Indirect Regulation of PTH by Estrogens May Require FGF23

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ABSTRACT

The mechanisms by which estrogens modulate PTH are controversial, including whether or not estrogen receptors (ERs) are present in the parathyroid glands. To explore these mechanisms, we combined a rat model of CKD with ovariectomy and exogenous administration of estrogens. We found that estrogen treatment significantly decreased PTH mRNA and serum levels. We did not observe ERα or ERβ mRNA or protein in the parathyroids, suggesting an indirect action of estrogens on PTH regulation. Estrogen treatment significantly decreased serum 1,25(OH)2 vitamin D3 and phosphorus levels. In addition, estrogens significantly increased fibroblast growth factor 23 (FGF23) mRNA and serum levels. In vitro, estrogens led to transcriptional and translational upregulation of FGF23 in osteoblast-like cells in a time- and concentration-dependent manner. These results suggest that estrogens regulate PTH indirectly, possibly through FGF23.


Estrogen deficiency is the main factor implicated in bone loss in postmenopausal osteoporosis.1 As a consequence of the lack of estrogens, bone turnover increases, leading to an imbalance between bone formation and bone resorption, favoring the latter.2,3 This imbalance affects calcium–phosphate metabolism and may increase serum parathyroid hormone (PTH) levels.4 Estrogen replacement therapy prevents bone loss and fractures,5,6 acting directly on bone cells through their specific estrogen receptors (ERs): α and β.7,8 In addition, in postmenopausal women, estrogens can also reduce PTH serum levels4,9 through an as of yet poorly understood mechanism.

A possible direct effect of estrogens reducing PTH acting through ERα and ERβ located in the parathyroid cells has been suggested, but the existence of ERα and ERβ in parathyroid tissue is still a controversial issue.10–11 Estrogens may also decrease PTH secretion by acting on other factors such as calcium,14,15 1,25(OH)2 D3 (calcitriol),15 and phosphorus,16,17 among others. Recently, fibroblast growth factor 23 (FGF23), involved in phosphorus and vitamin D metabolism,18 has been suggested to influence PTH synthesis and secretion.19

In women with chronic kidney disease (CKD), little is known about the role that estrogen deficiency plays in the pathogenesis and progression of bone disease.20,21 Understanding the mechanism through which estrogens act on PTH is also a subject of interest in these patients, because of the high prevalence of secondary parathyroid disorders.22 Because several aspects of the effects of estrogens on PTH remain unclear, the objective of this study was to investigate the factors and mechanisms involved in the likely effect of estrogens on the parathyroid gland.

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BASIC RESEARCH www.jasn.org
RESULTS

In Vivo Study
Renal Function, Estrogen Replacement, and Bone Mass.
Five different groups of rats were studied: CKD without ovariec-
tomy (OVX), CKD + OVX treated with placebo, CKD + OVX
treated with 17β-estradiol (E2) at doses of 15 and 45 ng/kg/d, and
a group of rats with normal renal function without OVX of the
same age. No differences in renal function (serum urea and creat-
ine) were observed among all groups with CKD (Table 1). As
expected, the placebo group showed significantly lower estrogen
serum levels, uterus weight (UW), and UW/body weight (BW)
ratio than the CKD-control group with no OVX. However, with
the administration of E2 (E2-15 and E2-45), significantly higher
values of UW and UW/BW were observed compared with the
placebo group (Table 1).

The placebo group showed significantly lower estrogen
levels (UW) and UW/body weight (BW) compared with the
placebo group (Table 1). As expected, the placebo group showed significantly lower estrogen
values of UW and UW/BW were observed compared with the
placebo group (Table 1).

Effect of Estrogens on PTH mRNA and Serum Levels.
The placebo group showed a significant increase in the serum
intact PTH (iPTH) levels compared with the CKD-control
group. The PTH increase was partially and totally blunted with
15 and 45 μg/kg body weight/d doses of E2, respectively (Figure
1A). Similar results were obtained at the transcriptional level
by quantitative real-time RT-PCR (qRT-PCR). A decreasing
trend in the PTH mRNA levels with E2 treatment was observed,
achieving similar values as the CKD-control group with the
high E2 dose (45 μg/kg body weight/d; Figure 1B).

Evaluation of the Likely Direct Effect of E2 on PTH Regulation:
Study of ERα and ERβ on Parathyroid Tissue.
To evaluate the putative direct effect of E2 on PTH regulation,
the presence of ERα and ERβ was analyzed by qRT-PCR in
each pool of parathyroid glands from the CKD-control, pla-
placebo, E2-15, and E2-45 groups. No signal for ERα and ERβ
was observed in any of the four studied groups.

To double check the absence of ERs on parathyroid tissue,
both ERα and ERβ were also assessed using parathyroid glands
extracted from normal rats (no CKD, no OVX) at mRNA and
protein levels. At the transcriptional level, three independent
qRT-PCR experiments and two independent RT-PCR ex-
periments showed no expression of either ERα or ERβ genes
in normal parathyroid tissue. As expected, ERα and ERβ
transcripts were observed in the uterus, used as a positive
control tissue in the qRT-PCR experiments (data not
shown) and also in the uterus and tibia used in RT-PCR
experiments (Figure 2A).

At the protein level, parathyroid glands did not show ERα
and ERβ expression as depicted in the Western blot and im-

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<th>Table 1. Serum biochemical markers and BMD in all groups at the end of the study</th>
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<td>Normal Group</td>
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<td>Creatinine (mg/dl)</td>
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<td>17 β-E2 (pg/ml)</td>
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<td>Proximal tibia BMD (g/cm²)</td>
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aP < 0.05 compared with the CKD-control group.
bP < 0.05 compared with the placebo group.
munohistochemistry analyses, despite that, in the latter, we applied a primary antibody concentration for ERα and ERβ 20 times higher than in the uterus tissue to the parathyroid glands. On the contrary, both receptors were highly expressed in the uterus (Figure 2B and C).

**Evaluation of the Putative Indirect Effect of E2 on PTH Regulation.**

To study the likely indirect mechanisms involved in the effect of E2 on PTH mRNA and serum levels, other serum biochemical parameters related to PTH regulation were studied.

E2 was able to significantly decrease 1,25(OH)2D3 levels in a dose-dependent manner, reaching lower values than the CKD-control group with both doses of E2 (Figure 3A). E2 also decreased serum phosphorus compared with the placebo group (Figure 3B), with a similar trend to that observed in the serum PTH results (Figure 1A).

Because of the known effect of FGF23 on phosphate metabolism and PTH function,18,19 serum FGF23 was also measured. FGF23 mRNA and serum levels decreased in the placebo group compared with the CKD-control group. Interestingly, rats treated with both E2 doses showed higher FGF23 mRNA and serum levels, achieving higher values than the CKD-control group (Figure 3C and D).

Because FGF23 requires Klotho as a coreceptor, the expression of this gene was measured in the pools of parathyroid glands and individual kidneys from all groups. An estrogen dose-dependent increase in Klotho mRNA levels in the parathyroid glands was observed (Figure 3E). However, no significant differences were found in kidney (data not shown).

In addition, FGF23 serum levels positively correlated with serum E2 levels ($r = 0.734$, $P = 0.001$) and negatively correlated with serum 1,25(OH)2D3 ($r = -0.791$, $P < 0.001$) in the three groups with CKD+OVX (Figure 4).

**In Vitro Study: Direct Effect of E2 on FGF23**

To confirm the finding that E2 might directly increase FGF23, the effect of E2 on FGF23 mRNA and protein levels was evaluated using UMR-106 osteoblasts.

E2 significantly increased FGF23 mRNA levels in a concentration- and time-dependent manner achieving the highest mRNA levels when cells were cultured for 48 h (Figure 5A).

To analyze FGF23 at the protein level, total extracts of proteins from cells cultured with E2 for 24 and 48 h were subjected to Western blot. A single band of 32 kDa corresponding to intact FGF23 was detected in all samples. In addition, FGF23 protein levels increased when both E2 concentration and time of culture increased, following the same pattern seen in qRT-PCR (Figure 5B).

![Figure 2. ERα and ERβ detection in parathyroid tissue. (A) Rat ERα (lanes 2, 3, and 4) and ERβ (lanes 5, 6, and 7) RT-PCR in different tissues resolved on agarose gel electrophoresis. Molecular weight markers (MWM; pUC18/HaeIII) (lane 1). (B) Western blot analysis of ERα and ERβ proteins in parathyroid glands and uterus. Anti-GAPDH was used as a loading control. (C) Immunohistochemical staining of the ERα and ERβ in parathyroid glands and uterus. Antibodies dilution: 1:50 for parathyroid tissue and 1:1000 for uterus). Hematoxylin counterstaining (magnification: ×20).](image-url)
DISCUSSION

Estrogen treatment prevents bone loss in postmenopausal women by a direct action on bone cells, but it might act also indirectly influencing the synthesis and secretion of calcitropic hormones, such as PTH, inhibiting PTH-dependent bone resorption. The effect of estrogens on PTH could be direct, acting on ERα and ERβ in the parathyroids; or indirect, influencing calcium, phosphorus, or other mediators, and then secondarily, reducing PTH levels. In this study, we showed that the parathyroid tissue does not express both ERα and ERβ and that FGF23 is upregulated by estrogens. As a result, we suggest that estrogens would not act on PTH directly but likely indirectly by a mechanism which may involve FGF23.

In our study, estrogen deprivation in CKD rats showed, as expected, a significant decrease in UW/BW ratio and serum estrogen levels, together with a significant decrease in BMD in the most trabecular area of the tibia, caused by the increased bone resorption from estrogen deprivation. To achieve a hormonal replacement equivalent to that used...
in postmenopausal women, two different estrogen concentrations were tested. Both doses were able to significantly increase the UW/BW ratio and the estrogen serum levels compared with the untreated group (Table 1). However, only the high estrogen dose completely reversed the estrogen insufficiency and totally avoided the loss of BMD (Table 1). Estrogen treatment was able not only to prevent BMD loss but also to avoid the increase in PTH mRNA and serum levels compared with the placebo group, achieving similar values to the CKD control group with no OVX (Figure 1). The latter is still a controversial issue, because previous experimental studies have shown contradictory results related to the effect of estrogens on PTH, likely explained by methodological differences such as renal function and the age of the rats.

The mechanisms by which estrogens may induce a reduction in PTH synthesis and secretion are still not well understood. If the parathyroid cells would possess ERs, estrogens might directly influence PTH; however, this aspect is still controversial. Some authors have described the lack of ERs in parathyroid glands, and others have reported that parathyroid glands are target organs for estrogens, showing that estrogens increased PTH gene expression. It is not easy to explain these controversial results. However, the different experimental approaches, doses of estrogens tested, periods of treatment, and procedures used for gland removal (parathyroidectomy or thyroparathyroidectomy) may explain these conflicting results.

In this study, the presence of ERα and ERβ in parathyroid tissue from the different groups studied, including normal rats, was tested by four different experimental approaches. Despite this careful and meticulous research, the ERs were not found in parathyroid tissue, even when using a primary antibody concentration 20 times higher in parathyroids than in uterus in the immunohistochemistry analysis (Figure 2).

This negative finding supports the hypothesis that the parathyroid glands do not express ERs, and thus estrogens cannot reduce PTH by a direct mechanism. Other receptors, such as the retinoid X receptor, might play a role in the effect of estrogens on different genes, but the definitive estrogen signaling pathways seem necessarily to involve the ERs.

Another possible factor involved in PTH regulation by estrogens could be calcitriol, but there is no clear evidence to support this hypothesis. Some experimental data suggest that estrogens do not influence calcitriol levels; meanwhile, others have shown that estrogens might modulate vitamin D receptor expression and decrease serum calcitriol levels. In our study, a significant dose-dependent decrease of serum calcitriol and PTH levels was observed with the use of estrogens (Figures 1A and B and 3A); this decrease was strikingly higher in the case of calcitriol. The reduction in serum calcitriol levels should have been accompanied by higher PTH levels unless a third player, in this case estrogens, was interfering in calcitriol–PTH regulation, decreasing both calcitriol and PTH.

Another mechanism by which estrogens can influence PTH levels is decreasing serum phosphorus levels. Several works have described that estrogens can downregulate the kidney sodium–phosphate cotransporter (Na–Pi), increasing phosphorus in urine and causing hypophosphatemia. In agreement with this view, we found that estrogen administration significantly decreased serum phosphorus levels to values similar to the CKD-control group (Figure 3B). Because phospho-
rus is well known to increase the synthesis and secretion of PTH, the reduction of phosphorus, secondary to the use of estrogens, could have been at least partly responsible for the reduction in PTH levels.

Finally, another possible factor linking the changes observed in phosphorus and PTH is FGF23, which has been identified as one of the most potent phosphatonin able to increase urinary phosphorus by inhibiting Na-Pi-dependent phosphate reabsorption in the proximal tubule. In addition, FGF23 also inhibits 1α-hydroxylase, leading to a decrease in calcitriol, and it can act directly on the mitogen activating protein kinase (MAPK) pathway of the parathyroid gland, leading to a decrease in PTH synthesis and secretion.

In our study, all of the previously described findings were present. The estrogen-treated rats showed significantly higher serum and bone FGF23 values and significantly lower serum calcitriol, phosphorus, and PTH values, the latter also confirmed by qRT-PCR.

Because the whole set of results strongly suggested that the estrogen effect on PTH may be at least partly driven by FGF23, and FGF23 requires Klotho as a coreceptor to suppress PTH expression and secretion, we also measured Klotho gene expression in parathyroid glands. A dose-dependent increase in Klotho mRNA levels was observed in the parathyroid glands from rats treated with estrogens, likely caused by the stimulatory effect of FGF23 on Klotho expression. However, previous findings suggest estrogens potentially suppress Klotho expression in estrogen target organs. The fact that estrogens did not suppress Klotho together with the finding of the lack of ERs in the parathyroid glands are in keeping with our findings that the parathyroid glands are not a direct target tissue for estrogens. No changes in kidney Klotho mRNA levels were found despite that the kidney is a target organ for estrogens. It may be speculated that the high levels of FGF23 may counterbalance or mask the estrogen effect in the kidney.

To further study the direct effect of estrogens on FGF23 metabolism, we performed in vitro experiments using osteoblast-like cells. The results showed that estrogens, in the presence of a constant concentration of phosphorus, increase FGF23 levels in a concentration- and time-dependent manner, measured at transcriptional and translational levels (Figure 5A and B). The mechanism by which estrogens signaling stimulate FGF23 is unknown. According to the classical estrogen signaling pathway, the nuclear ERs may bind putative estrogen response elements (EREs) in the gene promoter, acting as transcription factors; however, further studies are needed to fully understand the mechanisms by which estrogens may upregulate FGF23.

Therefore, taking all our experiments together, we postulate that PTH regulation by estrogens is mainly indirect, and FGF23 (a new factor never described as part of this axis until now) may be a candidate for potential factors linking estrogens and PTH. However, further studies are needed to confirm whether FGF23 stimulated by estrogens directly suppresses parathyroid function.

CONCISE METHODS

In Vivo Study: Animals, Drugs, and Experimental Design

Six-month-old female sexually mature Sprague-Dawley rats with a mean BW at the beginning of study of 325 ± 32 g (n = 24) were used. The animals were fed with a standard rodent chow containing 0.6% calcium and 0.6% phosphorus (Panlab, Barcelona, Spain) and housed in wire cages. Water and food administration was ad libitum.

E2 (Innovative Research of America, Sarasota, FL) was dissolved in ethanol and diluted with corn oil to a final volume of 0.8 ml corn oil/kg body weight/injection. The final doses of E2 administered to rats were 15 and 45 μg/kg body weight/d. This treatment was administered intraperitoneally 5 d/wk for 8 wk. Placebo (0.8 ml corn oil/kg body weight/d) was administered following the same procedure.

CKD was surgically induced using the modified technique by Ormrod and Miller (equivalent to 7/8 nephrectomy). Estrogen deprivation was surgically induced performing bilateral O VX. Both procedures were done in the same intervention using 42 mg/kg of intraperitoneally ketamine (Ketolar; Warner Lambert) and 0.16 mg/kg of medetomidine (Dontor; Orion, Espoo, Finland) as anesthetics. One week after surgery, a total of 20 animals with CKD + O VX were divided into three experimental groups. Group 1 (E2-15, n = 8) received intraperitoneal E2 (15 μg/kg body weight/d). Group 2 (E2-45, n = 5) received intraperitoneal E2 (45 μg/kg body weight/d). Group 3 (placebo, n = 7) received vehicle (corn oil at 0.8 ml/kg body weight/d) administered through intraperitoneal injections as described. A fourth group with CKD (same procedure) and no O VX was used as the CKD-control group (n = 4). A group of rats (n = 5) with normal renal function without O VX (normal group) was also included in the study.

After 8 wk of treatment, all rats were killed by exsanguination. Blood samples, tibias, uteri, parathyroid glands, and kidneys were removed and stored frozen at −80 °C until analysis. Blood samples were drawn for serum analyses, the right tibia was removed to perform BMD analyses, and uteri were collected to be weighed and used as a tissue marker of estrogen replacement. Because of the small size of each individual gland and to obtain enough total RNA for quantitation, the parathyroid glands from each group studied were pooled (8, 14, 16, 10, and 10 glands were pooled from the CKD-control, placebo, E2-15, E2-45, and normal groups, respectively), and each individual’s left tibia and kidney were used to extract total RNA. Parathyroid glands, left tibia, and uteri from normal rats were used both to extract total RNA and proteins. In addition, parathyroid glands and uteri were also embedded in paraffin. The protocol was approved by the Laboratory Animal Ethics Committee of Oviedo University.

In Vitro Study

To study the in vitro regulation of FGF23 by E2, a rat osteosarcoma cell line UMR-106 (Health Protection Agency Culture Collections, Salisbury, UK) was used. UMR-106 cells were grown in phenol red–free αMEM (Sigma-Aldrich, St. Louis, MO) containing 1 mM phosphorus with 10% charcoal-stripped FBS (Sigma-Aldrich), 100 U/ml penicillin, and 100 μg/ml streptomycin-sulfate (Biochrom, Berlin, Germany) at 37 °C in a humidified atmosphere with 5% CO2. Cells were
grown to subconfluence and were cultured in phenol red–free αMEM containing 0.25% (wt/vol) BSA (culture medium) for 24 h. At the end of this adaptation period, cells were exposed to vehicle (ethanol) or E$_2$ at $10^{-10}$, $10^{-8}$, or $10^{-6}$ M concentrations for 24 or 48 h in culture medium. After the period of exposure, cells were collected to extract total RNA and proteins to measure FGF23.

### Analytical and Technical Procedures

#### Serum Markers and BMD Analysis

Serum urea, creatinine, calcium, and phosphorus levels were measured using a multichannel autoanalyzer (Hitachi 717; Boehringer Mannheim, Berlin, Germany), serum E$_2$ levels were measured by RIA (Diagnostic Systems Laboratories, Webster, TX), serum iPTH by IRMA (Rat PTH kit Immunotopics, San Juan Capistrano, CA), serum 1,25(OH)$_2$D$_3$ by RIA (IDS, Boldon, Tyne & Wear, UK), and serum FGF23 with a sandwich ELISA kit (Kainos Laboratories, Tokyo, Japan), following the manufacturer’s protocol in all cases.

BMD was measured at the proximal one eighth of the right tibia using dual-energy x-ray absorptiometry (QDR-100; Hologic, Bedford, MA) with software specifically prepared and adapted to small animals.

RNA Extraction, cDNA Synthesis, and Quantitative Real-Time RT-PCR.

Total RNA extraction was performed by the method of Chomczynski. Total RNA concentration and purity were quantified by spectrophotometry UV-Vis (NanoDrop Technologies, Wilmington, DE), measuring the absorbance at 260 and 280 nm. RNA integrity was corroborated using formaldehyde/agarose gels. All RNA samples were stored in RNase-free tubes at $-80$ °C until analysis.

RT-PCR to synthesize cDNA was performed from 1 µg of total RNA previously extracted from parathyroid, tibia, uterus, kidney, and cell culture samples using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) following the manufacturer’s instructions. The cDNAs obtained were stored at $-20$ °C until required for analysis.

qRT-PCR was performed in the in vivo and in vitro studies on an ABI Prism 7000 Sequence Detection System using TaqMan Universal PCR Master Mix (Applied Biosystems). PTH, ER$_{α}$, ER$_{β}$, FGF23, and Klotho genes and rRNA 18s as endogenous control were analyzed using TaqMan pre-Developed assay reagents (TaqMan Gene Expression Assays-On-Demand; Applied Biosystems). All reactions were performed in triplicate, amplifying endogenous and target genes in the same plate. Relative quantitative evaluation of target genes was performed by comparing threshold cycles using $ΔΔC_{T}$ method, as described previously.

Detection by RT-PCR of ER$_{α}$ and ER$_{β}$.

Because of the controversy related to the existence of ER$_{α}$ and ER$_{β}$ in parathyroid glands, the presence of mRNA corresponding to the receptors in parathyroid tissue was also analyzed amplifying cDNA from normal rats with specific oligonucleotides for ER$_{α}$ (forward: 5’-GCA CAA GGC TCA GAG AGA TG-3’; reverse: 5’-GCA CTC TCT TTG CCC AGT TG-3’) and ER _β_ (forward: 5’-GGT GTG GTG ACC GTA TAG TG-3’; reverse: 5’-ATG ATG TGC ACC AGT TCC TTG-3’). The cDNA from normal tibia was used as positive control.

**Protein Extraction and Western Blot Analysis.**

To enrich the nuclear protein fraction to test the presence of ER$_{α}$ and ER$_{β}$, total proteins from a pool of 10 parathyroid glands and uteri (used as a positive control) from normal rats were extracted using a high-salts buffer containing 500 mM NaCl, 50 mM HEPES (pH 7.0), and 1× Protease Inhibitor Cocktail (Complete Mini; Roche Diagnostics, Mannheim, Germany).

To analyze the *in vitro* effect of E$_2$ on FGF23 protein, total proteins from UMR-106 cells exposed for 24 or 48 h to different E$_2$ concentrations were extracted using a standard RIPA buffer with protease inhibitors.

All samples of proteins were quantified by Bradford’s method (Bio-Rad, Hercules, CA).

For the study of ER$_{α}$ proteins, aliquots of 20 µg of protein from the parathyroids and uterus were electrophoresed on SDS-PAGE mini-gels and transferred to a Hybond P membrane (GE Healthcare UK, Buckinghamshire, UK) following standard protocols. ER$_{α}$ and ER$_{β}$ proteins were detected with a mouse anti-ER$_{α}$ monoclonal IgG1 antibody (dilution 1:1000; Acris Antibodies, Hidenhausen, Germany) and a rabbit anti-ER$_{β}$ polyclonal IgG antibody (dilution 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), respectively. Rabbit anti-GAPDH polyclonal antibody (dilution 1:25,000; Santa Cruz Biotechnology) was used as a loading control.

For the *in vitro* FGF23 protein assay, three independent experiments were performed using aliquots containing 30 µg of total proteins from E$_2$-treated UMR-106 cells. Total proteins were loaded on SDS-PAGE according to the same protocol described for the study of ERs with Western blot. FGF23 protein was detected using a goat anti-FGF23 polyclonal antibody (dilution 1:100; Santa Cruz Biotechnology) and rabbit anti-GAPDH polyclonal antibody (1:20,000; Santa Cruz Biotechnology) was used as a load control.

In both cases, chromogenic detection was performed with Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL). For relative Western blot quantification, Quantity One 1-D Analysis Software v4 (Bio-Rad) and a GS-800 Calibrated Densitometer (Bio-Rad) were used.

**Immunohistochemistry.**

The presence of ER$_{α}$ and ER$_{β}$ in parathyroid tissue was also determined by immunohistochemistry in 5-µm-thick serial sections from paraffin-embedded parathyroid glands and uteri from normal rats using the same specific antibodies used for Western blot and hematoxylin counterstaining (Dako REAL EnVision; Dako, Carpinteria, CA) following the manufacturer’s instructions. For ER detection in uterus tissue, a dilution of 1:1000 of both antibodies was used; however, to increase the sensitivity of the detection of ER$_{α}$ and ER$_{β}$ in parathyroid tissue, the dilution of both antibodies was 1:50.

**Statistical Analysis**

Biochemical markers, UW, BW, proximal tibia BMD, qRT-PCR, and Western blot quantitation were statistically analyzed using t test. Correlations between serum FGF23, serum E$_2$, and 1,25(OH)$_2$D$_3$ were performed using the Pearson correlation coefficient ($r$).

The results are expressed as mean ± SD. Differences were considered significant when $P < 0.05$. All statistical analyses were performed using SPSS 12.0 for Windows (SPSS, Chicago, IL).
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DISCLOSURES
None.

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