Estrogen enhances the bone regeneration potential of periodontal ligament stem cells derived from osteoporotic rats and seeded on nano-hydroxyapatite/collagen/poly(L-lactide)

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Abstract. This study investigated the effects of estrogen on the bone regeneration potential of periodontal ligament stem cells (PDLSCs) derived from osteoporotic rats and seeded on a collagen-based composite scaffold [nano-hydroxyapatite/collagen/poly(L-lactide) (nHAC/PLA)]. For this purpose, 48 healthy 3-month-old Sprague-Dawley female rats were divided into 2 groups as follows: the bilaterally ovariectomized (OVX) rats and sham-operated rats. The PDLSCs were isolated at 3 months after surgery (by which time postmenopausal osteoporosis had developed), and the effects of estrogen on the characteristics of these cells seeded in a culture plate and of the cells seeded on nHAC/PLA were then investigated. The PDLSC + nHAC/PLA constructs were implanted subcutaneously into the backs of severe combined immunodeficient (SCID) mice for 12 weeks in order to examine the role of estrogen in the bone formation ability of PDLSCs derived from osteoporotic rats. The results from methyl thiazolyl tetrazolium (MTT) assay revealed that the proliferation of the cells derived from the rats in the OVX group was significantly higher than that of the cells derived from the rats in the sham-operated group at the stage of logarithmic growth. The staining intensity of alkaline phosphatase (ALP) and the mineralization of the cells derived from the rats in the OVX group was significantly weaker than that of the cells from the rats in the sham-operated group. When the PDLSCs were seeded on nHAC/PLA, ALP activity, osteocalcin (OCN) secretion, mineral formation and the mRNA expression levels of ALP, OCN, estrogen receptor (ER) and ERβ in the cells derived from the rats in the OVX group were markedly decreased. Treatment with 17β-estradiol (E2) significantly weakened the proliferative ability of the cells derived from the OVX group rats, and enhanced their osteogenic differentiation ability and the mRNA expression levels of ALP, OCN, ERα and ERβ. When the constructs were implanted into the backs of SCID mice for 12 weeks, the results of histological analysis indicated that the constructs derived from the OVX group rats had a few newly formed bones and osteoids; however, a great number of newly formed bones and osteoids were present in the ones from the sham-operated group and the OVX + E2 group rats. Our findings further indicate that estrogen deficiency impairs the osteogenic differentiation potential of PDLSCs, and that ER plays an important role in the bone regeneration ability of PDLSCs. Estrogen enhances the bone regeneration potential of PDLSCs derived from osteoporotic rats and seeded on nHAC/PLA. This study may provide insight into the clinical management of periodontal bone tissue repair in postmenopausal women with the use of estrogen-mediated PDLSCs seeded on nHAC/PLA.

Introduction

Estrogens are hormones that are important for sexual and reproductive development, mainly in women. Estrogen is instrumental in bone formation, and together with vitamin D, calcium and other hormones, it effectively breaks down and rebuilds bones according to the body’s natural processes. As estrogen levels begin to decline in middled-aged women, the process of and the ability to rebuild bone diminishes. Consequently, in postmenopausal women, the amount of bone being broken down exceeds that of bone being rebuilt (1).

Estrogen deficiency, which is vital in the pathogenesis of postmenopausal osteoporosis (2), has received increasing attention in studies examining periodontal diseases. There have been reports of periodontitis-associated bone loss in the mandibular body in ovariectomized (OVX) animals (3-6); clinical observations in postmenopausal women have confirmed an increased prevalence of periodontal disease with low estrogen levels (7-11). Symptoms, such as thinning of the mandibular inferior cortex (12,13) and residual ridge reduction of the
edentulous jaw (14,15) have been reported in patients with postmenopausal osteoporosis, which suggest a link between postmenopausal osteoporosis and the loss of periodontal tissue.

Conventional periodontal tissue regenerative therapies, such as guided tissue regeneration, depending on the individual anatomy of the defects or the amount of resident vital periodontal ligament, can partially regenerate periodontal tissues (16-21). Until now, the most ideal strategy of periodontal tissue regeneration therapies is to control inflammation and stimulate stem progenitors to regenerate new periodontal tissues. The periodontal ligaments are very important in maintaining the integrity of the periodontal tissue, which is the connective tissue located between the alveolar bone and the root surface of the tooth. The periodontal ligament stem cells (PDLSCs) of this ligament exhibit osteoblast-like characteristics and are capable of differentiating into cementoblasts or osteoblasts, and can regenerate new functional periodontal support tissues, including the cementum, the alveolar bone and the periodontal ligament fiber (22,23), and are the most ideal seed cells of periodontal tissue regeneration.

Nowadays, bone grafting procedure limitations have shifted the focus of preclinical research to tissue engineering strategies, which are strictly dependent on the proliferation and differentiation ability of mesenchymal stem cells (MSCs); however, they may be affected by genetics, aging, hormone levels, drug consumption, or chronic systemic disease (24). MSCs have the ability to self-renew, proliferate and differentiate toward different lineages (osteoblasts, adipocytes, chondroblasts and myoblasts); however, MSCs derived from bone marrow (BM-MSCs), which are the most commonly employed cells in the clinical setting for different orthopedic diseases (25), are reported to be negatively influenced by various factors, most of which are also responsible for osteoporosis (26), such as systemic diseases, lifestyle, drug consumption and aging.

Several studies have also investigated the association between changes in levels of the female sex hormone, estrogen, and changes in PDLSCs (27,28), which modulates the activity of PDLSCs by binding to the intracellular estrogen receptor (ER) (29-31). Zhang et al (32) also reported that estrogen deficiency leads to the impaired osteogenic differentiation of PDLSCs in osteoporotic rats. Nonetheless, the mechanisms responsible for the effects of estrogen on PDLSCs are poorly understood. It is important to clarify the biological function of the female sex hormone, estrogen, in PDLSCs, as this hormone may affect periodontal health. Moreover, the effects of estrogen on the bone regeneration potential of PDLSCs isolated from osteoporotic rats in vivo have not yet been fully elucidated. Therefore, it remains undetermined as to whether functional periodontal tissue can be regenerated following the transplantation of autologous PDLSCs isolated from osteoporotic animals.

The nano-hydroxyapatite/collagen/poly(L-lactide) (nHAC/PLA), a three-dimensional (3D) nanostructured scaffold, is an attractive bone substitute, as the novel biomimetic strategy used to generate this scaffold endows it with properties similar to those of natural bone. Cell culture and animal model experiments have demonstrated that the composite material is highly osteoconductive, biocompatible and bioresorbable (33,34).

In the present study, we created an estrogen deficiency microenvironment by ovariectomy and examined the effects of estrogen deficiency and estrogen supplementation on the bone-forming capacity of PDLSCs derived from osteoporotic rats and seeded on nHAC/PLA, both in vitro and in vivo. This may provide an ideal approach for functional periodontal tissue regeneration in postmenopausal women with periodontal disease.

Materials and methods

Establishment of the animal model of osteoporosis. All surgical procedures and care administered to the animals were approved by the University Animal Care Committee and performed according to institutional guidelines. A total of 48 healthy 3-month old Sprague-Dawley (SD) female rats (Experimental Animal Center of the Academy of Military Medical Sciences) were randomly divided into 2 groups as follows: 24 animals were in the bilaterally ovariectomized (OVX) group, and the other 24 animals were subjected to sham surgery (sham-operated group, hereinafter referred to as the sham group). In the sham procedure, the ovaries were exteriorized and replaced intact to create a stress similar to that obtained with bilateral ovariectomy, in accordance with the method described in the study by Yu et al (35).

Measurement of body weight, bone mineral density (BMD) and the estrogen level. At 0 (baseline) and 3 months after surgery, the SD rats from the 2 groups were anesthetized with 10% chloral hydrate (400 mg/kg) and weighed; blood samples were obtained from the retro-orbital plexus to evaluate the estrogen level. The BMD of the lumbar spine was measured using a DEXA scanner (GE Healthcare Lunar; GE Healthcare, Madison, WI, USA). To measure the lumbar spine BMD, the rats were placed in the prone position on the DEXA plate and the X-ray tube was aligned along the longitudinal axis at the mid-point of the body. The BMD value was determined using small animal analysis software (GE Lunar Prodigy, Prodigy enCORE software version 10.50.086; GE Healthcare).

Cell culture and isolation of PDLSCs. The rat PDLCs were isolated in accordance with the method described in the study by Gay et al (36). Briefly, the sham-operated and ovariectomized SD rats after 3 months of surgery were sacrificed for gathering the periodontal ligament. The PDLCs were collected from the periodontal ligament which were rinsed and cut into trivial blocks under sterile conditions and digested in 0.3% collagenase (Invitrogen, Carlsbad, CA, USA) at 37°C for 4 h on a rotator set at 130 rpm. The cells obtained were then seeded in a 75 cm² culture flask and cultured in growth medium (GM) containing Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 IU/ml streptomycin (all from Invitrogen) in a humidified atmosphere (95% air, 5% CO₂) at 37°C. The cells were fed every 3 days with GM. After the cells reached confluence, they were trypsinized and plated in the culture flasks. Subsequently, PDLSCs were obtained as previously described (37). Briefly, STRO-1⁺ stem cells were isolated using immunomagnetic beads (Dynabeads; Dynal Biotech, Oslo, Norway) according to the manufacturer's instructions. After washing, bead-positive cells were segregated using a magnetic particle separator and subsequently seeded into a 75 cm² culture flask and cultured in GM in a humidified atmosphere (95% air, 5% CO₂) at 37°C. PDLSCs at passage 4 were used in each experiment. At least 3 replicates were included for analysis.
**Phenotypic analysis of PDLSCs.** The PDLSCs at passage 4 isolated from the rats in the sham group and OVX group were seeded into chamber slides, cultured for 7 days in GM, fixed with cold acetone for 10 min and immunostained for confocal laser scanning microscopy (CLSM). The cells were blocked and permeabilized 1 h at room temperature in Tris-buffered saline solution (TBS), pH 7.4, containing 10% FBS, 1% bovine serum albumin (BSA) and 0.5% Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA). Antibody labeling was performed overnight at room temperature [vimentin 1:100 (MAB2105), keratin 1:100 (MAB3165); R&D Systems, Minneapolis, MN, USA]. Secondary antibodies were applied for 2 h at room temperature [anti-mouse IgG TRITC 1:50 (sc-3796); Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA]. Following repeated washes with phosphate-buffered saline (PBS), nuclear staining was performed with 4',6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co.) at room temperature for 15 min, and Mowiol 4-88 confocal images were recorded using a Zeiss LSM5 Pascal system with a Zeiss Axiovert microscope (Carl Zeiss, Oberkochen, Germany).

**Methyl thiazolyl tetrazolium (MTT) assay.** MTT assay was performed as previously described in the study by E et al (38). For the experiment, the cells were divided into 3 groups as follows: the sham group, the OVX group and the OVX + E2 group. Briefly, the cells at passage 4 were plated in 96-well plates at 2x10^4 cells/ml with 200 µl GM for 24 h to allow attachment. The serum-containing medium was then removed and replaced with a medium without FBS for a further 12 h. The cells in the sham group and OVX group were cultured in GM, and the cells in the OVX + E2 group were cultured in GM + 10^−7 M E2 (Sigma Chemical Co.). Following culture for days 1, 2, 3, 4, 5, 6, 7 and 8, 20 µl 5 mg/ml MTT solution/well was added to the cells, followed by culture for 4 h. The cells were washed twice with PBS, and 150 µl dimethyl sulfoxide (Sigma Chemical Co.) was then added to each well, followed by shaking for 10 min, and the optical density values for each well were determined at 490 nm.

**Cell culture protocol for histochemical analysis of alkaline phosphatase (ALP) activity and mineralization.** For the experiment, the cells were divided into 3 groups: the sham group, the OVX group and the OVX + E2 group. Briefly, the cells at passage 4 were plated at 1x10^4 cells/ml in 6-well culture plates with GM for 24 h to allow attachment. The serum-containing medium was then removed and replaced with a medium without FBS for a further 12 h. The cells in the sham group and OVX group were cultured in an osteogenic medium (ODM) [growth medium containing 10 nM dexamethasone, 50 µg/ml ascorbic acid and 100 mM β-glycerophosphate (Sigma Chemical Co.)], and the cells in the OVX + E2 group were cultured in ODM + 10^−7 M E2. For the histochemical analysis of ALP activity and mineralization, the cells were cultured for 21 days. The medium was replaced every 4 days.

To determine ALP activity, the differentiated cells cultured for 21 days were fixed with 10% formalin and stained using the Gomori calcium-cobalt method. Briefly, the cells were washed with PBS for 5 min. An incubation solution containing 5 ml 2% barbital sodium, 5 ml 3% β-sodium glycerophosphate (Sigma Chemical Co.), 10 ml 2% calcium nitrate, 5 ml 2% magnesium sulfate and 25 ml distilled water was placed on each slide followed by incubation for 4 h at 37°C. The slides were then washed with distilled water and incubated in 2% calcium nitrate for 2 min at room temperature. Subsequently, the slides were incubated in 2% cobaltous nitrate for 2 min at room temperature. The slides were then washed with distilled water and incubated in 1% ammonium sulfide for 1 min at room temperature. The slides were then washed with running tap water and left to dry.

To detect extracellular matrix calcification with Alizarin red staining, on day 21 of differentiation, the osteogenic medium-cultured cells were fixed with 10% formalin. The cells were washed with PBS for 5 min. A 2% Alizarin red solution (Sigma Chemical Co.) was placed on each slide followed by incubation for 10 min at room temperature. The slides were then washed with running tap water for 5 min and left to dry.

**Biometrics preparation of nHAC/PLA grafts.** The nHAC/PLA material (Beijing Allgens Medical Science and Technology Co., Ltd., China, http://www.allgensmed.com/cn/index.aspx) mimics the composition and the microstructure characteristics of the natural bone, the porosity is 80±10%, the pore size is 300±250 µm and the calcium phosphate content is 45±5%. The mechanical strength is 20.8 Mpa. The nHAC/PLA materials were cut into 10x4x3 or 5x4x3 mm blocks. The samples were then rinsed with 100% alcohol in order to remove organic residues and with double distilled water in order to remove inorganic residues (each solution for 10 min). The samples were then sterilized by cobalt 60.

**Seeding of cells onto nHAC/PLA grafts.** The PDLSCs isolated from the rats in the sham and OVX groups were respectively seeded onto the nHAC/PLA scaffold. The constructs were incubated in ODM for 2 h at 37°C, allowing the cells to adhere to the scaffolds and 1 ml of additional ODM was then added. The medium was replaced by ODM with or without E2 on day 4 of incubation and the grafts were then ready for in vitro experiments and in vivo implantation.

**Scanning electron microscopy.** The PDLSCs isolated from the rats in the sham and OVX group were respectively seeded onto the nHAC/PLA scaffolds at 1x10^6 cells/cm² per graft, and were then cultured in ODM. Fixative was prepared from 2% paraformaldehyde and 2.5% glutaraldehyde (Sigma Chemical Co.) in 0.1 mol/l phosphate buffer. Following fixation (30 min, 37°C), the samples from nHAC/PLA, sham PDLSCs + nHAC/PLA, OVX PDLSCs + nHAC/PLA and...
OVX PDLSCs + nHAC/PLA + E2 cultured in ODM for 7 days were rinsed twice in PBS for 10 min and then washed 5 times (15 min each) in different ethanol concentrations (50, 75, 90 and 95% v/v ethanol) in distilled water and 3 times for 10 min each in analytical ethanol. After the ethanol washes, the samples were rinsed in a series of different hexamethyldisilazane (HMDS) concentrations (33.3, 50 and 66.6% v/v) in analytical ethanol and 3 times in 100% HMDS (1 min each). The morphological characterization of the cells and materials was carried out by means of scanning electron microscopy (SEM) using a Quanta 200 ESEM/SEM, FEI (Phillips, Madison, WI, USA) with beam energies of 6-25 kV and fitted with an energy dispersive spectroscopy (EDS) apparatus. The samples were glued with conducting paste to appropriate mounting stabs, which were then coated with a several nanometer-thick layer of gold. The samples were examined under a Hitachi S-520 scanning electron microscope (Hitachi, Tokyo, Japan).

Estimation of ALP activity and osteocalcin (OCN) secretion. For the experiment, the cells were divided into 3 groups: sham PDLSCs + nHAC/PLA, OVX PDLSCs + nHAC/PLA and OVX PDLSCs + nHAC/PLA + E2. The cells at passage 4 were plated in 6-well plates, at 1x10⁵ cells/ml, with DMEM containing 10% FBS for 12 h to allow attachment. The serum-containing medium was removed and replaced with a medium without serum for 12 h, and the cells were then cultured in ODM. After 1-7, 7-14 and 14-21 days, the medium was collected from the wells.

ALP activity in the medium was assayed using a biochemical automatic analyzer (Hitachi 7600; Hitachi).

OCN in the medium was assayed using a mouse-specific IRMA (Immutopics, Inc., San Clemente, CA, USA). Briefly, the sample containing mouse OCN was incubated simultaneously with an antibody-coated bead and the 125I-labeled antibody. OCN contained in the sample is immunologically bound by the immobilized antibody and the radio-labeled antibody to form a 'sandwich' complex: bead/anti-mouse OCN, mouse OCN, 125I-anti-mouse OCN. At the end of the overnight incubation period, the bead was washed to remove any unbound labeled antibody and other components. The radioactivity bound to the bead was measured in a gamma counter. The radioactivity of the bound antibody complex is directly proportional to the bound antibody complex in the sample. As the amount of extracellular matrix proteins interferes with the total cellular protein determination, the data were determined and expressed (as ng/ml) for each culture dish.

Mineral formation assay. For this experiment, the cells were divided into 3 groups as follows: the sham PDLSCs + nHAC/PLA, OVX PDLSCs + nHAC/PLA and OVX PDLSCs + nHAC/PLA + E2 groups. The PDLSCs were respectively seeded on nHAC/PLA at 1x10⁵ cells/cm² per well/well for 24 h to allow attachment in ordinary medium. The cells were starved using serum-free medium containing 2% BSA, 100 U/ml penicillin and 100 U/ml streptomycin for 24 h. The constructs were cultured in ODM with or without 10⁻⁷ M E2. After 14 days of culture, the cells were crushed in lysis buffer (Roche, Indianapolis, IN, USA) with an RNase-free piston (Pellet), vortexed and spun. The clear cell lysate was transferred to a ‘sandwich’ complex: bead/anti-mouse OCN, mouse OCN, 125I-anti-mouse OCN. At the end of the overnight incubation period, the bead was washed to remove any unbound labeled antibody and other components. The radioactivity bound to the bead was measured in a gamma counter. The radioactivity of the bound antibody complex is directly proportional to the bound antibody complex in the sample. As the amount of extracellular matrix proteins interferes with the total cellular protein determination, the data were determined and expressed (as ng/ml) for each culture dish.

Real-time-polymerase chain reaction (RT-PCR). For the experiment, the cells were divided into 3 groups as follows: the sham PDLSCs + nHAC/PLA, OVX PDLSCs + nHAC/PLA and OVX PDLSCs + nHAC/PLA + E2 groups. The PDLSCs were respectively seeded on nHAC/PLA at 1x10⁵ cells/cm² per graft for 24 h to allow attachment in ordinary medium. The cells were starved using serum-free medium containing 2% BSA, 100 U/ml penicillin and 100 U/ml streptomycin for 24 h. The constructs were then cultured in ODM with or without 10⁻⁷ M E2. After 14 days of culture, the cells were crushed in lysis buffer (Roche, Indianapolis, IN, USA) with an RNase-free piston (Pellet), vortexed and spun. The clear cell lysate was transferred to a ‘sandwich’ complex: bead/anti-mouse OCN, mouse OCN, 125I-anti-mouse OCN. At the end of the overnight incubation period, the bead was washed to remove any unbound labeled antibody and other components. The radioactivity bound to the bead was measured in a gamma counter. The radioactivity of the bound antibody complex is directly proportional to the bound antibody complex in the sample. As the amount of extracellular matrix proteins interferes with the total cellular protein determination, the data were determined and expressed (as ng/ml) for each culture dish.

Histological and morphometric analysis of the in vivo experiments. For this experiment, the cells were divided into 4 groups as follows: the sham PDLSCs + nHAC/PLA, OVX PDLSCs + nHAC/PLA and OVX PDLSCs + nHAC/PLA + E2 groups. The PDLSCs were seeded onto nHAC/PLA scaffolds at 1x10⁵ cells/cm² per graft. Following 7 days of culture in vitro, the engineered constructs were then implanted into the backs of 8 severe combined immunodeficient (SCID) mice (age, 6-8 weeks; weight, 20 g) for in vivo bone regeneration. Briefly, the SCID mice were anesthetized by an injection of pentobarbital sodium (70 mg/kg) to the abdominal cavity, and the skin on the backs of the mice was disinfected with iodine. After a small incision was made, the 4 grafts were transplanted into the backs of the SCID mice using tweezers, and the incision was then sutured. After 12 weeks, the SCID mice were sacrificed for removing the implanted constructs. The harvested constructs were then fixed
in 10% formalin neutral buffer solution at pH 7.4 for 2 days and were then trimmed using waterproof polishing paper without demineralization and cut into 5-µm-thick sections, and stained with hematoxylin and eosin (H&E) for light microscopic observation. All the samples were analyzed microscopically and compared with the controls. Digital images were recorded.

For morphometric analysis, 5 sequential sections per implant were selected for evaluation under low magnification, allowing the coverage of the entire implant. Using a Leica-Qwin 3.2 image analysis system (Leitz DMRD; Leica Microsystems Inc., Bannockburn, IL, USA), all slides were analyzed microscopically and compared with the controls. Digital images were recorded.

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Table I. Primers used for real-time PCR with SYBR-Green.

| Gene   | Primer sequence          | Species | Product size (bp) |
|--------|--------------------------|---------|------------------|
| ERα    | F: 5’-CAT CGA TAA GAA CCG GAG GA-3’  | Rat     | 190              |
|        | R: 5’-AAG GTT GGC AGC TCT CAT GT-3’ |         |                  |
| ERβ    | F: 5’-AGC AAC TGG TGC TCA CCC T-3’  | Rat     | 94               |
|        | R: 5’-GTC CGC CAG CTT AGT GAG G-3’ |         |                  |
| ALP    | F: 5’-TCC CAC GTT TTC ACG TTT-3’  | Rat     | 140              |
|        | R: 5’-GAG ACG TTC TCC CGT TCA C-3’ |         |                  |
| OCN    | F: 5’-TGA GGA CCC TCT CTC TGC TC-3 | Rat     | 150              |
|        | R: 5’-AGG TAGCGC CGG AGT CTA TT-3  |         |                  |
| β-actin| F: 5’-CCC ATC TAT GAG GGT TAC GC-3’ | Rat     | 150              |
|        | R: 5’-TTT AAT GTC ACG CAC GAT TTC-3’|         |                  |

F, forward; R, reverse; ER, estrogen receptor; OCN, osteocalcin.

Results

Effects of ovariectomy on body weight and BMD, and estrogen levels in rats. There were no significant differences in body weight, BMD and estrogen levels between the rats in the sham and OVX groups before the experiment. Three months after the ovariectomy, the body weight of the rats in the OVX group was significantly higher than that of the rats in the sham group; however, the BMD and estrogen levels of the rats in the OVX group were significantly lower than those of the rats in the sham group (Fig. 1).

Morphology and identification of rat PDLSCs. Morphologically, the PDLSCs isolated from the rats in the sham group (Fig. 2A) and OVX group (Fig. 2B) had a triangular, spindle and fusiform shape, and expressed STRO-1, as shown by immunohistochemical staining (Fig. 2C and D). They also expressed vimentin (Fig. 2E and G), but did not express keratin (Fig. 2F and H), as shown by immunofluorescence staining. Vimentin was labeled red with TRITC, and the nucleus was labeled blue with DAPI.

Effect of estrogen on the proliferation of PDLSCs isolated from OVX rats. To observe the differences in the proliferation of cells from the sham and OVX group, and to examine the effects of estrogen on the proliferation of cells from the OVX group, MTT assay was carried out to monitor the proliferation of the 3 groups of cells (sham group, OVX group and OVX + group). The results revealed that the proliferation of the cells in the 3 groups increased gradually with the extension of the culture time, and that the proliferation of the cells in the OVX and OVX + E2 groups reached peak levels on day 7. Following culture for 2, 3, 4, 5, 6 and 7 days, the proliferation of the cells in the OVX group was significantly greater than that of the cells in the sham group. The proliferation of the cells in the OVX + E2 group was significantly lower than that of the cells in the OVX group following culture for 2, 3, 4, 5, 6, 7 and 8 days, and was significantly greater than that of the cells in the sham group following culture for 2, 3, 4, 5 and 6 days (Fig. 3).

Effects of estrogen on the osteogenic differentiation of the PDLSCs isolated from OVX rats. To observe the differences in the proliferation of cells from the sham and OVX group, and to examine the effects of estrogen on the proliferation of cells from the OVX group, MTT assay was carried out to monitor the proliferation of the 3 groups of cells (sham group, OVX group and OVX + group). The results revealed that the proliferation of the cells in the 3 groups increased gradually with the extension of the culture time, and that the proliferation of the cells in the OVX and OVX + E2 groups reached peak levels on day 7. Following culture for 2, 3, 4, 5, 6 and 7 days, the proliferation of the cells in the OVX group was significantly greater than that of the cells in the sham group. The proliferation of the cells in the OVX + E2 group was significantly lower than that of the cells in the OVX group following culture for 2, 3, 4, 5, 6, 7 and 8 days, and was significantly greater than that of the cells in the sham group following culture for 2, 3, 4, 5 and 6 days (Fig. 3).
When the cells from the OVX group were cultured in ODM + 10^{-7} E2 for 21 days, the cells exhibited intense staining for ALP (Fig. 4C), and also exhibited calcium deposition, with formed and developed mineralization nodules (Fig. 4E).

**Scanning electron microscopy.** The results of SEM revealed that the nHAC/PLA had the micro-structure characteristics of natural bone (Fig. 5A). When the constructs were cultured in ODM for 7 days, a large number of cells in the sham...
PDLSCs + nHAC/PLA (Fig. 5B), OVX PDLSCs + nHAC/PLA (Fig. 5C) and OVX PDLSCs + nHAC/PLA + E2 (Fig. 5D) groups had adhered to, and had expanded and proliferated on the nHAC/PLA. There were many filamentous extracellular matrices on the surface of the cells. Some of the cells in the sham PDLSCs + nHAC/PLA and OVX PDLSCs + nHAC/PLA + E2 groups were covered by more mineral deposits.

**Effects of estrogen on ALP activity and OCN secretion in the PDLSCs isolated from OVX rats and seeded on nHAC/PLA.** The specific ALP activity of the PDLSCs from the 3 groups reached the highest levels during the culture period of 7-14 days. Compared with the cells isolated from the sham-operated rats, the ovariectomy significantly decreased ALP activity in the cells isolated from the OVX rats during the culture period of 1-7, 7-14 and 14-21 days. Nevertheless, E2 significantly increased ALP secretion from the cells isolated from the OVX rats, and the value of ALP secretion in the OVX PDLSCs + nHAC/PLA + E2 group was significantly higher than that of the sham PDLSCs + nHAC/PLA group during the culture period 7-14 days (Fig. 6A).

OCN secretion from the PDLSCs in the 3 groups gradually increased with the extension of the culture time. Compared with the cells isolated from the sham-operated rats, the ovariectomy significantly decreased OCN secretion from the cells isolated from the OVX rats during the culture period of 1-7, 7-14 and 14-21 days. Nevertheless, E2 significantly increased OCN secretion from the cells isolated from the OVX rats, and the value of OCN secretion in the OVX PDLSCs + nHAC/PLA + E2 group was significantly higher than that of the sham PDLSCs + nHAC/PLA group during the culture period of 14-21 days (Fig. 6B).

**Effect of estrogen on mineral formation in the PDLSCs isolated from OVX rats and seeded on the nHAC/PLA scaffold.** Alizarin red staining was used to quantify calcium phosphate mineral formation in the cells on the sham PDLSCs + nHAC/PLA, OVX PDLSCs + nHAC/PLA and OVX PDLSCs + nHAC/PLA + E2 constructs following culture for 21 days. The mineral formation in the cells on the OVX PDLSCs + nHAC/PLA construct was significantly less than that of the cells on the sham PDLSCs + nHAC/PLA and OVX PDLSCs + nHAC/PLA + E2 (Fig. 9A-C) constructs, and mineral formation in the cells on the OVX PDLSCs + nHAC/PLA + E2 construct was significantly greater than that of the cells on the sham PDLSCs + nHAC/PLA (Fig. 7A).

**Effect of estrogen on the mRNA expression levels of ALP, OCN, ERα and ERβ in the PDLSCs isolated from OVX rats and seeded on nHAC/PLA.** When the PDLSCs from the sham-operated and OVX rats were seeded on the nHAC/PLA and were cultured in ODM with or without E2 for 14 days, real-time PCR analysis revealed that the mRNA expression levels of ALP (Fig. 7B), OCN (Fig. 7C), ERα (Fig. 7D) and ERβ (Fig. 7E) in the PDLSCs from the OVX rats were significantly down-regulated due to the ovariectomy. E2 significantly increased the mRNA expression levels of ALP, OCN, ERα and ERβ in...
the PDLSCs from the OVX rats, and the expression levels in the OVX PDLSCs + nHAC/PLA + E2 group were significantly higher than those in the sham PDLSCs + nHAC/PLA group.

**Histological and morphometric analysis of in vivo experiments.** The PDLSCs isolated from the sham-operated and OVX rats were seeded onto the nHAC/PLA porous scaffolds and cultured in ODM or ODM + E2 for 7 days, and the cell/scaffold constructs were then implanted subcutaneously into SCID mice. After 12 weeks, the subcutaneous implants were removed from the mice and analyzed for new bone formation. H&E staining revealed that nHAC/PLA alone exhibited no new bone formation, while the marked ingrowth of soft connective tissue into the grafts was observed (Fig. 8A). Newly formed bones were observed in the sham PDLSCs + nHAC/PLA (Fig. 8B), OVX PDLSCs + nHAC/PLA (Fig. 8C) and OVX PDLSCs + nHAC/PLA + E2 (Fig. 8D) constructs. Newly formed blood vessels were observed in the micropore of the nHAC/PLA. Osteoblastic cells lined the surface of the newly formed bone.

The results of histomorphometric analysis are summarized in Fig. 9. After 12 weeks of implantation, the percentages of bone formation area between the groups differed significantly. The percentage of bone formation area in the sham PDLSCs + nHAC/PLA (5.498±0.65%) was significantly higher than that of the OVX PDLSCs + nHAC/PLA group (3.269±0.72%), but was significantly lower than that of...
the OVX PDLSCs + nHAC/PLA + E2 group (7.573±1.1%). The nHAC/PLA alone exhibited no bone formation.

Discussion

Autologous adult stem cell-based tissue engineering and regenerative medicine has been considered a promising substitute for current clinical treatments that restore tissue and organ deficiencies (39-41). Successful tissue regeneration requires a sufficient cell population with high differentiation potential. PDLSCs can regenerate new functional periodontal support tissue, including cementum, alveolar bone and periodontal ligament fibers. Therefore, PDLSC-based tissue engineering has now emerged as a promising and ideal alternative approach for the clinical treatment of periodontal tissue defects (22,23).

However, a number of factors (including advanced age, degenerative diseases of donors and the microenvironment of systemic disease, such as hyperglycemia and estrogen deficiency) affect the PDLSC population, the proliferation rate and the differentiation potential (32,37,42), indicating that the changes in the microenvironment are responsible for PDLSCs exhibiting different characteristics.

In light of the above findings, the aim of the present study was the detailed characterization of the properties of PDLSCs derived from OVX rats. An ovariectomy was performed in order to create an estrogen-deficient microenvironment. A number of studies have suggested that an ovariectomy can create an estrogen-deficient microenvironment (32,43-46). In this study, the weight, BMD of the lumbar spine and estrogen levels in the 2 groups of rats (sham-operated and OVX rats) were examined, and these values differed significantly between the sham and OVX group at the 3rd month after surgery, but not at day 0 after surgery. The OVX rats had a higher weight, and lower BMD and a lower estrogen level. These results demonstrated that we successfully created an estrogen-deficient microenvironment by ovariectomy.

Our first goal was to define the proliferative ability of the PDLSCs in regards estrogen depletion. In our primary PDLSC cultures, we observed that morphologically, the 2 groups of cells had a triangular, spindle and fusiform shape, with no significant differences between the 2 groups. The PDLSCs from the sham group attached more easily, and expanded earlier than those from the OVX group. After the primary cells were passaged, the PDLSCs from the OVX group proliferated more rapidly. This
indicated that the estrogen deficiency enhanced the proliferative ability of the PDLSCs. However, ALP staining and Alizarin red staining demonstrated that estrogen deficiency inhibited the osteogenic differentiation of the PDLSCs in monolayer culture conditions. These results were consistent with those reported in the study by Zhang et al (32). A possible explanation for the increased proliferation is that estrogen deficiency makes an organism produce more PDLSCs in a compensative manner. However, the increased proliferative ability of the PDLSCs failed to enhance the osteogenic differentiation ability, which is why postmenopausal women often suffer from osteoporosis. However, estrogen supplements can reverse the effects of estrogen deficiency. The results from real-time PCR further confirmed this by assessing the intrinsic expression of osteogenic markers. In order to further examine the effects of estrogen deficiency on the osteogenic differentiation of PDLSCs derived from OVX rats, we also examined the mineral formation of the cells cultured in monolayer conditions and on the nHAC/PLA. The results revealed that, in monolayer conditions, more positive strong Alizarin red staining existed in the cells derived from the sham group. In 3D nHAC/PLA nanostructured scaffolds, a number of studies have demonstrated that the population, proliferation rate and differentiation potential of adult stem cells are affected by systemic diseases, lifestyle, drug consumption and aging (24,26,32,37,42). The results from the study by Bressan et al (47) demonstrated that dental pulp stem cells derived from an aged group exhibited a low proliferative and osteogenic differentiation ability in monolayer culture conditions; when cultured on hydroxyapatite (HA) nanostructured granules and used in vivo to repair critical size defects, they exhibited the same ability as those of the younger group in terms of time to repair and the quality of extracellular matrix. Their study indicates that 3D HA nanostructured granules can reverse the effects of advanced age on the proliferative ability and the osteogenic differentiation ability of dental pulp stem cells.
estrogen deficiency significantly decreased mineral formation in the cells. Previous studies have demonstrated that estrogen plays an important role in the osteogenic differentiation of PDLSCs by binding specific intracellular ER (29-32). Thus, we also examined the mRNA expression of ER in the cells derived from OVX rats. Estrogen deficiency downregulated the mRNA expression of ERα and ERβ. There were no differences between the cells cultured on 3D nHAC/PLA nanostructured scaffolds in our study and the cells were cultured in monolayer conditions in a previous study (32). When the PDLSCs derived from OVX rats were treated with estrogen, the results revealed that the estrogen-treated PDLSCs exhibited increased ALP activity, OCN secretion and mineral formation, as well as an increased mRNA expression of ALP and OCN, and ERα and ERβ. These results indicated that the estrogen-deficient microenvironment impaired the osteogenic differentiation of PDLSCs derived from OVX rats. Estrogen enhanced the osteogenic differentiation of PDLSCs derived from OVX rats and seeded on nHAC/PLA in vivo. Both ERα and ERβ were involved in the osteogenic differentiation of the PDLSCs.

In in vitro experiments, we demonstrated that treatment with estrogen restored the osteogenic differentiation capacity of the PDLSCs seeded on nHAC/PLA, which had been impaired due to estrogen deficiency. As a final goal, we examined whether the PDLSCs derived from OVX rats and seeded on 3D nanostructured scaffolds were able to undergo osteogenesis in vitro. The cells seeded on the nHAC/PLA were cultured for 7 days in vitro, and the constructs were then implanted subcutaneously into the backs of SCID mice for 12 weeks, and we then observed that the impaired PDLSCs (OVX PDLSCs + nHAC/PLA) exhibited new bone formation abilities, but their regenerative ability was significantly lower than that of the other 2 groups (sham PDLSCs + nHAC/PLA and OVX PDLSCs + nHAC/PLA + E2). This ability was comparable between the estrogen-treated group and sham group.

In conclusion, to date, a number of studies have demonstrated that postnatal stem cells can be isolated from various adult tissues, such as bone marrow (25), skeletal muscle (48), brain (49), liver (50), pancreas (51), lungs (52), heart (53) and kidneys (54), and that these cells exhibit self-renewal capacity and multilineage differentiation potential. However, there are some disadvantages associated with harvesting stem cells, such as additional damage to the body. The harvesting of stem cells from various tissues or organs can damage those organs, such as in stem cells derived from bone marrow (25), skeletal muscle (48), brain (49), liver (50), pancreas (51), lungs (52), heart (53) and the kidneys (54). Therefore, we focused on utilizing tissues that can be obtained without additional injury, as well as on new stem cell sources, such as periodontal ligament stem cells from teeth extracted for orthodontic purposes, prophylactically extracted nondecayed third molar teeth. Periodontal ligament stem cells have many advantages, and the results to date suggest that teeth are a viable source of adult MSCs for a wide range of clinical applications (22,23,37). Thus far, no investigation on the stemness of PDLSCs cultured in 3D nHAC/PLA nanostructured scaffolds and its correlation with estrogen deficiency and estrogen supplements has been performed, at least to the best of our knowledge.

Our data provide three important insights. The first is that this study clearly demonstrates that PDLSCs exhibit a response to estrogen deficiency at the 3rd month following an ovarectomy. The PDLSCs derived from OVX rats exhibit an increased proliferative ability and a weaker osteogenic differential ability. The second important conclusion is that if cells from OVX rats are cultured on 3D nHAC/PLA nanostructured scaffolds, they regain the same biological properties as those of cells cultured in monolayer conditions. Both ERα and ERβ are involved in the osteogenic differentiation of PDLSCs. The third conclusion is that estrogen can enhance the bone regeneration potential of periodontal ligament stem cells derived from OVX rats and seeded on nHAC/PLA in vivo. The impaired PDLSCs from OVX rats can form new bone, but their regenerative ability is limited. Further studies are required to investigate the in situ bone regenerative capacity of estrogen-treated PDLSCs derived from OVX rats and seeded on 3D nHAC/PLA nanostructured scaffolds.

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