

MOL (11478)

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

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Running Title: Bone Formation Genes Suppressed by alendronate and estrogen

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Text Pages: 15

Tables: 7

Figures: 5

References: 43

Words in abstract: 385

Words in introduction: 508

Words in discussion: 1483

Non-standard Abbreviations: 17- α ethinyl estradiol (E2), selective estrogen receptor modulator (SERM), hydroxypropyl- β -cyclodextrin (CDX), ovariectomized vehicle controls (Ovx), false discovery rate (FDR), hierarchical clustering analysis (HCA), self-organizing map (SOM), collagen type I alpha 2 (Col1a2), collagen type V alpha 1, (Col5a1), osteocalcin (Ocn), osteonectin (Sparc), serpine proteinase inhibitor clade H (Serpinh1), procollagen C-proteinase enhancer protein (Pcolce), bone formation rate (BFR), carbonic anhydrase 4 (Ca4), cytochrome P450 family 27 subfamily A polypeptide 1 (Cyp 27a1), biglycan (Bgn), immunoglobulin superfamily member 4 (Igsf4), homeobox protein C8 (Hox3a), fibroblast growth factor receptor 1 (Fgfr1), bone mineral density (BMD), parathyroid hormone (PTH)

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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 utilized 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, EM652, and the aminobisphosphonate, alendronate. Ovariectomy resulted in 644 significant probe set changes relative to sham controls ($P < 0.05$) while E2, raloxifene, EM652, and alendronate regulated 613, 765, 652, 737 probe sets relative to ovariectomized controls, respectively. An intersection of these data sets yielded 334 unique genes that were altered following 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. Additionally, E2 and alendronate suppressed a cluster of genes associated with bone formation activity below that of Sham while 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.

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Ovariectomy of mature rats has been shown to induce cancellous bone loss from axial and appendicular sites, as observed with postmenopausal women (Kimmel, 1996; Turner et al., 1994) 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 (Anderson et al., 2004; Cauley et al., 2003; Turner et al., 1994), selective estrogen receptor modulators (SERMs) such as raloxifene and EM652, (Black et al., 1994; Delmas et al., 1997; Ettinger et al., 1999; Martel et al., 2000; Sato et al., 1995), and bisphosphonates, such as alendronate (Lieberman et al., 1995; Seedor et al., 1991; Toolan et al., 1992). Estrogen, Raloxifene and Alendronate have all been shown to inhibit bone resorption but they have very different mechanisms of action (Riggs and Parfitt, 2005; Sato et al., 1999).

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 and SERM treatment reduce 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).

While 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 following the loss of ovarian function, regardless of the mechanism by which the preservation of bone was achieved. Alternatively, 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 following ovariectomy, and are regulated by estrogen, by 2 SERMs from different structural classes (the benzothiophene SERM, raloxifene and the benzopyran SERM, EM652), and/or the

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aminobisphosphante, alendronate in the ovariectomized rat proximal femur. We sought to ascertain which genes are associated with ovariectomy at 40 days post 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 which genes were commonly regulated by all treatments. Finally, we sought to identify the similarities and differences between the agents that signal through the estrogen receptor (2 SERMs and estrogen) in regulating ovariectomy-induced gene changes.

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MATERIALS AND METHODS

Animal Study design

Six month old Sprague Dawley rats (Harlan, Indianapolis, IN) were group housed and maintained on a 12 hr light/dark cycle at 22°C with ad libitum access to food (TD 89222 with 0.5% Ca and 0.4% P, Teklad, Madison, WI) and water. Rats were randomized into 6 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/d 17- α -ethinyl estradiol (E2, Sigma) in CDX 4) ovariectomized animals administered 1.0 mg/kg/d raloxifene (Eli Lilly & Co.) in CDX 5) ovariectomized animals administered 0.1 mg/kg/d EM652 (Eli Lilly & Co.) in CDX, and 6) ovariectomized animals administered 8 ug/kg/d alendronate (Eli Lilly & Co.) in saline. The doses were chosen because they were shown previously to be fully efficacious in rats (Martel et al., 2000; Sato et al., 1996; Schenk et al., 1986).

The study was initiated 5 days post-ovariectomy and compounds were administered by oral gavage (except for the subcutaneous administration of alendronate) for 35 days (40 days post-surgery) after which the proximal femora were collected 24 hr post-dosing. Two additional studies were conducted for validation purposes and were executed exactly as the five-week study above (except for the omission of the EM652 group). The first validation study dosed the animals for five weeks and utilized femora for histomorphometric analysis while the proximal end of the contra lateral 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 CO₂ 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 Institute 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 utilizing a Roche/Hitachi 917 automated chemistry analyzer.

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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 (Biotex Houston, TX) according to the manufacturer's instructions. Twenty-five micrograms of total RNA was electrophoretically separated, transferred to nylon membranes, and probed with radiolabelled cDNA probes as previously described (Ma et al., 2001). Gene expression was normalized to either 18S ribosomal or cyclophilin expression.

For real-time quantitative RT-PCR, an ABI Prism Sequence Detection System 5700 was used and the primer-probe sets for the genes described were obtained from Applied Biosystems as Assay-on-Demand™ reagents (Foster City, CA). Before cDNA synthesis, 5µg of total RNA were Dnase-treated for 30 minutes at 37°C (DNA-free kits, Ambion, Austin, TX). RNA was reverse-transcribed from random hexamer primers using SUPERSCRIPT II RT kit (Invitrogen, Carlsbad, CA). Specific amplification reactions from the cDNAs were carried out via a 2-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 GeneChip Expression Technical Manual). Briefly, 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' GCCAGTGAATTGTA-ATACGACTCACTATAGGGA-GGCGG(dT)₂₄ 3' Genset Corp, Evry, France). Phase Lock Gel (1.5 ml) tubes (Eppendorf, Westbury, NY) were used to clean cDNA following phenol/chloroform/ isoamyl alcohol extraction. Biotin-labeled cRNA was synthesized from cDNA using BioArray High Yield RNA Transcript Labeling kit (Enzo, 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 pH8.1, 100 mM potassium acetate, and 30 mM

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magnesium acetate for 35 minutes. Ten μg of fragmented cRNA was hybridized to RG_U34A arrays for 16 hours 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

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

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) following distribution $N(0, \sigma_C^2)$.

Since 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 , $i = 1, 2, \dots, m$. 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

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

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

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

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

The ANOVA 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 co-regulation among a large number of genes while retaining as much as possible of the variation. 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, 1997). The first three principal components with at least 60% variation were exported into Spotfire (Sommerville, 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 Spotfire 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 Spotfire with a grid size of 4x4 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 5 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 representers are

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present in a sample size n , was calculated based on the hypergeometric cumulative distribution

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

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 $0 \leq M \leq N$, $0 < n \leq N$. These calculations were done on software written by Eli Lilly & Co., however, comparable software is publicly available (Boyle, 2004).

Bone Parameters

Quantitative computed tomography of the distal femur and biomechanical analyses of the proximal femur from the five-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 to the NCBI genome builds, to UniGene, and to 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 to the protein sequences in HumanPSD™. Alignments with at least 100 amino acids of 100% identity and/or a BLAST evaluate 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™.

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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 controls (Ovx) relative to Sham. Compounds signaling through the estrogen receptor demonstrated a decrease in body weight as expected, as 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 while 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 co-regulated genes, unsupervised clustering algorithms were utilized 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, while 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 2 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 (Figure 1).

To understand what 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 following each treatment regimen relative to OvX controls 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 supplementary data). The median of the intensity data for each treatment group was then subjected to hierarchical clustering analysis (HCA). The heat

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map visualization of the dataset (Figure 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 unique expression patterns compared to each other.

Given the complexity of the data we further used self-organizing maps (SOM) to identify unique patterns of expression for each treatment. SOM analysis (Figure 3) identified unique patterns of gene expression that could be assigned into 4 broad categories: a) genes altered by ovariectomy but were restored to or maintained near to Sham levels by all antiresorptive treatments (clusters 5,6,15,16) b) genes uniquely kept near Sham levels only by raloxifene while the other therapies had no effect (clusters 8,9,13,14) c) genes uniquely returned to or below that of Sham by all of the agents except raloxifene and partially by EM652 (cluster 1) d) 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 that no other treatment did 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 post-ovariectomy, the expected increase in expression from Sham level associated with bone formation genes [such as collagen type I $\alpha 2$ (Col1a2 +2.4 fold), collagen type V $\alpha 1$ (Col5a1 +1.4 fold), osteocalcin (Ocn +1.9 fold), osteonectin (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 as 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 following 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 (BFR) in the metaphysis, along with upregulation of osteoclastic activity (1). Co-regulated with these

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formation and resorption genes following ovariectomy were more than 300 genes. These ovariectomy-induced gene changes are implicated in a wide-variety of molecular functions and biological processes that are summarized by noting their Gene Ontology descriptor as outlined in 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 appeared to be stable changes as 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 V α 2 (+2.7 fold), serine proteinase inhibitor clade H (Serpinh1 +1.7 fold), lysyl oxidase (+1.8 fold), and collagen type XI α 1 (+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. Conversely, 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 and 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 controls 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 while the other 3 drug treatments had no effect on these genes (see Figure 2

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clusters 2 & 3). Sixty-three percent of the unique raloxifene genes were suppressed by ovariectomy and subsequently increased by raloxifene treatment; 41% of which were altered by at least 1.5-fold or more 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 five-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 or Ca4 expression changes following 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 Figure 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 5-6) 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 (Figure 2 cluster 1, Figure 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 (Figure 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

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(Fgfr1) implying that they may also serve an important role in osteoblastic activity because they are co-regulated 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 five-week array study. Biomechanical testing on the femoral neck (the site utilized for array analysis) from this 5 week validation study showed no differences in the strength of the bone from Ovx by any drug treatment (Figure 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 (Figure 5B).

The expression of the osteoblast activity genes in the proximal femur was re-evaluated in the five-week validation study and was observed to parallel the differential expression pattern observed on the array. While the fold change from control values were not identical between the array and the five-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 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) prior to when 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).

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DISCUSSION

Ovariectomized rats greater than 5 months of age have been shown to reproducibly lose cancellous bone from axial and appendicular skeletal sites due to estrogen deficiency, not unlike post-menopausal women (Turner et al., 1994). Upon pharmacologic administration of maximally efficacious doses of E2, raloxifene (Sato et al., 1995; Sato et al., 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 following ovariectomy (Sato et al., 1999), a gene array analysis was initiated in an effort to elucidate possible differences of these compounds on skeletal physiology.

Following 5 weeks of treatment, the overall expression profile of all genes on the microarray suggested that E2 and alendronate were most similar while the gene expression profiles of EM652 and raloxifene appeared to be distinctly different from E2 (Figure 1). This was an unexpected finding as we 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. In order to understand more fully what 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 following ovariectomy it would be expected 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 due to 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 following 12 days of ovariectomy but by 5 weeks these changes were no longer observed likely due to the lack of bone surface in the ovariectomized group which was lost by this time.

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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 likely represents a core set of molecular changes necessary to maintain bone mass post-ovariectomy in the rat and occur prior to any biomechanical effect in this bone site (Figure 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 as at least one of the other agents also regulated the genes associated with these treatments. Validation of 2 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 achieve statistical significance with either gene (Table 7) highlighting the importance of validating gene expression in independent assays.

While 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 following 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 while EM652 treatment resulted in an intermediate expression level (Figure 4). A replicated study (five-week treatment) confirmed that alendronate treatment was the most suppressive of the expression of these formation genes while 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). While the present study only evaluated RNA changes, the significantly enhanced suppression of Ocn protein by alendronate versus that of raloxifene has been observed in the serum of post-menopausal women following 1 year of treatment (Johnell et al., 2002). These data are consistent with previously published histomorphometric analyses in postmenopausal women where alendronate reduced bone formation activity (BFR per unit bone surface and activation frequency) by 90% (Chavassieux et al., 1997) while estrogen had milder suppressive

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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 in clinical studies the combined effects of alendronate and PTH were found to be less efficacious than PTH alone (Black et al., 2003; Finkelstein et al., 2003); 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 alendronate's suppression of osteoblastic function (as evidenced by the lack of expression of osteoblastic genes) antagonized PTH efficacy while raloxifene treatment did not impair osteoblast activity and thus can complement PTH in the skeleton.

Because *Hox3a*, *Fgfr1*, and *Syncam* clustered tightly (Figure 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 cranium 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 (Yang et al., 2000; Yueh et al., 1998), and mouse skeletal patterning (van den Akker et al., 2001). Our data support the importance of *Fgfr1* and *Hox3a* expression in the adult qualities that further differentiate antiresorptive therapies from each other. Because it has been demonstrated that BMD cannot completely predict fracture efficacy of a compound (Riggs and Melton, 2002; Sarkar, 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 (Figure 5), the underlying molecular events which collectively result in improved bone strength and density appear 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. 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

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al., 2002). While 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 implicates it as playing a role in bone formation of the adult rat skeleton.

The data in the current study demonstrate that while SERMs may be estrogen-like in their effects on BMD in the bone, the molecular events following prolonged treatment with raloxifene or EM652 show large differences in their RNA expression profiles from that of E2. It is not known whether these expression changes are the result of differences in levels of cellular activity, function, RNA stabilization or transcription, or cell number as 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 if some of the differences in gene expression observed in this study could contribute to architectural or biomechanical. bone density measurements) so that better indicators of fracture risk could be developed.

ACKNOWLEDGMENTS

The authors wish to thank John Calley and Amar Kumar for their excellent bioinformatics support; Harlan Cole, Rick Cain, Ellen Rowley, and Pam Shetler for their *in vivo* expertise; and Allan Schmidt for QCT and biomechanical assessments.

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FOOTNOTES

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This study was funded by Lilly Research Laboratories.

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FIGURE LEGENDS

Figure 1

Scatter plot of principal component analysis of all probe sets on the Affymetrix U34A chip following 35 days of treatment. Each square represents a microarray chip generated from individual animals (2 chips per animal) and the closer the proximity of the squares to each other the more closely related the chip expression profile. Note that the 2 EM652 outlier chips correspond to a single animal. Encircled are chips that appear to group together. Raloxifene (Ral), EM652 (EM), alendronate (ABP), 17- α ethinyl estradiol (E2)

Figure 2

Hierarchical clustering analysis of 380 probe sets altered by ovariectomy and by 1 or more drugs following 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 & 3 identify sets of genes altered by Ovx that only Raloxifene maintained at Sham levels. Clusters 4 & 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 is intermediate expression levels. Raloxifene (Ral), EM652 (EM), alendronate (ABP), 17- α ethinyl estradiol (E2)

Figure 3

Self-organizing map of the 380 probe sets altered by ovariectomy and by one or more drugs following 5 weeks of treatment. Y-axis represents range-scaled median expression values of various treatments. Raloxifene (Ral), EM652 (EM), alendronate (ABP), 17- α ethinyl estradiol (E2)

Figure 4

Detailed view of Cluster 1 from the hierarchical clustering analysis (Figure 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 is intermediate expression. Raloxifene (Ral), EM652 (EM), alendronate (ABP), 17- α ethinyl estradiol (E2)

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Figure 5

Physical properties of femora from the five-week validation study. A) Biomechanical analysis of the peak load to failure in the proximal femur. * indicates group mean is statistically significant ($P < 0.05$) from Ovx. B) Quantitative computed tomography analysis of bone mineral density (BMD) of the distal femur metaphysis. *** indicates group mean is statistically different from Ovx control ($P < 0.0001$). Values plotted represent group means \pm SEM. Raloxifene (Ral), alendronate (ABP), 17- α ethinyl estradiol (E2)

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Table 1 Study design, serum cholesterol, and body weight following 5 weeks treatment.

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

Mean body weight and serum cholesterol concentrations ± SEM for 5 animals per group.
o = $P < 0.05$ vs. Ovx control s = $P < 0.05$ vs. Sham control. Raloxifene (Ral), EM652 (EM), alendronate (ABP), 17- α ethinyl estradiol (E2)

Table 2 Genes Commonly Regulated by All Antiresorptive Treatments as Compared to Control*

| Affymetrix ID | <u>Ovx</u> | | <u>Raloxifene</u> | | <u>EM652</u> | | <u>Estrogen</u> | | <u>Alendronate</u> | | 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 beta B2 |
| AJ005396_at | 3.0 | 0.0 | -1.8 | 0.0 | -1.9 | 0.0 | -6.2 | 0.0 | -12.2 | 0.0 | Col11a1 | Alpha 1 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 | Alpha 2 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 (25kDa) |
| 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 |

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|------------------|-------------|-----|-------------|-----|-------------|-----|-------------|-----|-------------|-----|----------|---|
| 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 alpha 1 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 gamma 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 |
| A03913cde_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) beta 1 |
| 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 alpha subunit (P28 alpha) |
| rc_AA891785_at | 1.2 | 0.4 | -1.2 | 0.5 | -1.5 | 0.1 | -1.4 | 0.2 | -1.4 | 0.2 | ldh2 | 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 | Alpha 1 subunit of the Na ⁺ -K ⁺ -transporting ATPase |
| U77829mRNA_l_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 alpha |
| 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_Al639055_at | -2.1 | 0.2 | 2.3 | 0.2 | 2.2 | 0.3 | 2.2 | 0.3 | 1.8 | 0.9 | Al639055 | Unknown |
| rc_Al043796_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 | Pvr11 | 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 |
| 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_Al638972_at | -1.9 | 0.3 | 1.6 | 0.8 | 2.1 | 0.3 | 1.7 | 0.6 | 2.1 | 0.2 | Al638972 | 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 |

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|------------------|-------------|-----|------------|-----|------------|-----|------------|-----|------------|-----|---------------|---|
| 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 delta-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 alpha 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 | Gamma-aminobutyric acid (GABA-A) receptor subunit gamma 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 |

*Fold-change calculated for ovariectomized controls (Ovx) by comparing mean intensity data for each probe set to that of Sham controls while fold-change for each antiresorptive agent was calculated by comparing the mean intensity data of each to that of Ovx.

Table 3 Genes Uniquely Regulated by Raloxifene*

| Affymetrix ID | 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 beta-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 beta |
| rc_AA800296_at | 1.6 | 0.3 | -1.5 | 0.2 | Papola | Poly(A) polymerase alpha |
| 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 non-metastatic 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 | Cox8h | 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 alpha chain |
| rc_AA874803_g_at | -1.9 | 0.1 | 1.6 | 0.1 | AA874803 | Unknown |

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|------------------|------|-----|-----|-----|---------------|---|
| 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 | Beta 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 1 alpha |
| 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 alpha |
| 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 alpha |
| rc_AI178971_at | -1.4 | 0.1 | 1.3 | 0.2 | Hba1 | Hemoglobin alpha 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 | Dodecenoyl-Coenzyme A delta 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 alpha 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 | Delta-aminolevulinic acid 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 | Gamma-aminobutyric acid receptor associated protein |

*Fold-change calculated for ovariectomized controls (Ovx) by comparing mean intensity data for each probe set to that of Sham controls while fold-change for raloxifene was calculated by comparing the mean intensity data of each probe set to that of Ovx.

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Table 4 Genes Uniquely Regulated by Estrogen*

| 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 beta (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 beta-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 |

*Fold-change calculated for ovariectomized controls (Ovx) by comparing mean intensity data for each probe set to that of Sham controls while fold-change for estrogen was calculated by comparing the mean intensity data of each probes set to that of Ovx.

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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*

Gene Ontology Classification

| 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 | |
| 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** | |

Genes maintained at Sham level by all pharmaceutical agents and those uniquely maintained by E2 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 in materials and methods. Terms followed by an

** were significant at $p < 0.01$ and *** indicates a significance of $p < 0.005$. Raloxifene (Ral), EM652 (EM), alendronate (ABP), 17- α ethinyl estradiol (E2)

Molecular Pharmacology Fast Forward. Published on August 3, 2005 as DOI: 10.1124/mol.105.011478
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Table 7 Comparison of Gene changes following Treatment Relative to Ovariectomized Controls In Two Independent Studies

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

Study 1 analyzed genes by microarray analysis and Study 2 analyzed genes by real-time PCR in an independent five-week animal study as described in the materials and methods. ^o indicates a group is statistically different from Ovx control ($p < 0.05$), ^s indicates a group is statistically different from Sham control ($p < 0.05$). Values represent the ratio of mean intensity data for each probe set relative to that of ovariectomized controls within a given study.

Figure 1

- Sham
- Ovx
- Ral
- E2
- EM
- ABP

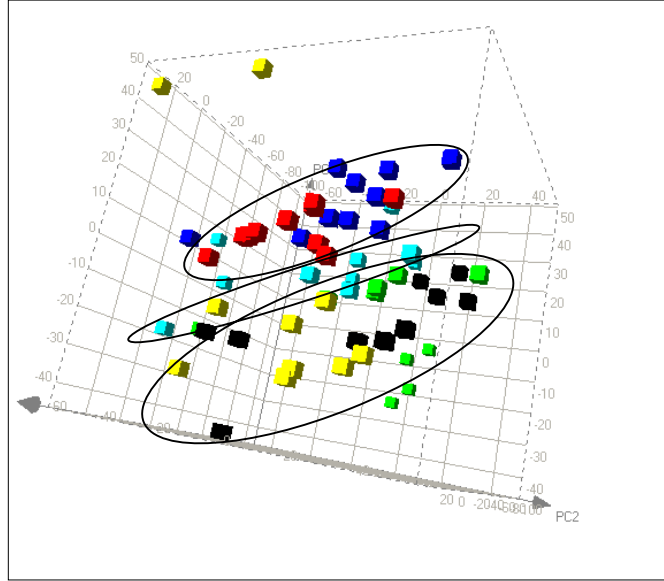


Figure 2

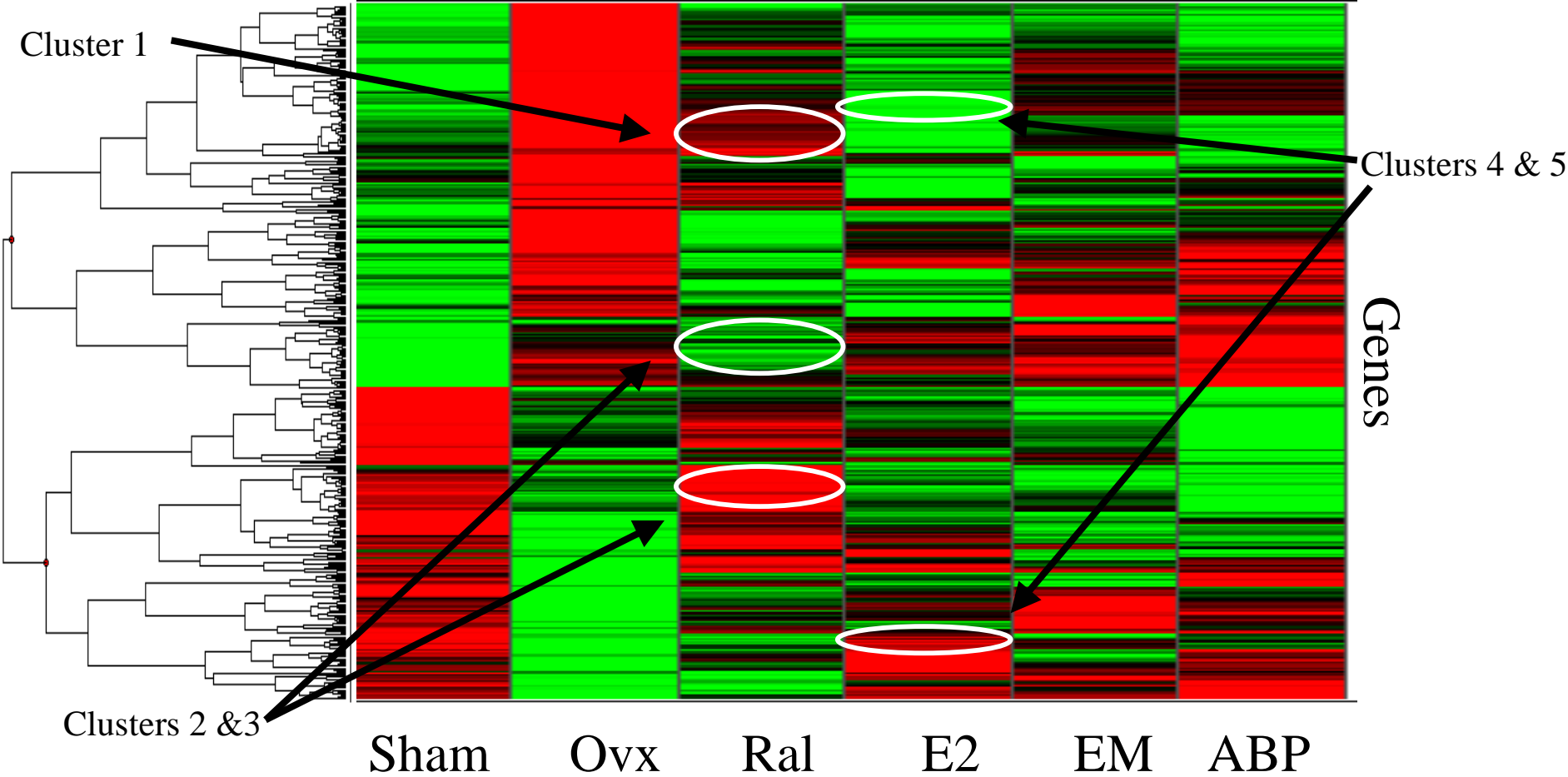


Figure 3

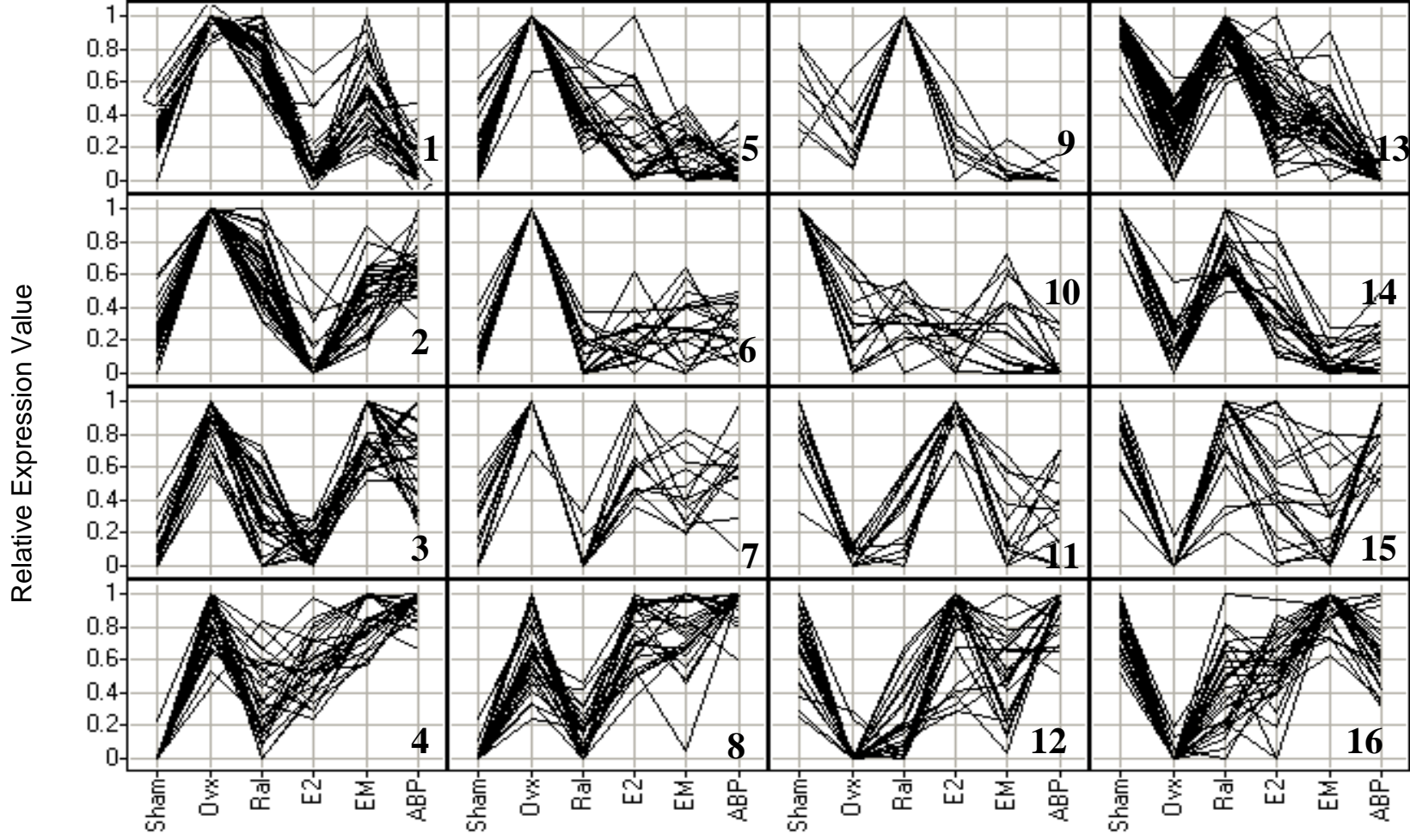


Figure 4

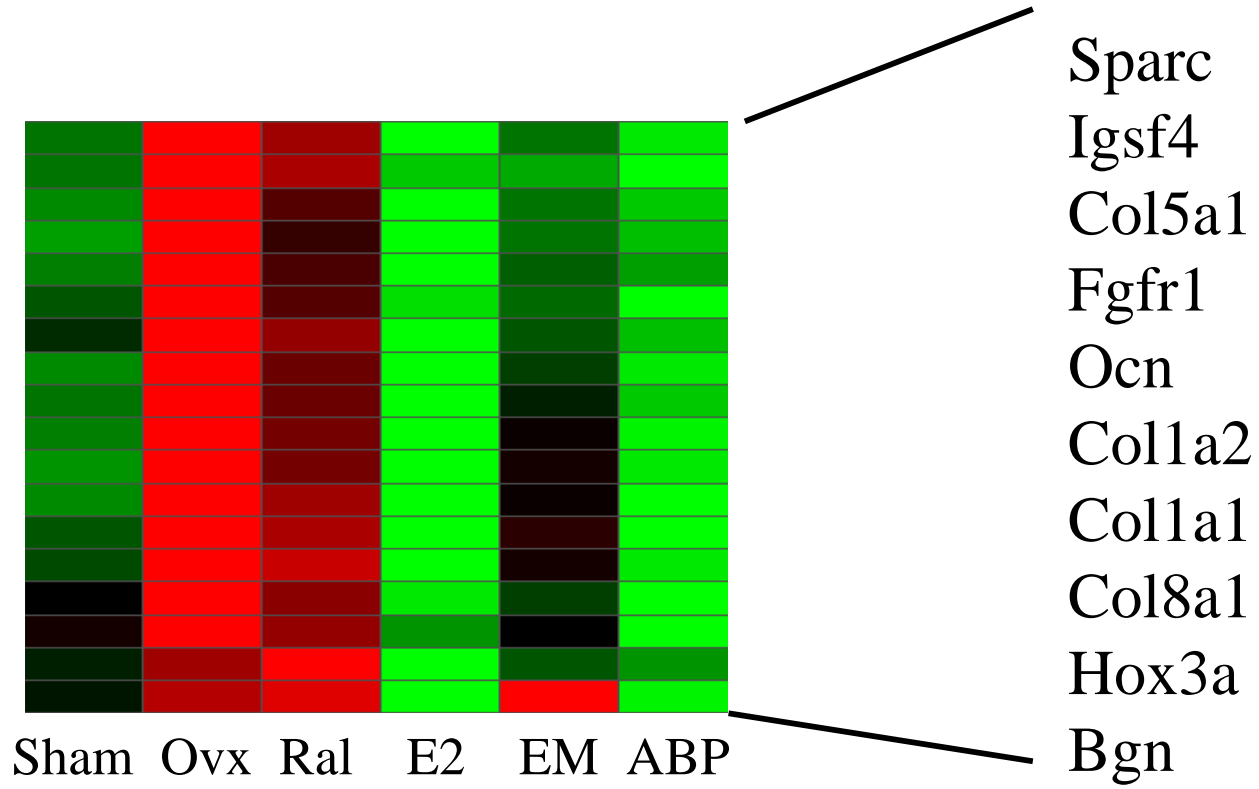


Figure 5

