J Periodont Res 2008; 43: 311–321

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2007.01031.x

Up-regulation of estrogen receptor-β expression during osteogenic differentiation of human periodontal ligament cells

X. Tang[1,2], H. Meng[1], J. Han[1], L. Zhang[1], J. Hou[1], F. Zhang[1]

[1]Department of Periodontology, Peking University School and Hospital of Stomatology, Beijing, China, [2]Department of Periodontology, School of Stomatology, China Medical University, Shenyang, China

Tang X, Meng H, Han J, Zhang L, Hou J, Zhang F. Up-regulation of estrogen receptor-β expression during osteogenic differentiation of human periodontal ligament cells. J Periodont Res 2008; 43: 311–321. © 2007 The Authors. Journal compilation © 2007 Blackwell Munksgaard

Background and Objective: Estrogen has been shown to up-regulate the expression of osteoblastic phenotypes of human periodontal ligament cells via binding to estrogen receptors and may also help periodontal tissue regeneration. However, which subtype of estrogen receptor (α or β) is predominately expressed in human periodontal ligament cells, and how estrogen receptor expression is regulated during the osteogenic differentiation of human periodontal ligament cells, is still unclear. This study aimed to explore the expression and regulation of estrogen receptor subtypes in human periodontal ligament cells and during their osteogenic differentiation.

Material and Methods: Human periodontal ligament cells derived from 10 individual age-matched donors (five male and five female donors) were cultured. Human periodontal ligament cells under osteogenic induction (group M) and the corresponding controls (group C) were harvested on days 7, 14 and 21 for estrogen receptor detection.

Results: Both estrogen receptor-α and estrogen receptor-β mRNAs were expressed in human periodontal ligament cells from all of the 10 donors. Protein only of estrogen receptor-β (not of estrogen receptor-α) was detected and was shown to be located in the nuclei of human periodontal ligament cells. The expression levels of estrogen receptor-β mRNA and protein from both male and female donors in group M were significantly higher compared with group C during the 21-d study period. In comparison, the expression level of estrogen receptor-α mRNA of the donors was not significantly different from that of the controls during osteogenic differentiation and no estrogen receptor-α protein was detected.

Conclusion: The results suggest that estrogen receptor-β may be the predominant subtype expressed in human periodontal ligament cells and may actively participate in the osteogenic differentiation process of human periodontal ligament cells, both in male and in female subjects.

Meng Huanxin, Department of Periodontology, Peking University School and Hospital of Stomatology, 22 Zhongguancun Nandajie, Haidian District, Beijing 100081 China
Tel: +86 10 62179977, ext 2522
Fax: +86 10 62173402
e-mail: kqhxmeng@bjmu.edu.cn

Key words: estrogen receptors; human periodontal ligament cells; osteogenic differentiation; stem cells

Accepted for publication June 27, 2007

Periodontitis is a type of chronic inflammatory disease affecting people worldwide, which is characterized by the loss of periodontal connective tissue and alveolar bone, eventually leading to tooth loss. Bone remodeling is one of the key factors in periodontal regeneration. Among all the factors affecting bone metabolism, the role of

estrogen is outstanding. For example, postmenopausal osteoporosis is closely associated with estrogen-deficiency status and can be ameliorated by hormone replacement therapy. Apart from regulating bone metabolism, estrogen can also relieve some chronic inflammatory diseases, such as intestinal and joint inflammation (1). Researchers have demonstrated that sufficient estrogen may help to improve the periodontal health of postmenopausal women by reducing gingival inflammation and the frequency of clinical attachment loss (2) and may help to improve tooth retention in postmenopausal women (3,4).It is well established that estrogen has multifunctional roles in influencing growth, differentiation and function in many tissues (5). The pleiotropic and tissue-specific effects of estrogen are likely to be mediated by the differential expression of distinct estrogen receptor subtypes (estrogen receptor-α and estrogen receptor-β) and their coregulators. Estrogen receptor is a type of nuclear hormone receptor, belonging to a family of transcription factors that can regulate the transcription of many genes. Researchers have demonstrated the complicated interaction between estrogen receptor-α and estrogen receptor-β. The ratio between the two isoforms may be important in mediating osteoblastic responses to estrogen, and estrogen receptor-β may function as a transdominant inhibitor of estrogen receptor-α transcriptional activity (6). Functional estrogen receptor-α/β heterodimers result in unique patterns of gene regulation, many of which are distinct from the genes regulated by the estrogen receptor homodimers (7). Furthermore, many studies have documented the important roles of both estrogen receptor subtypes in bone metabolism. It has been reported that both estrogen receptor-α and -β are expressed in osteoblasts, osteoblast-like cells and bones (8–11). Using knockout mice, Sims *et al*. found that in male mice, only estrogen receptor-α was shown to decrease bone turnover and increase trabecular bone volume, whereas in female mice both receptor subtypes influenced this process and could compensate for each other (12). Besides studies on association between estrogen receptor-α gene polymorphism and bone density, such studies on estrogen receptor-β are multiplying (13–16). The above *in vitro* and *in vivo* studies suggest important roles of both subtypes and their interaction in osteogenic differentiation and bone remodeling. Alveolar bone, as part of the whole skeleton, should be regulated by estrogen through the two estrogen receptors. Zhang *et al*., from the authors' laboratory, found that a certain genotype of estrogen receptor-α was associated with female patients with chronic periodontitis (17). However, the exact relationship between estrogen/estrogen receptor and the incidence and progression of periodontitis has not yet been fully elucidated. Therefore, it is necessary to determine the expression levels of the two subtypes in periodontal tissues, which may help to reveal the mechanism under which estrogen exerts its effects.

Periodontal ligament, which is a nonmineralized connective tissue between the cementum and alveolar bone, plays an important role in the maintenance and regeneration of periodontal tissue. It has been shown that periodontal ligament contains stem cells with the ability to differentiate into cementoblast-like cells, adipocytes and collagen-forming cells under defined culture conditions (18). A number of studies have demonstrated the osteoblastic properties of human periodontal ligament cells and their ability to form mineralized nodules (19,20). It has been shown that estrogen may up-regulate the expression of osteoblastic phenotypes of human periodontal ligament cells, which might help in the regeneration of cementum and alveolar bone. For instance, Morishita *et al*. found that 17-β estradiol enhanced the production of osteocalcin by human periodontal ligament cells in a time- and dose-dependent manner (21) and significantly enhanced the alkaline phosphatase activity and mineralized nodule formation compared with the controls (22).

In order to investigate the molecular mechanism by which estrogen exerts its biological effects in human periodontal ligament cells during their osteogenic differentiation, it is necessary to evaluate the expression and regulation of estrogen receptor subtypes in human periodontal ligament cells. However, which estrogen receptor subtype is predominately expressed in human periodontal ligament cells remains controversial (23–25). In addition, whether or not human periodontal ligament cells of women can express a higher level of estrogen receptors than those of men is questionable. Also, studies on estrogen receptor expression and regulation in human periodontal ligament cells during osteogenic differentiation have not yet been published in the literature.

This *in vitro* study was carried out to determine the subtypes and expression level, as well as the intracellular localization, of estrogen receptors in human periodontal ligament cells and to characterize the expression profile of estrogen receptors during the osteogenic differentiation of human periodontal ligament cells.

Material and methods

Cells from different donors were used in various experiments and the protocol is shown in Fig. 1.

Cell culture

The periodontal ligament tissues were obtained from healthy teeth extracted for orthodontic reasons from five male donors (age-range 12–24 years; mean ± standard deviation: 19.40 ± 6.31 years) and five age-matched female donors (age-range: 13–24 years; mean ± standard deviation: 19.20 ± 4.66 years). Informed consent was obtained from all of the patients before the extractions were carried out. Primary culture of human periodontal ligament cells was performed according to the method described by Somerman *et al*., with some modifications (26). In brief, the periodontal ligament tissues attached to the middle third of the roots were gently curetted with scalpels and contamination of gingiva and pulp was excluded with caution. After being cut into small pieces of $\approx$0.5–1 mm^3, the tissue explants from

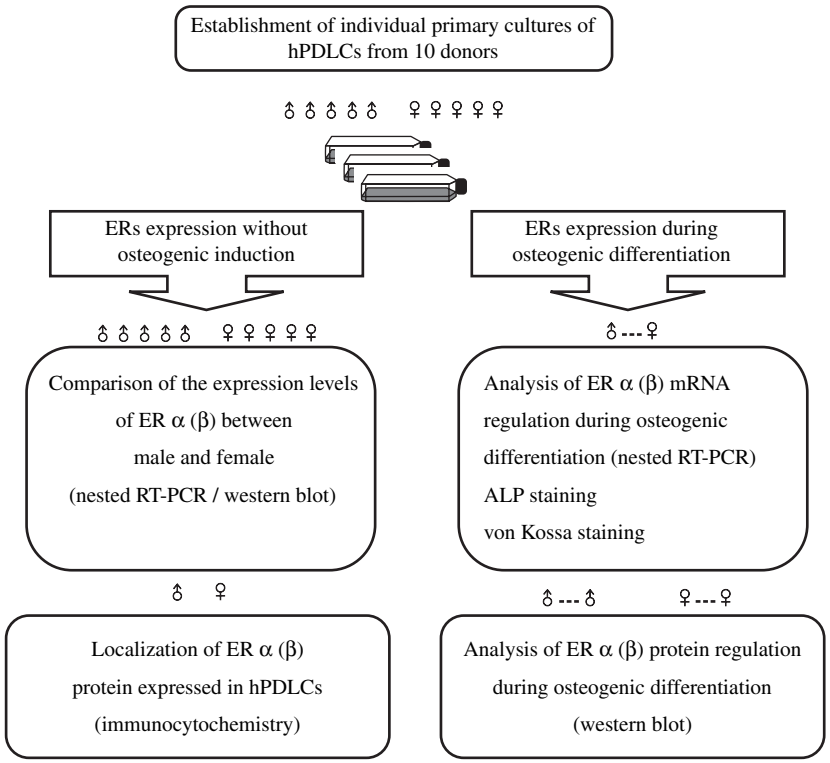

Fig. 1. Protocol for analysis of expression and regulation of estrogen receptors in human periodontal ligament cells. The primary cultures of human periodontal ligament cells were from five male donors and another five were from age-matched female donors. Cells between passages 3–5 were used in the various experiments. ♂♂♂♂♂, human periodontal ligament cells from individual male donors; ♀♀♀♀♀, human periodontal ligament cells from individual female donors; ♂(♀), human periodontal ligament cells from one male (one female) donor; ♂—♀, human periodontal ligament cells from all of the male and female donors; ♂—♂, human periodontal ligament cells from all of the five male donors; ♀—♀, human periodontal ligament cells from all of the five female donors. ALP, alkaline phosphatase; ER, estrogen receptor; hPDLCs, human periodontal ligament cells; RT-PCR, reverse transcription-polymerase chain reaction.

the individual donors were inoculated into one or more wells of a 24-well plate to exclude contamination from other donors. Tissue explants and the outgrown cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (PAA, Coelbe, Germany), 100 U/mL of penicillin and 100 μg/mL of streptomycin, and maintained in a humidified atmosphere of 5% (v/v) CO_2 at 37°C. After reaching confluence, cells were digested with 0.125% (w/v) trypsin and 0.02% (w/v) EDTA and subcultured at a 1 : 3 ratio. In the following experiments, cells from passages 3–5 were used. The MCF-7 cell line was used as a positive control for estrogen receptor protein detection and was cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and antibiotics.

Osteogenic induction

Osteogenic induction was performed to promote the osteogenic differentiation of human periodontal ligament cells. Cells were seeded at a density of 1.5×10^4 cells/cm^2 in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum and antibiotics. Forty-eight hours later, the medium was changed to Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum supplemented with 10 mM β-glycerophosphate, 50 μg/mL of ascorbic acid, 10^{-7} M dexamethasone (Sigma, St Louis, MO, USA) and antibiotics for osteogenic induction (group M), whereas control cells remained in the culture medium with Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum antibiotics (group C). The culture medium was replaced every 3 d. On days 7, 14 and 21, cells were harvested in Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA) and stored at −70°C until required for reverse transcription polymerase chain reaction (PCR) assay, whereas cells for protein detection were kept in liquid nitrogen until required for the western blot experiments. The three time-points selected were representative of the proliferation phase (day 7), the matrix maturation phase (day 14) and the mineralization phase (day 21), respectively, as described previously (27).

Alkaline phosphatase staining

Cells from all of the 10 donors were cultured and treated as described above. During the osteogenic induction, the Gomori calcium phosphate method (28), with some modification, was performed to demonstrate alkaline phosphatase in human periodontal

ligament cells on days 10, 16 and 21. First, cells on coverslips were fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline for 10 min. Second, cells were incubated in incubation solution containing 3% (w/v) β-glycerophosphate, 2% (w/v) calcium chloride and 2% (w/v) magnesium sulphate at 37°C for 5 min and then incubated in 2% (w/v) cobalt nitrate for 5 min at room temperature. Third, coverslips were immersed in 0.5% (w/v) ammonium sulfide for 2 min at room temperature. Coverslips were washed with distilled water after each of the foregoing steps. Finally, Mayer hematoxylin was used to stain the nuclei of the cells.

Von Kossa staining

Cells from all 10 donors were cultured and treated as described above. On day 28, mineralized nodules were revealed using von Kossa assay staining (29). Briefly, cells in six-well plates were fixed in 4% (w/v) paraformaldehyde (in phosphate-buffered saline) for 10 min and then stained with 5% (w/v) sodium silver nitrate under ultraviolet light for 1 h. Subsequently, cells were treated with 5% (w/v) sodium thiosulphate for 2 min and then washed with distilled water. Mineralized nodules were examined microscopically.

Nested reverse transcription-polymerase chain reaction

RNA was extracted according to the manufacturer's instruction for Trizol and quantified using a BioPhotometer (Eppendorf, Hamburg, Germany). Five micrograms of total RNA was used to synthesize first-strand cDNA by the ThermoSCRIPT^{TM} reverase transcriptase system (Invitrogen Life Technologies) in a final volume of 20 μL. Nested PCR was applied, according to a previously described method (8), with some modifications, described in detail below. Primer sequences for amplification of the DNA fragments encoding partial estrogen receptor-α and estrogen receptor-β are listed in Table 1. 18S rRNA was used as the internal control to evaluate total RNA input because it has been shown to demonstrate excellent consistency and nonregulation by treatments (30) and suitability in all the study models for studying differentiation of certain cells (31). The dynamics tests were carried out to find the linear range and identify the appropriate cDNA input amount and amplification circles. PCR was performed in reaction mixtures of 25 μL containing 0.5 μL of cDNA, 10 mM Tris-HCl, 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.2 μM forward and reverse primers, 200 μM dNTPs, 1.5 mM $MgCl_2$ and 1 U of *Taq* DNA polymerase. After predenaturation at 94°C for 5 min, 20 PCR cycles were performed (94°C for 30 s, 53°C for 30 s, 72°C for 30 s) followed by enlongation at 72°C for 7 min on a thermal cycler (Gene Amp, PCR system 2700; Applied Biosystems, Foster City, CA, USA). Next, for the nested amplification, 1 μL of the first amplification product was added to another 24 μL of reaction mixture containing the same components as those in the first reaction, except that the original primers were substituted with 0.2 μM nested forward and reverse primers. Another 20–25 PCR cycles (94°C for 30 s, 53°C for 30 s, 72°C for 30 s) were performed. As for 18S rRNA, normal PCR was carried out with 18 cycles of amplification (94°C for 30 s, 58°C for 30 s, 72°C for 30 s). PCR products were electrophoresed on a 1.5% (w/v) agarose gel and then stained with 0.2 μg/mL of ethidium bromide. MCF-7 cells were used as the positive control. A no-template control was included to show any DNA contamination in the PCR system. Digital photographs were taken under ultraviolet light, and bands on the images were analyzed using BAND LEADER 3.0 Software (Magnitec Ltd, Tel Aviv, Israel). The total band-volume of the estrogen receptor-α or estrogen receptor-β PCR product was normalized to that of the corresponding 18S rRNA. Samples of the PCR products were cloned into pGEM®-T vectors (Promega, Madison, WI, USA) and sequenced for verification.

Western blot

Cells were solubilized in lysis buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate and 2 mM Na_3VO_4 supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany) before use. Protein concentrations were determined with the bicinchoninic acid Protein Assay Kit (Pierce, Rockford, IL, USA), according to the manufacturer's instructions, and the absorbance was read at 595 nm on an enzyme-linked immunosorbent assay reader (EL-311SX; Bio-Tec Instruments, Burlington, VT, USA). Protein components of the cell lysate were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A 50-μg amount of total protein of each sample was loaded onto one gel

Table 1. Sequences of primers used in the nested polymerase chain reaction

Target genes	Type of primers	Primers for initial PCR (5'–3')	Primers for nested PCR (5'–3')	Product size (bp)
ERα	Forward	AATTCAGATAATCGACGCCAG	GACAAGGGAAGTATGGCTATGGA	248
	Reverse	GTGTTTCAACATTCTCCCTCCTC	TTCATCATTCCCACTTCGTAGC	
ERβ	Forward	TAGTGGTCCATCGCCAGTTAT	CGGAACCTCAAAAGAGTCCCTGG	323
	Reverse	GGGAGCCACACTTCACCAT	CCGAAGTCGGCAGGCCTGGCAGC	
18S rRNA	Forward	GTGGAGCGATTTGTCTGGTT	—	201
	Reverse	ACGCTGAGCCAGTCAGTGTA	—	

ERα, estrogen receptor-α; ERβ, estrogen receptor-β.

comprising a 5% (w/v) stacking gel and a 12.5% (w/v) running gel. At the end of electrophoresis, samples were transferred to nitrocellulose blotting membranes (Hybond™; Amersham Pharmacia, Little Chalfont, UK). Nonspecific binding sites were blocked by 5% (w/v) skimmed milk in 0.05% (v/v) tris-buffered salt solution (TBS)-Tween for 1 h at room temperature. Next, the membrane was incubated in the following diluted primary antibodies overnight. Dilution for rabbit monoclonal antibody to estrogen receptor-α (SRA1010; Neomarkers, Westinghouse, CA, USA) and for rabbit polyclonal antibody to estrogen receptor-α (HC-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was 1 : 400 and that for rabbit monoclonal antibody to estrogen receptor-β (05-824; Upstate, Lake Placid, NY, USA) was 1 : 500. Monoclonal antibody to β-actin (Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China), an internal control to determine the amount of total protein loaded, was used at a dilution of 1 : 5000 with 1 h of incubation at room temperature. The membrane was washed three times in 0.05% (v/v) TBS-Tween and incubated in diluted secondary antibody for 1 h at room temperature. The secondary antibody, anti-rabbit IgG, to detect estrogen receptor primary antibodies, was labeled with Alexa Fluor®780 (Odyssey, Lincoln, NE, USA), and the secondary antibody, anti-mouse IgG, to detect β-actin primary antibody, was labeled with Alexa Fluor® 680 (Odyssey). After thorough washing of the membrane, fluorescence emitted from the protein bands was captured, photographed and analyzed by the LI-COR Infrared Imaging System (Odyssey). MCF-7 cells were used as the positive control. The band volume of estrogen receptors was analyzed and normalized to that of the corresponding β-actin using QUANTITY ONE 4.31 software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Immunocytochemistry

MCF-7 cells and human periodontal ligament cells, without osteogenic induction, from one male and one female donor were seeded on coverslips at a density of 1.5×10^4 cells/cm^2 in Dulbecco's modified Eagle's medium with 10% (v/v) fetal bovine serum. After a period of 48 h, cells on coverslips were rinsed with phosphate-buffered saline and then fixed in cold acetone for 10 min. Next, endogenous perioxidase was blocked with 1% (w/v) H_2O_2 for 10 min. Subsequently, cells were permeated by 0.2% (v/v) Triton X-100 in phosphate-buffered saline for 10 min at room temperature followed by incubation in 5% (w/v) bovine serum albumin in phosphate-buffered saline to block nonspecific immunoreactions and were then incubated in the primary antibodies at 4°C overnight. The primary antibody to estrogen receptor-α (SRA1010; Stressgen Bioreagents) was used at a dilution of 1 : 200 and that to estrogen receptor-β (H-150; Santa Cruz) was used at a dilution of 1 : 100. The PV-9000 Polymer Detection System (Beijing Zhongshan Golden Bridge Biotechnology) was used for immunocytochemical staining, according to the manufacturer's recommendations, and finally a diaminobenzidine kit (Beijing Zhongshan Golden Bridge Biotechnology) was used to develop color. For negative controls, primary antibody was omitted and substituted with phosphate-buffered saline. All cell samples staining negative were stained with hematoxylin and eosin to complete the process.

Statistics

Semiquantitative data of nested PCR and western blot were analyzed with the Student's *t*-test for comparison of the expression levels of estrogen receptors in human periodontal ligament cells from male and female donors. Repeated Measurement in General Linear Model and Pairwise Comparisons were applied for comparison of the expression levels of estrogen receptors during osteogenic differentiation. Statistical analyses were carried out using the SPSS 11.5 software package (SPSS Inc., Chicago, IL, USA). Significance was considered to occur at $p < 0.05$.

Results

Expression of estrogen receptor subtypes in human periodontal ligament cells from male and female donors

Both estrogen receptor-α and estrogen receptor-β mRNA were expressed in human periodontal ligament cells from all 10 donors and there was no significant difference in expression levels between the male and the female donors. The band volume of estrogen receptor-α PCR products normalized to 18S rRNA was 1.288 ± 0.174 for male donors vs. 1.283 ± 0.880 for female donors (mean ± standard deviation, $p = 0.946$), and the band volume of estrogen receptor-β normalized to 18S rRNA was 1.249 ± 0.114 for males, vs. 1.073 ± 0.145 for females (mean ± standard deviation, $p = 0.104$). The positive-control MCF-7 cells expressed higher levels of estrogen receptor-α and estrogen receptor-β mRNA than human periodontal ligament cells, and no-template controls showed no PCR product band (Fig. 2).

Western blottong showed that estrogen receptor-β protein, not estrogen receptor-α protein, was expressed in human periodontal ligament cells from both male and female donors. No significant difference in band volume was found between human periodontal ligament cells from the two sexes. The band volume of estrogen receptor-β normalized to β-actin was 0.078 ± 0.018 for male donors vs. 0.065 ± 0.010 for female donors (mean ± standard deviation, $p = 0.317$). However, neither monoclonal or polyclonal antibodies to estrogen receptor-α detected estrogen receptor-α protein in any of the human periodontal ligament cell samples, but they did detect highly expressed estrogen receptor-α in the MCF-7 cells (Fig. 3).

Nuclear dominant staining of estrogen receptor-β immunoreactivity was shown in human periodontal ligament cells from both the male and the female donors and in MCF-7 cells. The immunoreactive staining of the controls was negative. However, no estrogen receptor-α immunoreactivity was observed in

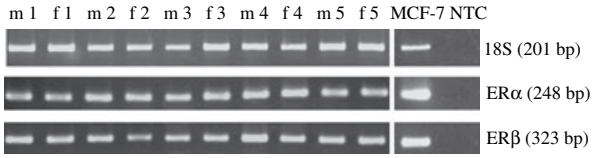

Fig. 2. mRNA expression of estrogen receptor-α and estrogen receptor-β (detected with a nested reverse transcription-polymerase chain reaction) in human periodontal ligament cells from five male and five female donors. Cells were harvested in Trizol and RNA was extracted. 18S rRNA was used as the internal control to evaluate total RNA input and MCF-7 cells were used as the positive control for estrogen receptor mRNA detection. Twenty-five cycles were performed in the second amplification in the nested reverse transcription-polymerase chain reaction. The polymerase chain reaction products were separated in a 1.5% (w/v) agarose gel. No significant difference of expression levels was found of estrogen receptor-α and estrogen receptor-β mRNA in human periodontal ligament cells between male and female donors. ERα, estrogen receptor-α; ERβ, estrogen receptor-β; f, female; NTC, no-template control; m, male.

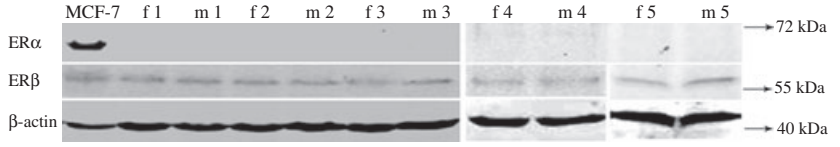

Fig. 3. Western blot analysis of estrogen receptor-α and estrogen receptor-β protein in human periodontal ligament cells from five male and five female donors. β-actin was used as the internal control to evaluate total protein of samples loaded. Two types of primary antibodies to estrogen receptor-α were applied in the experiments; this figure shows the result with the monoclonal antibody. Neither antibody was able to detect estrogen receptor-α protein in human periodontal ligament cells, whereas MCF-7 cells, the positive control, were found to express a high level of estrogen receptor-α. Both MCF-7 cells and human periodontal ligament cells expressed estrogen receptor-β protein. No significant difference in the band volume of estrogen receptor-β was detected in human periodontal ligament cells between male and female donors. ERα, estrogen receptor-α; ERβ, estrogen receptor-β; f, female; m, male.

human periodontal ligament cells, in contrast to the the positve staining of estrogen receptor-α in the nuclei of MCF-7 cells (Fig. 4A–F).

Alkaline phosphatase staining and von Kossa staining

Strong alkaline phosphatase staining occurred from day 10 after initial treatment, whereas only weak staining was detected earlier than day 10. Staining intensity and the number of positively stained cells gradually increased and become markedly higher in group M than in group C during the 21-d study period.

Mineralized nodules were seen from day 14 onwards after initial treatment in group M. On day 28, mineralized nodules were revealed by von Kossa staining in group M. No mineralized nodule was detected in group C.

Expression of estrogen receptor-α and estrogen receptor-β in human periodontal ligament cells during their osteogenic differentiation

Under osteogenic induction, the estrogen receptor-β mRNA expression level in human periodontal ligament cells gradually increased and was higher than that in controls on day 7 (2.34-fold, $p = 0.129$), day 14 (5.71-fold, $p = 0.014$) and day 21 (4.32-fold, $p = 0.006$). Furthermore, in group M, the expression level of estrogen receptor-β mRNA on day 21 was significantly higher than that on day 14 ($p = 0.034$) and on day 7 ($p = 0.042$). However, the expression level of estrogen receptor-α mRNA remained nearly constant during the differentiation process, although there was a slight, but not significant increasing trend compared with the controls (Fig. 5).

The results of western blotting showed that the expression level of estrogen receptor-β protein in group M was markedly elevated at all time-points compared with group C on day 7 (1.78-fold, $p = 0.004$), day 14 (2.73-fold, $p = 0.002$) and day 21 (2.86-fold, $p = 0.026$) in human periodontal ligament cells from the male donors, and on day 7 (1.66-fold, $p = 0.015$), day 14 (1.99-fold, $p = 0.011$) and day 21 (3.49-fold, $p = 0.024$) in human periodontal ligament cells from the female donors. No significant difference of expression level of estrogen receptor-β protein between the three time-points was found in group M of the two sexes. However, estrogen receptor-α protein was not detected in any of the cell samples with either of the two primary antibodies to estrogen receptor-α (Fig. 6).

Furthermore, the expression levels of estrogen receptors remained stable in group C during the 21-d study period.

Discussion

The present study demonstrates that estrogen receptor-β may be the dominant subtype expressed in the main constitutive cells of periodontal ligament, namely human periodontal ligament cells. By contrast to most related reports, which focused on either mRNA or protein expression of estrogen receptor subtypes in human periodontal ligament cells from one or a few donors, the present study analysed both mRNA and protein expression in human periodontal ligament cells from 10 donors and also compared the expression levels of estrogen receptors in human periodontal ligament cells between male and female donors. The results showed that although both estrogen receptor-α and -β mRNA were expressed in human periodontal ligament cells, only estrogen receptor-β protein was detected in human periodontal ligament cells and was found to be the dominant type in the nuclei of

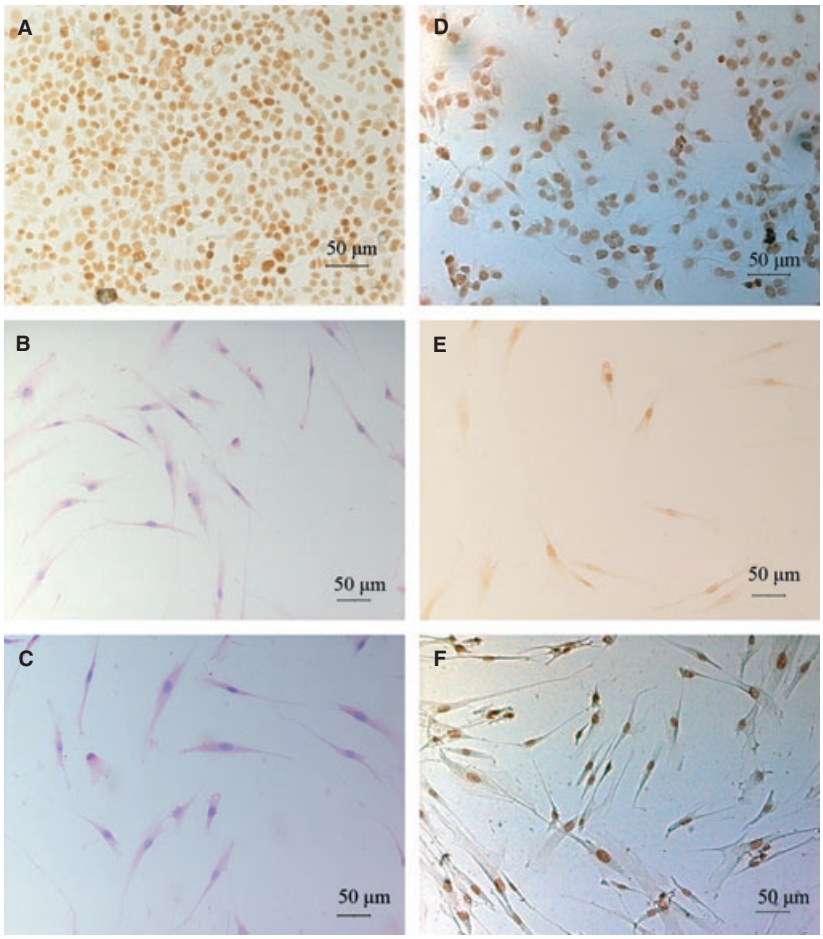

Fig. 4. Immunocytochemistry staining of estrogen receptor-α and estrogen receptor-β. Immunocytochemistry staining was carried out to localize estrogen receptor-α and estrogen receptor-β protein expressed in human periodontal ligament cells. Color was developed with diaminobenzidine in panels A, D, E and F, while the estrogen receptor-α- negative cell samples, shown in panels B and C, were stained with hematoxylin and eosin. Positive staining for estrogen receptor-α was shown in the nuclei of MCF-7 cells, the positive control (A). Human periodontal ligament cells from a male donor (B) and from a female donor (C) showed negative immunoreactivity for estrogen receptor-α. Positive staining for estrogen receptor-β was shown in the nuclei of MCF-7 cells (D), in human periodontal ligament cells from a male donor (E) and in human periodontal ligament cells from a female donor (F). Magnification: 200×.

human periodontal ligament cells. Because estrogen receptor belongs to the nuclear receptor superfamily, the positive staining of estrogen receptor in nuclei is not surprising. Furthermore, there was no significant difference in the estrogen receptor expression levels in human periodontal ligament cells between male and female donors, which indicates that estrogen may exert its biological effects in the periodontal tissues of both sexes. However, previous reports on the expression of estrogen receptor subtypes in human periodontal ligament cells have not reached the same conclusion. Parkar *et al*. could not detect estrogen receptor-α mRNA in periodontal tissues (23). However, Morishita *et al*. detected estrogen receptor-α mRNA in four sets of human periodontal ligament cells from two male and two female donors (24); moreover, positive immunostaining of estrogen receptor-β was found in the nuclei of ≈40% of human periodontal ligament cells, whereas no estrogen receptor-α immunostaining was detected (25), which supports our finding. To sum up, estrogen receptor-β may be the predominant subtype expressed in human periodontal ligament cells and may mediate estrogen's biological activities as a nuclear receptor in both men and women.

To date there is no evidence that estrogen receptor-α protein is expressed in human periodontal ligament cells, although in our study estrogen receptor-α mRNA was detected in these cells. This phemonenon may be explained as follows. First, too little estrogen receptor-α protein is expressed in human periodontal ligament cells to be detected with western blotting and immunocytochemistry. In support of this, Leimola-Virtanen *et al*. found that although estrogen receptor-α mRNA could be detected in the oral mucosa and salivary glands it could not be detected by immunohistochemistry staining, indicating a very low level of expressed protein (32). Second, human periodontal ligament cells may express some type of splice variant of estrogen receptor-α mRNA encoding a type of variant estrogen receptor-α protein that cannot be recognized by the commonly used antibodies, whereas the alternatively spliced mRNA may be easily checked by sequencing the products obtained by appropriately selected primers. The 46-kDa isoform (hERalpha46), lacking the 173 N-terminal amino acids, has been reported to be expressed in osteoblasts (33). To detect more epitopes of estrogen receptor-α protein, one monoclonal antibody and one polyclonal antibody were used in the present study. However, no estrogen receptor-α protein was detected in any of the human periodontal ligament cell samples. On the contrary, both antibodies detected highly expressed estrogen receptor-α protein in the positive-control MCF-7 cells with western blot and immunocytochemistry. Third, there may exist some manner of post-transcriptional and post-translational modification for estrogen receptor-α mRNA and protein, which may decrease estrogen receptor-α mRNA and protein stability or inhibit the protein translation process. Tateishi *et al*. found that the carboxyl terminus of Hsc70-interacting protein (CHIP) preferentially bound to misfolded unliganded estrogen

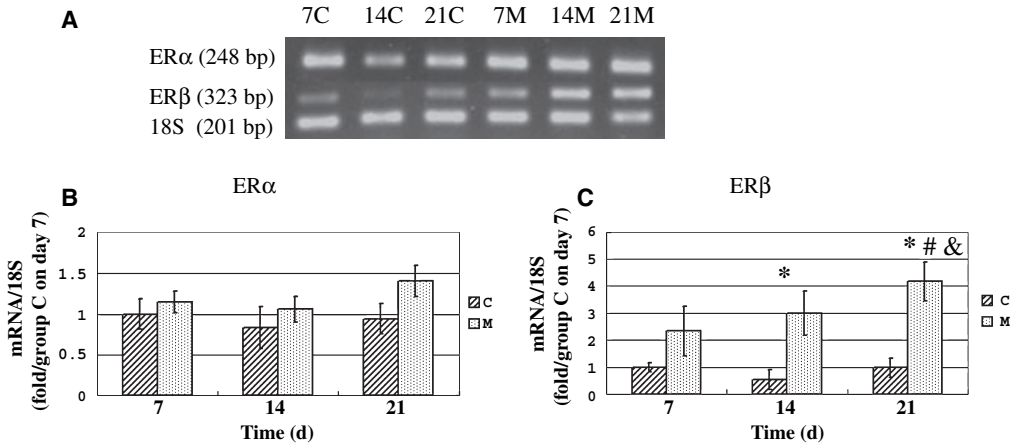

Fig. 5. mRNA expression of estrogen receptor-α and estrogen receptor-β in human periodontal ligament cells during their osteogenic differentiation, as detected using the nested reverse transcription-polymerase chain reaction. Cells were individually cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (group C) and in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 10 mM β-glycerophosphate sodium, 50 μg/mL of ascorbic acid and 10^{-7} M dexamethasone (group M). Twenty cycles were performed in the second amplification of the nested reverse transcription-polymerase chain reaction. The polymerase chain reaction products were separated in a 1.5% (w/v) agarose gel. 18S rRNA was used as the internal control to evaluate the total RNA input (A). mRNA levels of estrogen receptor-α (B) and estrogen receptor-β (C) were normalized to those of corresponding 18S rRNA. The amount of reverse transcription-polymerase chain reaction product present at different time-points was expressed relative to that found on day 7 in group C. The result is representative of three independent experiments. C, group C; M, group M. *Significant difference of expression level of estrogen receptor-β mRNA between group M and group C ($p < 0.05$). #Significant difference of the expression level of estrogen receptor-β mRNA between day 7 and day 21 in group M ($p < 0.05$). &Significant difference of the expression level of estrogen receptor-β mRNA between day 14 and day 21 in group M ($p < 0.05$). ERα, estrogen receptor-α; ERβ, estrogen receptor-β.

receptor-α protein and ubiquitinated it to induce degradation (34). As for estrogen receptor-β, the C-terminal F domain of estrogen receptor-β protects unliganded estrogen receptor-β bound with CHIP from proteolysis (35). Therefore, it is likely that unliganded estrogen receptor-α is liable to be degraded compared with unliganded estrogen receptor-β. Moreover, it is possible that estrogen receptor-α protein degradation may be active in human periodontal ligament cells so that estrogen receptor-α can be easily degraded. From the above, we conclude that human periodontal ligament cells may not express, or may express only an extremely low level of, estrogen receptor-α protein.

In the osteogenic induction experiment of this study, alkaline phosphatase staining and von Kossa staining demonstrated that human periodontal ligament cells underwent osteogenic differentiation. Both estrogen receptor-β mRNA and protein were significantly up-regulated under osteogenic induction compared with untreated controls. During osteogenic differentiation, the amount of estrogen receptor-β mRNA gradually increased with time, whereas the amount of estrogen receptor-β protein increased at the start and then remained nearly constant in human periodontal ligament cells from both male and female donors. In spite of the difference in expression profile between mRNA and protein, the up-regulation trend of estrogen receptor-β under osteogenic induction indicated that estrogen receptor-β may play an active part in the osteogenic differentiation of human periodontal ligament cells. As for estrogen receptor-α, the expression level of mRNA was slightly, and nonsignificantly, up-regulated under osteogenic induction compared with the controls, and estrogen receptor-α protein was not detected in any of the cell samples. Furthermore, the expression level of estrogen receptors remained stable in untreated cells. Therefore, it can be concluded that the up-regulation of estrogen receptor-β is induced by osteogenic induction, and estrogen receptor-β is the predominant estrogen receptor subtype that may play an active part in the osteogenic differentiation of human periodontal ligament cells, both in men and in women.

Many studies indicate the important role of estrogen receptor-β as a result of its high expression level in various osteoblast-like cell lines. Arts *et al.* found that estrogen receptor-β mRNA expression increased gradually during osteogenic induction, whereas estrogen receptor-α levels remained constant in an immortalized osteoblast-like cell line (8). Similar results were shown in our experiment. Another study reported that estrogen receptor-β mRNA was constantly and highly expressed in osteoblasts of rats during the differentiation of osteoblastic cells, whereas the estrogen receptor-α mRNA level was very low at the beginning, gradually increasing later (36). Ireland *et al.* concluded that the regulation of estrogen receptor subtype expression and the ability to mineralize depended on donor age and that the up-regulation of estrogen receptor-β is probably associated with the higher speed of mineralization and reduced proliferation (11). All these data demonstrate that both estrogen receptor subtypes play a role in osteogenic differentiation, but the regulation patterns differ with cell lines and vary with donor age and differen-

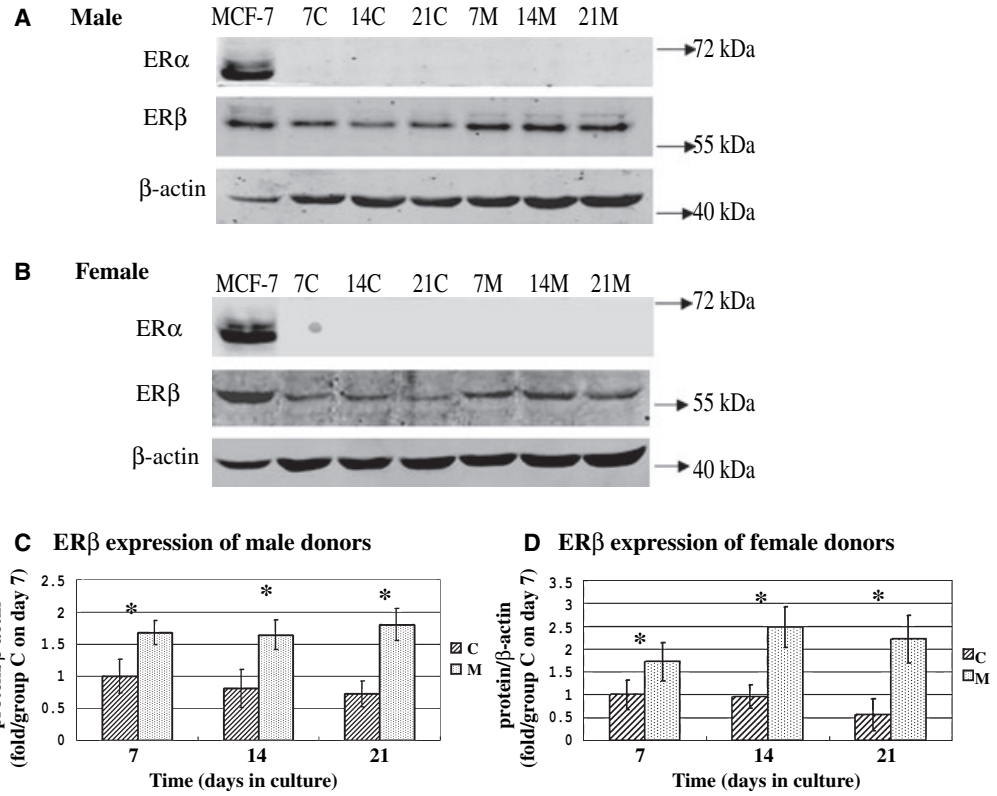

Fig. 6. Western blot analysis of estrogen receptor-α and estrogen receptor-β protein expression in human periodontal ligament cells (human periodontal ligament cells) from male (A) and female (B) donors during osteogenic differentiation. Cells were, respectively, cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (group C) and in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 10 mM β-glycerophosphate sodium, 50 μg/mL of ascorbic acid and 10^{-7} M dexamethasone (group M). On days 7, 14 and 21, cells were harvested for western blot analysis. MCF-7 cells were used as a positive control for estrogen receptor detection. β-actin was used as the internal control to evaluate total protein of the samples loaded. Details are stated in the text. The band density of estrogen receptor-β was normalized to that of the corresponding β-actin, and protein levels at different time-points were expressed relative to those found on day 7 in group C. No estrogen receptor-α protein was detected in any cell samples using two types of primary antibodies to estrogen receptor-α and the results here are shown using a polyclonal antibody (A,B). A higher level of estrogen receptor-β was expressed in group M compared with group C at various time-points, both in cells from male donors (A,C) and in cells from female donors (B,D). The findings are representative of three independent experiments. ERα, estrogen receptor-α; ERβ, estrogen receptor-β; f, female; m, male. 7C, 14C, 21C, group C on days 7, 14, or 21; 7M, 14M, 21M, group M on days 7, 14, or 21. *Significant difference of expression levels of estrogen receptor-β protein between group M and group C ($p < 0.05$).

tiation stages. It has also been shown that estrogen receptor-β may play an active part in the osteogenic differentiation of various cell lines.

The molecular mechanism by which estrogen receptors are regulated through osteogenic differentiation has not been elucidated. Many studies have shown that estrogen receptor-β is active in mediating the effects of estrogen and phytoestrogen on osteoblasts (37,38). Lindberg *et al.* concluded that estrogen receptor-β inhibits estrogen receptor-α-mediated gene transcription in the presence of estrogen receptor-α, whereas in the absence of estrogen receptor-α, estrogen receptor-β can partially replace estrogen receptor-α, which supports a 'Ying–Yang' relationship between estrogen receptor-α and estrogen receptor-β (39). In our study, because no estrogen receptor-α protein was detected in human periodontal ligament cells, estrogen receptor-β may replace estrogen receptor-α and mediate various estrogen-eliciting biological activities.

To our knowledge, this is the first report on estrogen receptor regulation during the osteogenic differentiation of human periodontal ligament cells. The results suggest that the expression and regulation of estrogen receptor-β may play an active part in the differentiation process. In further studies, estrogen receptor-β-specific agonists or estrogen receptor-β knockdown should be applied to address the function of estrogen receptor-β on the osteogenic differentiation of human periodontal ligament cells in order to understand, in greater detail, the relationship between estrogen receptor-β and periodontitis.

Acknowledgements

We especially thank Professor Ma Dalong, Associate Professor Zhao Hongshan and the staff of Peking University Center for Human Disease Genomics and Dr Li Shenglin from Laboratory of Oral and Maxillofacial Surgery Peking University School of Stomatology for their hearty help

during the study. We also would like to give our appreciation to teacher He Pingping from the Department of Epidemiology and Health Statistics, Peking University School of Public Health, for her assistance in statistics analysis. This work was supported by the Research Fund for the Doctoral Program of Higher Education of China (20060001103) and National Nature Science Foundation of China (30471882). Parts of this study was presented at 2006 FDI (Federation Dentaire International) Annual World Dental Congress (22–25 September 2006, Shenzhen, China), and the 85th General Session & Exhibition of the International & American Associations for Dental Research (21–24 March, 2007, New Orleans, Louisiana, USA).

References

1. Harris HA, Albert LM, Leathurby Y *et al.* Evaluation of an estrogen receptor-beta agonist in animal models of human disease. *Endocrinology* 2003;**144:**4241–4249.
2. Reinhardt RA, Payne JB, Maze CA, Patil KD, Gallagher SJ, Mattson JS. Influence of estrogen and osteopenia/osteoporosis on clinical periodontitis in postmenopausal women. *J Periodontol* 1999;**70:**823–828.
3. Birkenfeld L, Yemini M, Kase NG, Birkenfeld A. Menopause-related oral alveolar bone resorption: a review of relatively unexplored consequences of estrogen deficiency. *Menopause* 1999;**6:**129–133.
4. Grodstein F, Colditz GA, Stampfer MJ. Post-menopausal hormone use and tooth loss: a prospective study. *J Am Dent Assoc* 1996;**127:**370–377. quiz 392.
5. Moggs JG, Orphanides G. Estrogen receptors: orchestrators of pleiotropic cellular responses. *EMBO Rep* 2001;**2:**775–781.
6. Hall JM, McDonnell DP. The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ER-alpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology* 1999;**140:**5566–5578.
7. Monroe DG, Secreto FJ, Subramaniam M, Getz BJ, Khosla S, Spelsberg TC. Estrogen receptor alpha and beta heterodimers exert unique effects on estrogen- and tamoxifen-dependent gene expression in human U2OS osteosarcoma cells. *Mol Endocrinol* 2005;**19:**1555–1568.
8. Arts J, Kuiper GG, Janssen JM *et al.* Differential expression of estrogen receptors alpha and beta mRNA during differentiation of human osteoblast SV-HFO cells. *Endocrinology* 1997;**138:**5067–5070.
9. Bord S, Horner A, Beavan S, Compston J. Estrogen receptors alpha and beta are differentially expressed in developing human bone. *J Clin Endocrinol Metab* 2001;**86:**2309–2314.
10. Braidman IP, Hainey L, Batra G, Selby PL, Saunders PT, Hoyland JA. Localization of estrogen receptor beta protein expression in adult human bone. *J Bone Miner Res* 2001;**16:**214–220.
11. Ireland DC, Bord S, Beavan SR, Compston JE. Effects of estrogen on collagen synthesis by cultured human osteoblasts depend on the rate of cellular differentiation. *J Cell Biochem* 2002;**86:**251–257.
12. Sims NA, Dupont S, Krust A *et al.* Deletion of estrogen receptors reveals a regulatory role for estrogen receptors-beta in bone remodeling in females but not in males. *Bone* 2002;**30:**18–25.
13. Ogawa S, Hosoi T, Shiraki M *et al.* Association of estrogen receptor beta gene polymorphism with bone mineral density. *Biochem Biophys Res Commun* 2000;**269:**537–541.
14. Lau HH, Ho AY, Luk KD, Kung AW. Estrogen receptor beta gene polymorphisms are associated with higher bone mineral density in premenopausal, but not postmenopausal southern Chinese women. *Bone* 2002;**31:**276–281.
15. Scariano JK, Simplicio SG, Montoya GD, Garry PJ, Baumgartner RN. Estrogen receptor beta dinucleotide (CA) repeat polymorphism is significantly associated with bone mineral density in postmenopausal women. *Calcif Tissue Int* 2004;**74:**501–508.
16. Kung AW, Lai BM, Ng MY, Chan V, Sham PC. T-1213C polymorphism of estrogen receptor beta is associated with low bone mineral density and osteoporotic fractures. *Bone* 2006;**39:**1097–1106.
17. Zhang L, Meng H, Zhao H *et al.* Estrogen receptor-alpha gene polymorphisms in patients with periodontitis. *J Periodont Res* 2004;**39:**362–366.
18. Seo BM, Miura M, Gronthos S *et al.* Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* 2004;**364:**149–155.
19. Mukai M, Yoshimine Y, Akamine A, Maeda K. Bone-like nodules formed in vitro by rat periodontal ligament cells. *Cell Tissue Res* 1993;**271:**453–460.
20. Basdra EK, Komposch G. Osteoblast-like properties of human periodontal ligament cells: an in vitro analysis. *Eur J Orthod* 1997;**19:**615–621.
21. Morishita M, Yamamura T, Bachchu MA, Shimazu A, Iwamoto Y. The effects of oestrogen on osteocalcin production by human periodontal ligament cells. *Arch Oral Biol* 1998;**43:**329–333.
22. Morishita M, Yamamura T, Shimazu A, Bachchu AH, Iwamoto Y. Estradiol enhances the production of mineralized nodules by human periodontal ligament cells. *J Clin Periodontol* 1999;**26:**748–751.
23. Parkar MH, Newman HN, Olsen I. Polymerase chain reaction analysis of oestrogen and androgen receptor expression in human gingival and periodontal tissue. *Arch Oral Biol* 1996;**41:**979–983.
24. Morishita M, Shimazu A, Iwamoto Y. Analysis of oestrogen receptor mRNA by reverse transcriptase-polymerase chain reaction in human periodontal ligament cells. *Arch Oral Biol* 1999;**44:**781–783.
25. Jonsson D, Andersson G, Ekblad E, Liang M, Bratthall G, Nilsson BO. Immunocytochemical demonstration of estrogen receptor beta in human periodontal ligament cells. *Arch Oral Biol* 2004;**49:**85–88.
26. Somerman MJ, Archer SY, Imm GR, Foster RA. A comparative study of human periodontal ligament cells and gingival fibroblasts in vitro. *J Dent Res* 1988;**67:**66–70.
27. Owen TA, Aronow M, Shalhoub V *et al.* Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. *J Cell Physiol* 1990;**143:**420–430.
28. Gomori G. *Microscopic Histochemistry*. Chicago: University of Chicago Press, 1952:273.
29. Bellows CG, Aubin JE, Heersche JN, Antosz ME. Mineralized bone nodules formed in vitro from enzymatically released rat calvaria cell populations. *Calcif Tissue Int* 1986;**38:**143–154.
30. Tsuji N, Kamagata C, Furuya M *et al.* Selection of an internal control gene for quantitation of mRNA in colonic tissues. *Anticancer Res* 2002;**22:**4173–4178.
31. Ullmannova V, Haskovec C. The use of housekeeping genes (HKG) as an internal control for the detection of gene expression by quantitative real-time RT-PCR. *Folia Biol (Praha)* 2003;**49:**211–216.
32. Leimola-Virtanen R, Salo T, Toikkanen S, Pulkkinen J, Syrjanen S. Expression of estrogen receptor (ER) in oral mucosa and salivary glands. *Maturitas* 2000;**36:**131–137.
33. Flouriot G, Brand H, Denger S *et al.* Identification of a new isoform of the human estrogen receptor-alpha (hER-alpha) that is encoded by distinct transcripts and that is able to repress hER-alpha activation function 1. *EMBO J* 2000;**19:**4688–4700.

34. Tateishi Y, Kawabe Y, Chiba T *et al*. Ligand-dependent switching of ubiquitin-proteasome pathways for estrogen receptor. *EMBO J* 2004;**23:**4813–4823.
35. Tateishi Y, Sonoo R, Sekiya Y *et al*. Turning off estrogen receptor beta-mediated transcription requires estrogen-dependent receptor proteolysis. *Mol Cell Biol* 2006;**26:**7966–7976.
36. Onoe Y, Miyaura C, Ohta H, Nozawa S, Suda T. Expression of estrogen receptor beta in rat bone. *Endocrinology* 1997;**138:** 4509–4512.
37. De Wilde A, Lieberherr M, Colin C, Pointillart A. A low dose of daidzein acts as an ERbeta-selective agonist in trabecular osteoblasts of young female piglets. *J Cell Physiol* 2004;**200:**253–262.
38. Cao L, Bu R, Oakley JI, Kalla SE, Blair HC. Estrogen receptor-beta modulates synthesis of bone matrix proteins in human osteoblast-like MG63 cells. *J Cell Biochem* 2003;**89:**152–164.
39. Lindberg MK, Moverare S, Skrtic S *et al*. Estrogen receptor (ER)-beta reduces ERalpha-regulated gene transcription, supporting a 'ying yang' relationship between ERalpha and ERbeta in mice. *Mol Endocrinol* 2003;**17:**203–208.