., Lix, L., Prior, H. J., and Leslie, W. D. (2008) Tamoxifen use and osteoporotic fracture risk: a population-based analysis. *J. Clin. Oncol.* **26,** 5227–5232
6. Sugiyama, T., Galea, G. L., Lanyon, L. E., and Price, J. S. (2010) Mechanical loading-related bone gain is enhanced by tamoxifen but unaffected by fulvestrant in female mice. *Endocrinology* **151,** 5582–5590
7. Pead, M. J., Skerry, T. M., and Lanyon, L. E. (1988) Direct transformation from quiescence to bone formation in the adult periosteum following a single brief period of bone loading. *J. Bone Miner. Res.* **3,** 647–656
8. Sakai, D., Kii, I., Nakagawa, K., Matsumoto, H. N., Takahashi, M., Yoshida, S., Hosoya, T., Takakuda, K., and Kudo, A. (2011) Remodeling of actin cytoskeleton in mouse periosteal cells under mechanical loading induces periosteal cell proliferation during bone formation. *PLoS One* **6,** e24847
9. Robling, A. G., Niziolek, P. J., Baldridge, L. A., Condon, K. W., Allen, M. R., Alam, I., Mantila, S. M., Gluhak-Heinrich, J., Bellido, T. M., Harris, S. E., and Turner, C. H. (2008) Mechanical stimulation of bone *in vivo* reduces osteocyte expression of Sost/sclerostin. *J. Biol. Chem.* **283,** 5866–5875
10. Moustafa, A., Sugiyama, T., Prasad, J., Zaman, G., Gross, T. S., Lanyon, L. E., and Price, J. S. (2012) Mechanical loading-related changes in osteocyte sclerostin expression in mice are more closely associated with the subsequent osteogenic response than the peak strains engendered. *Osteoporos. Int.* **23,** 1225–1234
11. Tu, X., Rhee, Y., Condon, K. W., Bivi, N., Allen, M. R., Dwyer, D., Stolina, M., Turner, C. H., Robling, A. G., Plotkin, L. I., and Bellido, T. (2012) Sost down-regulation and local Wnt signaling are required for the osteogenic response to mechanical loading. *Bone* **50,** 209–217
12. Krause, C., Korchynskyi, O., de Rooij, K., Weidauer, S. E., de Gorter, D. J., van Bezooijen, R. L., Hatsell, S., Economides, A. N., Mueller, T. D., Löwik, C. W., and ten Dijke, P. (2010) Distinct modes of inhibition by sclerostin on bone morphogenetic protein and Wnt signaling pathways. *J. Biol. Chem.* **285,** 41614–41626
13. Padhi, D., Jang, G., Stouch, B., Fang, L., and Posvar, E. (2011) Single-dose, placebo-controlled, randomized study of AMG 785, a sclerostin monoclonal antibody. *J. Bone Miner. Res.* **26,** 19–26
14. Mödder, U. I., Clowes, J. A., Hoey, K., Peterson, J. M., McCready, L., Oursler, M. J., Riggs, B. L., and Khosla, S. (2011) Regulation of circulating sclerostin levels by sex steroids in women and in men. *J. Bone Miner. Res.* **26,** 27–34
15. Zhang, J., Lazarenko, O. P., Wu, X., Tong, Y., Blackburn, M. L., Gomez-Acevedo, H., Shankar, K., Badger, T. M., Ronis, M. J., and Chen, J. R. (2012) Differential effects of short term feeding of a soy protein isolate diet and estrogen treatment on bone in the pre-pubertal rat. *PLoS One* **7,** e35736
16. Ogita, M., Rached, M. T., Dworakowski, E., Bilezikian, J. P., and Kousteni, S. (2008) Differentiation and proliferation of periosteal osteoblast progenitors are differentially regulated by estrogens and intermittent parathyroid hormone administration. *Endocrinology* **149,** 5713–5723
17. Galea, G. L., Sunters, A., Meakin, L. B., Zaman, G., Sugiyama, T., Lanyon, L. E., and Price, J. S. (2011) Sost down-regulation by mechanical strain in human osteoblastic cells involves PGE2 signaling via EP4. *FEBS Lett.* **585,** 2450–2454
18. Yu, L., van der Valk, M., Cao, J., Han, C. Y., Juan, T., Bass, M. B., Deshpande, C., Damore, M. A., Stanton, R., and Babij, P. (2011) Sclerostin expression is induced by BMPs in human Saos-2 osteosarcoma cells but not via direct effects on the sclerostin gene promoter or ECR5 element. *Bone* **49,** 1131–1140
19. Zaman, G., Saxon, L. K., Sunters, A., Hilton, H., Underhill, P., Williams, D., Price, J. S., and Lanyon, L. E. (2010) Loading-related regulation of gene expression in bone in the contexts of estrogen deficiency, lack of estrogen receptor α, and disuse. *Bone* **46,** 628–642
20. Mantila Roosa, S. M., Liu, Y., and Turner, C. H. (2011) Gene expression patterns in bone following mechanical loading. *J. Bone Miner. Res.* **26,** 100–112
21. Li, Y., Ge, C., Long, J. P., Begun, D. L., Rodriguez, J. A., Goldstein, S. A., and Franceschi, R. T. (2012) Biomechanical stimulation of osteoblast gene expression requires phosphorylation of the RUNX2 transcription factor. *J. Bone Miner. Res.* **27,** 1263–1274
22. Jessop, H. L., Rawlinson, S. C., Pitsillides, A. A., and Lanyon, L. E. (2002) Mechanical strain and fluid movement both activate extracellular regulated kinase (ERK) in osteoblast-like cells but via different signaling pathways. *Bone* **31,** 186–194
23. Jessop, H. L., Sjöberg, M., Cheng, M. Z., Zaman, G., Wheeler-Jones, C. P., and Lanyon, L. E. (2001) Mechanical strain and estrogen activate estrogen receptor α in bone cells. *J. Bone Miner. Res.* **16,** 1045–1055
24. Aguirre, J. I., Plotkin, L. I., Gortazar, A. R., Millan, M. M., O'Brien, C. A., Manolagas, S. C., and Bellido, T. (2007) A novel ligand-independent function of the estrogen receptor is essential for osteocyte and osteoblast mechanotransduction. *J. Biol. Chem.* **282,** 25501–25508
25. Zaman, G., Cheng, M. Z., Jessop, H. L., White, R., and Lanyon, L. E. (2000) Mechanical strain activates estrogen response elements in bone cells. *Bone* **27,** 233–239
26. Armstrong, V. J., Muzylak, M., Sunters, A., Zaman, G., Saxon, L. K., Price, J. S., and Lanyon, L. E. (2007) Wnt/β-catenin signaling is a component of osteoblastic bone cell early responses to load-bearing and requires estrogen receptor α. *J. Biol. Chem.* **282,** 20715–20727
27. Sunters, A., Armstrong, V. J., Zaman, G., Kypta, R. M., Kawano, Y., Lanyon, L. E., and Price, J. S. (2010) Mechano-transduction in osteoblastic cells involves strain-regulated estrogen receptor α-mediated control of insulin-like growth factor (IGF) I receptor sensitivity to ambient IGF, leading to phosphatidylinositol 3-kinase/AKT-dependent Wnt/LRP5 receptor-independent activation of β-catenin signaling. *J. Biol. Chem.* **285,** 8743–8758
28. Jessop, H. L., Suswillo, R. F., Rawlinson, S. C., Zaman, G., Lee, K., Das-Gupta, V., Pitsillides, A. A., and Lanyon, L. E. (2004) Osteoblast-like cells from estrogen receptor α knockout mice have deficient responses to mechanical strain. *J. Bone Miner. Res.* **19,** 938–946
29. Damien, E., Price, J. S., and Lanyon, L. E. (2000) Mechanical strain stimulates osteoblast proliferation through the estrogen receptor in males as well as females. *J. Bone Miner. Res.* **15,** 2169–2177
30. Lee, K., Jessop, H., Suswillo, R., Zaman, G., and Lanyon, L. (2003) Endocrinology: bone adaptation requires oestrogen receptor-α. *Nature* **424,** 389
31. Callewaert, F., Bakker, A., Schrooten, J., Van Meerbeek, B., Verhoeven, G., Boonen, S., and Vanderschueren, D. (2010) Androgen receptor disruption increases the osteogenic response to mechanical loading in male mice. *J. Bone Miner. Res.* **25,** 124–131
32. Lee, K. C., Jessop, H., Suswillo, R., Zaman, G., and Lanyon, L. E. (2004)

The adaptive response of bone to mechanical loading in female transgenic mice is deficient in the absence of oestrogen receptor-α and -β. *J. Endocrinol.* **182,** 193–201
33. Saxon, L. K., Robling, A. G., Castillo, A. B., Mohan, S., and Turner, C. H. (2007) The skeletal responsiveness to mechanical loading is enhanced in mice with a null mutation in estrogen receptor-β. *Am. J. Physiol. Endocrinol. Metab.* **293,** E484–E491
34. Pettersson, K., Delaunay, F., and Gustafsson, J. A. (2000) Estrogen receptor β acts as a dominant regulator of estrogen signaling. *Oncogene* **19,** 4970–4978
35. Lindberg, M. K., Movérare, S., Skrtic, S., Gao, H., Dahlman-Wright, K., Gustafsson, J. A., and Ohlsson, C. (2003) Estrogen receptor (ER) β reduces ERα-regulated gene transcription, supporting a "ying yang" relationship between ERα and ERβ in mice. *Mol. Endocrinol.* **17,** 203–208
36. Somjen, D., Katzburg, S., Sharon, O., Knoll, E., Hendel, D., and Stern, N. (2011) Sex-specific response of cultured human bone cells to ERα and ERβ-specific agonists by modulation of cell proliferation and creatine kinase-specific activity. *J. Steroid Biochem. Mol. Biol.* **125,** 226–230
37. Kietz, S., Thomsen, J. S., Matthews, J., Pettersson, K., Ström, A., and Gustafsson, J. A. (2004) The Ah receptor inhibits estrogen-induced estrogen receptor β in breast cancer cells. *Biochem. Biophys. Res. Commun.* **320,** 76–82
38. Bartella, V., Rizza, P., Barone, I., Zito, D., Giordano, F., Giordano, C., Catalano, S., Mauro, L., Sisci, D., Panno, M. L., Fuqua, S. A., and Andò, S. (2012) Estrogen receptor β binds Sp1 and recruits a corepressor complex to the estrogen receptor α gene promoter. *Breast Cancer Res. Treat.* **134,** 569–581
39. Windahl, S. H., Hollberg, K., Vidal, O., Gustafsson, J. A., Ohlsson, C., and Andersson, G. (2001) Female estrogen receptor $\beta^{-/-}$ mice are partially protected against age-related trabecular bone loss. *J. Bone Miner. Res.* **16,** 1388–1398
40. Matthews, J., and Gustafsson, J. A. (2003) Estrogen signaling: a subtle balance between ERα and ERβ. *Mol. Interv.* **3,** 281–292
41. Sims, N. A., Dupont, S., Krust, A., Clement-Lacroix, P., Minet, D., Resche-Rigon, M., Gaillard-Kelly, M., and Baron, R. (2002) Deletion of estrogen receptors reveals a regulatory role for estrogen receptors-β in bone remodeling in females but not in males. *Bone* **30,** 18–25
42. Dupont, S., Krust, A., Gansmuller, A., Dierich, A., Chambon, P., and Mark, M. (2000) Effect of single and compound knockouts of estrogen receptors α (ERα) and β (ERβ) on mouse reproductive phenotypes. *Development* **127,** 4277–4291
43. Kudwa, A. E., and Rissman, E. F. (2003) Double oestrogen receptor α and β knockout mice reveal differences in neural oestrogen-mediated progestin receptor induction and female sexual behaviour. *J. Neuroendocrinol.* **15,** 978–983
44. Stauffer, S. R., Coletta, C. J., Tedesco, R., Nishiguchi, G., Carlson, K., Sun, J., Katzenellenbogen, B. S., and Katzenellenbogen, J. A. (2000) Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-α-selective agonists. *J. Med. Chem.* **43,** 4934–4947
45. Harrington, W. R., Sheng, S., Barnett, D. H., Petz, L. N., Katzenellenbogen, J. A., and Katzenellenbogen, B. S. (2003) Activities of estrogen receptor α- and β-selective ligands at diverse estrogen-responsive gene sites mediating transactivation or transrepression. *Mol. Cell. Endocrinol.* **206,** 13–22
46. Meyers, M. J., Sun, J., Carlson, K. E., Marriner, G. A., Katzenellenbogen, B. S., and Katzenellenbogen, J. A. (2001) Estrogen receptor-β potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. *J. Med. Chem.* **44,** 4230–4251
47. Compton, D. R., Sheng, S., Carlson, K. E., Rebacz, N. A., Lee, I. Y., Katzenellenbogen, B. S., and Katzenellenbogen, J. A. (2004) Pyrazolo[1,5-*a*]pyrimidines: estrogen receptor ligands possessing estrogen receptor β antagonist activity. *J. Med. Chem.* **47,** 5872–5893
48. Zaman, G., Suswillo, R. F., Cheng, M. Z., Tavares, I. A., and Lanyon, L. E. (1997) Early responses to dynamic strain change and prostaglandins in bone-derived cells in culture. *J. Bone Miner. Res.* **12,** 769–777
49. Sevetson, B., Taylor, S., and Pan, Y. (2004) Cbfa1/RUNX2 directs specific expression of the sclerosteosis gene (SOST). *J. Biol. Chem.* **279,** 13849–13858
50. Ghule, P. N., Medina, R., Lengner, C. J., Mandeville, M., Qiao, M., Dominski, Z., Lian, J. B., Stein, J. L., van Wijnen, A. J., and Stein, G. S. (2011) Reprogramming the pluripotent cell cycle: restoration of an abbreviated G_1 phase in human-induced pluripotent stem (iPS) cells. *J. Cell. Physiol.* **226,** 1149–1156
51. Becker, K. A., Stein, J. L., Lian, J. B., van Ijnen, A. J., and Stein, G. S. (2005) in *Cell Biology Handbook: A Laboratory Manual* (Celis, J., ed) pp. 103–110, Academic Press, San Diego
52. Becker, K. A., Stein, J. L., Lian, J. B., van Wijnen, A. J., and Stein, G. S. (2010) Human embryonic stem cells are premitotically committed to self-renewal and acquire a lengthened G_1 phase upon lineage programming. *J. Cell. Physiol.* **222,** 103–110
53. Ghule, P. N., Becker, K. A., Harper, J. W., Lian, J. B., Stein, J. L., van Wijnen, A. J., and Stein, G. S. (2007) Cell cycle-dependent phosphorylation and subnuclear organization of the histone gene regulator p220(NPAT) in human embryonic stem cells. *J. Cell. Physiol.* **213,** 9–17
54. Rozen, S., and Skaletsky, H. (2000) Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol.* **132,** 365–386
55. Kallio, A., Guo, T., Lamminen, E., Seppänen, J., Kangas, L., Väänänen, H. K., and Härkönen, P. (2008) Estrogen and the selective estrogen receptor modulator (SERM) protection against cell death in estrogen receptor α and β expressing U2OS cells. *Mol. Cell. Endocrinol.* **289,** 38–48
56. Xie, H., Sun, M., Liao, X. B., Yuan, L. Q., Sheng, Z. F., Meng, J. C., Wang, D., Yu, Z. Y., Zhang, L. Y., Zhou, H. D., Luo, X. H., Li, H., Wu, X. P., Wei, Q. Y., Tang, S. Y., Wang, Z. Y., and Liao, E. Y. (2011) Estrogen receptor α36 mediates a bone-sparing effect of 17β-estradiol in postmenopausal women. *J. Bone Miner. Res.* **26,** 156–168
57. Zhao, Y. Y., Guo, L., Zhao, X. J., Liu, H., Lei, T., Ma, D. J., and Gao, X. Y. (2009) Transcriptional activation of insulin-like growth factor binding protein 6 by 17β-estradiol in SaOS-2 cells. *Exp. Mol. Med.* **41,** 478–486
58. Zhao, L., and Brinton, R. D. (2007) Estrogen receptor α and β differentially regulate intracellular Ca^{2+} dynamics leading to ERK phosphorylation and estrogen neuroprotection in hippocampal neurons. *Brain Res.* **1172,** 48–59
59. Ivanova, M. M., Luken, K. H., Zimmer, A. S., Lenzo, F. L., Smith, R. J., Arteel, M. W., Kollenberg, T. J., Mattingly, K. A., and Klinge, C. M. (2011) Tamoxifen increases nuclear respiratory factor 1 transcription by activating estrogen receptor β and AP-1 recruitment to adjacent promoter binding sites. *FASEB J.* **25,** 1402–1416
60. Lindberg, K., Helguero, L. A., Omoto, Y., Gustafsson, J. Å., and Haldosén, L. A. (2011) Estrogen receptor β represses Akt signaling in breast cancer cells via down-regulation of HER2/HER3 and up-regulation of PTEN: implications for tamoxifen sensitivity. *Breast Cancer Res.* **13,** R43
61. Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R., and Ben-Ze'ev, A. (1999) The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 5522–5527
62. Glass, D. A., 2nd, Bialek, P., Ahn, J. D., Starbuck, M., Patel, M. S., Clevers, H., Taketo, M. M., Long, F., McMahon, A. P., Lang, R. A., and Karsenty, G. (2005) Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. *Dev. Cell* **8,** 751–764
63. Sawakami, K., Robling, A. G., Ai, M., Pitner, N. D., Liu, D., Warden, S. J., Li, J., Maye, P., Rowe, D. W., Duncan, R. L., Warman, M. L., and Turner, C. H. (2006) The Wnt co-receptor LRP5 is essential for skeletal mechanotransduction but not for the anabolic bone response to parathyroid hormone treatment. *J. Biol. Chem.* **281,** 23698–23711
64. Saxon, L. K., Jackson, B. F., Sugiyama, T., Lanyon, L. E., and Price, J. S. (2011) Analysis of multiple bone responses to graded strains above functional levels, and to disuse, in mice *in vivo* show that the human Lrp5 G171V high bone mass mutation increases the osteogenic response to loading but that lack of Lrp5 activity reduces it. *Bone* **49,** 184–193
65. Niziolek, P. J., Warman, M. L., and Robling, A. G. (2012) Mechanotransduction in bone tissue: The A214V and G171V mutations in Lrp5 enhance load-induced osteogenesis in a surface-selective manner. *Bone* **51,** 459–465
66. Ellies, D. L., Viviano, B., McCarthy, J., Rey, J. P., Itasaki, N., Saunders, S., and Krumlauf, R. (2006) Bone density ligand, *Sclerostin*, directly interacts with LRP5 but not LRP5G171V to modulate Wnt activity. *J. Bone Miner.*

Res. **21,** 1738–1749
67. Kushner, P. J., Agard, D. A., Greene, G. L., Scanlan, T. S., Shiau, A. K., Uht, R. M., and Webb, P. (2000) Estrogen receptor pathways to AP-1. *J. Steroid Biochem. Mol. Biol.* **74,** 311–317
68. McCarthy, T. L., Kallen, C. B., and Centrella, M. (2011) β-Catenin independent cross-control between the estradiol and Wnt pathways in osteoblasts. *Gene* **479,** 16–28
69. Ardawi, M. S., Al-Sibiany, A. M., Bakhsh, T. M., Rouzi, A. A., and Qari, M. H. (2012) Decreased serum sclerostin levels in patients with primary hyperparathyroidism: a cross-sectional and a longitudinal study. *Osteoporos. Int.* **23,** 1789–1797
70. Silvestrini, G., Ballanti, P., Leopizzi, M., Sebastiani, M., Berni, S., Di Vito, M., and Bonucci, E. (2007) Effects of intermittent parathyroid hormone (PTH) administration on SOST mRNA and protein in rat bone. *J. Mol. Histol.* **38,** 261–269
71. Cheng, M. Z., Rawlinson, S. C., Pitsillides, A. A., Zaman, G., Mohan, S., Baylink, D. J., and Lanyon, L. E. (2002) Human osteoblasts' proliferative responses to strain and 17β-estradiol are mediated by the estrogen receptor and the receptor for insulin-like growth factor I. *J. Bone Miner. Res.* **17,** 593–602
72. Williams, C., Edvardsson, K., Lewandowski, S. A., Ström, A., and Gustafsson, J. A. (2008) A genome-wide study of the repressive effects of estrogen receptor β on estrogen receptor α signaling in breast cancer cells. *Oncogene* **27,** 1019–1032
73. Chang, E. C., Frasor, J., Komm, B., and Katzenellenbogen, B. S. (2006) Impact of estrogen receptor β on gene networks regulated by estrogen receptor α in breast cancer cells. *Endocrinology* **147,** 4831–4842
74. Omoto, Y., Eguchi, H., Yamamoto-Yamaguchi, Y., and Hayashi, S. (2003) Estrogen receptor (ER) β1 and ERβcx/β2 inhibit ERα function differently in breast cancer cell line MCF7. *Oncogene* **22,** 5011–5020
75. Zhao, C., Dahlman-Wright, K., and Gustafsson, J. Å. (2010) Estrogen signaling via estrogen receptor β. *J. Biol. Chem.* **285,** 39575–39579
76. Keller, H., and Kneissel, M. (2005) SOST is a target gene for PTH in bone. *Bone* **37,** 148–158
77. Woo, S. M., Rosser, J., Dusevich, V., Kalajzic, I., and Bonewald, L. F. (2011) Cell line IDG-SW3 replicates osteoblast to-late-osteocyte differentiation *in vitro* and accelerates bone formation *in vivo*. *J. Bone Miner. Res.* **26,** 2634–2646
78. Tong, J. S., Zhang, Q. H., Wang, Z. B., Li, S., Yang, C. R., Fu, X. Q., Hou, Y., Wang, Z. Y., Sheng, J., and Sun, Q. Y. (2010) ER-α36, a novel variant of ER-α, mediates estrogen-stimulated proliferation of endometrial carcinoma cells via the PKCδ/ERK pathway. *PLoS One* **5,** e15408
79. Kim, J. H., Jeong, I. Y., Lim, Y., Lee, Y. H., and Shin, S. Y. (2011) Estrogen receptor β stimulates Egr-1 transcription via MEK1/Erk/Elk-1 cascade in C6 glioma cells. *BMB Rep.* **44,** 452–457
80. Setchell, K. D., Clerici, C., Lephart, E. D., Cole, S. J., Heenan, C., Castellani, D., Wolfe, B. E., Nechemias-Zimmer, L., Brown, N. M., Lund, T. D., Handa, R. J., and Heubi, J. E. (2005) S-equol, a potent ligand for estrogen receptor β, is the exclusive enantiomeric form of the soy isoflavone metabolite produced by human intestinal bacterial flora. *Am. J. Clin. Nutr.* **81,** 1072–1079
81. Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., van der Saag, P. T., van der Burg, B., and Gustafsson, J. A. (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. *Endocrinology* **139,** 4252–4263
82. Taku, K., Melby, M. K., Nishi, N., Omori, T., and Kurzer, M. S. (2011) Soy isoflavones for osteoporosis: an evidence-based approach. *Maturitas* **70,** 333–338
83. Bord, S., Horner, A., Beavan, S., and Compston, J. (2001) Estrogen receptors α and β are differentially expressed in developing human bone. *J. Clin. Endocrinol. Metab.* **86,** 2309–2314
84. Sugiyama, T., Saxon, L. K., Zaman, G., Moustafa, A., Sunters, A., Price, J. S., and Lanyon, L. E. (2008) Mechanical loading enhances the anabolic effects of intermittent parathyroid hormone (1–34) on trabecular and cortical bone in mice. *Bone* **43,** 238–248
85. Chung, C. J., Baik, H. S., and Soma, K. (2007) Bone formation and tooth movement are synergistically enhanced by administration of EP4 agonist. *Am. J. Orthod. Dentofacial. Orthop.* **132,** 427
86. Hagino, H., Kuraoka, M., Kameyama, Y., Okano, T., and Teshima, R. (2005) Effect of a selective agonist for prostaglandin E receptor subtype EP4 (ONO-4819) on the cortical bone response to mechanical loading. *Bone* **36,** 444–453
87. van der Eerden, B. C., Löwik, C. W., Wit, J. M., and Karperien, M. (2004) Expression of estrogen receptors and enzymes involved in sex steroid metabolism in the rat tibia during sexual maturation. *J. Endocrinol.* **180,** 457–467
88. Braidman, I. P., Hainey, L., Batra, G., Selby, P. L., Saunders, P. T., and Hoyland, J. A. (2001) Localization of estrogen receptor β protein expression in adult human bone. *J. Bone Miner. Res.* **16,** 214–220
89. Sen, B., Xie, Z., Case, N., Ma, M., Rubin, C., and Rubin, J. (2008) Mechanical strain inhibits adipogenesis in mesenchymal stem cells by stimulating a durable β-catenin signal. *Endocrinology* **149,** 6065–6075
90. Ahdjoudj, S., Lasmoles, F., Holy, X., Zerath, E., and Marie, P. J. (2002) Transforming growth factor β2 inhibits adipocyte differentiation induced by skeletal unloading in rat bone marrow stroma. *J. Bone Miner. Res.* **17,** 668–677
91. Jin, W. J., Jiang, S. D., Jiang, L. S., and Dai, L. Y. (2012) Differential responsiveness to 17β-estradiol of mesenchymal stem cells from postmenopausal women between osteoporosis and osteoarthritis. *Osteoporos. Int.* **23,** 2469–2478
92. Sen, B., Styner, M., Xie, Z., Case, N., Rubin, C. T., and Rubin, J. (2009) Mechanical loading regulates NFATc1 and β-catenin signaling through a GSK3β control node. *J. Biol. Chem.* **284,** 34607–34617
93. Case, N., Ma, M., Sen, B., Xie, Z., Gross, T. S., and Rubin, J. (2008) β-Catenin levels influence rapid mechanical responses in osteoblasts. *J. Biol. Chem.* **283,** 29196–29205
94. Okazaki, R., Inoue, D., Shibata, M., Saika, M., Kido, S., Ooka, H., Tomiyama, H., Sakamoto, Y., and Matsumoto, T. (2002) Estrogen promotes early osteoblast differentiation and inhibits adipocyte differentiation in mouse bone marrow stromal cell lines that express estrogen receptor (ER) α or β. *Endocrinology* **143,** 2349–2356
95. Waters, K. M., Rickard, D. J., Riggs, B. L., Khosla, S., Katzenellenbogen, J. A., Katzenellenbogen, B. S., Moore, J., and Spelsberg, T. C. (2001) Estrogen regulation of human osteoblast function is determined by the stage of differentiation and the estrogen receptor isoform. *J. Cell. Biochem.* **83,** 448–462
96. Almeida, M., Martin-Millan, M., Ambrogini, E., Bradsher, R., 3rd, Han, L., Chen, X. D., Roberson, P. K., Weinstein, R. S., O'Brien, C. A., Jilka, R. L., and Manolagas, S. C. (2010) Estrogens attenuate oxidative stress and the differentiation and apoptosis of osteoblasts by DNA-binding-independent actions of the ERα. *J. Bone Miner. Res.* **25,** 769–781
97. Leskelä, H. V., Olkku, A., Lehtonen, S., Mahonen, A., Koivunen, J., Turpeinen, M., Uusitalo, J., Pelkonen, O., Kangas, L., Selander, K., and Lehenkari, P. (2006) Estrogen receptor α genotype confers interindividual variability of response to estrogen and testosterone in mesenchymal-stem-cell-derived osteoblasts. *Bone* **39,** 1026–1034
98. Khalid, O., Baniwal, S. K., Purcell, D. J., Leclerc, N., Gabet, Y., Stallcup, M. R., Coetzee, G. A., and Frenkel, B. (2008) Modulation of Runx2 activity by estrogen receptor-α: implications for osteoporosis and breast cancer. *Endocrinology* **149,** 5984–5995
99. Barou, O., Palle, S., Vico, L., Alexandre, C., and Lafage-Proust, M. H. (1998) Hindlimb unloading in rat decreases preosteoblast proliferation assessed *in vivo* with BrdU incorporation. *Am. J. Physiol.* **274,** E108–E114
100. Turner, C. H., Owan, I., Alvey, T., Hulman, J., and Hock, J. M. (1998) Recruitment and proliferative responses of osteoblasts after mechanical loading *in vivo* determined using sustained release bromodeoxyuridine. *Bone* **22,** 463–469
101. Hong, L., Zhang, G., Sultana, H., Yu, Y., and Wei, Z. (2011) The effects of 17-β-estradiol on enhancing proliferation of human bone marrow mesenchymal stromal cells *in vitro*. *Stem Cells Dev.* **20,** 925–931
102. Hong, L., Sultana, H., Paulius, K., and Zhang, G. (2009) Steroid regulation of proliferation and osteogenic differentiation of bone marrow stromal cells: a gender difference. *J. Steroid Biochem. Mol. Biol.* **114,** 180–185
103. Cao, L., Bu, R., Oakley, J. I., Kalla, S. E., and Blair, H. C. (2003) Estrogen receptor-β modulates synthesis of bone matrix proteins in human osteo-

blast-like MG63 cells. *J. Cell. Biochem.* **89,** 152–164
104. Börjesson, A. E., Windahl, S. H., Lagerquist, M. K., Engdahl, C., Frenkel, B., Movérare-Skrtic, S., Sjögren, K., Kindblom, J. M., Stubelius, A., Islander, U., Antal, M. C., Krust, A., Chambon, P., and Ohlsson, C. (2011) Roles of transactivating functions 1 and 2 of estrogen receptor-α in bone. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 6288–6293
105. Stossi, F., Barnett, D. H., Frasor, J., Komm, B., Lyttle, C. R., and Katzenellenbogen, B. S. (2004) Transcriptional profiling of estrogen-regulated gene expression via estrogen receptor (ER) α or ERβ in human osteosarcoma cells: distinct and common target genes for these receptors. *Endocrinology* **145,** 3473–3486
106. Noble, B. S., Peet, N., Stevens, H. Y., Brabbs, A., Mosley, J. R., Reilly, G. C., Reeve, J., Skerry, T. M., and Lanyon, L. E. (2003) Mechanical loading: biphasic osteocyte survival and targeting of osteoclasts for bone destruction in rat cortical bone. *Am. J. Physiol. Cell Physiol.* **284,** C934–C943
107. Kousteni, S., Bellido, T., Plotkin, L. I., O'Brien, C. A., Bodenner, D. L., Han, L., Han, K., DiGregorio, G. B., Katzenellenbogen, J. A., Katzenellenbogen, B. S., Roberson, P. K., Weinstein, R. S., Jilka, R. L., and Manolagas, S. C. (2001) Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell* **104,** 719–730
108. Emerton, K. B., Hu, B., Woo, A. A., Sinofsky, A., Hernandez, C., Majeska, R. J., Jepsen, K. J., and Schaffler, M. B. (2010) Osteocyte apoptosis and control of bone resorption following ovariectomy in mice. *Bone* **46,** 577–583
109. Bradford, P. G., Gerace, K. V., Roland, R. L., and Chrzan, B. G. (2010) Estrogen regulation of apoptosis in osteoblasts. *Physiol Behav.* **99,** 181–185
110. Hawse, J. R., Subramaniam, M., Monroe, D. G., Hemmingsen, A. H., Ingle, J. N., Khosla, S., Oursler, M. J., and Spelsberg, T. C. (2008) Estrogen receptor β isoform-specific induction of transforming growth factor β-inducible early gene-1 in human osteoblast cells: an essential role for the activation function 1 domain. *Mol. Endocrinol.* **22,** 1579–1595
111. Cardoso, L., Herman, B. C., Verborgt, O., Laudier, D., Majeska, R. J., and Schaffler, M. B. (2009) Osteocyte apoptosis controls activation of intracortical resorption in response to bone fatigue. *J. Bone Miner. Res.* **24,** 597–605
112. Almeida, M., Iyer, S., Martin-Millan, M., Bartell, S. M., Han, L., Ambrogini, E., Onal, M., Xiong, J., Weinstein, R. S., Jilka, R. L., O'Brien, C. A., and Manolagas, S. C. (2013) Estrogen receptor-α signaling in osteoblast progenitors stimulates cortical bone accrual. *J. Clin. Invest.* **123,** 394–404
113. Mödder, U. I., Hoey, K. A., Amin, S., McCready, L. K., Achenbach, S. J., Riggs, B. L., Melton, L. J., 3rd, and Khosla, S. (2011) Relation of age, gender, and bone mass to circulating sclerostin levels in women and men. *J. Bone Miner. Res.* **26,** 373–379
114. Damien, E., Price, J. S., and Lanyon, L. E. (1998) The estrogen receptor's involvement in osteoblasts' adaptive response to mechanical strain. *J. Bone Miner. Res.* **13,** 1275–1282
115. Maran, A., Shogren, K., Zhang, M., Yaszemski, M. J., Hefferan, T. E., Spelsberg, T. C., Kloosterboer, H. J., and Turner, R. T. (2006) Effects of stable transfection of human fetal osteoblast cells with estrogen receptor-α on regulation of gene expression by tibolone. *Bone* **39,** 523–529
116. Monroe, D. G., Getz, B. J., Johnsen, S. A., Riggs, B. L., Khosla, S., and Spelsberg, T. C. (2003) Estrogen receptor isoform-specific regulation of endogenous gene expression in human osteoblastic cell lines expressing either ERα or ERβ. *J. Cell. Biochem.* **90,** 315–326
117. Saika, M., Inoue, D., Kido, S., and Matsumoto, T. (2001) 17β-Estradiol stimulates expression of osteoprotegerin by a mouse stromal cell line, *ST-2*, via estrogen receptor-α. *Endocrinology* **142,** 2205–2212
118. Krum, S. A., Miranda-Carboni, G. A., Hauschka, P. V., Carroll, J. S., Lane, T. F., Freedman, L. P., and Brown, M. (2008) Estrogen protects bone by inducing Fas ligand in osteoblasts to regulate osteoclast survival. *EMBO J.* **27,** 535–545
119. Quaedackers, M. E., Van Den Brink, C. E., Wissink, S., Schreurs, R. H., Gustafsson, J. A., Van Der Saag, P. T., and Van Der Burg, B. B. (2001) 4-Hydroxytamoxifen trans-represses nuclear factor-κB activity in human osteoblastic U2-OS cells through estrogen receptor (ER) α, and not through ER β. *Endocrinology* **142,** 1156–1166