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Effects of Estrogen on Osteoprogenitor Cells and Cytokines/ Bone-Regulatory Factors in Postmenopausal Women

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Abstract

Decreases in estrogen levels contribute not only to early postmenopausal bone loss but also to bone loss with aging. While estrogen is critical for the maintenance of bone formation, the mechanism(s) of this effect remain unclear. Thus, we assessed the effects of 4 months of transdermal estradiol treatment (0.05 mg/day) of postmenopausal women as compared to no treatment ($n = 16$ per group) on the expression of genes in pre-specified pathways in freshly isolated bone marrow osteoprogenitor cells (hematopoietic lineage [lin][−]/Stro1⁺). We also evaluated whether estrogen treatment modulated peripheral blood or bone marrow plasma levels of the Wnt antagonists, sclerostin and DKK1, as well as serotonin, OPG, RANKL, adiponectin, oxytocin, and inflammatory cytokines (TNF α , IL-1 β , IL-6), as each of these molecules have recently been shown to play an important role in regulating osteoblast function and/or being responsive to estrogen. We observed a significant decrease in the expression of several proliferation markers (cyclin B1, cyclin E1, E2F1) and increase in adhesion molecules (N-cadherin) in bone marrow lin[−]/Stro1⁺ cells from estrogen-treated compared to control women. None of the peripheral blood or bone marrow plasma marker levels differed between the two groups, with the exception of sclerostin levels, which were significantly lower in the estrogen-treated as compared to the control women in peripheral serum (by 32%, $P = 0.009$) and in bone marrow plasma (by 34%, $P = 0.017$). There were significant differences in bone marrow versus peripheral plasma levels of several factors: sclerostin and OPG levels were higher in bone marrow as compared to peripheral plasma, whereas serotonin and adiponectin levels were higher in peripheral as compared to bone marrow plasma. In summary, our data directly assessing possible regulation by estrogen of osteoprogenitor cells in humans indicate that, consistent with previous studies in mice, estrogen suppresses the proliferation of human bone marrow lin[−]/Stro1⁺ cells, which likely represent early osteoprogenitor cells. Further animal and human studies are needed to define the role of the changes we observed in mRNAs for adhesion molecules in these cells and in local sclerostin production in bone in mediating the effects of estrogen on bone metabolism in humans.

Keywords

Estrogen; osteoporosis; postmenopausal women

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Introduction

Estrogen mediates multiple signals that coordinate distinct cellular functions in bone. While estrogen is critical for the maintenance of bone formation [1], the mechanism(s) of this effect remain unclear. Estrogen reduces the proliferation of early mesenchymal progenitors [2], but also reduces apoptosis of mature osteoblasts [3], and may enhance osteoblast differentiation [4]. In addition, previous studies have shown that estrogen changes the adhesive properties of progenitor cells, thereby modulating their mode of interaction with the bone microenvironment. For example, osteoclasts have reduced adhesive properties following exposure to estrogen due to an inhibition of β -integrins [5]. Conversely, estrogen may enhance osteoblast adhesion to the extracellular matrix by increasing the level of cell adhesion proteins [6].

Although previous studies have used mouse or *in vitro* cell models to study estrogen action on bone (reviewed in [7]), it is important to directly define effects of estrogen on osteoblastic cells in humans. To do so, rapid isolation of osteoblast progenitor cells from human marrow aspirates is important in order to capture the complex relationships of these cells *in vivo* to their microenvironment.

The Stro1 antibody is produced by one of several hybridomas that were generated by immunizing mice intrasplenically with human CD34+ bone marrow cells [8]. These hybridomas were initially screened against T- and B-cell lines, and then further selected for reactivity with subpopulations of CD34-expressing cells. Additional studies defined the Stro1 antibody as of the IgM isotype and reacting with marrow stromal cells (MSCs) in the adherent layer of long-term bone marrow cultures [8]. Stro1 has been used predominantly for flow cytometry analysis and, to a much lesser extent, for immunocytochemical staining of candidate MSCs. Although the first report of the Stro1 antibody was 20 years ago [8], the Stro1 antigen remains unidentified, but this antibody is still one of the most widely recognized markers for MSCs [9].

In the present study, we used the established Stro1 antibody to isolate a population from human marrow enriched for osteoblast progenitor cells from untreated and estrogen-treated postmenopausal women and determined potential differences in gene expression for pre-specified pathways, including osteoblastogenesis, adipogenesis, proliferation, apoptosis, adhesion, stem cell markers, BMPs, BMP targets, chemokines, and Hif1 α targets. In addition, we assessed changes in levels of key cytokines/bone-regulatory factors in peripheral blood and bone marrow plasma following estrogen treatment. Specifically, we evaluated whether, in either compartment, estrogen treatment regulated levels of the Wnt antagonists, sclerostin and DKK1, as well as serotonin, OPG, RANKL, adiponectin, oxytocin, and inflammatory cytokines (TNF α , IL-1 β , and IL-6), as each of these molecules have recently been shown to play an important role in regulating osteoblast function and/or being responsive to estrogen, at least *in vitro* (for a review, see [10]).

Patients and Methods

Experimental subjects

For this blinded, randomized study, we recruited 32 healthy postmenopausal women who had cessation of menses for more than ten years. Screening laboratory studies included a complete blood count and serum levels of 25-hydroxyvitamin D (25OHD), follicle stimulating hormone (FSH), parathyroid hormone (PTH), creatinine, calcium, and phosphorus. Exclusion criteria were: 1) use of bisphosphonates, estrogen (oral or transdermal), raloxifene, or PTH (or other bone-active drugs) in the past 3 years; 2) history

of Paget's disease, other metabolic bone disease, diabetes, or significant cardiac, renal, or liver disease; 3) history of any fracture within the past 5 years; 4) hysterectomy; 5) abnormalities in the screening laboratory studies. The study was approved by the Mayo Institutional Review Board and all subjects provided written, informed consent prior to the study.

Study Design

The women were randomized to either a control (no treatment) group or to a 0.05 mg/d estradiol patch (Mylan technologies) group for 4 months (n = 16 per group). Fasting (8 AM) peripheral blood was collected to determine serum levels of estradiol (E₂), estrone (E₁), bone turnover markers, and other bone regulatory factors. Bone marrow was aspirated from the iliac crest to collect bone marrow plasma and to obtain lineage negative (lin⁻)/Stro1⁺ cells following magnetic activated cell sorting (MACS). Bone marrow plasma was used to determine levels of sclerostin, cytokines, and additional bone regulatory factors. Isolated RNA from MACS-sorted lin⁻/Stro1⁺ cells was used to determine gene expression patterns.

Cell Sorting

Bone marrow cells were initially subjected to Ficoll gradient centrifugation for mononuclear (MNC) cell enrichment. Depletion of mature hematopoietic cells such as T cells, B cells, NK cells, dendritic cells, monocytes, granulocytes, erythroid cells, and their committed precursors was accomplished by MACS (Miltenyi) using a lineage negative selection cocktail (Miltenyi) containing biotin-conjugated antibodies to CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, and CD235a (glycophorin A). Subsequently we enriched for lin⁻/Stro1⁺ cells by incubating the lin⁻ cells with a biotinylated Stro1 antibody (R&D Systems) and isolating positive cells by MACS.

Gene Expression Analysis

Total RNA from sorted bone marrow lin⁻/Stro1⁺ cells was isolated using microfuge columns (MicroColumns, Qiagen). DNase treatment to digest all genomic DNA that could lead to false positive gene expression results was done following RNA isolation using Turbo DNA-free DNase (Ambion). RNA quality and purity was confirmed with a Nanodrop spectrophotometer (Thermo Scientific). The overall number of the lin⁻/Stro1⁺ cells was on average 3×10^5 cells, which results in a limited quantity of total RNA to be able to perform in-depth gene expression analyses; therefore, we used the WT-OvationTM Pico RNA amplification system (NuGen Technologies, Inc) to synthesize μ g quantities of amplified cDNA starting with total RNA input of ~50 ng. In this linear amplification system, the relative representation of each transcript species in the original sample is maintained during and after amplification [11, 12].

For the QPCR analyses, we designed primers using the Primer Express program (Applied Biosystems). Primer sequences for any of the genes analyzed in this report are available on request. The PCR reactions were run in the ABI Prism 7900HT Real time System (Applied Biosystems) using SYBR Green (BioRad) as the detection method. Normalization for variations in input RNA was performed using a panel of 10 housekeeping genes (18S, G6PDH, GAPDH, HPRT, L13a, RPII, TBP, α -tubulin, β 2-microglobulin, β -actin) with the geNorm algorithm (<http://medgen.ugent.be/~jvdesomp/genorm/>) [13, 14] used to select the 3–4 most stable housekeeping genes from the 10 on the plate. The PCR Miner algorithm [15] was used to correct for variations in amplification efficiencies and the expression level in each subject for a given gene was normalized to the median expression level of that gene in the control subjects.

Biochemical Assays

Venous blood and bone marrow plasma was collected at 8 am at the end of the 4 months treatment period. Serum E₂ and E₁ were measured by LC-MS/MS (API 5000, Applied Biosystems-MDS Sciex; interassay CV 8%). For assessing bone formation, serum osteocalcin was measured using a two-site immunoradiometric assay (CIS-US; interassay CV, 8%) and serum amino-terminal propeptide of type I collagen (PINP) was measured by radioimmunoassay (Immunodiagnostic Systems; interassay CV, 9%). For assessing bone resorption, serum C-terminal telopeptide of type I collagen (CTX) and tartrate-resistant acid phosphatase 5b (TRAP5b) were measured by enzyme-linked immunosorbent assay (ELISA, Immunodiagnostic Systems; interassay CV, <10% and 4%, respectively). Serum and plasma sclerostin levels were measured using a recently available quantitative ELISA developed by Biomedica and obtained from ALPCO (interassay CV, 4%,) [16–19]. Serotonin levels were measured using a quantitative enzyme immunoassay (Immuno-Biological Laboratories; interassay CV, 7%). Additional proteins assessed included: OPG, using a quantitative enzyme immunoassay (ALPCO; interassay CV, 8%); RANKL, using a quantitative ELISA (ALPCO; interassay CV < 9.5%); TNF- α , using a quantitative sandwich enzyme immunoassay (R&D Systems; interassay CV < 7.5%); IL-1 β , using a quantitative sandwich enzyme immunoassay technique (R&D Systems; interassay CV < 8.5%); IL-6, using a quantitative sandwich enzyme immunoassay (R&D Systems; interassay CV < 6.5%); oxytocin, using an ELISA (Assay Designs; interassay CV < 15%); DKK1, using an ELISA (RayBiotech, Inc; interassay CV < 12%); and adiponectin, using a quantitative ELISA (R&D Systems; interassay CV < 7%).

Statistical Analyses

Statistical Analysis was performed using the JMP® Statistical Discovery Software (SAS Institute Inc). All of the data except for the gene expression data are presented as mean $\pm$ SEM and comparisons between groups were done using 2-sample t-tests. However, since much of the gene expression data was not normally distributed, these data are presented as medians and interquartile (25th–75th percentile) ranges (IQR), and pairwise comparisons between the estrogen-treated and control groups were done using the Wilcoxon Rank Sum test. Linear regression analysis was performed using Pearson correlation coefficients. To analyze the gene expression data, we also used Gene Set Enrichment Analysis (GSEA) [20, 21] as well as the O'Brien Umbrella test [22] to assess if changes in gene expression occurred along *a priori* defined pathways (osteoblastogenesis, adipogenesis, proliferation, apoptosis, adhesion, stem cell markers, BMPs, BMP targets, chemokines, and Hif1 α targets), based on knowledge of cellular signaling. These methods provide a more robust means to analyze gene expression data and increase the power of detecting changes in genes occurring in pre-specified clusters, rather than in isolation.

Results

Patient characteristics

The relevant anthropometric and biochemical data of the control and estrogen-treated postmenopausal women are presented in Table 1. All study subjects were vitamin D sufficient when entering the study, and there were no significant difference between the two groups in any of the baseline parameters.

Serum estrogen and bone turnover markers

As expected, serum estradiol and estrone levels were significantly higher in the estrogen-treated women as compared to the control women as determined in serum samples collected at the end of the treatment period (Table 2). In addition, bone turnover markers (PINP,

osteocalcin, CTX, and TRAP5b) were lower in the estrogen-treated as compared to the control women (Table 2), consistent with the known effects of estrogen in reducing bone resorption followed by a coupled decrease in bone formation [1].

Gene expression analysis of bone marrow lin[−]/Stro1⁺ cells

We used GSEA (see Statistical Methods) to evaluate if pre-specified clusters of genes in selected pathways were altered as a group in bone marrow lin[−]/Stro1⁺ cells from the estrogen-treated as compared to the control women. Table 3 list the genes evaluated in the clusters for osteoblastogenesis, adipogenesis, proliferation, apoptosis, adhesion, stem cell markers, BMPs, BMP targets, chemokines, and Hif1 α targets as well as the respective P-values. This analysis revealed that estrogen treatment significantly upregulated adhesion molecules ($P = 0.010$) but downregulated genes involved in proliferation ($P = 0.030$). The other pathways were not significantly regulated. In addition, we also used the O'Brien Umbrella test (which is another type of analysis used to evaluate changes in pre-specified gene clusters, see Statistical Methods) to analyze the gene expression data, and Table 3 also shows the respective P-values. As is evident, this analysis provided very similar results, with estrogen treatment significantly upregulating adhesion molecules ($P = 0.023$) while downregulating genes involved in proliferation ($P = 0.038$). Thus, two independent statistical analysis methods demonstrated that estrogen regulated genes important for cell proliferation and adhesion in bone marrow lin[−]/Stro1⁺ cells.

Within the cell adhesion pathway, N-cadherin was significantly upregulated when analyzed individually, whereas in the proliferation pathway, E2F1, cyclin B1, and cyclin E1 were also significantly individually downregulated (Table 4). Table 4 also shows several other genes in pathways that were not regulated using the cluster analysis (GSEA or O'Brien tests) but ones that were regulated on an individual basis. These included runx2 (downregulated by estrogen), VEGF B (upregulated), and lysl oxidase (downregulated).

Peripheral blood and bone marrow plasma sclerostin levels

Given the recent evidence that peripheral serum sclerostin levels may be regulated by estrogen [17, 23], we next examined peripheral serum, peripheral plasma, and bone marrow plasma sclerostin levels in the study subjects. As shown in Figure 1, sclerostin levels were lower in the estrogen-treated as compared to the control women in peripheral serum (by 32%), peripheral plasma (by 20%), and bone marrow plasma (by 34%); these differences were clearly significant for peripheral serum and bone marrow plasma, but were of borderline statistical significance for peripheral plasma. In all subjects combined, bone marrow plasma sclerostin levels were significantly correlated with peripheral plasma (Figure 2A) as well as peripheral serum (Figure 2B) sclerostin levels.

Levels of other cytokines/bone regulatory factors in peripheral and bone marrow plasma

In addition to sclerostin, we also measured levels of several other cytokines/bone regulatory factors for potential regulation by estrogen treatment *in vivo* (Table 5). Levels of another Wnt antagonist, DKK1, were similar in control and estrogen-treated women in peripheral and bone marrow plasma. Plasma serotonin, RANKL, and adiponectin levels were also similar in control and estrogen-treated women in peripheral and bone marrow plasma; there was a trend ($P = 0.095$) for OPG levels to be lower in estrogen-treated women in peripheral, but not bone marrow, plasma. Additional factors measured in bone marrow plasma only (oxytocin, TNF α , IL-1 β , IL-6) did not differ between the control and estrogen-treated women.

Comparison of bone marrow versus peripheral plasma levels of cytokines/bone regulatory factors

For the factors where we assessed both bone marrow and peripheral plasma levels, we compared these levels in all subjects combined (Table 6). As shown, bone marrow plasma sclerostin and OPG levels were significantly higher than peripheral plasma levels; by contrast, peripheral plasma serotonin and adiponectin levels were significantly higher than bone marrow plasma levels. DKK1 and RANKL levels did not differ in the two compartments.

Discussion

Our work provides “proof of concept” regarding the potential utility of bone marrow lin[−]/Stro1⁺ osteoprogenitor cells as a novel tool to study metabolic bone diseases. Stro1 is a cell surface marker that is expressed on early progenitor cells that express bone-related genes but low levels of collagen; as such, these cells likely represent early osteoblast progenitors that respond to estrogen with an attenuation in proliferation, consistent with previous data in mice [2]. The up-regulation of mRNAs for adhesion molecules that we observed may serve to anchor these progenitor cells to sites of bone remodeling. In addition, the consistent suppression of sclerostin by estrogen in peripheral blood and bone marrow plasma make it a potential candidate for mediating effects of estrogen on bone metabolism in humans.

As expected, treatment of postmenopausal women with a physiological dose of estradiol for 4 months led to a significant decrease in bone resorption markers with a coupled decrease in bone formation markers. Despite extensive data on effects of estrogen on bone turnover markers and bone mineral density in humans [24], there is little or no data available in humans on direct effect of estrogen on the bone marrow progenitor cells or active osteoblasts on bone surfaces. The study by Di Gregorio using a mouse model demonstrated that estrogen acts *in vivo* and *in vitro* to attenuate osteoblast precursor self-renewal by approximately 50% [2]. Similarly, in our study the human bone marrow lin[−]/Stro1⁺ osteoprogenitor cells expressed significantly lower levels of proliferation genes compared to women not treated with estrogen. Collectively, the previous mouse [2] and now our human data indicate that estrogen leads to a decrease in proliferation of osteoblast progenitor cells.

We also found a significant upregulation of adhesion molecules by the GSEA/O’Brien umbrella cluster tests and, in particular, upregulation of N-cadherin following estrogen treatment. Cell adhesion molecules may contribute to the migration of osteoblast precursor cells towards the bone surface and also to differentiation of these cells into fully mature osteoblasts, thus meeting the constant demand of bone-forming cells at sites of active remodeling. Cheng et al. found that human osteoblastic cells express abundant levels of N-cadherin [25], and these investigators further demonstrated that an N-cadherin antibody resulted in a significant reduction in cell-cell adhesion as well as in BMP-2-induced differentiation of bone marrow stromal cells [25]. Thus, N-cadherin mediated cell-cell adhesion may be necessary for normal differentiation of bone-forming cells. Additional work by Liu et al. [26] has demonstrated that cadherins are more abundantly expressed in human osteoblast progenitor cells following exposure to estrogen. Indirect support for our findings is also provided by the study of Tsutsumimoto et al. [27] which found that TNF α and IL-1 β , which are upregulated following estrogen deficiency, suppress N-cadherin expression in osteoblastic cells. Studies in heterozygous Cdh2^{+/-} mice, which have a 50% reduction in N-cadherin expression [28], lend further support to our findings. Bone mineral density is similar in these heterozygous mice to their wildtype littermates; however, bone loss after ovariectomy is accentuated by Cdh2 haplo-insufficiency as a result of an attenuated activation of bone formation following estrogen deprivation. The reduction in osteoblast recruitment from skeletal stem cells may be due to reduced cell-cell adhesion in

Cdh2 null heterozygous mice. Thus, the upregulation of adhesion molecules as a whole and the significant upregulation of N-cadherin we observed raise the possibility that estrogen may enhance recruitment of osteoblast progenitors and the cell-cell/cell-matrix adhesion of osteoblasts covering the bone surface to participate in active bone formation.

While osteoblast differentiation markers as a whole (using either the GSEA or O'Brien Umbrella analysis) were not regulated by estrogen, we did observe a significant reduction in the mRNA for *runx2* in *lin*⁻/*Stro1*⁺ cells from estrogen-treated as compared to control women. Previous studies on the effects of estrogen on osteoblast differentiation have varied with the cell models used. Thus, while Dang et al. [4] found that exposure of the osteoprogenitor cell line, KS483, to estrogen enhanced osteoblastic differentiation, Almeida and colleagues [29] reported that estrogen attenuated BMP-2-induced osteoblast differentiation in murine and human osteoblastic cells. In addition, since overall bone turnover was reduced following 4 months of estrogen therapy, it is possible that the reduction in *runx2* mRNA levels reflects changes secondary to this reduction in bone turnover rather than any direct effect of estrogen on the *lin*⁻/*Stro1*⁺ cells.

Mesenchymal stem cells have the capacity to differentiate into osteoblasts or adipocytes [30], and histological studies have shown that estrogen reduces the number of adipocytes in bone marrow following one year of treatment in postmenopausal women [31]. This raises the possibility that estrogen may inhibit adipocytic commitment and/or differentiation of mesenchymal stem cells. However, we did not detect any changes in adipogenic genes in *lin*⁻/*Stro1*⁺ cells, indicating that if estrogen does modulate the differentiation of precursor cells towards adipocytes, this effect is not via the stage of differentiation represented by the bone marrow *Stro1*⁺ cells.

We also extend our recent findings where we demonstrated that estrogen reduced circulating sclerostin levels following 4 weeks of therapy [17] to now show a similar effect of estrogen on bone marrow plasma sclerostin levels following 4 months of estrogen therapy. Indeed, of the 10 different candidate regulatory factors assessed in this study at the protein level in bone marrow plasma (sclerostin, DKK1, serotonin, OPG, RANKL, adiponectin, oxytocin, TNF α , IL-1 β , IL-6), only sclerostin was significantly regulated by estrogen. While it is possible that one or more of these (or other) factors change transiently early following estrogen treatment, the robust regulation of sclerostin production by estrogen in this and in our previous study [17] make it a strong candidate for mediating estrogen effects on the skeleton in humans.

We recognize that bone marrow plasma samples inevitably are contaminated by peripheral blood, and there is no rigorous way to "correct" for such contamination. However, as shown in Table 6, there were significant differences in bone marrow versus peripheral plasma levels of several factors: specifically, sclerostin and OPG levels were significantly higher in bone marrow as compared to peripheral blood plasma, whereas serotonin and adiponectin levels were significantly higher in peripheral as compared to bone marrow plasma. This is consistent with the skeleton being the major source for the production of sclerostin [32] and OPG [33], whereas enterocytes and peripheral adipose tissue are the major sources for the production of serotonin and adiponectin, respectively [34, 35]. Thus, while we cannot exclude some degree of peripheral blood contamination of our marrow aspirates, these data indicate that we were clearly sampling different compartments in the bone marrow versus peripheral blood plasma. Nonetheless, given the fairly strong correlations we observed between both peripheral serum and plasma sclerostin and bone marrow plasma sclerostin levels, peripheral blood sclerostin measurements likely do reflect changes in sclerostin levels occurring in the bone microenvironment.

In summary, our data directly assessing possible regulation by estrogen of osteoprogenitor cells in humans indicate that, consistent with previous studies in mice [2], estrogen suppresses the proliferation of human bone marrow lin⁻/Stro1⁺ cells, which likely represent early osteoprogenitor cells. Based on our work, further animal and human studies are also needed to define the role of the changes we observed in mRNAs for adhesion molecules (specifically, N-cadherin) in these cells and in local sclerostin production in bone in mediating the effects of estrogen on bone metabolism in humans.

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Figure 1.
(A) Peripheral serum, (B) peripheral plasma, and (C) bone marrow plasma sclerostin levels in the control and estrogen-treated subjects at the end of the study.

Figure 2. Plots showing the correlation of bone marrow plasma sclerostin levels with (A) peripheral plasma and (B) peripheral serum sclerostin levels in all subjects combined.

Table 1

Baseline anthropometric and biochemical variables in the control and estrogen-treated subjects. Data are means $\pm$ SEM.

	Control	Estrogen	P-value
N	16	16	-
Age, years	70.2 $\pm$ 1.0	72.9 $\pm$ 1.7	0.177
Height, cm	163 $\pm$ 1	162 $\pm$ 2	0.598
Weight, kg	73.0 $\pm$ 2.3	74.8 $\pm$ 3.3	0.611
BMI, kg/m ²	27.4 $\pm$ 0.8	28.6 $\pm$ 1.4	0.456
Serum			
Calcium, mg/dL	9.69 $\pm$ 0.08	9.67 $\pm$ 0.09	0.874
Phosphorus, mg/dL	3.66 $\pm$ 0.06	3.84 $\pm$ 0.11	0.152
Creatinine, mg/dL	0.81 $\pm$ 0.04	0.79 $\pm$ 0.04	0.746
25-hydroxyvitamin D, ng/mL	35.4 $\pm$ 2.1	38.0 $\pm$ 5.2	0.644

Table 2

Serum estrogen and bone turnover markers in the control and estrogen-treated subjects at the end of the study.

	Control	Estrogen	P-value
E ₂ , pg/mL	6.2 ± 0.7	86.9 ± 11.4	<0.0001
E ₁ , pg/mL	27.1 ± 2.7	52.1 ± 4.3	<0.0001
PINP, µg/mL	50.6 ± 3.8	39.0 ± 4.2	0.051
Osteocalcin, ng/mL	21.2 ± 3.5	15.0 ± 1.2	0.105
CTX, ng/mL	0.43 ± 0.05	0.25 ± 0.03	0.005
TRAP5b, U/L	3.24 ± 0.272	2.24 ± 0.13	0.003

Table 3

List of genes examined in each of the pathways analyzed along with the results of the cluster analysis testing for changes in the genes in each pathway as a group, using two different statistical approaches (GSEA and O'Brien Umbrella test, see Methods).

Pathway	Genes	GSEA P-value	O'Brien Umbrella P-value
Osteoblastogenesis	AP, Col1 α 1, Col1 α 2, Osteocalcin, Osteonectin, Osteopontin, Osterix, OPG, RANKL, Runx2	0.215	0.722
Adipogenesis	Adiponectin, adipon, aP2, LPL, Perilipin, PPAR γ 2	0.475	0.268
Proliferation	CDK2, CDK6, CyclinA, CyclinB1, CyclinB2, CyclinC, CyclinD1, CyclinD2, CyclinE1, E2F1, Ki67, Lef1, Tcf7	0.030	0.038
Apoptosis	Bad, Bax, Bcl-2, Bcl-xl, Caspase	0.264	0.160
Adhesion	ALCAM, N-cadherin, ICAM-1, VCAM1	0.010	0.023
Stem cell markers	ABCG2, BMP-4, Egfr, KITL, Nanog, Pou5f1, SDF1	0.240	0.502
BMPs	BMP2, BMP4, BMP6	0.080	0.984
BMP targets	Areb6, Hes1, Id1, Id2, JunB, Lysl Oxidase, SMAD6, SMAD7, Sox4, TIEG	0.105	0.138
Chemokines	CXCL1, CXCL4, CXCL9, CXCL13, CXCL16	0.295	0.937
Hif1 α and targets	ABCB1, CITED2, CXCR3, DEC2, EDN1, ENG, EPO, ETS1, Flt-1, HIF-1 α , HIF-1 α inhibitor, IGFBP1, KDR, LDHA, Leptin, Nos2, P4HA1, Serpine1, VEGF A, VEGF B, VEGF C	0.375	0.707

Table 4

Individually regulated genes within the pathways in the estrogen-treated compared to the control women. Data are median (25th–75th percentile [IQR]), with the expression level for each gene in the estrogen group normalized to the median expression level in the control group.

	Control	Estrogen	P-value
N-Cadherin	1.0 (0.3–1.6)	2.7 (1.2–23.7)	0.006
E2F1	1.0 (0.8–1.4)	0.5 (0.2–1.0)	0.048
CyclinB1	1.0 (0.7–1.1)	0.7 (0.6–0.9)	0.022
CyclinE1	1.0 (0.8–1.7)	0.8 (0.6–1.1)	0.027
Runx2	1.0 (0.9–1.2)	0.8 (0.6–0.9)	0.033
VEGF B	1.0 (0.7–1.2)	1.6 (1.1–4391)	0.011
Lysl oxidase	1.0 (0.5–9.8)	0.3 (0.0–1.7)	0.036

Table 5

Peripheral and bone marrow plasma levels of cytokines/bone regulatory factors in the control and estrogen-treated subjects at the end of the study.

	Control	Estrogen	P-value
Peripheral plasma			
DKK1, ng/mL	3.63 ± 3.49	8.50 ± 3.51	0.334
Serotonin, ng/mL	3.16 ± 0.88	2.29 ± 0.58	0.416
OPG, pmol/L	4.04 ± 0.30	3.35 ± 0.26	0.095
RANKL, pmol/L	5415 ± 2013	6607 ± 2257	0.698
Adiponectin, ng/mL	12549 ± 1530	12919 ± 1544	0.866
Bone marrow plasma			
DKK1, ng/mL	5.99 ± 5.95	3.88 ± 3.74	0.766
Serotonin, ng/mL	0.22 ± 0.02	0.22 ± 0.02	0.908
OPG, pmol/L	4.33 ± 0.43	3.90 ± 0.30	0.411
RANKL, pmol/L	4203 ± 2063	6364 ± 2286	0.488
Adiponectin, ng/mL	8939 ± 1484	9615 ± 1268	0.731
Oxytocin, pg/mL	1906 ± 275	1509 ± 217	0.264
TNFα, pg/mL	7.49 ± 0.30	7.90 ± 0.24	0.287
IL-1β, pg/mL	1.65 ± 0.09	1.54 ± 0.09	0.416
IL-6, pg/mL	2.88 ± 0.21	3.47 ± 0.68	0.423

Table 6

Comparison of peripheral versus bone marrow plasma levels of cytokines/bone regulatory factors in all subjects combined.

	Bone marrow	Peripheral blood	P-value
Sclerostin, pmol/L	68.9 ± 6.0	54.0 ± 3.6	0.002
Serotonin, ng/mL	0.22 ± 0.015	2.72 ± 0.52	<0.001
DKK1, ng/mL	4.9 ± 3.5	6.1 ± 2.5	0.621
OPG, pmol/L	4.1 ± 0.26	3.7 ± 0.20	0.011
RANKL, pmol/L	5283 ± 1527	6030 ± 1497	0.280
Adiponectin, ng/mL	9277 ± 962	12734 ± 1069	<0.001