Estrogen Deficiency Leads to Impaired Osteogenic Differentiation of Periodontal Ligament Stem Cells in Rats

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Estrogen deficiency in post-menopausal women is considered as one of the risk factors for periodontal diseases. The periodontal ligament is a connective tissue that connects cementum and alveolar bone to constrain teeth within the jaw. Periodontal ligament stem cells (PDLSCs) isolated from the periodontal ligament can differentiate into many types of specialized cells, including osteoblast-like cells that can be used to regenerate alveolar bone. However, little is known about the effect of estrogen-deficient microenvironment on the osteogenic differentiation of PDLSCs. The aim of this study was to explore the role of estrogen on the potential for osteogenic differentiation of PDLSCs using a rat model of osteoporosis. Three-month-old female Sprague-Dawley rats were divided into two groups (n = 6 for each): ovariectomized (OVX) and sham-operated rats (Sham). Then the characteristics of PDLSCs isolated from these rats were investigated. Real-time PCR analysis showed the lower expression levels of estrogen receptors (ERα and ERβ) mRNAs in PDLSCs of OVX animals compared to Sham control. Mineralization assay demonstrated fewer calcium deposits in PDLSCs from OVX group than those from Sham group. Treatment with 17β-estradiol (E2) significantly enhanced the osteogenic differentiation of PDLSCs from both groups in vitro. Furthermore, by means of lentivirus-mediated siRNA targeting ERα or ERβ, the expression of ERα or ERβ was down-regulated (> 50% reduction), which impaired the estrogen-induced osteogenic differentiation of PDLSCs from both groups (> 50% reduction). These results indicate that estrogen plays an important role in maintaining osteogenic differentiation of PDLSCs, which acts through both ERα and ERβ.

Keywords: ovariectomized rats; periodontal ligament stem cells; estrogen; estrogen receptor; osteogenic differentiation


Estrogen deficiency, which typically happens in post-menopause, has been proved effective in accelerating the bone remodeling process which usually results in higher levels of bone resorption than bone formation, hence a net loss in bone mass (Robert and Heaney 2003). Nowadays, osteoporosis is considered as one of the risk factors for periodontal disease and tooth loss (Brennan et al. 2007; Gomes-Filho et al. 2007). Clinical observations in post-menopausal women have confirmed an increased prevalence of periodontal disease with low estrogen levels, even when oral hygiene remains unchanged (Krall et al. 1994; Reinhardt et al. 1999). Animal experiments with ovariectomized (OVX) rats have also clarified that estrogen deficiency may result in low mineral density in the mandible (Kuroda et al. 2003). However, the precise effects of estrogen depletion on periodontal tissues at the molecular level are unknown, so we would like to provide more valuable clues to discover the mechanism of the effects of estrogen deficiency on periodontal regeneration.

The periodontal ligament (PDL) is a specialized, vascular, and highly cellular connective tissue attaching cementum to the inner wall of alveolar bone and maintaining teeth in situ. It has been demonstrated that human PDL contains a heterogeneous population of cells (Lekic et al. 2001; Murakami et al. 2003) capable of differentiating into cementoblasts or osteoblasts in vitro. Recently, Seo et al. (2004) proved that human periodontal ligament cells contained stem cells (periodontal ligament stem cells, PDLSCs) and the application of these stem cells is a promising therapeutic approach for reconstruction of tissues destroyed by periodontal diseases.

Estrogen actively participates in bone metabolism and

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exhibits multifunctional regulation in many types of cells including periodontal ligament cells (PDLCs) (Morishita et al. 1999). It modulates the activity of target cells by binding specific intracellular estrogen receptors (ER). ER exists in two different isoforms: ERα and ERβ (Kuiper et al. 1996; Tremblay et al. 1997). Previous studies have shown that both ERα and ERβ were expressed in rat bone marrow mesenchymal stem cells (BMSCs) and human PDLCs (Tang et al. 2008; Hong et al. 2009). Also, in a transgenic mouse model, it was demonstrated that both ERs play important roles in the bone metabolism (Sniekers et al. 2009). Therefore, it is necessary to determine the expression levels of the two receptors in periodontal tissues, which may help to reveal the mechanism under which estrogen exerts its effects.

Insufficient estrogen is believed to be one of the major causes of postmenopausal osteoporosis and it is also considered to be one of the risk factors for periodontitis. However, to the best of our knowledge, there is no sufficient information concerning the osteogenic differentiation of PDLSCs under estrogen-deficient microenvironments. Therefore, it is of great interest to explore the role of estrogen on the osteogenic differentiation of PDLSCs in OVX rats. In the present study, we harvested PDLSCs from OVX rats and investigated the molecular mechanism of their osteogenic differentiation. We believe that this research will help us to know the effects of estrogen and its receptors on periodontal tissues at the molecular level.

**Materials and Methods**

**Animals and osteoporosis model**

All procedures were carried out according to the guidelines of Animal Care Committee of Fourth Military Medical University (Xi'an, Shaanxi, China). Twelve three-month-old female Sprague-Dawley (SD) rats were divided into two groups and randomly assigned to the OVX group and Sham group. The acclimatized rats were either sham-operated or bilaterally ovariectomized using the dorsal approach (Wronski et al. 1989). The rats were anesthetized with ether. A single longitudinal skin incision was made on the dorsal midline at the level of the kidneys. Both ovaries were ligated and removed in the OVX. The rats in the Sham group underwent sham surgery. Three months later, molars were extracted for cells culture and the assay was repeated 3 times.

**Osteogenic induction**

PDLSCs were plated at a density of 2 × 10³ cells/well in 96-well plates for 4 h at 37°C. Then the supernatant was removed and 150 μl of dimethyl sulfoxide (DMSO) was added following 10 min of oscillation, and the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] assay to evaluate metabolic rate of cells as previously described (Ma et al. 2009). PDLSCs were plated at a density of 2 × 10³ cells/well in 96-well plates for 4 h at 37°C. Then the supernatant was removed and 150 μl of dimethyl sulfoxide (DMSO) was added following 10 min of oscillation, and the MTT assay was carried out at day 1, 3, 5, 7, 9, 11 and 13 according to the manufacturer’s instructions. After washing, positive cells were segregated with a magnetic particle separator and subsequently seeded into 75 cm² culture flasks (Costar at 37°C in 5% CO₂. PDLSCs at passage 3 were used in each experiment. Totally 6 parallel cultures were performed and for each subsequent experiment, at least 3 replicates were included for analysis.

**Flow cytometry analysis**

PDLSCs and PDLCs were collected and washed with phosphate buffered-saline (PBS), then were re-suspended in PBS containing 2% BSA and 0.1% sodium azide. Cell aliquots (3 × 10³ cells/100 μl) and incubated on ice with fluorescein isothiocyanate (FITC)-coupled antibodies against STRO-1 and CD146 (Beckman Coulter, Fullerton, USA) or FITC- coupled nonspecific mouse IgG (isotype control) for 30 min. The cells were then washed with PBS and resuspended in 1 ml of PBS for FACS analysis. The data were analyzed, and positive expressions were defined as a level of fluorescence greater than 95% of the corresponding isotype-matched control antibodies. Detection of each marker was repeated three times.

**Cell culture and PDLSCs isolation**

The PDL cells were isolated as described elsewhere (Kaneda et al. 2006; Gay et al. 2007). All procedures were carried out according to the guidelines of the Animal Care Committee of Fourth Military Medical University. Six OVX and six Sham SD rats were included. We extracted four first molars from each rat. Periodontal ligament tissue was gently scraped from the surface of the middle part of the root, minced into 1 mm³ cubes, and placed into 6-well culture dishes (Costar Company, Cambridge, MA). The explants were grown in phenol-red free Eagle's minimum essential medium alpha modification (α-MEM; Sigma Chemical, St. Louis, MO, USA) supplemented with 10% charcoal stripped and heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA), 100 U/ml penicillin G (Sigma, St. Louis, MO, USA) and 100 μg/ml streptomycin (Sigma). The cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂.

Then, PDLSCs were obtained as previously described (Zheng et al. 2009). Briefly, STRO-1⁺ stem cells were obtained by using immunomagnetic beads (Dynabeads; Dynal Biotech, Oslo, Norway) according to the manufacturer’s instructions. After washing, positive cells were segregated with a magnetic particle separator and subsequently seeded into 75 cm² culture flasks (Costar) at 37°C in 5% CO₂. PDLSCs at passage 3 were used in each experiment. Totally 6 parallel cultures were performed and for each subsequent experiment, at least 3 replicates were included for analysis.

**Cell growth rate assay**

Cells were subjected to MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] assay to evaluate metabolic rate of cells as previously described (Ma et al. 2009). PDLSCs were plated at a density of 2 × 10³ cells/well in 96-well plates for 4 h at 37°C. Then the supernatant was removed and 150 μl of dimethyl sulfoxide (DMSO) was added following 10 min of oscillation, and the MTT assay was carried out at day 1, 3, 5, 7, 9, 11 and 13 according to the manufacturer’s instructions. The optical density (OD) values for each well were measured spectrophotometrically at 490 nm, and the assay was repeated three times.

**Osteogenic induction**

PDLSCs were plated at 5 × 10³ cells/100 cm² in 24-well plates. At 24 h the medium was changed to an osteogenic-inducing medium (CambrexBio Science, Walkersville, MD, USA) for 15 days to induce mineralization. Alizarin red staining was performed to assess mineralization ability as previously reported (Wu et al. 2008). The nodule area per well was measured quantitatively with an image analysis system (Image-Pro Plus 5.0; Media Cybernetics, Baltimore, MD) to quantify mineralization.
Measurement of alkaline phosphatase activity

The standard protocol was manipulated as previously described (Ma et al. 2009). Briefly, Cells were seeded at a density of 1 × 10^5 cells/well in 96-well plates. After incubation in the medium for 24 h, they were changed with fresh medium, respectively. The alkaline phosphatase (ALP) activity of cells was determined using an ALP assay kit (JianCheng Co, Nanjing, China) according to the manufacturer’s instructions. The optical density value was detected spectrophotometrically at 520 nm by using a microplate reader (Bio Tek Instruments, Winooski, VT), and the measurement was repeated 3 times.

Real-time Polymerase Chain Reaction

We harvested PDLSCs in osteogenic media for 5, 7, 9, 11, and 13 days. Total cellular RNA was extracted by using the TRizol Reagent (Invitrogen Life Technology, Carlsbad, CA), and first-strand cDNA synthesis was performed by High Capacity cDNA Archive Kit (Applied Biosystems, Warrington, UK) according to the manufacturer’s protocol. Real-time PCR products were detected with Quantitect Sybr Green Kit (Toyobo, Osaka, Japan) by using Light Cycler Instrument (Roche, Basel, Switzerland). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified as internal control. Primer sequences for ERα (GenBank accession no. NM012689), ERβ (GenBank accession no. NM012754), ALP (GenBank accession no. NM013059), bone sialoprotein (BSP) (GenBank accession no. NM 012587), and GAPDH (GenBank accession no. NM 017008) were as follows: (1) ERα-sense, 5’-GCG CCT CCC TTC TTG TA -3’, and ERα-antisense, 5’-GGA GAG TCT CTC TGC GCC ATT -3’; (2) ERβ-sense, 5’-AAC TCT ATG CTG CTT CTC TCT CAC -3’, and ERβ-antisense, 5’-CTT CAT GCT GAG CAG ATG TTC C-3’ (Braniste et al. 2010); (3) ALP-sense, 5’-AAC GTG GCC ACG AAC ATC ATC A-3’, and ALP-antisense, 5’-TGT CCA TCT CCA GCC GTG TC-3’; (4) BSP-sense, 5’-TGT GGA ATG GTG CTA CGG TCT C-3’, and BSP-antisense, 5’-GAT CAA CAG CCC TGA TTA ACG ATG-3’; (5) GAPDH-sense, 5’-GAC AAT TTT GGC ATC GTG GA-3’, and GAPDH-antisense, 5’-ATG CAG GGA TGA TGT TCT GG-3’ used as an internal control. Relative quantization was done by using the ΔCt method by taking the difference (∆Ct) between the ∆Ct of GAPDH and ∆Ct of each transcript and computing ∆Ct. The amplification condition for ALP, BSP, and GAPDH was denaturing at 95°C for 30 s, 35 cycles by Takara (Bio Co Ltd, Kyoto, Japan). The amplification condition for ERα, ERβ, and GAPDH and ∆Ct of each transcript and computing ∆Ct. The culture supernatants containing the lentivirus were harvested and ultra-acentrifuged. Then, cells were grown to 70-80% confluence for cell infection. They were infected with lentivirus-siRNA-ERα, lentivirus-siRNA-ERβ or lentivirus-siRNA-Ctr. The culture supernatants containing the lentivirus were harvested and ultra-centrifuged. Then, cells were treated with E2 (10^{-7} M). Three separate experiments were performed.

Western blot analysis

We confirmed the gene silencing effect by Western blot analysis as previously described (Li et al. 2008). Briefly, cell extracts containing 30 ug total protein were directly subjected to SDS-PAGE and transferred. The membranes were blocked and probed with primary antibodies that recognize ERα, ERβ, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were chosen according to the species of origin of the primary antibodies and detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA). Three separate experiments were performed.

Statistical analysis

Descriptive statistics were reported as mean ± s.d. The differences of body weight, estrogen level, BMD and nodule area between two groups were analyzed with Student’s t-test. Repeated measures ANOVA analyses (with Bonferroni confidence interval adjustment) were used and conducted for MTT assay and dynamic changes of ALP activity, mRNA expression, which were justified as normal distribution by one-sample Kolmogorov–Smirnov test (data not shown). Analysis of variance followed by least significant difference (LSD) test was the method used to compare the different study groups for the results of E2 treatment and genetic manipulation. P < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 16.0 software (SPSS Inc., Chicago, IL, USA).

Results

Body weight, estrogen level and femoral BMD

There were no significant differences in body weight, estrogen level and femoral BMD between two groups before the experiment. Three months later after ovariec-
tomy, the data of two groups were as follows. Body weight: 359.1 ± 26.9 g (OVX group) and 310.5 ± 15.2 g (Sham group); Estrogen level: 40.78 ± 3.73 ng/L (OVX group) and 69.80 ± 5.48 ng/L (Sham group); Femoral BMD: 0.059 ± 0.01 g/cm^2 (OVX group) and 0.091 ± 0.01 g/cm^2 (Sham group) (Fig. 1A, B, C). All data showed significant differences between two groups (p < 0.05), indicating that we have established a rat model of osteoporosis successfully.

Characterization and morphological appearance of rat PDLSCs

By flow-cytometric analysis we observed PDLSCs...
exhibited a high expression profile for the markers STRO-1 and CD146 compared with PDLCs. This result showed that we obtained the enriched PDLSCs from rat PDLCs (Fig. 2A).

By microscope we observed PDLSCs from two groups at day 5. Both presented a polymorphic appearance, some of which were spindle-shaped and fibroblast-like, and some were cuboidal or polygonal in appearance. The number of PDLSCs from OVX group was larger than that of Sham group (Fig. 2B, C).

Proliferation of induced and untreated PDLSCs was described with a growth curve using the MTT method (Fig. 2D). At the first day, cells of both groups were at the same low level. Next, with a short period, exponential growth began. Both cell types were plated and measured at day 1, 3, 5 and 7. At day 5, PDLSCs from OVX group presented a statistically significant \((p < 0.05)\) difference in cell number (nearly twice as much as Sham group). At day 7 and day 9 the difference between two groups became small but still remained significant \((p < 0.05)\). Then, the metabolic rate of OVX cells decreased while that of Sham cells increased and at day 11, 13 there was no significant difference between two cell populations.

**Dynamic changes of ALP activity, ER and relative makers**

It is well known that ALP is a marker for osteogenic differentiation. Our data clearly demonstrated that there was significantly lower ALP activity in the OVX group compared to that of Sham group at almost every time point except for day 1 and day 13 (Fig. 3A). ALP activity in two groups quickly increased as they differentiated and reached a peak level at day 11, and then declined gradually.

Real-time PCR analysis presented that the mRNA expressions of ER\(\alpha\) and ER\(\beta\) associated with ALP, BSP were all up-regulated when immersed in osteogenic-induced media. The up-regulation trend indicated that both ER\(\alpha\) and ER\(\beta\) may play an active part in the osteogenic differentiation of PDLSCs from two groups. However, the expression level of these makers was different between OVX and Sham groups. In each day, OVX PDLSCs seemed to show lower expression of either ERs or osteogenic makers than Sham cells (Fig. 3B, C, D, E).

**Mineralization ability**

When grown in osteogenic media for 15 days, Alizarin red staining was performed to assess mineralization ability as previously reported. Under the microscope cells from OVX group demonstrated fewer calcium deposits than those from Sham group (Fig. 3F, G), which was also proved by the results of nodule area analysis (Fig. 3H).

**Effects of E2 treatment and genetic manipulation**

To determine the effect of estrogen treatment on the proliferation and osteogenic differentiation of PDLSCs, E2 \((10^{-7} \text{M})\) was added into culture media of two groups (OVX and Sham). We found E2-treated PDLSCs showed increased activity of ALP level in two groups. The effects were tested by real-time PCR analysis (Fig. 4A). The results suggest estrogen may play important role in the osteogenic differentiation of PDLSCs.

Next, we tested biological function of ER\(\alpha\) and ER\(\beta\) for further study. Lentivirus-mediated specific gene siRNA was applied to down-regulate the expression of ER\(\alpha\) and ER\(\beta\). The GFP reporter in the lentivirus vector helped us to detect the transfection efficiency of our delivery system, and the gene silencing effect was confirmed by Western blot analysis (Fig. 4B). After cells were treated with E2 \((10^{-7} \text{M})\) for 10 days, ALP activity assay was used to determine whether ER\(\alpha\) and ER\(\beta\) functioned in the estrogen-induced osteogenic differentiation of PDLSCs. The results showed that with the down-regulation (> 50% reduction) of either ER\(\alpha\) or ER\(\beta\) estrogen-induced ALP activity was attenuated (> 50% reduction) compared with the control group, suggesting that both ER\(\alpha\) and ER\(\beta\) were involved in estrogen-
Estrogen Maintains Osteo-Differentiation Ability of PDLSC

Fig. 2. Characterization and morphologic appearance of rat PDLSCs.
(A) Characterization of PDLSC immunophenotype in vitro. A representative diagram is given for STRO-1 and CD146 expressions in PDLSCs and PDLCs. We observed PDLSCs exhibited a high expression profile for these markers compared with PDLCs. Morphologic appearance of PDLSCs isolated from OVX (B) and Sham (C) rats. (Both groups of cells at day 5; bars = 100 um). (D) Proliferative rates of PDLSC from two groups. PDLSCs were cultured for 1, 3, 5, 7, 9, 11, and 13 days in 96 well plates with media changes every other day. All results are presented as means ± standard deviation. *Indicates statistically significant difference with $p < 0.05$ compared Sham group.
Fig. 3. Dynamic changes of ALP activity, ER and relative makers of PDLSCs.
(A) We detected ALP activity for 13 days, at day 1, 13 there was no significant difference between two groups. But on the whole, the ALP activity in OVX group was lower than that in Sham group. (B-E) Expression of ERα, ERβ, ALP, and BSP mRNA was lower in cells from OVX group than those in Sham group. (F-H) Mineralization assay in PDLSCs of two groups. (F) PDLSCs of OVX rat. (G) PDLSCs of Sham rat. Bars = 100 μm. (H) Graph represents a statistically significant difference in the number of mineralization nodules between two groups. All results are presented as means ± standard deviation. *Indicates statistically significant difference with \( p < 0.05 \) compared Sham group.
Fig. 4. Effects of E2 treatment and genetic manipulation.

(A) Estradiol-induced ALP activity in PDLSCs under the treatment of estrogen. Cells were divided into four groups: OVX-control group, OVX-estrogen treated group (10^{-7} M E2); Sham-control group, Sham-estrogen treated group. 10 days after the treatment, ALP activity was measured respectively. The effects were tested by real-time PCR analysis. All results are presented as means ± standard deviation. *Indicates statistically significant difference with p < 0.05 compared control group. (B) Western blot analysis of lentivirus-mediated downregulation of ERs in PDLSCs of two groups. In pLenti-ERα infected cells ERβ was used to show the specific silencing effect of ERα siRNA and in pLenti-ERβ infected cells ERα was used to show the specific silencing effect of ERβ siRNA. (C) Lentivirus-infected PDLSCs of two groups were treated with 10^{-7} M E2 and were cultured for 10 days. Subsequently, ALP activity was determined and Real-time PCR analysis confirmed the results in PDLSCs of two groups. All results are presented as means ± standard deviation. *Indicates statistically significant difference with p < 0.05 compared OVX-E2 Plenti-GFP group. #Indicates statistically significant difference with p < 0.05 compared Sham-E2 Plenti-GFP group.
induced osteogenic differentiation of PDLSCs. The results were also tested by real-time PCR analysis (Fig. 4C).

**Discussion**

Previous findings have shown that stem cells act as one of the fundamental underpinnings of tissue biology. They allow blood, bone, epithelia, nervous system, muscle, and myriad other tissues to be replenished by fresh cells throughout life. Stem cells lie dormant, but can be activated at particular stages of life cycle, or following injury. These potent agents are controlled within restricted tissue microenvironments known as “niches” (Spradling et al. 2001; Morrison and Spradling 2008). Since PDLSCs are similar to other mesenchymal stem cells in terms of their multi-differentiated capacity and expression of genes (Seo et al. 2004), we can assume that there be some factors in periodontal microenvironments that modulate the key molecular pathways which regulate the properties of PDLSCs. Based on this assumption, we created estrogen-deficient microenvironment by ovariectomy and investigated the effect of estrogen depletion on osteogenic differentiation of PDLSCs as well as the dynamic changes of ERα and ERβ.

A number of studies have suggested that estrogen may play an important role in the pathogenesis of periodontal diseases (Chapple 2009; Reynolds et al. 2009). Periodontal disease is more prevalent and severe in males than females. Gingival inflammation and hyperplasia frequently occur during puberty, pregnancy, and menstruation (Nyman 1971; Mombelli et al. 1989, 1990). To our knowledge, two different isoforms (ERα and ERβ) have been found. Both proteins are physiologically relevant, they bind estradiol with high affinity and activate transcription of estrogen responsive gene constructs expressed in mammalian cell lines (Kuiper et al. 1996; Tremblay et al. 1997).

Recent reports have demonstrated the importance of estrogen in the osteogenic differentiation of adult stem cells (Zhou et al. 2001; Wang et al. 2006). In vitro, they found a dose of estrogen stimulation enhanced osteogenic differentiation ability by detecting the expression of ER and osteogenic genes (Syed and Khosla 2005; Wang et al. 2006). Similarly, estrogen may modulate cytokine expression and regulate the character of human PDLCs (Cao et al. 2007; Shu et al. 2008). These findings made us wonder whether PDLSCs have the same character when exposed to specific microenvironment such as estrogen-deficient environment. The effects of increased age on periodontal ligament fibroblasts have been reported, indicating that the changes of microenvironment make PDLCS exhibit different features. However, little is known about whether PDLSCs exhibit different features in OVX and Sham rats.

In our study, we successfully isolated PDLSCs from OVX and Sham rats. While they were cultured in the same medium in vitro, we found the difference between them. Firstly, morphologically, PDLSCs from OVX group seemed to show higher proliferation rate than PDLSCs from Sham group, which was also corroborated by the results of MTT assay. But osteogenic differentiation including the ALP activity and mineralization ability decreased in OVX PDLSCs. Real-time PCR results further supported this by assessing the intrinsic expression of osteogenic markers (ALP, BSP). Also, the mRNAs of ERα and ERβ were up-regulated in two groups when immersed in osteogenic-induced media. Secondly, due to the uncertainty about the effect of estrogen and ERs in the process of osteogenic differentiation of PDLSCs, we used estrogen stimuli and RNA interference to test above findings. We found E2-treated PDLSCs showed increased activity of ALP and cells seemed insensitive to estrogen stimulation after siRNA of ERs was induced into PDLSCs of two groups. All of these indicated that estrogen-deficient microenvironment decreased osteogenic differentiation of PDLSCs from OVX rats. Both ERα and ERβ were involved in estrogen-induced effects on osteoblastic differentiation of PDLSCs.

Our study demonstrated that PDLSCs of OVX rats still kept the character originating from their special microenvironment at the beginning and presented decreased osteogenic differentiation ability. Besides, the results presented obvious time dependence which indicated by MTT assay and ALP activity. At first, cells from two groups were seeded at the same density and needed adaptation to growth conditions. So there were no differences at day 1. Then, within a short period delay, exponential growth began as well as the synthesis of RNA, enzymes and other molecules occur. The significant difference could be seen in the next several days. At last, the equalization of the two groups demonstrated that the difference became small. This phenomenon was consistent with the previous reports which indicated that the character of cells would change due to different extracellular environment. More interestingly, PDLSCs of OVX rats showed an increased proliferation but decreased osteogenic differentiation as compared with Sham group. A possible explanation for this difference is that PDLSCs are located in a specific microenvironment, and estrogen is one of the critical factors that regulate the character of proliferation. When the estrogen level is below normal, organism produces more PDLSCs in a compensatory way. However, the increased proliferative ability of the PDLSCs failed to treat the disorder caused by low estrogen level. That is why estrogen-deficient patients often suffer from osteoporosis. At the same time, we detected the dynamic changes of ERα and ERβ and found their mRNAs were expressed in PDLSCs of two groups. This result was similar to previous research (Tang et al. 2008; Hong et al. 2009), which indicated that the mRNA of ERα and ERβ were expressed in rat BMSCs and human PDLSCs. Although we have revealed this phenomenon in PDLSCs from OVX rats, the underlying mechanism is still elusive. It has been known that ERα and ERβ act on their respective roles in organism. However, it is very difficult to identify the specific role of ERα or ERβ during this process because many molecules are involved in OVX niches. Moreover, in
the present study, ER mRNA levels were not assessed in a non-osteogenic inducing media and changes in mRNA levels may not be correlated with corresponding changes in protein levels. These are key points to be explored in our future study.

In summary, the data presented here show that decreased osteogenic differentiation of PDLSCs in OVX rats may be due to deficient estrogen. Both ERα and ERβ are involved in estrogen-induced effects on osteoblastic differentiation function of PDLSCs. How ER participates in this process remains unknown. The specific mechanism is still to be explored. Elucidation of these issues will guide us toward a better understanding of PDLSCs differentiation and their optimized use in regenerative medicine.

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Conflict of Interest

The authors declare no conflict of interest.

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