Expression of Estrogen Receptors α and β in Human Osteoblasts: Identification of Exon-2 Deletion Variant of Estrogen Receptor β in Postmenopausal Women

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Background: Postmenopausal osteoporosis is associated with estrogen deficiency. Estrogens have effects on bone metabolism, which are mediated by estrogen receptors (ERs). If estrogen responsiveness is related to the ER expression level, ER expression in postmenopausal women should be different from previous studies using osteoblast lineage. We investigated the expression of variant isoforms of ER messenger ribonucleic acid (mRNA) in osteoblasts (OB) from postmenopausal women and a human osteosarcoma cell line, MG 63.

Methods: Osteoblast cultures were prepared from the upper femur of postmenopausal patients or MG 63. For OB cultures at 5, 10, 15, 20, and 25 days, the expressions of ERα and β mRNA were examined using reverse transcriptase-polymerase chain reaction.

Results: In MG 63, ERβ mRNA was constantly and highly expressed during the 25-day culture, whereas ERα mRNA was barely detected. In the primary OB cells, both ERα and β mRNA were transcribed during the 25-day culture, but expression of ERα mRNA was much stronger than that of ERβ mRNA. A splice variant form of ERβ mRNA that was missing the entire exon 2 (ERβ Δ2) was detected and heterogeneously expressed in OB cultures from 16 postmenopausal women.

Conclusion: Differential expressions of these ER isoforms suggest that they may have different functions or that they interact with each other during bone metabolism. The different ratio of ERβ to ERβΔ2 mRNA or ERα to ERβ mRNA expressions in osteoblast cultures may be related to different bone conditions. Whether the presence of ERβΔ2 in postmenopausal women influences the biological properties of bone needs to be determined.
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Key words: **human osteoblast, postmenopausal women, ERα mRNA, ERβ mRNA, exon 2 deletion of ERβ mRNA.**

It has been well established that estrogen deficiency is associated with bone loss in postmenopausal women, and these changes can be entirely prevented using estrogen replacement therapy.[1,2] In experi-

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mental studies,[2,3] estrogen inhibited the synthesis and secretion of certain cytokines, namely interleukin-1, tumor necrosis factor-α, and interleukin-6, by osteoblasts. These cytokines stimulated bone resorption by increasing osteoclastic activity. Osteoblast exposure to estrogen also resulted in increased cellular proliferation, as well as the secretion of alkaline phosphatase, which is associated with an increase in bone formation.[2] However, the effects of estrogens on bone metabolism is still hypothetical. It has been suggested that estrogens decreased bone resorption by directly inhibiting lysosomal enzyme production in osteoclasts and by decreasing the synthesis of osteoclast-stimulating cytokines in osteoblasts. Therefore, estrogens are important for maintaining skeletal structure and skeletal remodeling in women.

Estrogen exerts its effects on target cells by interacting with specific estrogen receptors (ERs). Following the cloning of ERα in 1986,[4,5] ERβ was cloned initially from rodent tissues and later from human tissues.[6-8] This finding has raised the question of the relative importance that estrogen receptor subtypes have in different target tissues. ERα and ERβ have almost identical deoxyribonucleic acid (DNA)-binding domains, and in vitro studies have demonstrated that the two receptors have similar affinities for estrogenic compounds.[7-9] Since the amino acid sequence of ERβ differs from that of ERα in the N- and C-terminal trans-activating regions, the transcriptional activation mediated by ERβ may be distinct from that of ERα.[10] Recently, various alternative specie forms of messenger ribonucleic acid (mRNA) were found in both human ERα and ERβ genes, including 20 different variants for ERα and 10 different variants for ERβ mRNAs that have deletions in various combinations of exons.[11-13] Poola et al. identified 10 exon deleted ERβ mRNAs in the human ovary, breast, uterus and bone tissues in which alternate splicing patterns of ERβ mRNA were distinct from that of ERα.[13]

Estrogen receptors are present in low numbers in osteoblasts in vitro.[2,3] ERα has been reported to express in murine,[14] rat,[15] and human osteosarcoma cell lines,[16-19] as well as in cultured human osteoblast-like cells.[20] ERβ has also been detected in rat osteoblasts, a rat osteosarcoma cell line (ROS 17/2.8), and cancellous and cortical bone from 8-week-old rats,[21] as well as in a human osteoblast cell line, SV-HFO.[22] Although, as aforementioned, the low numbers of estrogen receptors in osteoblasts in vitro and the effects of estrogen on cells of osteoblast lineage have been demonstrated, it is still unclear whether these effects are mediated by ERα, ERβ, or both receptor subtypes. The aims of this study were two fold. First, we characterized the differential expressions of ERα and ERβ mRNA in both human osteoblast cell lines and primary cultures using reverse transcriptase-polymerase chain reaction (RT-PCR) during the 25-day culture period. Next, we demonstrated exon 2 deletion variants of ERβ (ERβ Δ E2) in human osteoblast primary cultures.

METHODS

Culture of primary human osteoblast-like cells and established human osteosarcoma cell lines

Human primary osteoblast-like cells (hOB cells) were obtained from the upper femur of female patients undergoing bipolar endoprosthesis arthroplasty for a fracture neck of the femur, which occurred after menopause. All patients were postmenopausal and aged 60 to 74 years. None of them had received any medication, including hormone replacement therapy, which may have influenced bone metabolism. This study was approved by the Ethical Medicine Committee of our hospital and supported by the National Science Council (Taiwan, R.O.C.).

First, connective tissue was carefully dissected from the bone fragments, which were then extensively washed with phosphate-buffered saline, diced into small pieces (3-5 mm in diameter) with a scalpel, and subjected to a 2-hour digestion at 37˚C in a shaking water bath with crude bacterial collagenase at 1 mg/mL in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA). The fragments were then seeded into 75-cm^2 culture flasks and cultured in a calcium-free, phenol red-free medium (Gibco) consisting of a 1:1 mixture of penicillin and streptomycin (100 U/ml and 100 μg/ml; Gibco). We replaced the medium with an equal volume (10 ml) of fresh medium every week the first 2 weeks, and then twice weekly thereafter. In all cultures, at least 60% of the cells showed intense staining for alkaline phosphatase activity.

A human osteosarcoma cell line (MG-63) and passage primary hOB cells were cultured in DMEM,

at a 1:1 ratio, with the addition of 10% fetal calf serum (Gibco), penicillin (100 U/ml; Gibco), streptomycin (100 μg/ml; Gibco), L-glutamine (2 mmol/l; Gibco), and L-ascorbic acid (100 mg/l; Sigma, St. Louis, Mo, USA), in a humidified, 5% CO_2 atmosphere at 37˚C.

RT-PCR analysis of osteoblastic cells

Total RNA was isolated from cells using the single-step guanidinium thiocyanate-phenol-chloroform procedure described previously by Chomczynski and Sacchi[23] and quantified spectrophotometrically at 260 nm. One microgram of total RNA was reverse transcribed (RT) into single-strand cDNA using Moloney murine leukemia virus (MMLV) reverse transcriptase (Epicentre Tech., Madison, Wis, USA) with 2.5 μM oligo $(dT)_{16}$ as the primer. The RT reaction was carried out for 90 min at 37˚C in 1X MMLV-RT buffer, 10 mM DTT, and 2.5 mM dNTP. The single-strand cDNA was split into two aliquots, which were PCR-amplified each in 50-μl reactions with primers as follows:
ERα (forward) 5'-AATTCAGATAATCGACGCCAG-3'
ERα (reverse) 5'-GTGTTTCAACATTCTCCCTCCTC-3'
ERβ (forward) 5'-TAGTGGTCCATCGCCAGTTAT-3'
ERβ (reverse) 5'-GGGAGCCACACTTCACCAT-3'
β-actin (forward) 5'-CTGGCACCACACCTTCT-3'
β-actin (reverse) 5'-GCTCGAAGTCCAGGGCG-3'

PCR amplifications were carried out with 2 U/μl Taq DNA polymerase (Promega, Madison, Wis, USA), 0.6 μM of each primer, and 0.5 mM dNTP with a cycle profile of 94˚C (denaturing) for 30 s, 60˚C (annealing) for 30 s, and 72˚C (elongation) for 30 s. Both ERα and ERβ were PCR-amplified for 35 cycles and β-actin was PCR-amplified for 25 cycles to ensure that the PCR reaction was carried out in the linear portions of the PCR amplification. Therefore, 345-bp, 393-bp, and 419-bp fragments were obtained using primers for ER α, ER β, and β-actin, respectively. PCR products were separated using electrophoresis on a 1.5% agarose gel and visualized with ethydium bromide staining.

To construct internal standards for ERα and ERβ PCR reactions, pairs of gene-specific primers (as described above) were inserted into pBluescript SK-plasmid at *Pvu*II sites (nt 532 and 977). Therefore, 498-bp and 490-bp fragments were obtained using ERα- and ERβ-specific primers, respectively.

RESULTS

Expression of wild type ERα and ERβ mRNA in human osteoblastic cells

In this study, the expression of ER mRNA was monitored in MG 63 human osteosarcoma cells and in primary human osteoblast-like cells cultured for various time periods. To make a more accurate estimation of ER mRNA expression in cells at different growth stages, a fixed amount of internal standard constructed for ERα (ISα) or ERβ (ISβ) was added to every amplification reaction to serve as the basis for quantifying the level of ER mRNA using RT-PCR. Under a near-constant level of ISβ, a significant amount of a 393-bp fragment of ERβ cDNA was obtained from the osteosarcoma cells cultured for 5 to 25 days, indicating a constitutive ERβ mRNA expression that was independent of cell

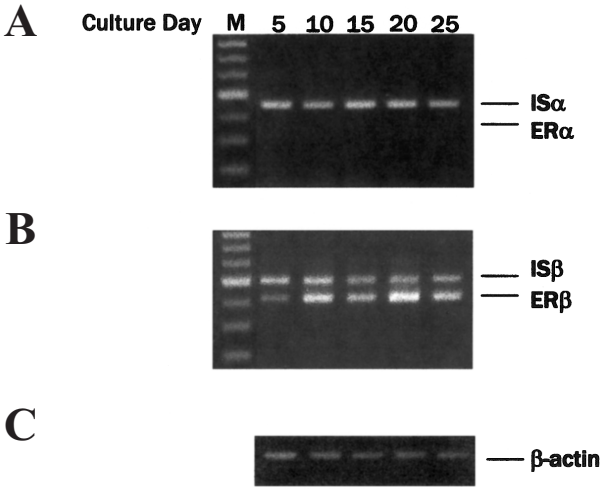

Fig. 1 RT-PCR analysis of ERα and ERβ mRNA expression in the human osteosarcoma cell line, MG63. Total RNA (1 μg) isolated from MG63 cells at the indicated time period in the culture was reverse-transcribed, and a 35-cycle PCR was performed using gene-specific primers for ERα (A) or ERβ (B). Each PCR mixture contained a pBluescript SK plasmid (0.1 ng) carrying the constructed internal standard for ERα or ERβ (ISα and ISβ). The efficiency of RNA isolation was determined using RT-PCR reaction of the house keeping gene β-actin (C). The amplified products were analyzed using 1.5% agarose gel electrophoresis and ethidium bromide staining. The expected PCR products, 345-bp, 393-bp, and 419-bp fragments obtained using primers for ER α, ER β, and β-actin, are indicated by the solid lines.

growth. In contrast, ERα mRNA was not detected in the MG 63 osteosarcoma cells during the 25-day culture (Fig. 1). The ERα mRNA was still barely detectable when even 3 μg RNA was used for RT-PCR (data not shown).

Compared with the MG 63 osteosarcoma cells, a different pattern of ER mRNA expression was found in primary osteoblast-like cells isolated from the upper femur of postmenopausal women. As revealed using RT-PCR, we detected a constant level of ERα mRNA in the primary cultured osteoblast-like cells throughout the 25-day period, while a very low level of ERβ mRNA was observed in these cells (Fig. 2).

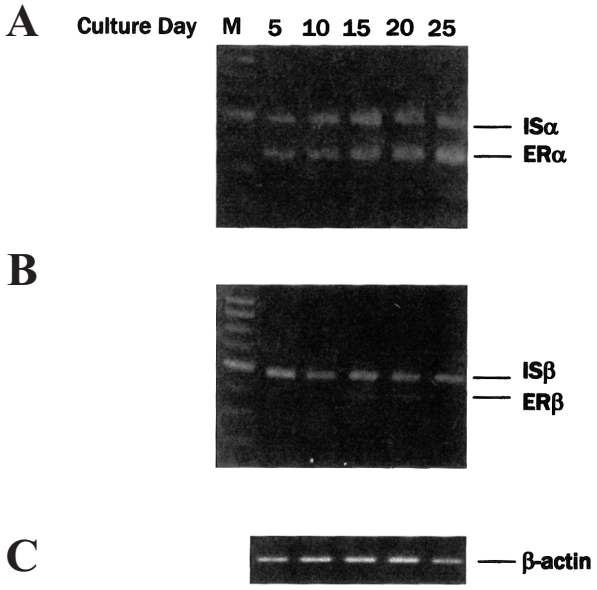

Fig. 2 RT-PCR analysis of ERα and ERβ mRNA expression in primary osteoblast-like cells taken from the femur of a postmenopausal woman, Subject 44. Total RNA (1 μg) isolated from primary cultured cells at the indicated time period was reverse-transcribed, and a 35-cycle PCR was performed using gene-specific primers for ERα (A) or ERβ (B). Each PCR mixture contained a pBluescript SK plasmid (0.1 ng) carrying the constructed internal standard for ERα or ERβ (ISα and ISβ). The efficiency of RNA isolation was determined using the RT-PCR reaction of the house keeping gene β-actin (C). The amplified products were analyzed using 1.5% agarose gel electrophoresis and ethidium bromide staining. The expected PCR products, 345-bp, 393-bp, and 419-bp fragments obtained using primers for ER α, ER β, and β-actin, are indicated by the solid lines.

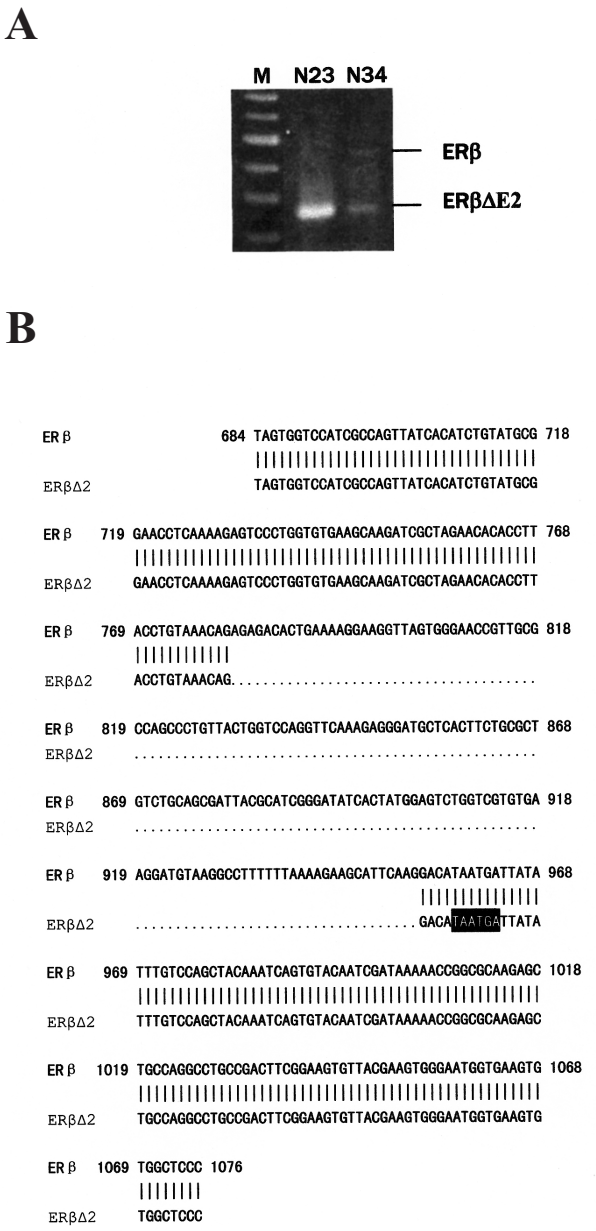

Fig. 3 Detection of a 173-bp truncated form of ERβ mRNA (ERβΔ2) in primary human osteoblast-like cells. (A) Heterogeneous expressions of the truncated mRNA in different human subjects as detected using RT-PCR. The amplified products were analyzed using 1.5% agarose gel electrophoresis and ethidium bromide staining. (B) Nucleotide sequence comparison between the 220- and 393-bp PCR products. The truncated173 nucleotides are labeled as dots. The corresponding positions of ERβ cDNA (GeneBank Accession number NM 001437) are indicated in numbers. The stop codons generated by exon 2 deletion are labeled in the black box.

Detection and characterization of exon 2-deleted ERβ mRNA variants in human osteoblastic cells

In addition to the wild type ERβ mRNA, a number of variant splicing forms of ERβ mRNA were found in human tissues.[11-13] Here, a low level of a variant splicing form of ERβ mRNA that generated a 220-bp cDNA fragment was detected in the primary osteoblast-like cells derived from 16 postmenopausal women. As shown using a representative agarose gel, the ERβ variant was predominantly expressed in subject N23, but the same type of bone cells in subject N34 produced both the wild type and the variant forms of ERβ mRNA (Fig. 3A). Nucleotide sequence comparison revealed that the 220-bp PCR product was the result of a 173-bp exon 2 deletion from the 393-bp product (Fig. 3B and 4). Owing to the appearance of an early stop codon generated after the exon 2 deletion, this exon 2-deleted ERβ mRNA would encode only a 122-a.a peptide at the N-terminal region of wild-type ERβ (Fig. 4B), resulting in the production of immature ERβ. A similar exon 2 deletion form of ERβ mRNA was occasionally found in the cultured MG 63 osteosarcoma cells (data not shown). However, the exon 2 deletion form of ERβ mRNA was not detected in some human primary osteoblast cells (Fig. 2).

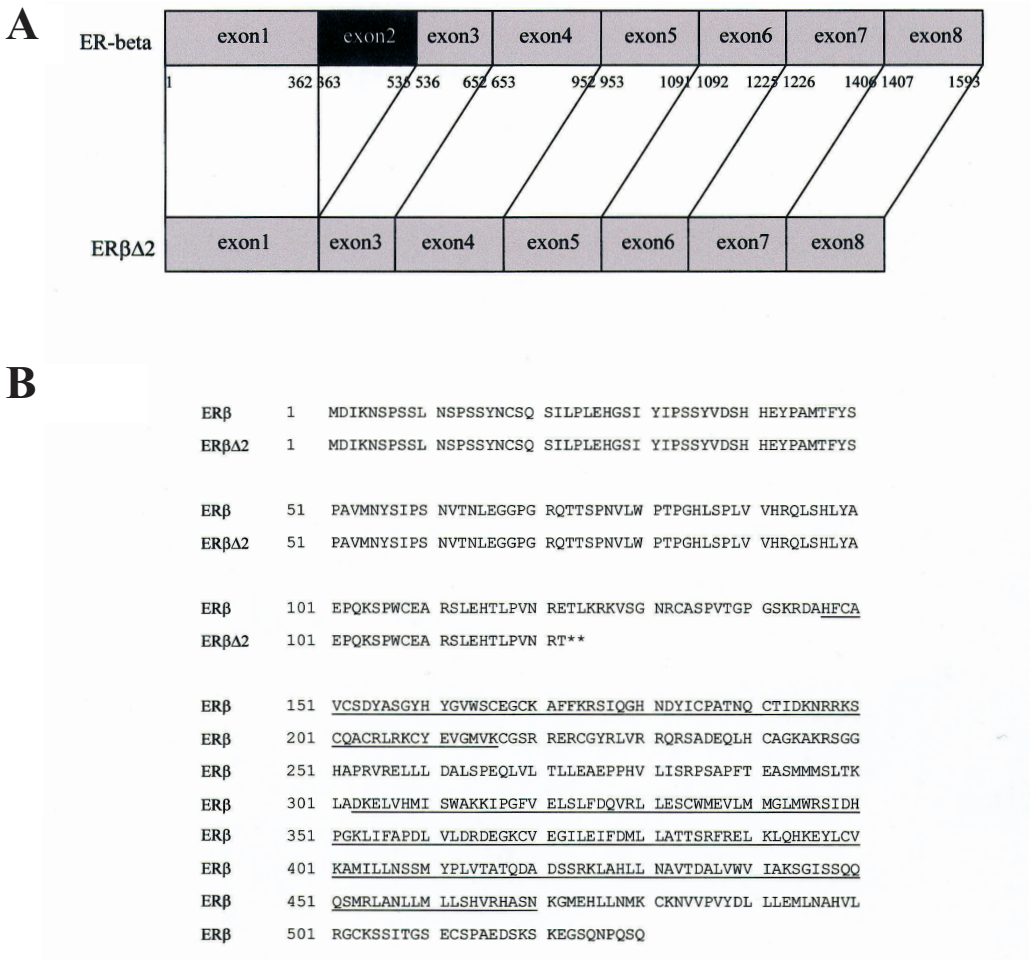

Fig. 4 The translated amino acid sequences of ERβ and ERβΔ2. (A) Sequence comparison indicates that the deleted nucleotide sequence is located at exon 2 of the ERβ gene. (B) The exon 2-truncated form of ERβ mRNA encodes only a polypeptide of 122 amino acids due to the presence of two early stop codons generated after the exon 2 deletion. The regions of zinc finger DNA binding domain (amino acids 147-216) and ligand binding domain (amino acids 303-460) are underlined.

DISCUSSION

In the present study, we examined the expression of ERα and ERβ in primary cultured hOB cells and a human osteosarcoma cell line (MG 63). During the 25-day culture period, the strength of expression of ERα and ERβ mRNA differed between the human osteosarcoma cell lines (MG 63) and the primary hOB cells. The presence of ER mRNA in human osteoblastic cells indicates that bone is a target for estrogen. In addition, we have, for the first time, demonstrated the presence of an ERβ exon 2 deletion in human osteoblastic cells. We also found that the strength of expression of the ERβ exon 2 deletion differed between human osteosarcoma cell lines and primary hOB cells, as well as between primary hOB cells from different subjects. It remains to be determined whether the expression of these isoforms may influence the biological properties of bone.

Previous studies have indicated the physiological importance of ERα in humans. Estrogen resistance due to a point mutation in the ERα gene was reported in a 28-year-old male.[24] This patient had increased bone turnover and osteopenia, indicating that ERα was important for normal bone remodeling in humans. Similar effects have also been described in patients deficient in estrogen due to failure of the aromatase enzyme to convert testosterone into estrogen.[25] In cultured rat calvarial-derived osteoblasts, Bodine et al.[26] also found that expression of ERα mRNA correlated with progressive osteoblast differentiation and may be a contributing factor to the differential regulation of bone cell gene expression by 17β-E2. From the aforementioned studies, similar effects between the ERα mutation in humans and aromatase deficiency, as well as the expression of ERα mRNA in cultures of rat osteoblasts, suggest that ERα is important for normal bone metabolism in humans. However, this does not rule out a functional role for ERβ, because it has been speculated that the ratio between ERα and ERβ determines the downstream activities of estrogens in target tissues.[27-30] In recent reports, ERα and ERβ were demonstrated using RT-PCR in primary rat osteoblastic cells and rat osteosarcoma cells (ROS 17/2.8),[21] as well as in the human osteosarcoma cell lines SV-HFO.[22] Using RT-PCR, we confirmed that a human osteosarcoma cell line (MG-63) and primary hOB cells express ERα and ERβ mRNA. These findings point out that ERα might not be the sole mediator of the estrogen response in bone. ERβ may by itself or in association with ERα also be involved in this process.

As with studies of rat osteosarcoma cells (ROS 17/2.8)[21] and the human osteosarcoma cell lines SV-HFO,[22] in the present study, we demonstrates that ERα and ERβ were differentially expressed during human osteoblast differentiation. It appears that the ERβ mRNA exhibited higher levels of transcription in the cultured MG 63 osteosarcoma cells than the ERα mRNA during the 25-day culture period. However, the expression of ERα and ERβ greatly differed in the primary hOB cells, in which the ERα mRNA expression was more prominent than in human osteosarcoma cell line (MG-63) culture. Since primary hOB cells were obtained from the femoral neck of postmenopausal women, it is possible that aging was associated with the alterations in the levels or functioning of ER in the bone. The results were compatible with the study of Ankrom et al.,[31] in which it was demonstrated that ERα levels in osteoblast-like cells were up-regulated by an age-dependent decrease in estrogen. Age-associated diminution of signal transduction might therefore be a likely mechanism to explain our observation, in which the increase in ERα mRNA levels might be a consequence of a decreased ER response reflecting a compensatory mechanism of the cells. Furthermore, we demonstrated that the primary hOB cell culture exhibited much lower level of ERβ mRNA transcription than the MG-63 osteosarcoma cell line did. However, since the number of postmenopausal women in this study was small, we still could not demonstrate that the expression of ERβ exon 2 deletion mRNA was related to the age or menopausal period of postmenopausal women. In addition to the consideration of aging factors in primary hOB cells, characteristics of osteosarcoma cell lines might not represent actual osteoblast differentiation. Differential expressions of ERα and ERβ mRNA during osteoblastic differentiation suggest the possibility that ERα and ERβ may act in conjunction with each other.

As shown by Shupnik et al.[11] and Poola et al.,[13] most of the estrogen-responsive tissues also expressed a number of ERs that had deletions in the portions of the molecules in addition to ERα and

ERβ. For human osteoblast cells, we are the first to identify ERβ exon 2 deletion mRNA co-expressed with wild-type ERα and ERβ mRNA. Although there has been a debate for a long time whether the ER splice variant mRNAs are translated into proteins and the truncated ERs are functionally active, recent reports have shown that both ERα and ERβ splice variant mRNAs are translated into proteins.[32,33] Therefore, in human osteoblast cells, deletion of exon 2 in ERβ mRNA may cause a frame shift mutation resulting in premature termination of translation. The biological activity of this severely truncated protein from the exon 2-deleted mRNA, if any, is difficult to predict. Further evaluation is needed to determine whether the presence of exon 2-deleted ERβ mRNA affects the activity of ER. In the present study, differences in the expression of ER isoforms in primary human osteoblast cells from various postmenopausal women, as well as in an osteosarcoma cell line, were noted. Thus it remains to be established whether the biologic effects of differential expression of types and relative levels of various ER isoforms occur during differentiation of various human osteoblast cells.

In conclusion, this study provides evidence for the presence of ERα and ERβ, as well as the ERβ exon 2 deletion, in human bone cells. Differential expressions of these ER isoforms suggest that they may have different functions or that they interact with each other. In addition, the strength of expression of these ER isoforms may be related to age and bone cell conditions. Further evaluation is needed to determine whether different expression of these ER isoforms in osteoblast differentiation influenced the response to estrogen.

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甲型及乙型雌激素感受器在人類造骨細胞之表現：在停經後婦女發現缺損Exon-2之乙型雌激素感受器變異型

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背 景： 停經後骨質疏鬆症與雌激素缺乏有關。雌激素藉由雌激素感受器影響骨之代謝。如果雌激素的效應與雌激素感受器表現的量有關，雌激素感受器在停經後婦女的表現應會不同於以往使用造骨細胞群的研究。本篇研究在顯示取自停經後婦女和人類骨癌細胞系 (MG63) 之造骨細胞，其不同雌激素感受器傳遞醋栗糖核酸 (ERmRNA) 在分化中的表現。

方 法： 造骨細胞之培養取自停經後婦女和人類骨癌細胞系(MG63)，在第5、10、15、20和25日造骨細胞培養中，以RT-PCR檢視造骨細胞中，甲型及乙型雌激素感受器傳遞醋栗糖核酸(α and β ERmRNA) 之表現。

結 果： MG63造骨細胞在25天的培養中，乙型雌激素感受器傳遞醋栗糖核酸都呈現穩定且明顯之表現，而甲型雌激素感受器傳遞醋栗糖核酸則很難偵測到。由停經後婦女培養之造骨細胞，在25天的培養中都可看到甲型及乙型雌激素感受器傳遞醋栗糖核酸，但甲型雌激素感受器傳遞醋栗糖核酸之表現較乙型雌激素感受器傳遞醋栗糖核酸爲強。在這16位停經後婦女之造骨細胞中，同時偵測到不同強度表現的缺損exon-2之乙型雌激素感受器傳遞醋栗糖核酸變異型。

結 論： 這些雌激素感受器傳遞醋栗糖核酸不同的表現，意謂著它們在骨的代謝中有不同的作用或互相影響。在造骨細胞培養中，乙型雌激素感受器傳遞醋栗糖核酸之於缺損exon-2之乙型雌激素感受器變異型、或甲型雌激素感受器傳遞醋栗糖核酸之於乙型雌激素感受器傳遞醋栗糖核酸，其不同比例之表現，可能與不同的骨質之狀況有關。缺損exon-2之乙型雌激素感受器變異型存在於停經後婦女是否會影響骨之生物特質，仍待研究確認。

(長庚醫誌 2004;27:107-15)

關鍵字： 人類造骨細胞，停經婦女，甲型雌激素感受器傳遞醋栗糖核酸，乙型雌激素感受器傳遞醋栗糖核酸，缺損exon-2之乙型雌激素感受器。

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