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Expression Profiling of Rat Femur Revealed Suppression of Bone Formation Genes by Treatment with Alendronate and Estrogen but Not Raloxifene[S]

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ABSTRACT

The pharmacological preservation of bone in the ovariectomized rat by estrogen, selective estrogen receptor modulators (SERMs), and bisphosphonates has been well described. However, comprehensive molecular analysis of the effects of these pharmacologically diverse antiresorptive agents on gene expression in bone has not been performed. This study used DNA microarrays to analyze RNA from the proximal femur metaphysis of sham and ovariectomized vehicle-treated rats, and ovariectomized rats treated for 35 days with maximally efficacious doses of 17-α ethinyl estradiol, the benzothiophene SERM, raloxifene, the benzopyran SERM, (*S*)-3-(4-hydroxyphenyl)-4-methyl-2-[4-[2-(1-piperidinyl)ethoxy]phenyl]-2*H*-1-benzopyran-7-ol (EM652), and the aminobisphosphonate, alendronate. Ovariectomy resulted in 644 significant probe set changes relative to sham control rats ($p < 0.05$), whereas E2, raloxifene, EM652, and alendronate regulated 613, 765, 652, and 737 probe sets, respectively, relative to ovariectomized control rats. An intersection of these data sets yielded 334 unique genes that were altered after ovariectomy and additionally changed by one or more antiresorptive treatment. Clustering analysis showed that the transcript profile was distinctly different for each pharmaceutical agent and that raloxifene maintained more genes at sham levels than any other treatment. In addition, E2 and alendronate suppressed a cluster of genes associated with bone formation activity below that of sham, whereas raloxifene had little effect on these genes. These data indicate stronger suppressive effects of E2 and alendronate on bone formation activity and that ovariectomy plus raloxifene resembles sham more closely than ovariectomized animals treated with E2, EM652, or alendronate.

Ovariectomy of mature rats has been shown to induce cancellous bone loss from axial and appendicular sites, as observed with postmenopausal women (Turner et al., 1994; Kimmel, 1996), and this model has been used widely to study the prevention of ovariectomy-induced bone loss (Kimmel, 1996; Sato et al., 1999). Efficacy of antiresorptive agents in ovariectomized rats has been predictive of skeletal benefit in postmenopausal women for estrogens (Turner et al., 1994; Cauley et al., 2003; Anderson et al., 2004), selective estrogen receptor modulators (SERMs), such as raloxifene and EM652 (Black et al., 1994; Sato et al., 1995; Delmas et al., 1997; Ettinger et al., 1999; Martel et al., 2000), and bisphosphonates, such as alendronate (Seedor et al., 1991; Toolan et al., 1992; Liberman et al., 1995). Estrogen, raloxifene, and alendronate have all been shown to

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ABBREVIATIONS: SERM, selective estrogen receptor modulator; E2, 17-α-ethinyl estradiol; EM652, (*S*)-3-(4-hydroxyphenyl)-4-methyl-2-[4-[2-(1-piperidinyl)ethoxy]phenyl]-2*H*-1-benzopyran-7-ol; CDX, hydroxypropyl-β-cyclodextrin; PCR, polymerase chain reaction; FDR, false discovery rate; HCA, hierarchical clustering analysis; SOM, self-organizing map; Ovx, ovariectomized vehicle control rats; BFR, bone formation rate; Cyp27a1, cytochrome P450 family 27 subfamily A polypeptide 1; Ca4, Carbonic anhydrase 4; BMD, bone mineral density; PTH, parathyroid hormone; Col1a2, collagen type I α2; Col5a1, collagen type V α1; Ocn, osteocalcin; Sparc, osteonectin; Serpinh1, serpine proteinase inhibitor clade H; Pcolce, procollagen C-proteinase enhancer protein; Ca4, carbonic anhydrase 4; Cyp 27a1, cytochrome P450 family 27 subfamily A polypeptide 1; Bgn, biglycan; Igsf4, immunoglobulin superfamily member 4; Hox3a, homeobox protein C8; Fgfr1, fibroblast growth factor receptor 1.

inhibit bone resorption but they have very different mechanisms of action (Sato et al., 1999; Riggs and Parfitt, 2005).

The loss of ovarian function dramatically reduces circulating levels of estrogen and results in an increased rate of bone resorption in animals and humans. Estrogen or SERM treatment reduces skeletal turnover by maintaining the important estrogen receptor-signaling component in bone (Sato et al., 1999). SERMs, however, achieve this benefit in a tissue-specific manner and behave as estrogen agonists in bone while exerting antagonistic effects in various other estrogen target tissues, thereby avoiding the adverse effects associated with estrogen (Riggs and Hartmann, 2003). The bisphosphonates achieve a dramatic reduction in bone turnover rates by physically complexing with the bone mineral and thus inhibiting the ability of osteoclasts from resorbing the bone away (Rodan and Fleisch, 1996).

Although the histological and biomechanical changes associated with these antiresorptive therapies have been described in the ovariectomized rat, a comparison of the detailed molecular changes for each drug has not been reported. Understanding the common molecular signature that results from drug treatment could provide clues as to the most essential gene changes associated with the preservation of bone integrity after the loss of ovarian function, regardless of the mechanism by which the preservation of bone was achieved. On the other hand, observing the differences in gene expression resulting from the different treatments could yield information about the mechanisms of bone preservation resulting from estrogen receptor signaling versus that of bisphosphonate treatment.

In this report, we describe the expression profile of genes that are changed after ovariectomy and are regulated by estrogen, by two SERMs from different structural classes (the benzothiophene SERM raloxifene and the benzopyran SERM EM652), and/or by the aminobisphosphonate alendronate in the ovariectomized rat proximal femur. We sought to ascertain which genes are associated with ovariectomy 40 days after surgery when bone formation and resorption activity are known to be elevated. We also evaluated the ability of various antiresorptive agents to maintain genes that had been altered by ovariectomy near to sham levels and to determine the genes that were commonly regulated by all treatments. Finally, we sought to identify the similarities and differences among the agents that signal through the estrogen receptor (two SERMs and estrogen) in regulating ovariectomy-induced gene changes.

Materials and Methods

Animal Study Design. Six-month-old Sprague-Dawley rats (Harlan, Indianapolis, IN) were group-housed and maintained on a 12-h light/dark cycle at 22°C with access to food ad libitum (TD 89222 with 0.5% Ca and 0.4% P; Teklad, Madison, WI) and water. Rats were randomized into six groups (n = 5 rats/group): 1) sham-operated animals treated with vehicle [20% hydroxypropyl-β-cyclodextrin (CDX)] (Sigma, St. Louis, MO) 2) ovariectomy group treated with CDX (Ovx); 3) ovariectomized animals administered estrogen [0.1 mg/kg/day 17-α-ethinyl estradiol (E2; Sigma)] in CDX; 4) ovariectomized animals administered 1.0 mg/kg/day raloxifene (Eli Lilly and Co., Indianapolis, IN) in CDX; 5) ovariectomized animals administered 0.1 mg/kg/day EM652 (Eli Lilly and Co.) in CDX; and 6) ovariectomized animals administered 8 μg/kg/day alendronate (Eli Lilly and Co.) in saline. The doses were chosen because they were shown previously to be fully efficacious in rats (Schenk et al., 1986; Sato et al., 1996; Martel et al., 2000).

The study was initiated 5 days after ovariectomy, and compounds were administered by oral gavage (except for the subcutaneous administration of alendronate) for 35 days (40 days after surgery), after which the proximal femora were collected 24 h after dosing. Two additional studies were conducted for validation purposes and were executed exactly as the 5-week study above (except for the omission of the EM652 group). The first validation study dosed the animals for 5 weeks and used femora for histomorphometric analysis; the proximal end of the contralateral femur was subjected to RNA isolation. The second validation study dosed animals for 9 days and analyzed the RNA from the distal end of the femur. At sacrifice, anesthetized rats were subjected to cardiac puncture and asphyxiated by CO_2 inhalation. Animals were fasted the night before termination of each study, and all animal procedures were reviewed by an internal animal welfare committee to ensure compliance with National Institutes of Health guidelines.

Blood samples were allowed to clot at 4°C for approximately 2 h and then centrifuged at 2000g for 10 min. Serum samples were collected and stored at −70°C for subsequent analysis of serum cholesterol. Quantitative determination of cholesterol levels was achieved by measurement of cholesterol esterase/cholesterol oxidase activity using a Roche/Hitachi 917 automated chemistry analyzer.

RNA Isolation and Northern Analysis. The epiphysis and periosteum were removed from the proximal femora, and a 3-mm section of the metaphysis was collected and directly immersed in liquid nitrogen. Samples were then stored in liquid nitrogen until subjected to RNA analysis, at which time they were mechanically homogenized in Ultraspec RNA Isolation reagent (Biotexc Laboratories, Houston, TX) according to the manufacturer's instructions. Twenty-five micrograms of total RNA was electrophoretically separated, transferred to nylon membranes, and probed with radiolabeled cDNA probes as described previously (Ma et al., 2001). Gene expression was normalized to either 18S ribosomal or cyclophilin expression.

For real-time quantitative reverse transcription PCR, an ABI Prism Sequence Detection System 5700 was used and the primer-probe sets for the genes described were obtained as Assay-on-Demand reagents (Applied Biosystems, Foster City, CA). Before cDNA synthesis, 5 μg of total RNA were DNase-treated for 30 min at 37°C (DNA-free kits; Ambion, Austin, TX). RNA was reverse-transcribed from random hexamer primers using SuperScript II reverse transcription kit (Invitrogen, Carlsbad, CA). Specific amplification reactions from the cDNAs were carried out via a two-step, real-time PCR, and relative quantities were obtained by generating a standard curve for each gene. For normalization, amplification of 18S ribosomal RNA was performed for each sample in the same PCR run.

Microarray Analysis. Affymetrix rat genome U34A microarrays were used to determine transcript abundance from total RNA samples of individual metaphyseal samples. Total RNA was labeled according to manufacturer's instructions (Affymetrix, Santa Clara, CA). In brief, all samples were cleaned using RNeasy spin columns (QIAGEN, Valencia, CA). Double-stranded cDNA was synthesized from 10 μg of clean total RNA using SuperScript II cDNA synthesis kit (Invitrogen) and the T7-$(dT)_{24}$ primer containing a T7 promoter [5′-GCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG $(dT)_{24}$-3′; Genset Corp, Evry, France]. Phase Lock Gel tubes (1.5 ml; Eppendorf, Westbury, NY) were used to clean cDNA after phenol/chloroform/isoamyl alcohol extraction. Biotin-labeled cRNA was synthesized from cDNA using BioArray High Yield RNA Transcript Labeling kit (Enzo Diagnostics, Farmingdale, NY) and cleaned by RNeasy spin columns (QIAGEN). Clean cRNA was fragmented by incubation at 94°C in the presence of 40 mM Tris-acetate, pH 8.1, 100 mM potassium acetate, and 30 mM magnesium acetate for 35 min. Fragmented cRNA (10 μg) was hybridized to RG_U34A arrays for 16 h at 45°C with rotation (60 rpm). Each microarray was washed and stained using an Affymetrix Fluidics Station 400 and scanned in an Affymetrix confocal GeneArray scanner. Affymetrix MAS 4.0

software was used to scale data to a target intensity of 1500 and calculate transcript abundance.

Statistical Analysis. Five animals were used in each treatment group to control for biological variation. Each biological sample was then hybridized to duplicate chips to account for the variation caused by chip performance, hybridization quality, and other differences. To test whether a gene is differentially expressed, a mixed effect model was fitted on each of the 8799 probe sets on the chip. The intensity value (Affymetrix MAS4 signal) of a particular gene was modeled as

$$Y_{kij} = \mu_k + \alpha_{i(k)} + \varepsilon_{kij} \quad k = 1, \ldots, 4; i = 1, \ldots, 5; j = 1, 2. \quad (1)$$

where Y_{kij} is the signal of the j^{th} replicate of animal i from treatment group k, μ_k is the group mean of treatment k, $\alpha_{i(k)}$ is the animal variation (random effect) having distribution $N(0, \sigma_A^2)$, and ϵ_{kij}, independent of $\alpha_{i(k)}$, is the measurement error (chip-to-chip variation) after distribution $N(0, \sigma_C^2)$.

Because thousands of hypotheses were tested simultaneously, the issue of multiplicity is a big concern. To eliminate the false positives, Benjamini and Hochberg's false discovery rate (FDR) was used to adjust the p values derived from the above mixed model (Benjamini and Hochberg, 1995). The algorithm of FDR calculation could be simplified as follows: Suppose that being tested are m hypotheses H_i with corresponding p value P_i, $i = 1, 2, \ldots, m$. Let $P_{(i)}$ be the i^{th} p value ranked from the smallest to the largest with the corresponding hypothesis noted as $H_{(i)}$. Let the adjusted p value be labeled as $\tilde{P}$, they are calculated as

$$\text{Step 1. } \tilde{P}_{(m)} = P_{(m)};$$

$$\text{Step 2. } \tilde{P}_{(i)} = \min\left(\frac{m}{i}P_{(i)}, \tilde{P}_{(i+1)}\right), \quad \text{for } i = 1, 2, \ldots, m-1.$$

The FDR is controlled at level q if we reject all $H_{(j)}, j = 1, 2, \ldots, k$, where

$$k = \max\left\{i: P_{(i)} \le \frac{i}{m}q\right\} \quad (2)$$

The analysis of variance tests were done with treatment groups being the fixed effect and animals being the random effect. The p values for the pair-wise comparisons were derived based on the corresponding t statistics.

Bioinformatics Analysis. Principal component analysis was employed to reduce the dimensionality of the data and to assess any animal-to-animal variability by taking advantage of coregulation among a large number of genes while retaining as much variation as possible. The reduction is achieved by transforming the data into a new set of independent variables, the principal components. The principal components are ordered in such a way that the first few retain most of the variation present in all original genes. The analysis was performed in R using the principal component analysis function with standard data (Venables and Ripley, 1997). The first three principal components with at least 60% variation were exported into DecisionSite (Spotfire, Somerville, MA) to generate sample scatter plots for visually examining the data structure.

Differentially expressed genes were identified as significant if $p < 0.05$ and the median signal was larger than 500 for each animal within one or more treatment groups. Hierarchical clustering analysis (HCA) was done in DecisionSite in Euclidean space by the complete linkage method. Heat map visualization of clusters formed was generated using range-scaled expression values. To compensate for local minima often seen in HCA, self-organizing maps (SOM) were also employed to cluster genes. SOM analysis was carried out in DecisionSite with a grid size of 4 × 4 with default parameters. Clusters from SOM analysis were compared with those from HCA and similar clusters were merged into one.

To determine the statistical significance of the numbers of genes maintained at sham level by drug treatment, the proportion of genes in a given gene ontology (Ashburner et al., 2000) term relative to the total number of genes that had changed by ovariectomy was calculated. For each of five groups, the proportion of genes in a given term relative to the total number of genes in that group was calculated. Over-representation significance, when k representors are present in a sample size n, was calculated based on the hypergeometric cumulative distribution function:

$$p = 1 - \sum_{i=0}^{k-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{i}} \quad (3)$$

where N is the number of genes that had changed with ovariectomy, M is the number of genes in the gene ontology term of interest, n is the number of genes in the treatment group, and k is the number of genes in the treatment group that are in the gene ontology term of interest. N, M, n, and k are integers such that $0 \le M \le N$, $0 < n \le N$. These calculations were done on software written by Eli Lilly and Co.; however, comparable software is publicly available (Boyle et al., 2004).

Bone Parameters. Quantitative computed tomography of the distal femur and biomechanical analyses of the proximal femur from the 5-week validation study were performed as described previously (Sato et al., 1997).

Gene Annotation. Target sequences for each chip were downloaded from the Affymetrix web site and then compared with the NCBI genome builds, with UniGene, and with RefSeq transcripts with BLAT. Annotations from these sources were used to map the probe sets both to LocusLink IDs and to full-length sequence IDs. The LocusLink IDs were mapped into the HumanPSD database (Hodges et al., 2002) via indices provided by that database. Note that this database aspires to contain the full protein complement of mouse and rat as well as human. For probe sets without a LocusLink based mapping, the full-length sequences were compared with the protein sequences in HumanPSD. Alignments with at least 100 amino acids of 100% identity and/or a BLAST e value of better than 1e-20 were recorded. Multiple identifications at the same reliability level were suppressed as potential conflicts. Functional information, including gene ontology classification, gene names, and descriptions were retrieved from HumanPSD.

Results

Ovariectomy and treatment by all compounds were well tolerated, with no clinical issues observed. Study outcomes (body weight and serum cholesterol levels) of each group used to collect femoral metaphyseal RNA for the microarray are detailed in Table 1. Ovariectomy increased serum cholesterol and body weight in vehicle control rats (Ovx) relative to sham. Compounds signaling through the estrogen receptor

TABLE 1

Study design, serum cholesterol, and body weight after 5 weeks of treatment

Mean body weight and serum cholesterol concentrations ± S.E.M. for five animals per group.

Group	Treatment	Body Weight	Serum Cholesterol
		g	*mg/dl*
Sham	Vehicle p.o.	285 ± 18	111 ± 8*
Ovx	Vehicle p.o.	309 ± 9	123 ± 9
Ral	1.0 mg/kg/day p.o.	253 ± 7*†	42 ± 1*†
E2	0.1 mg/kg/day p.o.	231 ± 10*†	41 ± 3*†
EM	1.0 mg/kg/day p.o.	290 ± 10	51 ± 7*†
ABP	8 μg/kg/day s.c.	292 ± 15	130 ± 4†

Ral, raloxifene; EM, EM652; ABP, alendronate.
* $P < 0.05$ vs. Ovx control.
† $P < 0.05$ vs. Sham control.

demonstrated a decrease in body weight as expected; it is known that estrogen receptor agonism reduces the ovariectomy-induced hyperphagia associated with estrogen loss (Geary, 2001; Meli et al., 2004). E2 and raloxifene treatment significantly reduced both body weight and cholesterol below Ovx and sham, whereas alendronate had no effect relative to Ovx (Black et al., 1994; Frolik et al., 1996). EM652 significantly lowered cholesterol below that of sham and Ovx, but the lowering of body weight did not achieve statistical significance in our study; however, this has been demonstrated by others (Martel et al., 2000).

Bioinformatics Analyses of Gene Changes. To explore hidden patterns and to visually identify coregulated genes, unsupervised clustering algorithms were used for data analysis. Principal component analysis evaluated the entire microarray data from all animals and all treatments without any filtering of the data. A subtle pattern emerged in the overall expression profile that could be used to distinguish various treatment groups. The duplicate microarray chips from each alendronate and E2 animal appeared to cluster together, whereas the EM652, raloxifene, and Ovx chip profiles seemed to be more closely associated. The sham group chips clustered together and were positioned intermediate between the other two groupings. It should be noted that duplicate chips from one animal in the EM652 group did not cluster with the remainder of its treatment group (Fig. 1).

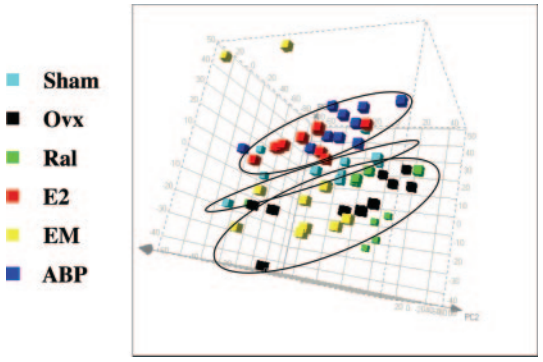

Fig. 1. Scatter plot of principal component analysis of all probe sets on the Affymetrix U34A chip after 35 days of treatment. Each square represents a microarray chip generated from individual animals (two chips per animal). The closer the proximity of the squares to each other, the more closely related the chip expression profile. Note that the two EM652 outlier chips correspond to a single animal. Chips that seem to group together are encircled. Ral, raloxifene; EM, EM652; ABP, alendronate.

To understand which genes relevant to ovariectomy-induced bone loss were driving these associations, we limited our subsequent analysis to those 644 probe sets that were significantly changed ($p < 0.05$) by Ovx relative to sham. Genes changed after each treatment regimen relative to Ovx control rats were determined for each antiresorptive agent: E2 (613 probe sets), raloxifene (765), EM652 (652), and alendronate (737) and compared with the list of ovariectomy-induced gene changes. The intersection of these data generated 380 probe sets that represent 334 unique genes changed by ovariectomy that are additionally modulated by one or more of the antiresorptive agents (see Supplemental data). The median of the intensity data for each treatment group was then subjected to HCA. The heat map visualization of the data set (Fig. 2) illustrates that gene changes associated with Ovx were almost equally distributed between induction or repression from sham levels, and each of the antiresorptive therapies had a unique expression pattern compared with the others.

Given the complexity of the data, we further used SOMs to identify unique patterns of expression for each treatment. SOM analysis (Fig. 3) identified unique patterns of gene expression that could be assigned into four broad categories: 1) genes altered by ovariectomy but restored to or maintained near sham levels by all antiresorptive treatments (clusters 5, 6, 15, and 16); 2) genes uniquely kept near sham levels only by raloxifene whereas the other therapies had no effect (clusters 8, 9, 13, and 14); 3) genes uniquely returned to or below that of sham by all of the agents except raloxifene and partially by EM652 (cluster 1); and 4) genes uniquely kept near sham levels only by E2 (clusters 2 and 11). The genes that were commonly regulated by all the antiresorptives are listed in Table 2. E2 and raloxifene uniquely regulated genes back toward sham level (i.e., no other treatment did so) and are listed as "unique" genes in Tables 3 and 4. In contrast, there were no specific clusters of genes that were solely regulated by EM652 or by alendronate.

Gene Changes Resulting from Ovariectomy. At 40 days after ovariectomy, the expected increase in expression from sham level associated with bone formation genes [such as collagen type I α2 (Col1a2 + 2.4-fold), collagen type V α1 (Col5a1 + 1.4-fold), osteocalcin (Ocn + 1.9-fold), osteonectin

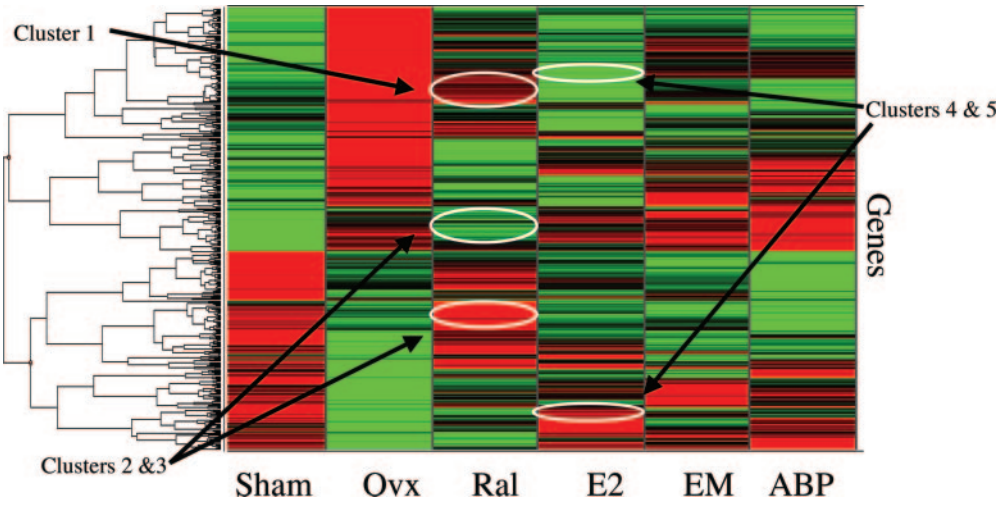

Fig. 2. Hierarchical clustering analysis of 380 probe sets altered by ovariectomy and by one or more drugs after 5 weeks of treatment. Cluster 1 identifies a set of genes that was elevated by Ovx and suppressed by all treatments *except* raloxifene to or below that of sham. Clusters 2 and 3 identify sets of genes altered by Ovx that only raloxifene maintained at sham levels. Clusters 4 and 5 identify sets of genes altered by Ovx that only E2 maintained at sham levels. The group median intensity values from MAS4 analysis were scaled so that red indicates high, green indicates low, and black indicates intermediate expression levels. Ral, raloxifene; EM, EM652; ABP, alendronate.

(Sparc + 2.1-fold), and decorin (+2.3-fold)] were observed by microarray. Increases in osteoclastic genes (i.e., cathepsin K, tartrate resistant acid phosphatase, and calcitonin receptor) were either not expressed above background levels or were not significantly changed by ovariectomy. This absence was not surprising; the 5-week time point was well beyond the peak of osteoclastic activity (2 weeks), and the trabecular bone volume at the metaphyseal site was reduced by ~40% (Wronski et al., 1989). However, because the sample region is rich in osteoblasts, the osteoblastic marker of bone resorption (RANK ligand) was measured to demonstrate that signature molecular changes could be observed after ovariectomy by microarray. Because probe sets for RANK ligand were not included on the chip, its expression was analyzed by Northern blot and found to be elevated (+2-fold) with ovariectomy (data not shown). These data are consistent with bone histomorphometry of the Ovx rat that showed rapid increase in bone formation rates (BFRs) in the metaphysis, along with up-regulation of osteoclastic activity (Turner et al., 1994). More than 300 genes were coregulated with these formation and resorption genes after ovariectomy. These ovariectomy-induced gene changes are implicated in a variety of molecular functions and biological processes that are summarized by noting their gene ontology descriptor as outlined under *Materials and Methods*. Table 5 details the gene ontology assignment of gene changes altered by Ovx relative to sham.

Genes Regulated by All Antiresorptive Agents. Seventy genes listed in Table 2 were commonly regulated by all antiresorptives relative to Ovx after 5 weeks of treatment as identified by SOM analysis. These 70 genes were maintained near sham levels with all treatments and were either elevated (n = 42) or suppressed (n = 28) by ovariectomy. The majority of these changes were small, but 44% of the genes altered by ovariectomy were changed 1.5-fold or more from sham. Genes that were changed by less than 1.5-fold seemed to be stable changes in that the FDR was less than 0.3 for 77% of the genes. Genes affecting matrix production and mineralization that were significantly elevated by ovariectomy [collagen type Vα2 (+2.7-fold), serine proteinase inhibitor clade H (Serpinh1 + 1.7-fold), lysyl oxidase (+1.8-fold), and collagen type XIα1 (+3-fold), Sparc (+3.0 fold), and tissue inhibitor of metalloproteinase 1 (+2.1-fold)] were suppressed near to sham levels by all test compounds. On the other hand, each drug increased a subset of genes that were suppressed by ovariectomy, such as fibroblast growth factor 9 (−2-fold), mucin (−1.7-fold), K-cadherin (−1.9-fold), and retinoic acid receptor α (−5.8-fold). The molecular function and biological processes potentially affected by these gene changes were evaluated by assessing the gene ontology terms associated with each gene as shown in Table 5. Statistical testing of these common gene changes relative to those changed by Ovx was performed to determine which categories were more significantly affected; these are listed in Table 6. Three genes regulated commonly by all treatments were validated in a separate animal study [lysyl oxidase, Serpinh1, and procollagen C-proteinase enhancer protein (Pcolce)]. Comparisons of their regulation relative to ovariectomized control rats in the original microarray study and in a second independent study are presented in Table 7.

Genes Uniquely Regulated by Raloxifene. Raloxifene maintained more genes near sham level than any other treatment. In addition to the commonly regulated genes (Table 2), raloxifene uniquely returned 67 genes (Table 3) near to sham level, whereas the other three drug treatments had no effect on these genes (Fig. 2, clusters 2 and 3). Sixty-three percent of the unique raloxifene genes were suppressed by ovariectomy and subsequently increased by raloxifene treatment; 41% of these were altered

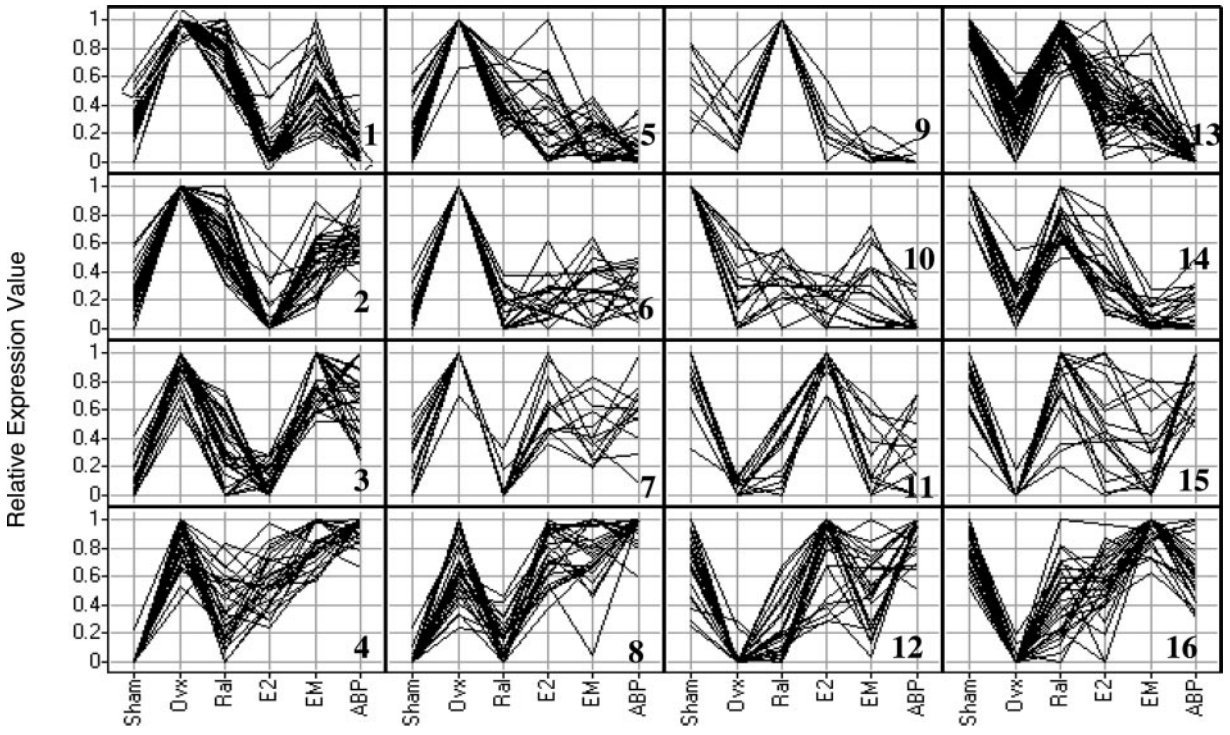

Fig. 3. Self-organizing map of the 380 probe sets altered by ovariectomy and by one or more drugs after 5 weeks of treatment. The y-axis represents range-scaled median expression values of various treatments. Ral, raloxifene; EM, EM652; ABP, alendronate.

TABLE 2

Genes commonly regulated by all antiresorptive treatments as compared to control

Fold-change calculated for ovariectomized control rats (Ovx) by comparing mean intensity data for each probe set with that of sham control rats; -fold change for each antiresorptive agent was calculated by comparing the mean intensity data of each with that of Ovx.

Affymetrix ID	Ovx		Raloxifene		EM652		Estrogen		Alendronate		Gene Symbol	Gene Name
	-Fold Change	FDR	-Fold Change	FDR	-Fold Change	FDR	-Fold Change	FDR	-Fold Change	FDR		
X83671cds_i_at	21.6	0.1	−2.5	0.3	−2.4	0.3	−2.5	0.3	−3.3	0.2	CRYBB2	Crystallin β B2
AJ005396_at	3.0	0.0	−1.8	0.0	−1.9	0.0	−6.2	0.0	−12.2	0.0	Col11a1	α1 Subunit of type XI collagen
AF097593_at	3.0	0.1	−2.4	0.1	−1.7	0.7	−2.2	0.3	−1.5	0.7	Cdh2	Cadherin-2 type 1 (N-cadherin)
rc_AA891204_s_at	3.0	0.0	−1.6	0.1	−2.4	0.0	−4.4	0.0	−3.8	0.0	Sparc	Osteonectin
rc_AI179399_at	2.7	0.0	−1.8	0.0	−1.9	0.0	−3.8	0.0	−3.8	0.0	Col5a2	α2 Subunit of type V collagen
AB002393_at	2.4	0.3	−2.5	0.5	−1.8	0.7	−1.8	0.4	−2.4	0.3	Hal	Histidine ammonia-lyase
L07281_at	2.3	0.0	−1.5	0.1	−1.6	0.1	−3.3	0.0	−2.9	0.0	Cpe	Carboxypeptidase E
rc_AI169327_g_at	2.1	0.0	−1.5	0.1	−2.4	0.0	−3.2	0.0	−2.1	0.0	Timp1	Tissue inhibitor of metalloproteinase 1
X94185cds_s_at	2.0	0.4	−1.5	0.5	−1.7	0.8	−2.3	0.3	−1.9	0.7	Dusp6	Dual specificity phosphatase 6
U67915_at	1.9	0.2	−1.4	0.8	−2.8	0.1	−1.4	0.6	−2.4	0.1	Mcpt1	Mast cell protease 1
M81639_at	1.9	0.1	−2.5	0.2	−1.4	0.3	−2.2	0.1	−1.5	0.2	Snn	Protein with very strong similarity to stannin
S77494_s_at	1.8	0.0	−1.6	0.1	−1.5	0.2	−1.7	0.0	−2.1	0.0	Lox	Lysyl oxidase
rc_AA799745_at	1.8	0.1	−1.4	0.4	−1.7	0.1	−1.7	0.1	−1.8	0.1	C53	CDK5 activator-binding protein C53
M69246_at	1.7	0.1	−1.7	0.3	−2.0	0.0	−3.2	0.0	−2.6	0.0	Serpinh1	Serine proteinase inhibitor clade H (heat shock protein 47) member 1
rc_AA800881_at	1.7	0.1	−1.4	0.5	−1.7	0.1	−1.8	0.1	−1.8	0.0	Sultx1	Protein containing a sulfotransferase domain
J02669_s_at	1.6	0.0	−1.2	0.2	−1.6	0.0	−1.6	0.0	−1.7	0.0	Cyp2a1	Cytochrome P450 IIA1
Z34004exon_g_at	1.6	0.1	−1.3	0.3	−1.4	0.2	−1.6	0.0	−1.9	0.0	Ghrh	Growth hormone releasing hormone
AF073891_at	1.5	0.3	−1.7	0.2	−1.2	0.9	−1.3	0.6	−1.3	0.5	Kcnh5	Potassium voltage-gated channel subfamily H (EAG-related) member 5
S69206_s_at	1.5	0.4	−1.3	1.0	−1.3	0.5	−1.3	0.5	−1.5	0.4	Mcpt4	Mast cell protease
X80290cds_s_at	1.5	0.1	−1.2	0.6	−1.3	0.2	−1.4	0.1	−1.4	0.1	Adcyap1	Adenylyl cyclase activating polypeptide 1
AB017170_s_at	1.4	0.2	−1.3	0.4	−1.4	0.3	−1.3	0.4	−1.4	0.4	Slit1	Slit homolog 1
rc_AA893406_at	1.4	0.1	−1.3	0.4	−1.5	0.1	−1.1	0.4	−1.5	0.1	AA893406	Unknown
rc_AA894305_at	1.4	0.3	−1.3	0.3	−1.4	0.2	−1.5	0.1	−1.5	0.1	AA894305	Unknown
rc_AA800850_at	1.4	0.3	−1.5	0.4	−1.3	0.6	−1.4	0.6	−1.4	0.5	Bmi1	B lymphoma Mo-MLV insertion region 1
rc_AI103874_at	1.4	0.3	−1.2	0.3	−1.3	0.5	−1.5	0.2	−1.4	0.3	Fkbp3	FK506-binding protein 3 (25 kDa)
U65656_at	1.4	0.3	−1.3	0.4	−1.7	0.2	−2.1	0.2	−1.3	0.7	Mmp2	Matrix metalloproteinase 2
rc_AA892300_at	1.4	0.4	−1.4	0.3	−1.2	0.5	−1.4	0.4	−1.2	0.7	Pex5	Peroxin 5
U38180_g_at	1.4	0.1	−1.3	0.2	−1.2	0.3	−1.2	0.1	−1.3	0.1	Slc19a1	Protein with strong similarity to solute carrier family 19
rc_AA818593_at	1.4	0.5	−1.4	0.2	−1.2	0.3	−1.3	0.5	−1.3	0.6	Ppap2a	Phosphatidic acid phosphatase type 2a
rc_aa859757_at	1.3	0.1	−1.1	0.1	−1.3	0.1	−1.4	0.2	−1.4	0.1	Col5a1	collagen type 5 α1 chain
S79711_at	1.3	0.3	−1.4	0.1	−1.3	0.1	−2.0	0.0	−1.2	0.3	Cd3g	CD3 antigen γ polypeptide
AF016503_s_at	1.3	0.1	−1.3	0.3	−1.4	0.1	−1.6	0.0	−1.7	0.0	Pcolce	Procollagen C-proteinase enhancer protein
A03913cds_s_at	1.2	0.2	−1.5	0.1	−1.3	0.1	−1.4	0.1	−1.3	0.2	Serpine2	Serine (or cysteine) proteinase inhibitor clade E member 2
D13623_at	1.2	0.2	−1.2	0.1	−1.2	0.3	−1.2	0.3	−1.2	0.3	AA959742	Protein containing three leucine rich repeats
U88324_at	1.2	0.3	−1.2	0.2	−1.2	0.5	−1.2	0.4	−1.2	0.5	Gnb1	Guanine nucleotide binding protein (G protein) β1
rc_AA892270_at	1.2	0.2	−1.4	0.1	−1.5	0.0	−1.3	0.1	−1.4	0.1	AA892270	Unknown
D45249_at	1.2	0.2	−1.1	0.4	−1.2	0.3	−1.1	0.4	−1.2	0.2	Psme1	Proteasome activator α subunit (P28 α)
rc_AA891785_at	1.2	0.4	−1.2	0.5	−1.5	0.1	−1.4	0.2	−1.4	0.2	Idh2	Isocitrate dehydrogenase 2 (NADP+) mitochondrial
U26595_at	1.2	0.2	−1.2	0.3	−1.3	0.2	−1.3	0.2	−1.2	0.2	Ptgfrn	Prostaglandin F2 receptor negative regulator
M28647_g_at	1.2	0.3	−1.1	0.3	−1.2	0.4	−1.2	0.3	−1.1	0.6	Atp1a1	α1 Subunit of the Na^+-K^+-transporting ATPase
U77829mRNA_I_at	1.1	0.3	−1.1	0.3	−1.1	0.2	−1.2	0.2	−1.1	0.6	U77829	Unknown
m64986_g_at	1.1	0.4	−1.2	0.3	−1.3	0.3	−1.1	0.4	−1.2	0.4	Hmgb1	High mobility group 1
U15211_g_at	−5.8	0.1	5.6	0.0	6.8	0.3	3.9	0.0	5.5	0.1	Rara	Retinoic acid receptor α
rc_AA891447_at	−2.2	0.2	1.9	0.2	2.0	0.3	1.5	0.6	2.1	0.4	AA891447	Unknown
rc_AI639055_at	−2.1	0.2	2.3	0.2	2.2	0.3	2.2	0.3	1.8	0.9	AI639055	Unknown
rc_AI043796_s_at	−2.0	0.4	1.7	0.5	2.7	0.0	1.8	0.3	1.9	0.4	Slc18a2	Solute carrier family 18 A2 (vesicular monoamine transporter 2)
rc_AA891735_at	−2.0	0.1	1.7	0.2	1.7	0.2	1.6	0.4	1.6	0.1	Pvrl1	Poliovirus receptor-related 1 (nectin-1)
D14839_at	−2.0	0.1	1.4	0.4	2.2	0.0	1.9	0.1	1.9	0.0	Fgf9	Fibroblast growth factor 9

TABLE 2
Continued

Affymetrix ID	Ovx		Raloxifene		EM652		Estrogen		Alendronate		Gene Symbol	Gene Name
	-Fold Change	FDR	-Fold Change	FDR	-Fold Change	FDR	-Fold Change	FDR	-Fold Change	FDR		
X83671cds_i_at												
D25290_at	−1.9	0.2	1.5	0.3	2.0	0.1	1.5	0.2	1.6	0.4	Cdh6	K-cadherin
rc_AI638972_at	−1.9	0.3	1.6	0.8	2.1	0.3	1.7	0.6	2.1	0.2	AI638972	Unknown
AF032872_at	−1.8	0.3	1.7	0.3	1.9	0.2	1.7	0.5	1.7	0.3	83614	Protein inhibitor of activated STAT3
AF007554_at	−1.7	0.2	1.4	0.5	1.7	0.1	1.4	0.5	1.6	0.1	Muc1	Mucin (episialin)
U93092_at	−1.6	0.3	1.2	0.9	2.1	0.1	1.5	0.6	1.9	0.3	Hoxa1	Homeo box A1
L07380_at	−1.4	0.3	1.1	0.8	1.3	0.4	1.3	0.3	1.1	0.8	Ghrhr	Growth hormone-releasing hormone receptor
L10072_at	−1.4	0.1	1.3	0.6	1.3	0.3	1.5	0.2	1.3	0.3	Htr5a	5-hydroxytryptamine (serotonin) receptor 5A
rc_AA859990_s_at	−1.4	0.3	1.5	0.5	1.4	0.6	1.4	0.4	1.4	0.5	1500003N18Rik	Protein with strong similarity to polymerase δ-interacting protein 1
M92074_g_at	−1.3	0.4	1.4	0.2	1.3	0.5	1.2	0.6	1.2	0.8	Tnni3	Cardiac troponin I
rc_AI639107_at	−1.3	0.2	1.3	0.1	1.1	0.7	1.3	0.4	1.3	0.4	AI639107	Unknown
M26643_g_at	−1.3	0.3	1.2	0.4	1.3	0.5	1.2	0.7	1.3	0.4	Scn4a	Voltage-gated sodium channel type IV α polypeptide
M18529cds_f_at	−1.3	0.2	1.1	0.8	1.3	0.1	1.3	0.2	1.1	0.4	Scop	Suprachiasmatic nucleus circadian oscillatory protein
U73142_g_at	−1.3	0.1	1.2	0.4	1.4	0.1	1.3	0.1	1.3	0.2	Mapk14	Mitogen activated protein kinase 14
X63995_at	−1.3	0.5	1.2	0.9	1.5	0.3	1.2	0.7	1.3	0.6	Slc6a4	Solute carrier family 6 member 4
rc_AI011376_at	−1.3	0.3	1.1	0.6	1.3	0.2	1.3	0.1	1.3	0.3	AI874665	Protein with strong similarity to mitogen-activated protein kinase-activated protein kinase 3
S77900_g_at	−1.3	0.3	1.3	0.2	1.2	0.3	1.2	0.6	1.1	0.8	Myl9	Protein with strong similarity to myosin regulatory light chain
AF034896_f_at	−1.2	0.3	1.1	0.8	1.2	0.2	1.1	0.4	1.1	0.7	Scrd8	Spermatid chemoreceptor D−8
U02506UTR#1_s_at	−1.2	0.4	1.1	0.8	1.2	0.4	1.1	0.7	1.1	0.5	Pigr	Polymeric immunoglobulin receptor (secretory component)
X57514_at	−1.2	0.3	1.1	0.8	1.3	0.1	1.1	0.8	1.2	0.5	Gabrg1	GABA-A receptor subunit γ 1
U06713_at	−1.1	0.4	1.1	0.8	1.2	0.2	1.1	0.4	1.1	0.6	Egln3	EGL nine homolog 3
M19936_at	−1.1	0.3	1.1	0.2	1.0	0.8	1.1	0.8	1.1	0.8	Psap	Prosaposin
X05566_i_at	−1.1	0.4	1.1	0.5	1.0	0.6	1.1	0.3	1.1	0.4	Mrlcb	Myosin regulatory light chain

by at least 1.5-fold from Ovx. The gene ontology categorization of these genes are listed in Table 5 and the results of statistical testing of gene ontology terms most significantly maintained near to sham levels by raloxifene are listed in Table 6.

Two of the raloxifene unique genes with larger -fold change values were evaluated in an independent 5-week validation study. Carbonic anhydrase 4 (Ca4) and cytochrome P450 family 27 subfamily A polypeptide 1 (Cyp27a1) were reduced by ovariectomy on the microarray and then uniquely increased by raloxifene. Ovariectomy did reduce the levels of both of these genes in the follow-up study, but only the Cyp27a1 reduction was significantly ($p < 0.05$) lowered. Neither the Cyp27a1 nor the Ca4 expression changes after antiresorptive treatment achieved statistical significance (Table 7).

Genes Uniquely Regulated by Estrogen. Estrogen also uniquely maintained a small subset of genes near sham level in addition to the 70 commonly regulated genes. Genes that were altered by ovariectomy and were returned toward sham levels solely by E2 treatment are listed in Table 4 and highlighted in clusters 4 and 5 of Fig. 2. The changes were nearly equivalently divided between genes that were increased and those decreased by Ovx and uniquely regulated to sham levels by E2. Fifty percent of these E2 unique genes were changed by 1.5-fold or more relative to Ovx. The gene ontology classification of these genes (Tables 5 and 6) revealed that E2 significantly maintained genes near sham levels that were associated with perception of a stimulus (mechanical and pain stimuli), feeding behavior, and antigen binding.

Suppression of Bone Formation Genes by Alendronate and Estrogen but Not Raloxifene. Cluster analysis of the microarray data identified an additional group of genes that were elevated by Ovx and were suppressed to or below that of sham levels by all treatments *except* raloxifene (Fig. 2 cluster 1, Fig. 3 cluster 1). The identities of the genes in this cluster were those associated with osteoblastic activity [several collagens, osteocalcin, Sparc, and biglycan (Bgn)]. E2 and alendronate suppressed the expression of these genes below that of sham; however, EM652 only partially suppressed their expression (Fig. 4). Three additional genes clustered together with these osteoblastic genes: immunoglobulin superfamily member 4 (Igsf4 or syncam), homeobox protein C8 (Hox8c or Hox3a), and fibroblast growth factor receptor 1 (Fgfr1) implying that they may also serve an important role in osteoblastic activity because they are coregulated with the collagens Ocn, Bgn, and Sparc.

Additional animal studies were conducted, and representative genes from this cluster were analyzed to determine the consistency of treatment effects in independent assays. In addition, the femora were subjected to biomechanical testing in the first validation study that was an exact replicate of the original 5-week array study. Biomechanical testing on the femoral neck (the site used for array analysis) from this 5-week validation study showed no differences in the strength of the bone from Ovx by any drug treatment (Fig.

TABLE 3

Genes uniquely regulated by raloxifene

-Fold change calculated for ovariectomized control rats (Ovx) by comparing mean intensity data for each probe set with that of sham control rats; -fold change for raloxifene was calculated by comparing the mean intensity data of each probe set with that of Ovx.

Affymetrix	Ovx		Raloxifene		Gene Symbol	Gene Name
	-Fold Change	FDR	-Fold Change	FDR		
rc_H33629_at	3.6	0.3	−1.7	0.8	4930451A13Rik	Member of the TBC domain containing family
S83279_g_at	2.3	0.3	−1.8	0.3	Hsd17b4	Type IV 17 β-hydroxysteroid dehydrogenase
Y11321cds_at	2.3	0.2	−1.9	0.2	Foxe1	Forkhead box E1
rc_AA891851_at	2.2	0.1	−1.7	0.2	C6.1A	Member of the Mov34
U68562mRNA#2_s_at	2.1	0.3	−1.4	0.3	Hsp60	Chaperonin 60
rc_AI011706_at	2.0	0.1	−1.9	0.2	Sfrs3	Splicing factor arginine/serine-rich 3
rc_AA892391_at	1.8	0.4	−1.7	0.3	FLJ20531	Protein containing five C2H2 type zinc finger domains
rc_AA799576_at	1.7	0.3	−1.6	0.3	Ddx50	RNA helicase II/Gu β
rc_AA800296_at	1.6	0.3	−1.5	0.2	Papola	Poly(A) polymerase α
rc_AA875054_at	1.5	0.3	−1.4	0.2	Tcp1	T-complex 1
rc_AA891069_at	1.5	0.3	−1.4	0.3	Srpk2	Serine/arginine-rich protein specific kinase 2
S75730_at	1.5	0.3	−1.4	0.4	Scd2	Stearoyl-CoA desaturase 2
M80550_at	1.4	0.3	−1.5	0.3	Adcy2	Adenylyl cyclase type 2
rc_AA859502_at	1.4	0.2	−1.2	0.3	AA859502	Unknown
rc_AI045440_at	1.4	0.3	−1.4	0.4	Spn	Sialophorin (leukosialin)
rc_AA875559_at	1.4	0.2	−1.2	0.7	AA875559	Unknown
rc_AA799751_at	1.4	0.1	−1.3	0.3	AA799751	Unknown
rc_H31128_at	1.4	0.4	−1.2	0.3	H31128	Unknown
AF093139_s_at	1.4	0.3	−1.3	0.5	Nxf1	Nuclear RNA export factor 1
rc_AA875253_at	1.4	0.2	−1.2	0.3	Arl1	ADP-ribosylation factor 1
rc_AI176052_at	1.3	0.4	−1.3	0.3	Ak3	Adenylyl kinase isoenzyme 3
AF051943_at	1.3	0.3	−1.4	0.0	Nme6	Nucleoside diphosphate kinase 6 (expressed in nonmetastatic cells 6)
rc_AA800855_at	1.3	0.2	−1.5	0.1	AA800855	Unknown
S68944_r_at	1.3	0.2	−1.3	0.3	Slc7a4	Member of the amino acid permease family of membrane transporters
M32783cds_i_at	1.3	0.4	−1.4	0.3	Pdyn	Prodynorphin
rc_AI237016_at	1.2	0.2	−1.1	0.4	H2afy	H2A histone family member Y
AF091566_f_at	−2.9	0.3	3.9	0.2	LOC287000	Protein with high similarity to olfactory receptor 50
Y07534cds_s_at	−2.8	0.3	2.1	0.5	Cyp27a1	Cytochrome P450 family 27 subfamily A polypeptide 1
S68245_at	−2.4	0.2	2.0	0.5	Ca4	Carbonic anhydrase 4
U40836mRNA_s_at	−2.1	0.4	1.6	0.4	Cox8 h	Cytochrome c oxidase subunit VIII-H
rc_AA799861_g_at	−2.1	0.4	2.3	0.2	Irf7	Interferon regulatory factor 7
X03015_at	−1.9	0.2	1.9	0.3	Cd8a	CD8 antigen α chain
rc_AA874803_g_at	−1.9	0.1	1.6	0.1	AA874803	Unknown
J02722cds_at	−1.8	0.2	1.7	0.3	Hmox1	Heme oxygenase
X56327cds_s_at	−1.7	0.3	1.7	0.4	Hbb-y	Hemoglobin Y (epsilon 3 globin)
AF020618_g_at	−1.7	0.3	1.5	0.3	Myd116	Myeloid differentiation primary response gene 116 (progression elevated gene 3)
rc_AA892378_at	−1.6	0.4	1.4	0.4	Ttc11	Protein with strong similarity to tetratricopeptide repeat domain 11
M36151cds_s_at	−1.6	0.1	1.6	0.2	RT1.B−1	β chain of RT1.B−1
rc_AA859870_f_at	−1.6	0.1	1.3	0.2	1110007C05Rik	Member of the Josephin family
M34134_s_at	−1.5	0.1	1.4	0.2	Tpm1	Tropomyosin 1α
U53855_at	−1.5	0.4	1.5	0.2	Ptgis	Prostaglandin I2 (prostacyclin) synthase
rc_H31887_at	−1.5	0.3	1.5	0.3	1700037H04Rik	Protein of unknown function
D42148_at	−1.4	0.4	1.4	0.1	Gas6	Growth arrest-specific 6
rc_AI232783_s_at	−1.4	0.1	1.4	0.2	Glns	Glutamine synthetase 1
D28557_s_at	−1.4	0.1	1.5	0.1	Csda	Muscle Y-box protein YB2
S61973_g_at	−1.4	0.4	1.4	0.2	Grina	NMDA receptor glutamate-binding chain
AF007758_at	−1.4	0.2	1.2	0.7	Snca	Synuclein α
rc_AI235890_s_at	−1.4	0.2	1.2	0.7	H2-T24	Protein with high similarity to human HLA-B
rc_AA894008_at	−1.4	0.3	1.4	0.2	AA894008_at	Unknown
X07551cds_s_at	−1.4	0.2	1.5	0.0	H2-Aa	Histocompatibility 2 class II antigen A α
rc_AI178971_at	−1.4	0.1	1.3	0.2	Hba1	Hemoglobin α 1
rc_AA892773_at	−1.4	0.3	1.3	0.2	AA892773	Unknown
X14254cds_at	−1.4	0.3	1.5	0.1	Cd74	CD74 antigen
X71127_at	−1.3	0.4	1.3	0.1	C1qb	B chain of complement subcomponent C1q
X76697_at	−1.3	0.2	1.2	0.4	Cd52	Campath-1 antigen
rc_AA894318_at	−1.3	0.3	1.1	0.2	B230312A22Rik	Protein of unknown function
AF102552_s_at	−1.3	0.3	1.3	0.1	Ank3	Ankyrin 3 (ankyrin G)
U06099_at	−1.2	0.2	1.2	0.5	Prdx2	Thioredoxin peroxidase 1
rc_AI170568_s_at	−1.2	0.3	1.2	0.2	Dci	Dodecanoyl-Coenzyme A δ isomerase
rc_AA892821_at	−1.2	0.5	1.3	0.5	Akr7a2	Aldo-keto reductase family 7 member A2
rc_AA891695_f_at	−1.2	0.4	1.3	0.2	Ly6b	Lymphocyte antigen 6 complex locus A
M15562_g_at	−1.2	0.1	1.3	0.0	RNRT1DAU	MHC class II α chain
AJ005642_at	−1.2	0.4	1.2	0.4	4733401N09Rik	Protein with high similarity to serine protease 22
X82396_at	−1.2	0.1	1.2	0.2	Ctsb	Cathepsin B
D86297_at	−1.2	0.1	1.2	0.2	Alas2	δ-aminolevulinate synthase (erythroid-specific)
D16554_at	−1.1	0.4	1.2	0.3	Loc192255	Polyubiquitin
rc_AA892649_at	−1.1	0.3	1.2	0.2	Gabarap	GABA receptor associated protein

5A); however, the sham group was significantly stronger than Ovx ($p < 0.05$). At a different bone site (the distal femur), pharmacological efficacy was observed for all treatments ($p < 0.001$) with the maintenance of bone mineral density (BMD) at sham levels (Fig. 5B).

The expression of the osteoblast activity genes in the proximal femur was reevaluated in the 5-week validation study and was observed to parallel the differential expression pattern observed on the array. Although the -fold change from control values were not identical between the array and the 5-week Taqman validation experiment, the rank order at which these agents suppressed the bone formation activity genes was the same. Alendronate was the most suppressive of all agents tested and consistently suppressed the expression of the bone formation genes (i.e., Ocn, collagens, Sparc, and Bgn) below that of sham in the array and the repeated

TABLE 4

Genes uniquely regulated by estrogen

-Fold change calculated for ovariectomized control rats (Ovx) by comparing mean intensity data for each probe set with that of sham control rats; -fold change for estrogen was calculated by comparing the mean intensity data of each probes set with that of Ovx.

Affymetrix ID	Ovx		Estrogen		Gene Symbol	Gene Name
	-Fold Change	FDR	-Fold Change	FDR		
rc_AA874848_s_at	1.9	0.0	−2.0	0.0	Thy−1	Thymus cell surface antigen
AB004831cds_at	1.7	0.0	−1.8	0.0	Igb	Protein with strong similarity to immunoglobulin-associated β (mouse Cd79b)
U49062_at	1.7	0.0	−2.1	0.0	Cd24	CD24 antigen
X68782cds_at	1.7	0.1	−1.9	0.0	RNIGMC	Immunoglobulin heavy constant mu
rc_AA849769_g_at	1.5	0.2	−1.5	0.2	Fstl	Follistatin-like (follistatin-related protein precursor)
rc_AA891872_at	1.4	0.0	−1.5	0.0	Nnt	Nicotinamide nucleotide transhydrogenase
rc_AA800318_at	1.4	0.1	−1.5	0.0	Serping1	Serine (or cysteine) proteinase inhibitor
rc_H32977_at	1.4	0.0	−1.4	0.0	2010015J01Rik	Protein with high similarity to actin related protein 2/3 complex subunit 5 16kDa
rc_AA893584_at	1.3	0.2	−1.6	0.1	MGC10120	Unknown
rc_AA799440_g_at	1.3	0.3	−1.2	0.6	Mrpl13	Protein with strong similarity to mitochondrial ribosomal protein L13
U77829mRNA_i_at	1.1	0.3	−1.2	0.2	U77829	Unknown
rc_AA866435_at	−2.7	0.1	3.1	0.0	AA866435	Unknown
U18982_s_at	−2.3	0.2	2.2	0.3	Fosl2	FOS-like antigen 2
rc_AI008423_at	−1.9	0.0	1.6	0.3	Uncl	Unc-50 related protein (UNCL)
M91595exon_s_at	−1.8	0.2	2.1	0.1	Igfbp2	Insulin-like growth factor binding protein 2
S49491_s_at	−1.6	0.0	1.6	0.2	Penk-rs	Proenkephalin (preproenkephalin A)
X90651_at	−1.5	0.2	1.5	0.4	P2rx3	Purinergic receptor P2X ligand gated ion channel 3
L07407_at	−1.5	0.0	1.4	0.1	RATIGKAB	Kappa light chain
rc_AA924084_at	−1.4	0.4	1.3	0.5	Ywhab	14-3-3 β-subtype
X52196cds_at	−1.3	0.3	1.6	0.1	Alox5ap	Arachidonate 5-lipoxygenase-activating protein
U60976_s_at	−1.3	0.4	1.3	0.4	Flot1	Flotillin 1
D10587_g_at	−1.3	0.2	1.3	0.3	Cd36l2	CD36 antigen-like 2
X13527cds_s_at	−1.1	0.4	1.5	0.0	Fasn	Fatty acid synthase
rc_AA955477_g_at	−1.1	0.3	1.1	0.3	AI874665	Protein with strong similarity to mitogen-activated protein kinase-activated protein kinase 3

TABLE 5

Gene ontology classification of genes altered by ovariectomy and drug treatment

	Ovx	Common	Raloxifene Unique	Estrogen Unique
Biological process				
Physiological process	246	56	47	16
Metabolism	135	23	30	6
Cell cycle	12	2	2	0
Cell growth & maintenance	118	31	18	5
Cell motility	24	8	1	1
Development	117	32	20	7
Death	41	12	4	2
Cell communication	99	27	13	10
Transport	46	13	6	3
Response to stress	71	29	14	6
Molecular function				
Binding	135	21	19	15
Catalytic activity	101	16	21	2
Signal transducer activity	54	13	6	4
Motor activity	1	0	0	0
Transporter activity	36	10	6	2
Chaperone activity	5	1	2	0
Nucleic acid binding	29	4	4	1
Cell adhesion molecule activity	7	3	0	1
Transcription regulator activity	23	4	4	1
Defense/immunity protein activity	16	1	6	4
Enzyme regulator activity	18	7	2	2
Structural molecule activity	17	3	1	1
Number of genes	334	70	67	24

TABLE 6

Significant gene ontology terms maintained at sham levels by drug treatment relative to Ovx

Genes maintained at Sham level by all pharmaceutical agents and those uniquely maintained by estrogen and raloxifene were compared to Ovx-induced gene changes. All gene ontology terms listed were determined to be significant at a confidence level of $p < 0.05$ as described under *Materials and Methods*.

Treatment	Biological Process						Molecular Function			
	Metabolism	Response to Stimulus	Secretion	Transport	Cell Communication	Behavior	Catalytic Activity	Transporter Activity	Binding	Other
Common (E2, Ral, EM, ABP)	cAMP biosynthesis		Growth hormone	Ion transport** (metal ion transport & K^+ transport)	Cell-to-cell signaling, cell-to-cell adhesion, G protein signal coupled to a cyclic nucleotide 2nd messenger		Metallopeptidase	Neurotransmitter, ion channel activity		Enzyme regulatory activity
Ral	Coenzyme and prosthetic group metabolism*	Response to hypoxia, response to cell defense, response to bacteria					Oxidoreductase	Electron transporter activity	Cytoskeletal protein binding*	Chaperone (heat shock protein)
E2	Protein complex assembly**	Perception of pain, sensory perception of mechanical stimulus				Feeding behavior**			Antigen binding*	

Ral, raloxifene; EM, EM652; ABP, alendronate.
* Significant at $p < 0.01$.
** Significant at $p < 0.005$.

study. Estrogen was less suppressive than alendronate but suppressed the expression of these genes more than raloxifene. Raloxifene lowered the expression of some of these genes in the validation study more than had been observed on the microarray; however, levels were not different from sham in nearly each case (Table 7).

A second validation study evaluated the expression profile of bone formation genes in the distal femur at an earlier time point (9 days) before an effect on BMD would be observed (Sato et al., 1994). The increased expression of Col 5a1, Sparc, and Bgn induced after 5 weeks of ovariectomy were not yet altered at this earlier time point. However, Ocn and Col1a2 were significantly elevated by ovariectomy, and the suppression of Ocn by E2 and alendronate was also observed by 9 days in the distal femur (data not shown).

Discussion

Ovariectomized rats greater than 5 months of age have been shown to reproducibly lose cancellous bone from axial and appendicular skeletal sites as a result of estrogen deficiency, not unlike postmenopausal women (Turner et al., 1994). Upon pharmacologic administration of maximally efficacious doses of E2, raloxifene (Sato et al., 1995, 1996), and alendronate (Toolan et al., 1992), a similar preservation of BMD can be achieved in femora of ovariectomized rats. Given the differences in the mechanism of action by which estrogens/SERMs and alendronate sustain BMD after ovariectomy (Sato et al., 1999), a gene array analysis was initiated in an effort to elucidate possible differences of these compounds on skeletal physiology.

After 5 weeks of treatment, the overall expression profile of all genes on the microarray suggested that E2 and alendronate were most similar, whereas the gene expression profiles of EM652 and raloxifene seemed to be distinctly different from E2 (Fig. 1). This was an unexpected finding, in that we had hypothesized that because of the similarity in mechanism of action (i.e., estrogen receptor agonism), the SERMs and E2 would more closely resemble each other. However, array analyses showed that EM652 and raloxifene were quite different from E2 in their gene expression profile in bone. To understand more fully which genes were driving this association between the compounds, we looked in more detail at only those genes (334 unique genes or 380 probe sets), which were significantly modulated by ovariectomy *and* were maintained near sham levels by any of the drug treatments.

Ovariectomy induced the expected changes in bone formation and resorption genes such as Ocn, Sparc, Col1a2 and Col5a1, and Rank ligand. Because osteoclastic activity is elevated after ovariectomy, one would expect to observe increases in osteoclastic genes such as calcitonin receptor, cathepsin K, and tartrate-resistant acid phosphatase. However, these genes were either not significantly altered by ovariectomy or were so lowly expressed they did not meet our exclusion criteria. The likely explanation for the lack of detection of these gene changes is the kinetics of bone loss in the ovariectomized rat model. The 5-week time point at which we collected femoral RNA was well beyond the peak of osteoclastic activity, and the cancellous bone volume was reduced by nearly 40% (Wronski et al., 1989). In unpublished data from our laboratory, we have measured increases in these osteoclast genes after 12 days of ovariectomy, but by 5 weeks, these changes were no longer observed, probably because of the lack of bone surface in the ovariectomized group, which was lost by this time.

As expected, there was a subset of genes ($n = 70$) commonly regulated toward sham levels by all antiresorptive agents irrespective of their differences in mechanism of action, representing ~20% of the total ovariectomy-induced and drug-responsive genes. The regulation of these common genes probably represents a core set of molecular changes necessary to maintain bone mass after ovariectomy in the rat and occur before any biomechanical effect in this bone site (Fig. 5). Validation in an independent experiment of three genes commonly regulated by all treatments (Pcolce, lysyl oxidase, and Serpinh1) confirmed a similar down-regulation by all agents tested (Table 7). In addition to the 70 commonly regulated genes, only two of the treatments (raloxifene and E2) could modulate subsets of genes uniquely by their treatment. There were not any genes uniquely maintained near sham level by EM652 or alendronate in that at least one of the other agents also regulated the genes associated with these treatments. Validation of two raloxifene unique genes (Cyp27a1 and Ca4) in a repeated 5-week experiment was not successful. The change in expression by ovariectomy was statistically lowered for only one of the genes (Cyp27a1), and subsequent increases in expression by raloxifene did not

TABLE 7

Comparison of gene changes after treatment relative to ovariectomized control rats in two independent studies

Study 1 analyzed genes by microarray analysis and study 2 analyzed genes by real-time PCR in an independent 5-week animal study as described under *Materials and Methods*. Values represent the ratio of mean intensity data for each probe set relative to that of ovariectomized controls within a given study.

PID	Gene Symbol	Sham		Raloxifene		Estrogen		EM652		Alendronate	
		Study 1	Study 2	Study 1	Study 2	Study 1	Study 2	Study 1	Study 2	Study 1	Study 2
AF050214_at	Col1a2	0.41*	0.44*	0.77	0.42*	0.18*†	0.30*	0.55*	na	0.28*†	0.10*†
AJ005394_at	Col5a1	0.69*	0.59*	0.85†	0.55*	0.62*	0.43*	0.73*	na	0.67*	0.24*†
S54008_i_at	Fgfr1	0.51*	0.94	0.78*	0.72*	0.34*	0.56*†	0.54*	na	0.47*	0.40*†
U17834_at	Bgn	0.81*	0.69	1.08†	0.84	0.60*	0.59*	0.76*	na	0.70*	0.33*†
Y13714_at	Sparc	0.47*	0.59*	0.80*†	0.38*	0.30*†	0.29*†	0.57*	na	0.34*†	0.17*†
M25490_at	Ocn	0.54*	0.59*	0.84†	0.31*†	0.20*†	0.25*†	0.47*	na	0.32*†	0.09*†
AF104362_at	Omd	0.71*	0.90	0.84*	0.65*	0.64*	0.37*†	0.88	na	0.73*	0.30*†
AF016503_s_at	Pcolce	0.80*	0.86	0.78*	0.54*†	0.61*	0.55*†	0.72*	na	0.60*	0.35*†
S77494_s_at	Lox	0.55*	0.86	0.62*	0.64*†	0.60*	0.50*†	0.70†	na	0.50*	0.30*†
M69246_at	Serpinh1	0.58*	0.75	0.60	0.90*	0.32*†	0.55*	0.50*	na	0.38*†	0.41*†
Y07534cds_s_at	Cyp27a1	2.79*	1.78*	2.10	1.67	0.80†	1.34	0.70†	na	0.50*†	0.97
S68245_at	Ca4	2.40*	1.88	2.00*	0.84	1.00†	1.15	1.00†	na	1.00	0.88†

* Statistically different from Ovx control ($p < 0.05$).
† Statistically different from sham control ($p < 0.05$).

achieve statistical significance with either gene (Table 7), highlighting the importance of validating gene expression in independent assays.

Although raloxifene treatment was the most efficacious agent at maintaining gene expression near sham levels in the microarray, there was a small cluster of genes that raloxifene did not suppress or only slightly lowered after their ovariectomy-induced increase. The genes which remained elevated by raloxifene, were identified as those associated with bone formation activity (Ocn, Col1a1, Col1a2, Col5a2, Bgn, and Sparc). E2 and alendronate suppressed the expression of these osteoblastic genes below that of sham, whereas EM652 treatment resulted in an intermediate expression level (Fig. 4). A replicated study (5-week treatment) confirmed that alendronate treatment was the most suppressive of the expression of these formation genes, whereas raloxifene was the least (Table 7). An additional validation study revealed that the suppression of Ocn by E2 and alendronate occurred as early as 9 days of treatment (data not shown). Although the present study evaluated only RNA changes, the significantly enhanced suppression of Ocn protein by alendronate versus that of raloxifene has been observed in the serum of postmenopausal women after 1 year of treatment (Johnell et al., 2002). These data are consistent with previously published histomorphometric analyses in postmenopausal women in which alendronate reduced bone formation activity (BFR per unit bone surface and activation frequency) by 90% (Chavassieux et al., 1997), whereas estrogen had milder suppressive effects (Lufkin et al., 1992; Weinstein et al., 2003). Raloxifene had no effect on BFR per unit of bone volume or on the formation period in clinical samples (Ott et al., 2002). These array data suggest a possible explanation as to why the combined effects of alendronate and PTH were found to be less efficacious than PTH alone (Black et al., 2003; Finkelstein et al., 2003) in clinical studies; but when PTH was given in combination with raloxifene, an additive skeletal effect was observed (Cosman et al., 2004; Deal et al., 2004). A possible explanation is that suppression by alendronate of osteoblastic function (as evidenced by the lack of expression of osteoblastic genes) antagonized PTH efficacy, whereas raloxifene treatment did not impair osteoblast activity and thus can complement PTH in the skeleton.

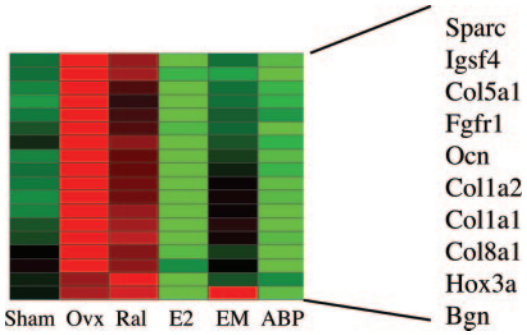

Fig. 4. Detailed view of Cluster 1 from the hierarchical clustering analysis (Fig. 2). The genes identified in this cluster were elevated by Ovx but were suppressed by E2 and alendronate to levels below sham. The group median intensity values from MAS4 analysis were scaled so that red indicates high, green indicates low, and black indicates intermediate expression. Ral, raloxifene; EM, EM652; ABP, alendronate.

Because Hox3a, Fgfr1, and Syncam clustered tightly (Fig. 4) with several known bone formation activity genes (Ocn, Bgn, and collagens) there is great interest in pursuing a deeper understanding of their relevance to bone formation in the ovariectomized rat. Fgfr1 is critical to embryonic craniofacial development (Rice et al., 2003) and has been shown to be elevated robustly during the formation of fracture callus in rat femur (Nakajima et al., 2001), and activation mutations in this gene lead to increased bone formation in the crania of both human and mice (Zhou et al., 2001). Hox3a, a transcriptional repressor of the group 8 Hox family, is also known to be involved with osteoblast and cartilage differentiation (Yueh et al., 1998; Yang et al., 2000) and mouse skeletal patterning (van den Akker et al., 2001). Our data support the importance of Fgfr1 and Hox3a expression in the adult rat proximal femur, and we have now demonstrated their regulation during ovariectomy-induced bone formation. Syncam (Igsf4) has been described as an important intracellular adhesion protein and as a possible tumor suppressor in lung (Masuda et al., 2002). Although the roles of Igsf4 in bone formation have never been studied, the tight association of its regulation with that of known bone formation genes in this study implies that it plays a role in bone formation of the adult rat skeleton.

The data in the current study demonstrate that although SERMs may be estrogen-like in their effects on BMD in the bone, the molecular events after prolonged treatment with raloxifene or EM652 show large differences in RNA expression profiles compared with that of E2. Whether these expression changes are the result of differences in levels of cellular activity, function, RNA stabilization or transcription, or cell number is unknown; any of these parameters could be changed as a result of prolonged antiresorptive treatment that achieves changes in bone strength and architecture. Further study is necessary to understand

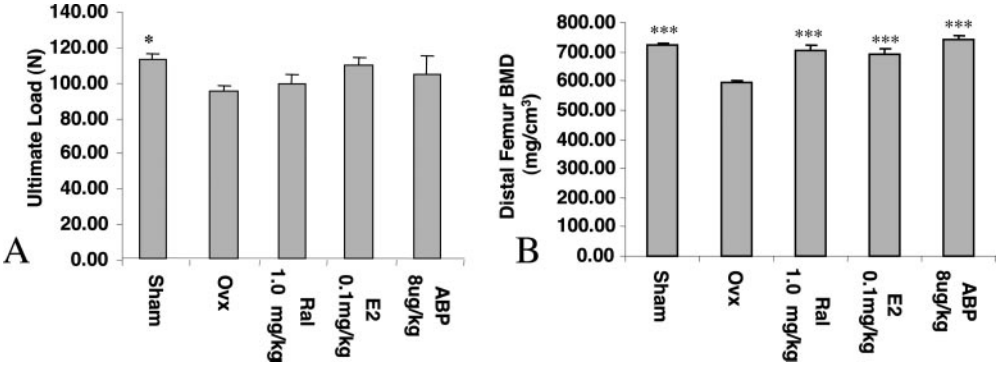

Fig. 5. Physical properties of femora from the 5-week validation study. A, biomechanical analysis of the peak load to failure in the proximal femur. *, group mean is statistically significant ($p < 0.05$) from Ovx. B, quantitative computed tomography analysis of BMD of the distal femur metaphysis. ***, group mean is statistically different from Ovx control ($p < 0.0001$). Values plotted represent group means ± S.E.M. Ral, raloxifene; ABP, alendronate.

whether some of the differences in gene expression observed in this study could contribute to architectural or biomechanical qualities that further differentiate these antiresorptive therapies. Because it has been demonstrated that BMD cannot completely predict fracture efficacy of a compound (Riggs and Melton, 2002; Sarkar et al., 2004), there is great interest in furthering our understanding of what parameters define bone quality and what molecular events could help to predict bone fracture. Profiling the RNA changes associated with antiresorptive therapy demonstrate that even though these drugs have similar efficacy at maintaining BMD in the ovariectomized rat (Fig. 5), the underlying molecular events that collectively result in improved bone strength and density seem to be complex. This study helps to uncover some of the possible mechanistic changes resulting from various drug treatments and provides new avenues for investigation of the role these differences may play in determining bone quality. Gene expression changes may help to complement current methods of assessing bone quality (i.e., bone density measurements) so that better indicators of fracture risk could be developed.

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