Biochemical and Biophysical Research Communications **268,** 60–64 (2000)
doi:10.1006/bbrc.1999.2063, available online at http://www.idealibrary.com on IDEAL®

Effect of Interleukin-1β on Aromatase Activity and Cell Proliferation in Human Osteoblast-like Cells (HOS)

Miki Morioka,[1] Kazuhisa Shimodaira, Yuzuru Kuwano, Hiroshi Fujikawa, Hiroshi Saito, and Takumi Yanaihara

Department of Obstetrics and Gynecology, Showa University School of Medicine, 1-5-8, Hatanodai, Shinagawa-ku, Tokyo, 142-8666, Japan

Received December 14, 1999

Osteoblast cells have a capacity to produce estrogen from androgen. It is known that inflammatory cytokines in bone increase during estrogen deficiency. In the present study, the effect of interleukin-1β (IL-1β) on aromatase (Arom) activity in human osteoblast-like cells (HOS) was investigated. We also investigated the effect of IL-1β and estradiol (E2) on cell proliferation in HOS. [^{3}H] water method was employed to measure Arom activity. Expression of Arom mRNA was determined by the reverse-transcription polymerase chain reaction (RT-PCR) method. The PCR products were confirmed by Southern blot analysis. Cell proliferation was measured by an ELISA–bromo deoxyuridine (BrdU) kit. Addition of IL-1β increased Arom activity in a dose-dependent manner and addition of IL-1β (10 ng/ml) resulted in 40% greater activity than control. Addition of 500 ng/ml of human recombinant IL-1 receptor antagonist neutralized the increased Arom activity to control level. Stimulation of Arom mRNA expression by IL-1β was also found. IL-1β and E2 stimulate osteoblastic cell proliferation significantly. These findings suggest for the first time that IL-1β stimulates Arom activity through the IL-1 receptor and also cell proliferation in osteoblast-like cells. It is also demonstrated that this stimulatory effect may be through the IL-1 receptor. Cell proliferation stimulated by IL-1β was reduced by the addition of the Arom inhibitor fadrozole–HCL (CGS-16949A). These results imply that IL-1β has a stimulatory effect on estrogen formation and sequentially cell proliferation in bone, and this mechanism may play an important role in osteoblastic function especially in postmenopausal women.

A number of studies have shown that estrogen deficiency is one of the main risk factors for osteoporosis in postmenopausal women. Pacifici and coworkers (1) found that the stimulated release of interleukin 1β (IL-1β) from human peripheral blood monocytes increased in the first decade after menopause and became normal after treatment with estrogen. IL-1 receptor antagonist (IL-1 ra) blocks bone loss in ovariectomized rats through the inhibition of bone resorption (2). Ralston (3) reported that gene expression of inflammatory cytokines including IL-1α, IL-1β, tumor necrosis factor-α (TNF-α) and IL-6 were significantly greater in the bone samples obtained from the postmenopausal women with osteoporotic fructures than in from postmenopausal women with normal bone density or who received estrogen replacement therapy. These findings indicated the possibility that local release of these cytokines contributes to bone loss in estrogen deficiency conditions. It is known that IL-1 and IL-6 are osteoclast-activating-factors and the studies have been reported on bone resorbing effects of these cytokines in murine and rat models (4–6). Though IL-1β is a potent stimulator for bone resorption, stimulates the activity of mature osteoclasts and plays an important role on the pathogenesis of postmenopausal osteoporosis, the effect of IL-1β on osteoblast cells has not been fully understood (6).

Although it has been known that estrogen deficiency-induced bone loss results in excessive bone resorption relative to bone formation, cellular mechanisms involving a characteristic osteoblastic function in estrogen deficiency have not been elucidated. Since estrogen and androgen receptors are found in osteoblast cells, sex steroids are thought to have a positive effect on osteoblastic function. Recently, osteoblast cells have been shown to have steroid-converting enzymes, namely steroid sulphatase (7, 8), 5α-reductase (9, 10), 17β-hydroxysteroid dehydrogenase (7, 9), 3β-hydroxysteroid dehydrogenase (11) and aromatase (Arom) (7, 10, 12). Thus the local conversion of steroids in osteoblast cells might modulate bioactivities of sex steroids as well as cellular functions.

[1] To whom correspondence should be addressed. Fax: +81-33784-8355.

0006-291X/00 $35.00

Arom, a cytochrome P450 enzyme system that converts androgens to estrogens, is preasent in a number of tissues (13–18). The effect of IL-1β on Arom activity has been reported in some cell types and tissues, including placental cytotrophoblast (19), breast cancer (20). Therefore, the effect of IL-1β on Arom activity in osteoblast cells and the effect of IL-1β and estrogen on proliferation in human osteoblast-like cells were studied.

MATERIALS AND METHODS

Materials. Fetal bovine serum (FBS) and Eagle's minimal essential medium (MEM) were purchased from GIBCO (Grand Island, NY). [1β-^{3}H]androstenedione (A) (SA = 2.2 GBq/mmol) was purchased from New England Nuclear Corp. (Boston, MA). Non-labeled steroids were purchased from SIGMA Chemical Co. (St. Louis, MO). Anti-human IL-1 receptor antagonist (IL-1 ra) was purchased from Pepro Tech EC LTD (London, UK). IL-1β was a a generous gift from Otsuka Pharmaceutical Corp. Ltd. (Tokyo, Japan).

Fadrozole–HCL (CGS-16949A) was provided by NOVARTIS Pharmacy (Basel, Switzerland). All other chemicals were of reagent grade and obtained commercially.

Cell culture. Human osteoblast-like cell line HOS (American Type Culture Collection, Rockville, MD) was used in this study. Cells were cultured in 10-cm-diameter tissue culture dishes at 37°C in 5% CO_2–95% air. The medium was MEM with 10% (v/v) FBS, penicillin (0.1 unit/ml) and streptomycin (100 μg/ml).

For the determination of messenger ribonucleic acid (mRNA) of Arom, medium was removed and cells were kept frozen at −80°C until analysis.

Measurement of Arom activity. When cells reached 80% of confluence, medium was changed to phenol red free and FBS free MEM, and cells were cultured for a further 24 h. The medium was changed and the cells were incubated with IL-1β and/or hrIL-1ra for an additional 24 h. [1β-^{3}H]-A (25 nM) was added to culture media as a substrate and Arom activity was measured by [^{3}H] water assay for an additional 24 h culture (10). The medium was collected and steroids were removed by 10% dextran coated charcoal. The tritium radioactivity was measured by a liquid scintilation counter (Aloca LSC-652, Tokyo).

Determination of Arom mRNA levels. Three ml of Isogen (Nippon gene Co., Kanazawa, Japan) were added to the frozen culture dish and the total RNA was extracted by the method of Chomcynski and Sacci (21). We used the method of hemi-nested RT-PCR and synthesized Arom c-DNA using an RNA-PCR kit (Perkin–Elmer, Branchburg, NJ) (11). Aliquots of total RNA (1 μl) were added to a reverse-transcriptase reaction mixture (10 μl) resulting in final concentrations of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$ and deoxyribonucleotide 5′-triphosphate (each at 1 mM). To this mixture were added 1 IU/μl RNase inhibitor, 16 IU/μl oligo d(T) and 2.5 IU/μl Murine Leukaemia Virus reverse transcriptase to give a final volume of 20 μl at room temperature and the mixture was incubated at 42°C for 15 min, 99°C for 5 min and 5°C for 5 min. PCR procedure and product analysis were performed as previously described (11). One-microliter aliquots of the cDNA were added to the PCR mixture resulting in a final concentration of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 0.001% (w/v) gelatin, 0.25 μl of each dNTP, 0.25 mM of each primer and 1.25 IU of *Taq* polymerase in a total volume of 25 μl. The sequence of sense and antisense primers to amplyfy the open reading frame of cDNA were 5′-AGGTCAAGGAACACAAGATGG-3′ and 5′-TAGTAGTGCAGGCACTGCC-3′ (outer) and 5′-CCGAATCGAGAGCTGTAATGA-3′ (inner), respectively. Samples were covered with mineral oil and subjected to 30 cycles of PCR. One PCR cycle consisting of denaturation at 94°C for 30 s, annealing at 59°C for 30 s and polymerization at 72°C for 2 min. The expected size of the PCR products for Arom are 247 bp. PCR products were subjected to electrophoresis on a 2.5% agarose gel in 0.5× Tris borate buffer, visualized by staining with ethidium bromide (0.5 μg/ml gel) and photographed using an ultraviolet transilluminator. We used the human term placenta for positive control and same cells without reverse-transcription steps for negative control. The Southern hybridization procedure was performed to confirm the PCR products. The RT-PCR products on agarose gel were transfered to a blotting membrane (Hybond-N, Amersham, UK) by capillary elution. The membranes were backed, prehybridized and hybridized with the 5′-digoxigenin labeled oligonucleotide probe. The sequence of the probe used was 5′-TTTGGAAATCGTGAACCCGATACATTATAACAT-3′.

The blotting membranes were blocked using a DIG DNA Labeling and Detection Kit (Beohringer Mannheim Corp., Indianapolis, IN).

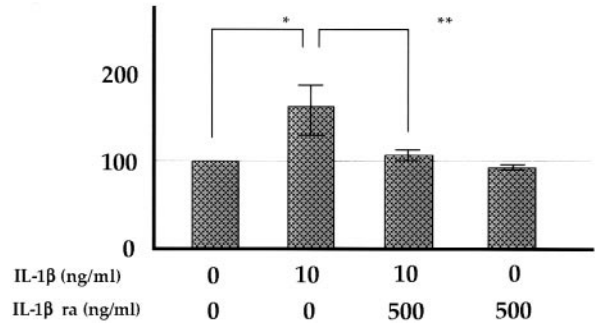

FIG. 1. Effect of IL-1β and hrIL-1ra on the aromatase activity in HOS. [1β-^{3}H]-A (25 nM) was added into culture media as a substate and Arom activity was measured by [^{3}H] water assay for 24 h culture. Results are expressed as a percent of the control which was set to 100%. Values represent the mean ± SD of 3 experiments. *P < 0.05, **P < 0.01. IL-1ra, IL-1 receptor antagonist.

Measurement of cell proliferation. Cell proliferation was measured by a Bromodeoxyuridine (BrdU) ELISA kit (Boehringer-Mannheim Corp., Indianapolis, IN). When cells reached 80% of confluence, HOS was plated at a concentration of 5000 cells/well in phenol red free and FBS free MEM with estradiol or IL-1β in 96-hole tissue culture dishes. And the cells were incubated for 24 to 72 h. The fluorescence obtained in control wells was set to 100% and effects of IL-1β and E2 on cell proliferation were compared to this value. In addition, fadrozole HCL (1 nM), an Arom inhibitor, was added to investigate its effects on both Arom and cell proliferation.

Statistics. Analysis of variance (ANOVA) was used for significant differences between groups. A P value <0.05 was considered to indicate significance.

RESULTS

Effect of IL-1β and hrIL-1ra on the Arom activity in HOS. The effect of IL-1β and hrIL-1ra on Arom activity in HOS was shown in Fig. 1. When IL-1β at the concentration of 10 ng/ml was added into the medium, the aromatase activity was enhanced 40% compared to the control in which none of these substances was added. On the other hand, the presence of hrIL-1ra 500 ng/ml neutralized the increased Arom activity to the control level.

Effect of IL-1β on the expression of Arom mRNA in HOS. Arom mRNA in HOS was detected by RT-PCR. We used the human placenta as a positive control and

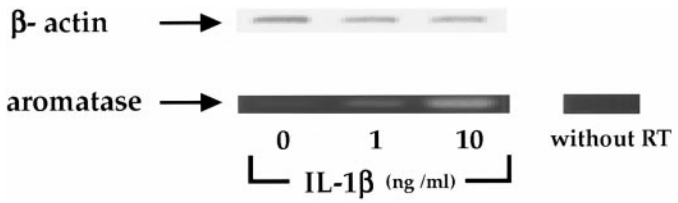

FIG. 2. Induction of $P450_{Arom}$ gene expression by IL-1β in HOS. Arom mRNA in HOS was detected by RT-PCR. The PCR products were confirmed by Southern blot analysis. Cells were incubated with 0–10 ng/ml IL-1β. The effect of IL-1β on the expression of Arom mRNA was evaluated.

the mRNA in HOS without reverse transcription steps as a negative control. The PCR products were confirmed by Southern blot analysis.

The effect of IL-1β on expression of Arom mRNA was evaluated. The expression of Arom mRNA was increased by the treatment of IL-1β in a dose dependent fashion (Fig. 2).

Effect of estrogen and IL-1β on cell proliferation in HOS. The addition of 1 nM E2 into the medium significantly ($P < 0.05$) enhanced cell proliferation. However, the addition of 30 nM E2 showed no significant effect on cell proliferation. When 50 ng/ml IL-1β was added to the medium, BrdU incorporation into osteoblastic cells increased significantly ($P < 0.01$) compared to control (Fig. 3).

Effect of IL-1β and fadrozole HCL on cell proliferation in HOS. The effect of IL-1β and fadrozole–HCL on cell proliferation in HOS was shown in Fig. 4. The

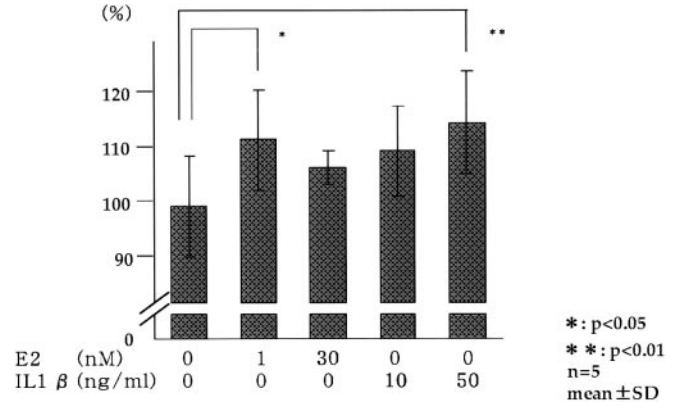

FIG. 3. Effect of estrogen and IL-1β on cell proliferation in HOS. Cell proliferation was measured by bromo-deoxyuridine (BrdU) method. When cells reached 80% of confluence, HOS was plated at a concentration of 5000 cells/well in phenol red free and FBS free MEM with E2 or IL-1β in 96-hole tissue culture dishes. The cells were incubated for 24 to 72 h. The fluorescence obtained in control wells was set to 100% and effects of IL-1β and E2 on cell proliferation were compared to this value. The addition of 1 nM E2 into the medium significantly ($P < 0.05$) enhanced the fluorescence. When 50 ng/ml IL-1β was added into the medium, the fluorescence significantly ($P < 0.01$) increased.

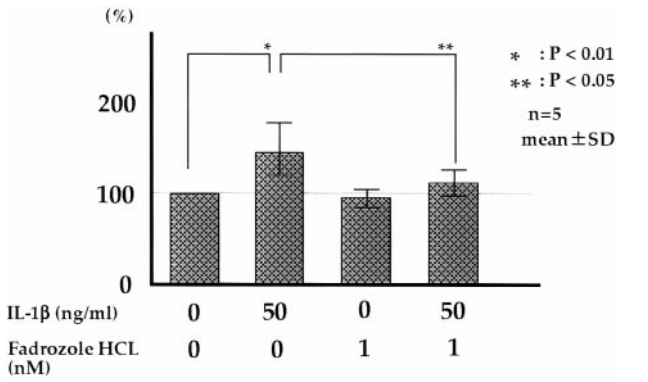

FIG. 4. Effect of IL-1β and Fadrozole HCl (CGS-16949A) on cell proliferation in HOS. Cell proliferation was measured by bromodeoxyuridine (BrdU) method. When cells reached 80% of confluence, HOS was plated at a concentration of 5000 cells/well in phenol red free and FBS free MEM with IL-1β and/or fadrozole–HCl in 96-hole tissue culture dishes. The cells were incubated for 24 h. The fluorescence obtained in control wells was set to 100% and effects of IL-1β and/or fadrozole–HCl on cell proliferation were compared to this value. When 50 ng/ml IL-1β was added into the medium, the fluorescence was significantly ($P < 0.01$) increased. The presence of 1 nM fadrozole–HCl neutralized the stimulation of IL-1β to the control level.

fluorescence obtained in control wells was set to 100% and effects of IL-1β and/or fadrozole–HCL on cell proliferation were compared to this value.

When 50 ng/ml IL-1β was added into the medium, the fluorescence was significantly ($P < 0.01$) increased.

The presence of fadrozole–HCL (1 nM) neutralized the stimulation of IL-1β to the control level (Fig. 4).

DISCUSSION

It is known that bone loss related to aging is caused by diminished osteoblast proliferation and differentiation due to estrogen deficiency. In contrast, it was reported that estrogen deficiency increases osteoblast proliferation without altering cell differentiation in rats. In postmenopausal women, an increase in bone formation was associated with increased proliferation of osteoblastic cells with no change in cell differentiation (22). Marie (23) reviewed the discrepancy of intracellular mechanisms between age-related and estrogen deficiency-induced bone loss. The author speculated about the involvement of local growth factors such as IGF-I and TGF-β in this mechanism. Estrogen replacement therapy together with androgen markedly increased markers for bone formation when compared with estrogen replacement alone in postmenopausal women (24). However, intracellular mechanisms of sex steroid actions in osteoblastic function are not fully understood. Osteoblast cells possesses the capacity to convert circulating androgen to estrogen as well as to more biologically potent androgen such as 5 α-reduced androgen (10, 13). It is known that estrogen levels

dramatically decrease while androgen levels gradually decreased in postmenopausal women (25).

Nawata *et al.* (12) reported that dexamethasone stimulated Arom activity as well as the expression of Arom mRNA in isolated human osteoblast cells. Recently the Arom gene was cloned and the regulations of tissue-specific expression of this gene has been investigated. It was found that expression of the Arom is regulated by a variety of tissue-specific promoters with alternative splicing mechanism. Transcription of Arom gene is induced by glucocorticoid. Shouzu *et al.* (26) report that IL-1β is one of the most potent stimulators of Arom expression in osteoblast-like cells obtained from the human fetus and the presence of dexamethasone is necessary for the induction of Arom by this cytokine. They reported that exon 1.4-specific transcripts are predominant under this circumstance, with exon 1.3- and exon 1.6-specific transcripts occurring to a much lesser extent.

In the present study, direct stimulatory effect of IL-1β on Arom activity and Arom mRNA expression was demonstrated for the first time in HOS. Because of the small amaunt of protein, Northern/Western blot analysis would not be obtained. IL-1β significantly enhanced the Arom activity and the levels of Arom mRNA expression in a dose dependent manner without addition of dexamethasone. The stimulated expression of Arom was neutralized by addition of 500 ng/ml of human recombinant IL-1 receptor antagonist (hrIL-1ra) to the control level. This result implies that IL-1β stimulates Arom activity through the IL-1 receptor.

The effects of estrogen and IL-1β on cell proliferation in osteoblast cells are still controversial. Robinson *et al.* (27) demonstrated that during the stage of rapid cell proliferation, estradiol treatment of human fetal osteoblastic cells resulted in a dose dependent decrease in [^{3}H]thymidine incorporation. Ernst *et al.* (28) reported that 1.0 nM of estrogen showed the greatest effect on the proliferation of rat osteoblast-like cells while the addition of 10 nM E2 showed no effect. The discrepancies may, at least in part, be the result of how closely each of these models reflects estrogenic action on bone *in vivo* and *in vitro* (29). In the present study, using human osteoblastic cells, a similar effect of estrogen on cell proliferation was observed. In addition, inflammatory cytokines have pronounced effects on osteoblastic function and proliferation. Recently, Frost *et al.* (30) reported that IL-1β and TNF-α enhanced the proliferation of human osteoblast time- and dose-dependently in primary cell culture. In the present study, we measured the cell proliferation by BrdU incorporation and found for the first time that 50 ng/ml IL-1β significantly enhanced cell proliferation in human osteoblast-like cells. Furthermore it is also demonstrated that this stimulatory effect of IL-1β may be through IL-1 receptor. Also the addition of 1 nM E2 significantly enhanced cell proliferation. However, the addition of 30 nM E2 did not show any effect. This fact may suggest that E2 in physiological concentrations should be consider to exert stimulatory effect on cell proliferation (28). When fadrozole–HCL, an Arom inhibitor, was added into the medium with IL-1β, cell proliferation stimulated by IL-1β was neutralized. From the results obtained above, it is suggested that IL-1β stimulates the Arom activity in osteoblast to promote estrogen synthesis and regulates the cell proliferation of osteoblasts through the stimulation of Arom activity. Our results imply that inflammatory cytokines are part of the intracellular mechanisms of cell proliferation through the regulation of steroidogenesis in osteoblast cells as autocrine agents.

Further study has to be performed to define proper concentration of steroid to regulate cell proliferation.

REFERENCES

1. Pacifici, R., Rifas, L., McCracken, R., Vered, I., McMury, C., Avioli, L. V., and Peck, W. A. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 2398–2402.
2. Kimble, R. B., Hopfer, W., Brownfieid, C., Vannice, J. L., Kung, V., and Pacifici, R. (1993) *J. Bone Miner. Res.* **8**(Suppl. 1), S122.
3. Ralston, S. H. (1994) *J. Bone Miner. Res.* **9,** 883–890.
4. Kitazawa, R., Kimble, R. B., Vannice, J. L., Kung, V., and Pacifici, R. (1994) *J. Clin. Invest.* **94,** 2397–2406.
5. Miyaura, C., Kusano, K., Masuzawa, T., Chaki, O., Onoe, Y., Aoyagi, M., Sasaki, T., Tamura, T., Koisihara, Y., Ohsugi, Y., and Suda, T. (1995) *J. Bone Miner. Res.* **10,** 1365–1373.
6. Akatsu, T., Takahashi, N., Debari, K., Morita, I., Murota, S., Nagata, N., Takatani, O., and Suda, T. (1986) *J. Bone Miner. Res.* **4,** 29–35.
7. Purohit, A., Flanagan, A. M., and Reed, M. J. (1992) *Endocrinology* **131,** 2027–2029.
8. Fujikawa, H., Okura, F., Kuwano, Y., Sekizawa, A., Chiba, H., Shimodaira, K., Saito, H., and Yanaihara, T. (1997) *Biochem. Biophys. Res. Commun.* **231,** 42–47.
9. Bruch, H. R., Wolf, L., and Budde, R. (1992) *J. Clin. Endocrinol. Metab.* **75,** 101–105.
10. Shimodaira, K., Fujikawa, H., Okura, F., Shimizu, Y., Saito, H., and Yanaihara, T. (1996) *Biochem. Mol. Biol. Int.* **39,** 109–116.
11. Kuwano, Y., Fujiawa, H., Watanabe, A., Shimodaira, K., Sekizawa, A., Saito, H., and Yanaihara, T. (1997) *Endocr. J.* **44,** 847–853.
12. Nawata, H., Tanaka, S., Tanaka, S., Takayanagi, R., Sakai, Y., Yanase, T., Ikuyama, S., and Haji, M. (1995) *J. Steroid Biochem. Mol. Biol.* **53,** 165–174.
13. Saito, H., and Yanaihara, T. (1998) *J. Intern. Med. Res.* **26,** 1–12.
14. Ryan, K. J. (1956) *J. Biol. Chem.* **234,** 268–278.
15. McNatty, K. P., Baird, D. T., Bolton, A., Chambers, P., Corker, C. S., and Mclean, H. (1976) *J. Endocrinol.* **71,** 77–85.
16. Valladares, L. E., and Payne, A. H. (1979) *Endocrinology* **105,** 431–436.
17. Roselli, C. E., Horton, L. E., and Resco, J. A. (1985) *Endocrinology* **117,** 2471–2477.
18. Ackerman, G. E., Smith, M. E., Mendeison, C. R., MacDonald, P. C., and Shimpson, E. R. (1981) *J. Clin. Endocrinol. Metab.* **53,** 412–417.
19. Nestler, J. E. (1993) *Endocrinology* **132,** 566–570.

20. Reed, M. J., Topping, L., Coldham, N. G., Purohit, A., Ghilchik, M. W., and James, V. H. T. (1993) *J. Steroid Biochem. Mol. Biol.* **44,** 589–596.
21. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
22. Marie, P. J., Sabbagh, A., Vernejoul, M. C., and Lomri, A. (1989) *J. Clin. Endocrinol. Metab.* **69,** 272–279.
23. Marie, P. J. (1997) *Rev. Rhum.* **64**(1), 44–53.
24. Raisz, L. G., Wiita, B., Artis, A., Bowen, A., Schwartz, S., Trahiotis, M., Shoukri, K., and Smith, J. (1996) *J. Clin. Endocrinol. Metab.* **81,** 37–43.
25. Akamatsu, T., Chiba, H., Kamiyama, H., Hirose, K., Saito, H., and Yanaihara, T. (1992) *Asia–Oceania J. Obstet. Gynecol.* **18,** 271–276.
26. Shozu, M., and Shimpson, E. R. (1998) *Mol. Cell. Endocrinol.* **139,** 117–129.
27. Robinson, J. A., Harris, S. A., and Riggs, B. L. (1997) *Endocrinology* **138,** 2919–2927.
28. Ernst, M., Schmid, C. H., and Froesch, E. R. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 2307–2310.
29. Oursler, M. J. (1998) *Crit. Rev. Eukaryotic Gene Expr.* **8**(2), 125–140.
30. Frost, A., Jonsson, K. B., Nilsson, O., and Ljunggren, O. (1997) *Acta Orthop. Scand.* **68**(2), 91–96.