J Periodont Res 2005; 40; 401–406

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2005.00821.x

Differential effects of estrogen on DNA synthesis in human periodontal ligament and breast cancer cells

D. Jönsson[1,2], Å. Wahlin[1,2], I. Idvall[3], I. Johnsson[3], G. Bratthall[2], B-O. Nilsson[1]
[1]Department of Physiological Sciences, Lund University, Lund, [2]Department of Periodontology, Faculty of Odontology, Malmö University, Malmö and [3]Department of Pathology, Helsingborg Hospital, Helsingborg, Sweden

Jönsson D, Wahlin Å, Idvall I, Johnsson I, Bratthall G, Nilsson B-O: Differential effects of estrogen on DNA synthesis in human periodontal ligament and breast cancer cells. J Periodont Res 2005; 40: 401–406. © *Blackwell Munksgaard 2005*

Background: It is important to clarify the biological function of the female sex hormones estrogen and progesterone in periodontal ligament cells, as these hormones may affect periodontal health. We have previously shown that human periodontal ligament cells express estrogen receptor β (ERβ) but not ERα, whereas human breast cancer cells (MCF7) express both ERα and ERβ. Data on progesterone receptor (PgR) expression in human periodontal ligament cells have not been reported.

Objectives: Determine PgR expression in human periodontal ligament and MCF7 cells and to investigate how estrogen affects DNA and collagen synthesis in these two cell types showing different pattern of expression for ERα and β.

Methods: Periodontal ligament cells were obtained from the periodontal ligament of premolars extracted for orthodontic reasons and MCF7 cells from the American Type Culture Collection (ATCC). PgR expression was determined by immunocytochemistry. DNA and collagen synthesis was determined by [^{3}H]thymidine and L-[^{3}H]proline incorporation, respectively.

Results: PgR immunoreactivity was observed in nuclei of MCF7 but not periodontal ligament cells. Treatment with estrogen (17β-estradiol, E_2) at physiological concentrations for 24 h stimulated DNA synthesis by more than two times in MCF7 cells, whereas there was no effect on periodontal ligament cell DNA synthesis. The ER blocker ICI 182780 fully reversed the stimulatory effect of E_2. Not only short-term (24 h) but also long-term (5 days) treatment with E_2 lacked effect on DNA synthesis in periodontal ligament cells. Neither periodontal ligament cell viability nor collagen synthesis was affected by E_2 treatment. Identical results were observed in periodontal ligament cells from male and female subjects.

Conclusions: Human MCF7 but not periodontal ligament cells express PgR, suggesting that progesterone via PgR affects MCF7 but not periodontal ligament cells. Further, estrogen stimulates breast cancer MCF7 cell proliferation, whereas it has no effect on proliferation of periodontal ligament cells, probably reflecting cell type specific ER expression pattern in these two cell types.

Dr Bengt-Olof Nilsson, Department of Physiological Sciences, Section for Molecular and Cellular Physiology, Lund University, BMC F12, SE-221 84 Lund, Sweden
Tel. +46 46 2227769
Fax: +46 46 2224546
e-mail: bengt-olof.nilsson@mphy.lu.se

Key words: breast cancer cells; DNA synthesis; estrogen; periodontal ligament cells

Accepted for publication March 6, 2005

Two estrogen receptor subtypes, designated ERα and ERβ, have been identified (1–3). These receptors are coded by individual genes and they show a tissue specific expression pattern (4–6). We have recently shown by immunocytochemistry that human periodontal ligament cells express ERβ but not ERα, whereas the human breast cancer cell line MCF7 expresses both ER subtypes, suggesting that ERβ in periodontal ligament cells and ERα together with ERβ in MCF7 cells mediate estrogen-induced effects (7). The different cellular expression of ER subtypes in periodontal ligament and MCF7 cells suggests that estrogen may have different effects in these two cell types. When investigating the effects of female sex hormones on periodontal ligament cell function, not only estrogen but also progesterone have to be taken into consideration. To our knowledge there are no reports on progesterone receptor (PgR) expression in periodontal ligament cells.

The periodontal ligament is a connective tissue, which is composed of extracellular matrix and periodontal ligament cells. The ligament connects the tooth with the surrounding alveolar bone tissue. The periodontal ligament cells possess some osteoblast-like features, e.g. production of the bone-associated protein osteonectin, as described by Somerman *et al.* (8). Estrogen has been reported to stimulate bone formation capacity in cultured periodontal ligament cells by increasing alkaline phosphatase activity, the production of osteocalcin and the formation of mineralized nodules (9, 10). Estrogen might, however, also affect other important biological properties of periodontal ligament cells, such as collagen synthesis and growth. It is well known that estrogen influences cellular proliferation in many of its classical target tissues such as uterus (4), but less information is available regarding effects of estrogen on cell-growth and protein synthesis in non-classical target tissues such as periodontal ligament tissue.

The aims of the present study were to determine PgR expression in human periodontal ligament and MCF7 cells and to investigate how estrogen affects DNA and collagen synthesis in these two cell types showing different pattern of expression for ERα and β. We determined PgR expression in human periodontal ligament and MCF7 cells by immunocytochemistry and elucidated the effects of estrogen on DNA synthesis and collagen formation by measuring incorporation of radiolabelled thymidine and proline.

Material and methods

Preparation of periodontal ligament cells and experimental procedure

The periodontal ligament cells were obtained from the root surface of premolars extracted for orthodontic reasons from four patients (two males and two females) 13–15 years of age. The patients and their parents were informed and the parents gave written consent. The study was approved by the Human Ethical Committee at Lund University, Lund, Sweden. The periodontal ligament was gently scraped off from the middle third of the root surface to avoid contamination from the gingival and apical tissues. The tissue explants were transferred to cell culture dishes with Dulbecco's modified Eagle's medium supplemented with antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml), glutamine (1.16 g/l) and 10% fetal calf serum. The dishes were placed in a water-jacketed cell/tissue incubator with 5% CO_2 in air. Cells were allowed to migrate from the explants and after reaching confluence the cells were trypsinated (0.25%) and reseeded at a density of 80,000 cells/ml. Experiments were performed on subconfluent cells in passages 3–5. The human breast cancer cell line MCF7 cells (ATCC, Manassas, VA, USA) were cultured as the periodontal ligament cells and used for experiments in passages 13–15.

For immunocytochemistry the cells were allowed to grow on polylysine-coated glass coverslips. The normal culture medium was exchanged for phenol red-free culture medium containing dextran-coated charcoal stripped fetal calf serum before experiments to remove the estrogen-like activity of phenol red and the estrogens derived from fetal calf serum. In order to have the cells in the same phase of the cell cycle, the cells were made quiescent by omitting fetal calf serum from the culture medium for 24 h. Then the cells were pre-treated for 2 h with or without 0.1, 10 or 100 nM 17β-estradiol (E_2, Sigma Chemicals, St. Louis, MO, USA) and thereafter submaximal concentrations (1 or 5%) of charcoal stripped fetal calf serum was introduced. The ER blocker ICI 182780 (kind gift from Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK) was introduced 1 h before E_2 and was then present throughout the incubation. Controls received ethanol (< 0.1%) as vehicle. The periodontal ligament and MCF7 cells were treated with or without E_2 for 24 h. In some experiments the cells were treated with E_2 for 5 days.

Immunocytochemistry

The cells were fixed in a mixture of 2% formaldehyde and 0.2% picric acid in phosphate buffer (pH 7.2) followed by rinsing in Tyrode's solution with 10% sucrose. The PgR antibody was a monoclonal mouse anti-human PgR antibody (DAKO, M3569, DAKO A/S, Glostrup, Denmark) at dilution 1:50. This concentration was shown to achieve optimal nuclear staining with minimal unspecific staining. The cells were stained using the APAAP procedure. Counterstaining to delineate nuclei was performed with Mayer's haematoxylin. For negative controls, the PgR antibody was omitted. At each staining, periodontal ligament and MCF7 cells were run in parallel. No immunoreactivity was observed when the PgR antibody was omitted.

Determination of DNA and collagen synthesis

DNA and collagen synthesis was determined by measuring the incorporation of methyl-[^{3}H]thymidine (10 μCi, Amersham Biosciences Europe, Uppsala, Sweden) and L-[^{3}H]proline (10 μCi, Amersham) into newly synthetized DNA and collagen, respectively, during the last hour of incubation with E_2 as described by Liang *et al.* (11). Collagen synthesis

was not determined in MCF7 cells, as these cells do not synthesize collagen (12). The incubation with radiolabelled thymidine and proline was stopped by placing the flasks on ice. After washing, the cells were trypsinated and then centifuged at 4500 ***g*** for 5 min at 4°C. Thereafter, the cells were were sonicated in 5 mM NaOH for 2×10 s. Aliquots of the homogenate was precipitated with 5% trichloroacetic acid and centrifuged at 10,621 ***g*** for 2 min at 4°C. After washing twice with trichloroacetic acid the pellet was dissolved in soluene. Liquid scintillation cocktail was added and radioactivity measured in a liquid scintillation counter (Beckman LS6500, Beckman Instruments Inc., Fullerton, CA, USA). Radioactivity was expressed as d.p.m. and normalized to the total protein concentration. Protein was determined using Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) based on the Lowry method (13).

Determination of cell viability with trypan-blue exclusion test

Cell viability was determined by the trypan-blue exclusion test. After removing culture medium and washing with 0.9% NaCl the cells were incubated for 2 min with 0.4% trypan-blue. The cells were then washed three times to remove unspecific staining and the number of cells that contained trypan-blue was determined as a measure of dead/dying cells.

Statistics

Values are presented as means ± SEM. Student's two-tailed *t*-test for unpaired data was used to evaluate statistical significance. *P*-values less than 0.05 were regarded as statistically significant.

Results

Immunocytochemistry

No nuclear PgR immunoreactivity was observed in the periodontal ligament cells (Fig. 1A). The staining shown in Fig. 1 is from cells derived from a female subject. Also periodontal ligament cells from male subjects lacked PgR immunoreactivity. In contrast to periodontal ligament cells, the human breast cancer cell line MCF7 cells expressed nuclear PgR immunoreactivity (Fig. 1B).

Effects of estrogen on collagen and DNA synthesis in periodontal ligament cells

The effects of treatment with E_2 on cellular collagen and DNA synthesis were determined in periodontal ligament cells derived from male and female subjects stimulated with submaximal concentrations (1 or 5%) of the well-known growth promotor fetal calf serum. The effects of E_2 on collagen and DNA synthesis were identical whether the cells were stimulated with 1 or 5% fetal calf serum. As seen in Fig. 2A, treatment with 100 nM E_2 for 24 h had no effect on collagen synthesis.

Treatment with E_2 (100 nM) for 24 h had no effect on DNA synthesis (Fig. 2B). Also, at lower concentrations (0.1 and 10 nM) E_2 had no effect on DNA synthesis. Long-term (5 days) treatment with 10 nM E_2 caused no change in DNA synthesis compared to controls (15629 ± 6437 d.p.m. per mg protein in control cells vs. 15583 ± 6912 d.p.m. per mg protein in E_2 treated cells, $n = 3$ in each group). Identical results were observed in cells derived from male and female subjects. Trypan-blue exclusion test showed very few trypan-blue containing periodontal ligament cells both in control and E_2 treated (100 nM) culture flasks, suggesting high cell viability. No difference was observed between control cells and cells treated with E_2, showing that E_2 has no effect on periodontal ligament cell viability via an unspecific toxic effect.

Effects of estrogen on DNA synthesis in MCF7 cells

In MCF7 cells, treatment with 100 nM E_2 for 24 h enhanced the fetal calf serum (1%) induced proliferation by

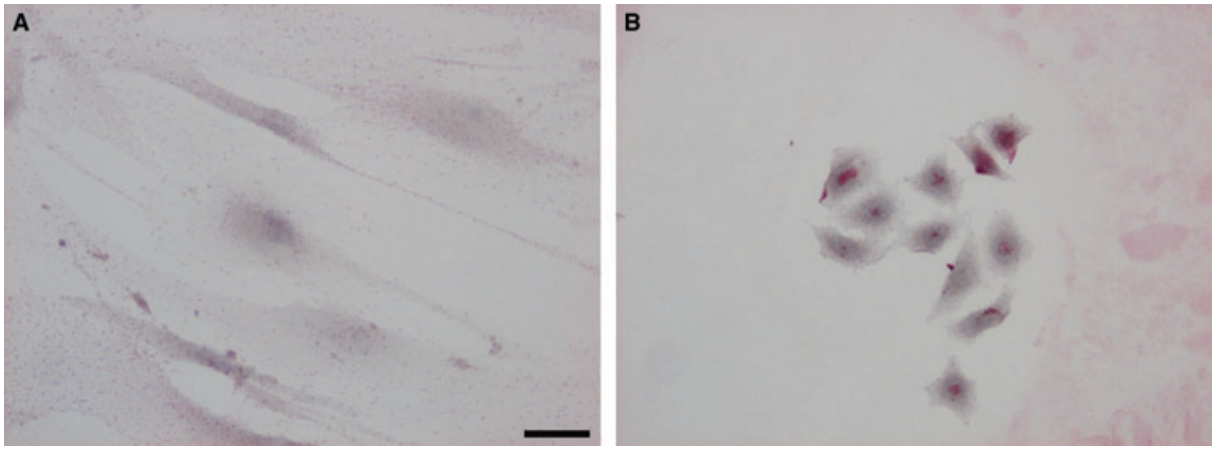

Fig. 1. Human periodontal ligament cells lack nuclear progesterone receptor (PgR) immunoreactivity (A). In contrast to periodontal ligament cells, the human breast cancer cell line MCF7 cells show nuclear PgR immunoreactivity (red-stained nuclei in B). The cells were immunostained with a PgR antibody at dilution 1:50 (monoclonal mouse anti-human PgR antibody, DAKO, M3569). These periodontal ligament cells are derived from a female subject. Identical results were obtained in cells from male subjects. Bar in A represents 20 μm (for A and B).

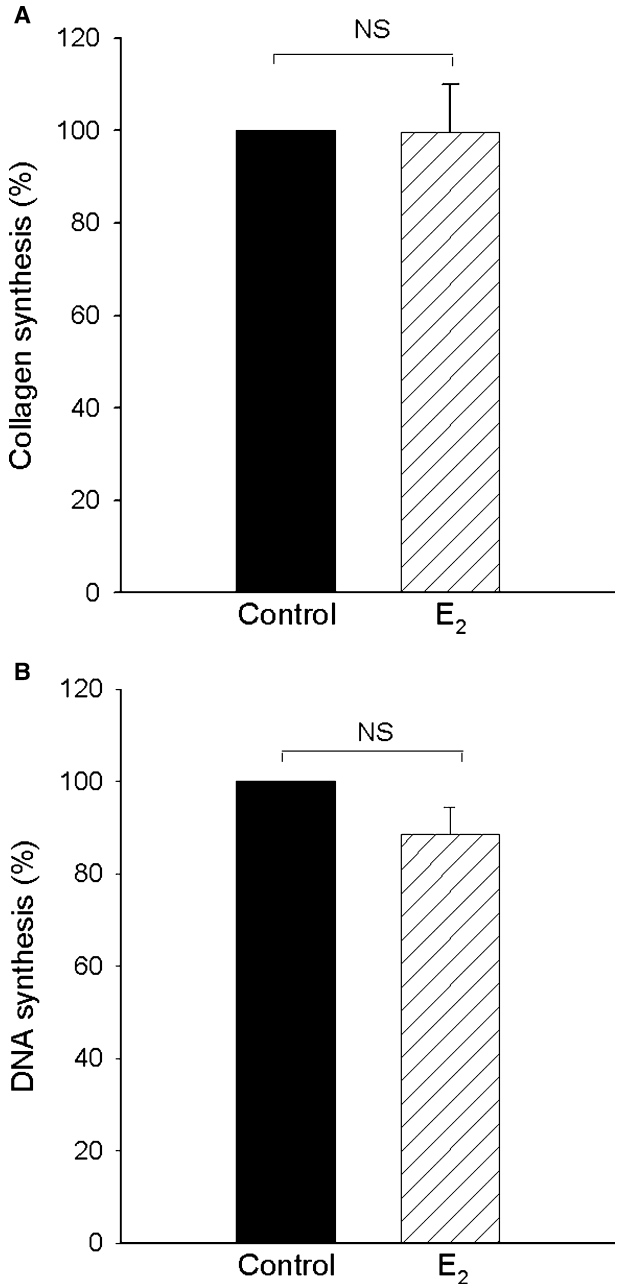

Fig. 2. Effects of treatment with 17β-estradiol (E_2, 100 nM) for 24 h on collagen (A) and DNA (B) synthesis in human periodontal ligament cells. The cells were derived from both male and female subjects. Collagen synthesis was determined as [^{3}H]proline incorporation into newly synthetized collagen, whereas DNA synthesis was determined as [^{3}H]thymidine incorporation into newly synthetized DNA. Radioactivity was determined as d.p.m. and normalized to total protein concentration. Control values were set to 100%. Values are means ± SEM of 6–10 determinations in each group. NS = no significant difference.

more than two times (Fig. 3). The effect of E_2 was fully blocked by the pure and specific ER blocker ICI 182780 (10 μM), showing that E_2-induced stimulation of proliferation is ER dependent (Fig. 3). In fact, co-incubation of E_2 with ICI 182780 reduced DNA synthesis below the control level, suggesting that fetal calf serum stimulated breast cancer cell proliferation involves ER regulated mechanisms and pathways. Also at a lower (10 nM) concentration E_2 augmented DNA synthesis (6785 ± 515 d.p.m. per ng protein in control cells vs. 18419 ± 2974 d.p.m. per ng protein in E_2 treated cells, $n = 3$ in each group, $p < 0.05$).

Discussion

Periodontal ligament cell function may be affected by the female sex hormones estrogen and progesterone. We have recently reported that human periodontal ligament cells express ERβ but not ERα, whereas human breast cancer cell line MCF7 expresses both ERα and ERβ (7). These data suggest that estrogen influences functional properties of periodontal ligament and MCF7 cells via the ERβ and ERα/ERβ, respectively. In the present study we show that the estrogen E_2 at physiological concentrations stimulates DNA synthesis in MCF7 cells, whereas it has no effect on periodontal ligament cell DNA synthesis. E_2 activates both ERα and ERβ (14, 15), and thus by using this ligand, we cannot discriminate between ERα and ERβ mediated effects. The difference in proliferation-response to estrogen between MCF7 and periodontal ligament cells probably reflects different cellular distribution of ER subtypes. In vascular smooth muscle cells, another cell type with specific expression pattern of ERα and ERβ (16–20), estrogen has been reported to inhibit proliferation (21–23). These different effects of estrogen on growth, reported in different cell systems, might be attributed to different tissue distribution of ERα and ERβ. However, it cannot be excluded that other tissue specific mechanisms are also involved.

In the present study, we demonstrate nuclear PgR immunoreactivity in MCF7 but not in periodontal ligament cells, suggesting that MCF7 but not periodontal ligament cells are under the influence of progesterone via the PgR. In previous studies MCF7 cells have been reported to express PgR (24–27), whereas no data on PgR expression in human periodontal ligament cells have been reported. Kawahara and Shimazu (27) have reported that occasional human gingival fibroblasts contain nuclear PgR immunoreactivity, whereas others are

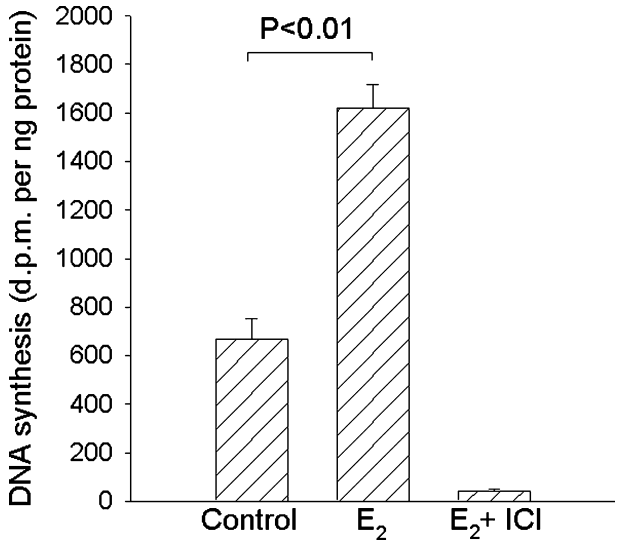

Fig. 3. Treatment with 17β-estradiol (E_2, 100 nM) for 24 h caused a pronounced stimulation of DNA synthesis (d.p.m. per ng protein) in human breast cancer cell line MCF7 cells. Co-incubation of E_2 and the pure ER blocker ICI 182780 (ICI, 10 μM) fully reversed the effect of E_2. Values are means ± SEM of three determinations in each group.

PgR negative. Our data and those reported by Kawahara and Shimazu thus suggest that PgR expression is absent in human periodontal ligament cells and low in gingival fibroblasts, and that cellular distribution of PgR may vary somewhat between cells from different oral tissues originating from the same germ layer.

Estrogen has been suggested to influence the periodontal tissues and the development of periodontal disease (28). During pregnancy, which is associated with high level of estrogen in plasma, and after menopause, which is associated with low level of estrogen in plasma, periodontal tissue properties are affected. Many pregnant women develop gingivitis and after menopause changes in periodontal tissue structure occur that may affect tooth attachment (28). The mechanisms responsible for these changes are not fully known. Periodontal ligament cells and also other cell types in the periodontal tissues may be involved.

The results presented by us in the present study show that estrogen has no effect on periodontal ligament cell collagen and DNA synthesis. Estrogen has been reported to lack effect on collagen synthesis in rat periodontal ligament demonstrated in an *in vivo* system (29). Thus, both our study and that by Dyer *et al.* (29), performed in different experimental systems, suggest that estrogen lacks effect on collagen synthesis in periodontal ligament tissue. In rat embryonic fibroblasts and skin fibroblasts, estrogen has been reported to stimulate collagen synthesis (30, 31), whereas estrogen has been reported to attenuate collagen synthesis in vascular smooth muscle cells (32). Taken together these data suggest that the effects of estrogen on collagen synthesis are cell/tissue specific.

The genes regulated by the estrogen–ER complex in periodontal ligament cells remain to be identified in order to understand how estrogen can influence periodontal function and health. Based on the data presented in this study we conclude that these genes are probably not associated with periodontal ligament cell proliferation and collagen formation.

Acknowledgements

This study was supported by grants from the Swedish Research Council, the Crafoord foundation, the Swedish Dental Society, the Medical Faculty at Lund University, Lund, Sweden and the Odontological Faculty at Malmö University, Malmö, Sweden. We thank Eva Karlsson for skilled technical assistance with cell culture work.

References

1. Green S, Walter P, Kumar V *et al.* Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature* 1986;**320:**134–139.
2. Greene GL, Gilna P, Waterfield M, Baker A, Hort Y, Shine J. Sequence and expression of human estrogen receptor complementary DNA. *Science* 1986;**231:** 1150–1154.
3. Kuiper GGJM, Enmark E, Pelto-Huiko M, Nilsson S, Gustafsson J-Å. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* 1996;**93:**5925–5930.
4. Nilsson S, Mäkelä S, Treuter E *et al.* Mechanisms of estrogen action. *Physiol Rev* 2001;**81:**1535–1565.
5. Muramatsu M, Inoue S. Estrogen receptors: how do they control reproductive and nonreproductive functions? *Biochem Biophys Res Commun* 2000;**270:**1–10.
6. Couse JF, Korach KS. Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr Rev* 1999;**20:** 358–417.
7. Jönsson D, Andersson G, Ekblad E, Liang M, Bratthall G, Nilsson B-O. Immunocytochemical demonstration of estrogen receptor β in human periodontal ligament cells. *Arch Oral Biol* 2004;**49:** 85–88.
8. Somerman MJ, Young MF, Foster RA, Moehring M, Imm GR, Sauk JJ. Characteristics of human periodontal ligament cells in vitro. *Arch Oral Biol* 1990;**35:**241–247.
9. Morishita M, Yamamura T, Bachchu MAH, Shimazu A, Iwamoto Y. The effects of estrogen on osteocalcin production by human periodontal ligament cells. *Arch Oral Biol* 1998;**43:**329–333.
10. 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.
11. Liang M, Ekblad E, Gustafsson J-Å, Nilsson B-O. Stimulation of vascular protein synthesis by activation of oestrogen receptor β. *J Endocrinol* 2001;**171:** 417–423.
12. Noel A, Munaut C, Boulvain A *et al.* Modulation of collagen and fibronectin synthesis in fibroblasts by normal and malignant cells. *J Cell Biochem* 1992;**48:** 150–161.
13. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;**193:**265–275.

14. Kuiper GGJM, Carlsson B, Grandien K *et al*. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. *Endocrinology* 1997;**138:**863–870.
15. Kuiper GGJM, Lemmen JG, Carlsson B *et al*. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. *Endocrinology* 1998;**139:**4252–4263.
16. Lindner V, Sung KK, Karas RH, Kuiper GGJM, Gustafsson J-Å, Mendelsohn ME. Increased expression of estrogen receptor-β mRNA in male blood vessels after vascular injury. *Circ Res* 1998;**83:** 224–229.
17. Aavik E, du Toit D, Myburgh E, Frösen J, Häyry P. Estrogen receptor beta dominates in baboon carotid after endothelial denudation injury. *Mol Cell Endocrinol* 2001;**182:**91–98.
18. Andersson C, Lydrup M-L, Fernö M, Idvall I, Gustafsson J-Å, Nilsson B-O. Immunocytochemical demonstration of oestrogen receptor β in blood vessels of the female rat. *J Endocrinol* 2001;**169:** 241–247.
19. Critchley HO, Brenner RM, Henderson TA *et al*. Estrogen receptor β, but not estrogen receptor α, is present in the vascular endothelium of the human and nonhuman primate endometrium. *J Clin Endocrinol Metab* 2001;**86:**1370–1378.
20. Liang M, Nilsson B-O. Proteasome-dependent degradation of ERα but not ERβ in cultured mouse aorta smooth muscle cells. *Mol Cell Endocrinol* 2004;**224:**65–71.
21. Watanabe T, Akishita M, Nakaoka T *et al*. Estrogen receptor β mediates the inhibitory effect of estradiol on vascular smooth muscle cell proliferation. *Cardiovasc Res* 2003;**59:**734–744.
22. Morey AK, Pedram A, Razandi M *et al*. Estrogen and progesteron inhibit vascular smooth muscle proliferation. *Endocrinology* 1997;**138:**3330–3339.
23. Iafrati MD, Karas RH, Aronovitz M *et al*. Estrogen inhibits the vascular injury response in estrogen receptor α-deficient mice. *Nat. Med.* 1997;**3:**545–548.
24. Jin R, Bay BH, Chow VT, Tan PH, Lin VC. Metallothionein 1E mRNA is highly expressed in oestrogen receptor-negative human invasive ductal breast cancer. *Br J Cancer* 2000;**83:**319–823.
25. Zabaglo L, Ormerod MG, Dowsett M. Measurement of markers for breast cancer in a model system using laser scanning cytometry. *Cytometry* 2000;**41:**166–171.
26. Kanai H, Barrett JC, Metzler M, Tsutsui T. Cell-transforming activity and estrogenicity of bisphenol-A and 4 of its analogs in mammalian cells. *Int J Cancer* 2001;**93:** 20–25.
27. Kawahara K, Shimazu A. Expression and intracellular localization of progesterone receptors in cultured human gingival fibroblasts. *J Periodont Res* 2003;**38:**242–246.
28. Mascarenhas P, Gapski R, Al-Shammari K, Wang H-L. Influence of sex hormones on the periodontium. *J Clin Periodontol* 2003;**30:**671–681.
29. Dyer RF, Sodek J, Heersche JNM. The effect of 17β-estradiol on collagen and noncollagenous protein synthesis in the uterus and some periodontal tissues. *Endocrinology* 1980;**107:**1014–1021.
30. Hurst AG, Goad DW, Mohan M, Malayer JR. Independent downstream gene expression profiles in the presence of estrogen receptor alpha or beta. *Biol Reprod* 2004;**71:**1252–1261.
31. Surazynski A, Jarzabek K, Haczynski J, Laudanski P, Palka J, Wolczynski S. Differential effects of estradiol and raloxifene on collagen biosynthesis in cultured human skin fibroblasts. *Int J Mol Med* 2003;**12:**803–809.
32. Barchiesi F, Jackson EK, Imthurn B, Fingerle J, Gillespie DG, Dubey RK. Differential regulation of estrogen receptor subtypes α and β in human aortic smooth muscle cells by oligonucleotides and estradiol. *J Clin Endocrinol Metab* 2004;**89:**2373–2381.