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# Estrogen Receptor (ER)-$\beta$ Reduces ER$\alpha$-Regulated Gene Transcription, Supporting a "Ying Yang" Relationship between ER$\alpha$ and ER$\beta$ in Mice

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**Estrogen is of importance for the regulation of adult bone metabolism. The aim of the present study was to determine the role of estrogen receptor-$\beta$ (ER$\beta$) *in vivo* on global estrogen-regulated transcriptional activity in bone. The effect of estrogen in bone of ovariectomized mice was determined using microarray analysis including 9400 genes. Most of the genes (95% = 240 genes) that were increased by estrogen in wild-type (WT) mice were also increased by estrogen in ER$\beta$-inactivated mice. Interestingly, the average stimulatory effect of estrogen on the mRNA levels of these genes was 85% higher in ER$\beta$-inactivated than in WT mice, demonstrating that ER$\beta$ reduces estrogen receptor-$\alpha$ (ER$\alpha$)-regulated gene transcription in bone. The average stimulatory effect of estrogen on estrogen-regulated bone genes in ER$\alpha$-inactivated mice was intermediate between that seen in WT and ER$\alpha\beta$ double-inactivated mice. Thus, ER$\beta$ inhibits ER$\alpha$-mediated gene transcription in the presence of ER$\alpha$, whereas, in the absence of ER$\alpha$, it can partially replace ER$\alpha$. In conclusion, our *in vivo* data indicate that an important physiological role of ER$\beta$ is to modulate ER$\alpha$-mediated gene transcription supporting a "Ying Yang" relationship between ER$\alpha$ and ER$\beta$ in mice. (*Molecular Endocrinology* 17: 203–208, 2003)**

ESTROGEN IS OF importance for the regulation of adult bone metabolism. We and others have previously demonstrated that female estrogen receptor-$\beta$ (ER$\beta$)-inactivated or ER$\alpha^{+/+}\beta^{-/-}$ mice (BERKO) have an increased amount of cortical bone and are partly protected against age-related trabecular bone loss that normally occurs in old female wild-type (WT) mice. Therefore, one may speculate that ER$\beta$ represses the bone-protective effect of estrogen mediated via estrogen receptor-$\alpha$ (ER$\alpha$) (1–3). A possible interaction between ER$\alpha$ and ER$\beta$ in the regulation of transcriptional activity has previously been investigated in different *in vitro* systems. It is clear that ER$\beta$ and ER$\alpha$ can form heterodimeric complexes with retained DNA-binding ability (4). Transient transfection assays have demonstrated that ER$\beta$ has the capacity to repress the transcriptional activity of ER$\alpha$ (5, 6). Furthermore, when complexed with estradiol, ER$\alpha$ and ER$\beta$ signal in opposite directions from an activator protein 1 site (7). An inhibitory effect of ER$\beta$ might be explained by the finding that ER$\beta$ does not contain a strong activation function 1 within its amino terminus but, rather, contains a repressor domain; when this particular segment is removed, the overall transcriptional activity of the receptor increases (6). Thus, previous *in vitro* data indicate that ER$\beta$ can sometimes act as a dominant negative regulator of ER$\alpha$ activity. Gene expression profiling opens up possibilities to investigate the interplay between ER$\alpha$ and ER$\beta$ in a global sense. The aim of the present study was to determine, *in vivo*, the role of ER$\beta$ and its possible interactions with ER$\alpha$ in global estrogen-regulated transcriptional activity in bone.

Abbreviations: BERKO, ER$\alpha^{+/+}\beta^{-/-}$; DERKO, ER$\alpha^{-/-}\beta^{-/-}$; ER, estrogen receptor; ERKO, ER$\alpha^{-/-}\beta^{++}$; EST, expressed sequence tag; GR, glucocorticoid receptor; ovx, ovariectomized; PR, progesterone receptor; WT, wild-type.

## RESULTS AND DISCUSSION

### ER$\beta$ Reduces ER$\alpha$-Regulated Gene Transcription in Bone and Liver

The effects of estrogen on global gene expression in humerus from ovariectomized (ovx) mice were determined by microarray analysis. Most of the genes (95%, 240 genes) that were increased by estrogen in WT mice were also increased by estrogen in BERKO mice, indicating that ER$\beta$, in the presence of ER$\alpha$, is generally not required for estrogen-regulated gene transcription in bone. The relative magnitude of the effect of estrogen in WT and BERKO mice was compared for these genes. Interestingly, the average stimulatory effect of estrogen on the expression of these genes was 85% higher in BERKO than in WT mice and, when considering the individual genes, it was found that 80% of the genes were more regulated by

estrogen in BERKO than in WT mice (Fig. 1A and Table 1). ERα mRNA levels in bone were unchanged in 3-month-old estrogen-treated female BERKO mice compared with WT mice (WT, 100 ± 13%, BERKO, 132 ± 29% of WT mice, n = 7–8) as determined by real-time PCR.

To study whether the effect of ERβ to reduce estrogen-stimulated gene transcription was specific for bone or whether it could be found in other tissues as well, the effect of estrogen on estrogen-regulated genes was analyzed in the liver. In accordance with the results from the humerus, the average stimulatory effect of estrogen in liver was increased in BERKO compared with WT mice (184% of WT) and when the individual genes were considered it was found that 76% of the genes were more regulated by estrogen in BERKO than in WT mice (Fig. 1B and Table 1). As a negative control, we compared the expression of all genes not regulated by estrogen (n = 9060) without finding any difference between WT and BERKO mice (data not shown). The stimulatory effect of estrogen on the expression of 13 genes in bone and 8 genes in liver was confirmed by real-time PCR analysis (Fig. 2 and data not shown). Thus, in the presence of ERα, it is clear that ERβ reduces the global estrogen-stimulated gene transcription in bone and liver. This is the first demonstration that ERβ is a global inhibitor of ERα-regulated gene transcription in mice. In contrast, a minority of the estrogen-regulated genes were more regulated in WT than in BERKO mice, which might indicate that ERβ has an additional role as a potent activator of a small specific gene set. However, we believe, rather, that these results represent a random distribution of several estrogen-regulated genes, where most genes are more regulated and very few are less regulated in BERKO than in WT mice.

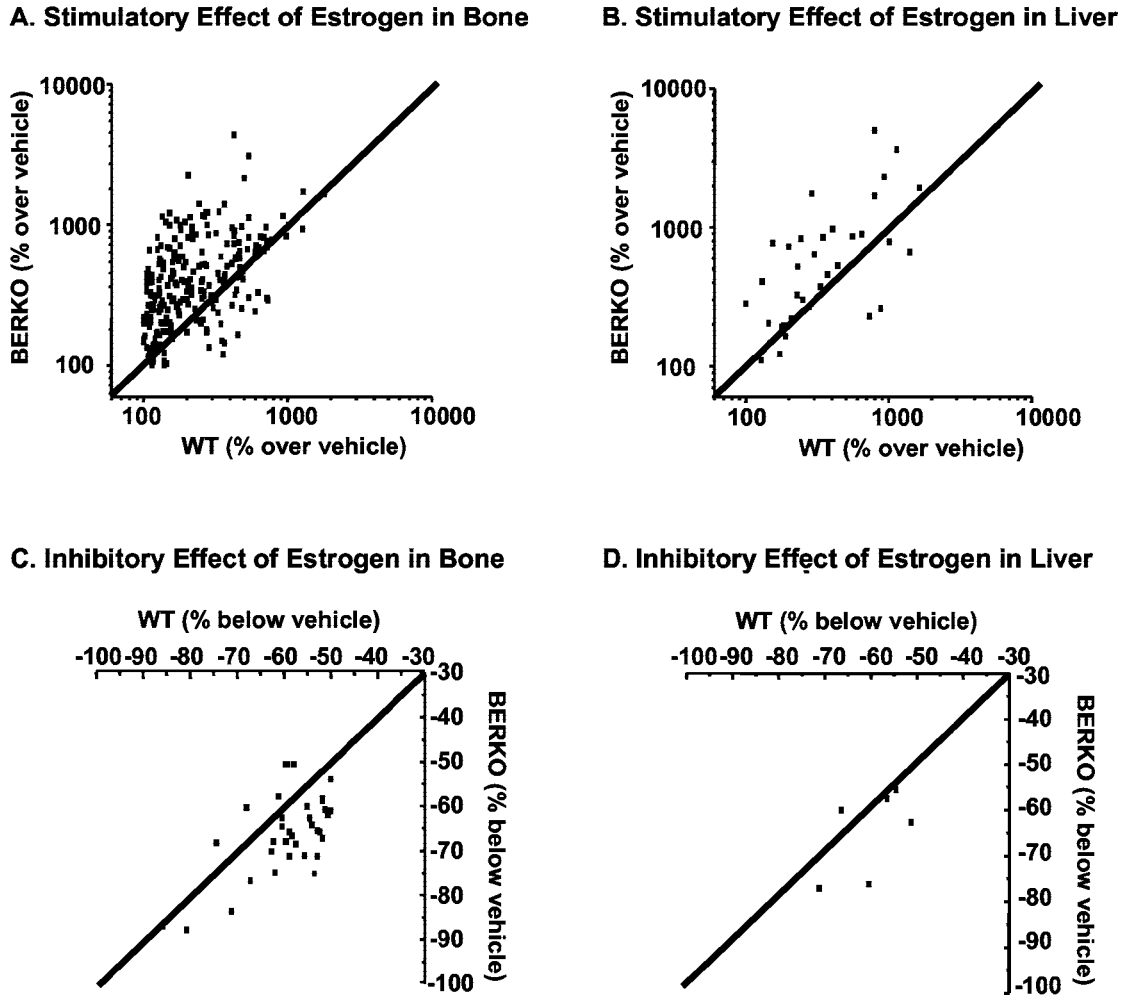


**Fig. 1.** ERβ Reduces ERα-Regulated Gene Transcription in Bone and Liver

Stimulatory (A and B) and inhibitory (C and D) effects of estrogen on estrogen-regulated gene transcription in bone (A and C) and liver (B and D) of ovx WT and BERKO mice as measured using microarray analysis. The percent increase over vehicle (A and B) or decrease below vehicle (C and D) for each individual estrogen-regulated gene is given for WT (x-axis) and BERKO (y-axis) mice. The *black line* indicates the theoretical position of genes regulated by estrogen to the same magnitude in WT and BERKO mice.

A global ERα-repressing effect of ERβ on transcriptional activity is supported by recent functional *in vivo* studies demonstrating that ERβ represses some ERα-mediated effects, including effects on female bone, thymus involution, fat reduction, and proliferative effects in uterus and prostate (8, 9).

The number of genes inhibited by estrogen in bone from both WT and BERKO mice was lower than the number of genes stimulated by estrogen (stimulated, 240; inhibited, 35). The average inhibitory effect of estrogen on these genes was more pronounced in BERKO compared with WT mice and, when considering the individual genes, it was found that 83% of the genes were more regulated by estrogen in BERKO than in WT mice (Fig. 1C and Table 1). The number of genes inhibited by estrogen in liver (n = 6) was too low to permit any statistical difference between WT and BERKO mice even though a similar tendency as in bone was found (Fig. 1D and Table 1). These data indicate that ERβ, in the presence of ERα, not only reduces the stimulatory but also the inhibitory effect of estrogen on gene transcription.

**Table 1.** Effect of Estrogen in WT and BERKO Mice

| | WT (%) | BERKO (%) |
|---|---|---|
| Stimulation in bone (n = 240) | 275 ± 14 | 511 ± 30[a] |
| Stimulation in liver (n = 34) | 472 ± 67 | 868 ± 179[b] |
| Inhibition in bone (n = 35) | −59 ± 1 | −66 ± 2[a] |
| Inhibition in liver (n = 6) | −60 ± 3 | −65 ± 41 |

Effect of estrogen on estrogen-regulated gene transcription in bone and liver of ovx mice as studied by microarray analysis. Values are the average effect of estrogen expressed as percent stimulation above or inhibition below vehicle and are given as mean ± SEM. n = number of genes regulated by estrogen in both WT and BERKO mice.
[a] $P < 0.001$ BERKO *vs.* WT, Student's paired *t* test.
[b] $P < 0.05$ BERKO *vs.* WT, Student's paired *t* test.

### ERβ Can Partially Replace ERα in Estrogen-Regulated Gene Transcription

The function of ERβ on estrogen-regulated gene transcription in the absence of ERα was investigated by comparing the effect of estrogen in bone from ovx WT, $ER\alpha^{-/-}\beta^{+/+}$ (ERKO), BERKO, and $ER\alpha^{-/-}\beta^{-/-}$ (DERKO) mice. We could, by microarray analysis, clearly demonstrate that more than 98% of the genes stimulated by estrogen in WT and BERKO mice were unchanged by estrogen in DERKO mice, demonstrating that, for most genes, either ERα or ERβ is required for the mediation of the effect of estrogen (Fig. 3). Less than 2% of the estrogen-regulated genes were regulated by estrogen also in DERKO mice, indicating that these few genes were regulated in an ERα/ERβ-

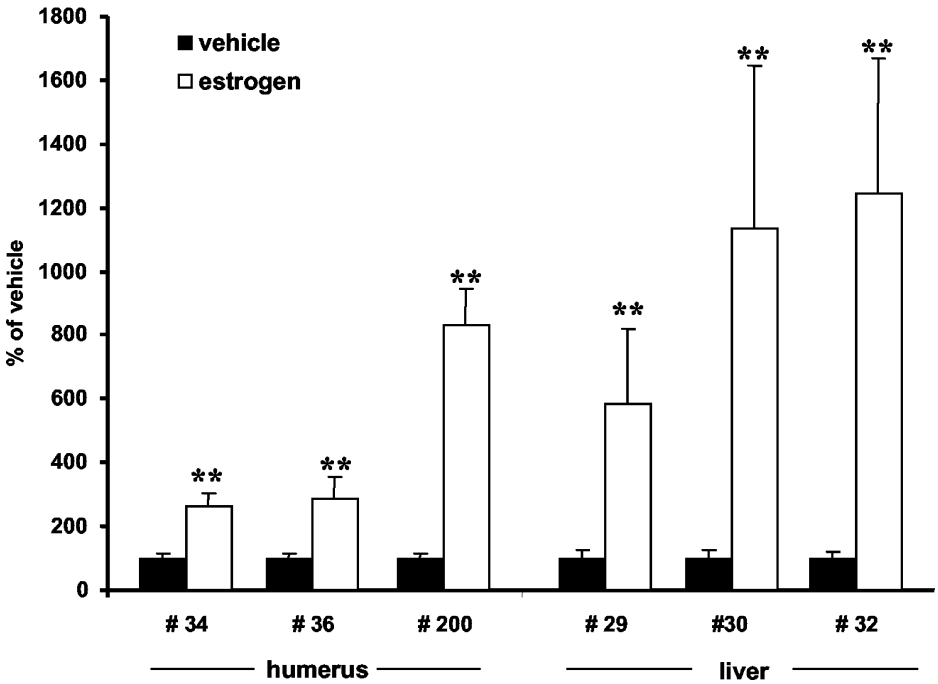


**Fig. 2.** TaqMan Verification of DNA Microarray Analysis

Verification of DNA microarray analysis with relation to the stimulatory effect of estrogen on three different genes in bone (gene 34, 36, and 200 according to the supplemental data file) and three other genes in liver (gene 29, 30, and 32 according to the supplemental data file) as measured by real-time PCR on individual mice. Values are shown as percent of vehicle-treated WT ± SEM. Student's *t* test. **, $P < 0.01$, estrogen *vs.* vehicle.

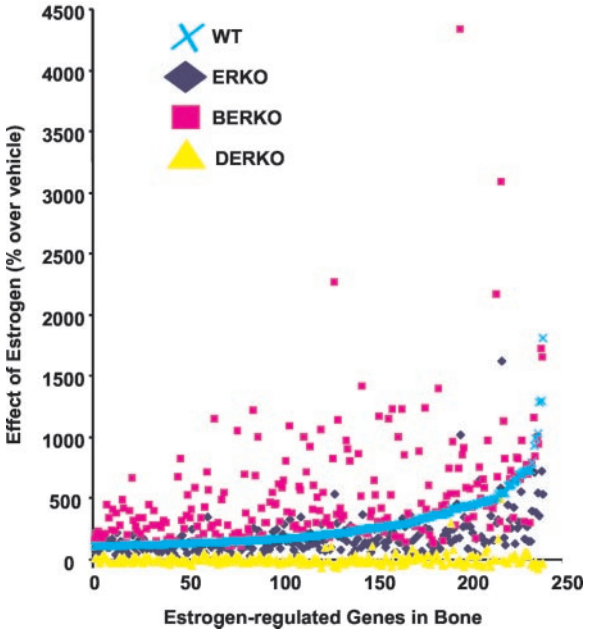


**Fig. 3.** ERβ Can Partially Replace ERα in Estrogen-Regulated Gene Transcription

Stimulatory effects of estrogen on estrogen-regulated gene transcription in bone of ovx WT, ERKO, BERKO, and DERKO mice as measured using microarray analysis. The percent increase over vehicle for each individual estrogen-regulated gene (n = 240) is given.

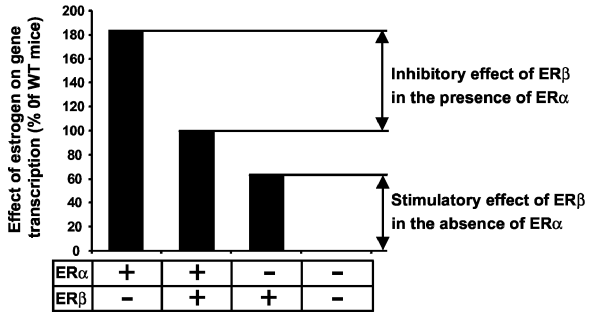


**Fig. 4.** ERβ inhibits ERα-Mediated Gene Transcription in the Presence of ERα, Whereas It Can Partially Replace ERα in the Absence of ERα

The figure is based on the effects of estrogen in bone of ovx WT, ERKO, BERKO, and DERKO mice. These effects were investigated by a statistical model using three-way ANOVA (gene, n = 240; ERα + or −, ERβ + or −) with gene as block effect, demonstrating that all four groups are significantly different from each other ($P < 0.00001$). The average stimulatory effect of estrogen on the 240 estrogen-regulated genes is given and expressed as percent of WT.

independent manner, but one cannot exclude a minor ERα activity remaining in these mice (10, 11). Alternatively, estrogen has been suggested to exert nongenomic actions via cell membrane receptors in a variety of cell types (12–14), and the effect might also be due to some low-affinity binding to other known nuclear receptors (14). Interestingly, the average stimulatory effect of estrogen in ERKO (176 ± 11% over vehicle) was intermediate between that seen in WT (275 ± 14% over vehicle) and DERKO (−7 ± 3% over vehicle) mice (Fig. 3).

Furthermore, the effects of estrogen in humerus of ovx WT, ERKO, BERKO, and DERKO mice were investigated by a statistical model using three-way ANOVA (gene, n = 240; ERα + or −, ERβ + or −) with gene as block effect, demonstrating that all these four groups are significantly different from each other ($P < 0.00001$). Similar results were also seen using three-way ANOVA applied on ranks within blocks, with gene as block effect ($P < 0.00001$). These data demonstrate that ERβ, in the absence of ERα, is able to partially mediate the effect of estrogen on gene transcription (Fig. 3). However, ERα is more efficient than ERβ in mediating the effect of estrogen on gene transcription. These analyses also demonstrated that there is a clear negative interaction between the effect of ERα and ERβ, which could be interpreted as meaning that ERβ significantly reduces the stimulatory effect of ERα (Figs. 3 and 4). Thus, ERβ inhibits ERα-mediated gene transcription in the presence of ERα, whereas it can partially replace ERα in the absence of ERα (Fig. 4).

It has previously been shown, both for the two progesterone receptor (PR) subtypes (PR-A and PR-B), and for the two glucocorticoid receptor (GR) subtypes (GRα and GRβ), that one of these two subtypes can act as a dominant negative inhibitor of the transcriptional activity of the other subtype [PR-A inhibits PR-B and GRβ inhibits GRα (15–17)]. Our present *in vivo* study together with previous *in vitro* studies suggest that ERα and ERβ provide yet another example of two nuclear receptor subtypes that demonstrate distinct transcriptional activities. The contrasting transcriptional activities of the different subtypes of PR, GR, and ER indicate that the relative tissue distributions of receptor subtypes are a major determinant of the biological effects of their respective ligands.

The increased estrogen-regulated gene transcription in BERKO mice compared with WT mice might be explained by the fact that ERβ is a transdominant repressor of ERα. An alternative, and more probable, explanation is a competition between two activators, ERα and ERβ, which exhibit different intrinsic activities. A competition between the more active ERα and the less active ERβ in WT mice results in a reduced gene activation compared with the situation in BERKO mice where only the more active ERα is present. The interesting finding that ERβ can reduce the transcriptional activity of ERα *in vivo* was, in the present study, obtained from two tissues, liver and bone, which both seem to be mainly ERα controlled. In other tissues, including prostate, lung, and ovary, it is clear that ERβ is a major regulator in its own right (8, 9, 18). Accordingly, we cannot extrapolate the present data to conclude, in general, that repression of ERα is the major physiological function of ERβ in all tissues.

In the present study, mice were treated for 3 wk with estradiol. Therefore, it is not clear which genes were directly transcriptionally regulated by estradiol and which genes were secondarily regulated by other genes regulated by estradiol. Furthermore, the microarray data do not distinguish between changes in transcription *vs.* changes in RNA processing or RNA stability. Thus, the present result should be interpreted as the net result of estrogen on mRNA levels after chronic estradiol treatment.

In conclusion, our data indicate that an important role of ERβ is to modulate ERα-mediated gene transcription in bone and liver, supporting a "Ying Yang" relationship between ERα and ERβ in mice.

## MATERIALS AND METHODS

### Animals

ER-inactivated mice were bred as previously described (19). Male double-heterozygous ($ER\alpha^{+/-}\beta^{+/-}$) mice were mated with female double-heterozygous ($ER\alpha^{+/-}\beta^{+/-}$) mice, resulting in $ER\alpha^{+/+}\beta^{+/+}$ (WT), $ER\alpha^{-/-}\beta^{+/+}$ (ERKO), $ER\alpha^{+/+}\beta^{-/-}$ (BERKO), and $ER\alpha^{-/-}\beta^{-/-}$ (DERKO) offspring (20–22). The diet, housing, and genetic background were as previously described (22). The mice were ovx at 2 months of age. Ovaries were removed after a flank incision and the incisions were closed with metallic clips. After recovery for 4 d, mice were injected sc with 2.3 μg/mouse/d of 17β-estradiol benzoate (Sigma, St. Louis, MO) for 5 d/wk during a 3-wk period. Control mice received injections of vehicle oil (olive oil, Apoteksbolaget, Gothenburg, Sweden). The study protocol was reviewed and approved by the ethical committee at the University of Gothenburg.

### DNA Microarray Analysis

RNA from humerus (n = 6: WT vehicle, DERKO estrogen; n = 7: WT estrogen; n = 8: BERKO vehicle, BERKO estrogen; n = 4: DERKO vehicle; n = 3: ERKO vehicle, ERKO estrogen) and liver (n = 6: WT vehicle, WT estrogen, BERKO vehicle, BERKO estrogen, DERKO vehicle and DERKO estrogen; n = 4: ERKO vehicle, ERKO estrogen) were prepared as described elsewhere (23). The RNA was further purified using RNeasy Kit (QIAGEN, Chatsworth, CA). For microarray assays the RNA samples were divided into two pools per animal group, whereas for the confirmatory real-time PCR analysis each individual animal was analyzed separately. The pooled RNA was reverse transcribed into cDNA, labeled, and analyzed by DNA microarray (MG-U74A Array; Affymetrix, Santa Clara, CA). The array represents approximately 5700 characterized mouse genes and approximately 3700 uncharacterized expressed sequence tags (ESTs) (in total, 9400). In the present article we do not distinguish between characterized mouse genes and uncharacterized ESTs. Preparation of labeled cRNA and hybridization was done according to the Affymetrix Gene Chip Expression Analysis manual.

### Bioinformatics

Scanned output files were analyzed using Affymetrix Micro Array Suite Version 4.0.1 software. To allow comparison of gene expression, both between groups and between tissues, the GeneChips were globally scaled to an average intensity of 500. The estrogen-regulated genes were determined by calculating the fold change in average between vehicle-treated and estrogen-treated samples. Comparisons were made between the two vehicle-treated and the two estrogen-treated GeneChips, generating a total of four comparisons. We defined very strict criteria for genes to be regarded as regulated; 1) the absolute call for the gene had to be present (*i.e*. regarded by the software to be expressed at a detectable level; Affymetrix Micro Array Suite Version 4.0.1) for all GeneChips; 2) at least three of the four comparisons had to be considered increased (I) or decreased (D) according to Affymetrix algorithms; 3) the average fold increase or decrease of the four comparisons should be at least 2.0-fold. Thus, we may have excluded some estrogen-regulated genes but this was done to avoid false positives among the regulated genes. The average coefficient of variation for the four microarray comparisons was 22.8% for the 240 estrogen-regulated genes in bone.

For determination of the ER specificity of the genes regulated by estrogen in WT mice, the same rules as above were applied for comparisons between vehicle and estrogen-treated ERKO, BERKO, and DERKO GeneChips.

### Real-Time PCR Analysis

The confirmatory real-time PCR analyses were, as described in the microarray section, run on each individual sample. The analyses were performed using the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Stockholm, Sweden) using probes labeled with the reporter fluorescent dye FAM. Predesigned primers and a probe labeled with the reporter fluorescent dye VIC, specific for 18S rRNA, were included in the reactions as an internal standard. The oligonucleotide primers and probes were purchased from PE Applied Biosystems. The cDNA was amplified at the following conditions: 1 cycle at 50 C for 2 min and 95 C for 10 min, followed by 40 cycles at 95 C for 15 sec and 60 C for 1 min. The mRNA amount of each gene was calculated using the Standard Curve Method (multiplex reaction, following the instructions in User Bulletin no. 2, PE Applied Biosystems) and adjusted for the expression of 18S rRNA.

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