Y. Liu[1,2], L. Wang[1], S. Liu[1], D. Liu[1,3], C. Chen[1,3], X. Xu[1], X. Chen[2], and S. Shi[1,3]*

[1]Center for Craniofacial Molecular Biology, Ostrow School of Dentistry, University of Southern California, 2250 Alcazar Street, CSA 103, Los Angeles, CA 90033, USA; [2]Department of Pediatric Dentistry, School of Stomatology, China Medical University, Shenyang, China; and [3]Department of Anatomy and Cell Biology, University of Pennsylvania, School of Dental Medicine, Philadelphia, PA 19104, USA; *corresponding author, songtaos@usc.edu

J Dent Res DOI: 10.1177/0022034514552675

Transplantation of SHED Prevents Bone Loss in the Early Phase of Ovariectomy-induced Osteoporosis

APPENDIX

MATERIALS & METHODS

Antibodies and Reagents

Anti-mouse alkaline phosphatase (ALP), peroxisome proliferator-activated receptor γ (PPARγ), and lipoprotein lipase (LPL) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-mouse runt-related transcription factor 2 (Runx2) antibody was purchased from Cell Signaling (Danvers, MA, USA). Anti-β-actin antibody was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). APC-conjugated anti-CD3, PerCP-conjugated anti-CD4, PE-conjugated anti-FoxP3, and PE-conjugated anti-IL17 antibodies and APC-conjugated anti-IFNγ were purchased from eBioscience (San Diego, CA, USA). The EdU imaging kit was purchased from Invitrogen (Carlsbad, CA, USA).

MicroCT Analysis

After being harvested and fixed in 4% paraformaldehyde (PFA), femurs were analyzed in a high-resolution Scanco μCT50 scanner (Scanco Medical AG, Bruttisellen, Switzerland). The specimens were scanned at a voxel size of 20 μm at 70 kVp and 200 μA. Scanned data were reconstructed with Scanco software. Datasets were loaded into Amira 5.3.1 software (Visage Imaging, Berlin, Germany) for visualization. μCT analysis followed the guidelines of the ASBMR (Bouxsein *et al*., 2010).

Isolation and Culture of SHED and Human BMMSCs

Human exfoliated deciduous incisors were obtained as discarded biological samples from children (6-8 yr old) at the Dental Clinic of the University of Southern California following the approved Institutional Review Board guidelines. Human bone marrow aspirates from healthy human adult volunteers (20-35 yr of age) were purchased from AllCells LLC (Alameda, CA, USA). Stem cells from human exfoliated deciduous teeth (SHED) and human bone marrow mesenchymal stem cells (hBMMSCs) were cultured as reported previously (Shi *et al*., 2002; Miura *et al*., 2003).

Isolation and Culture of Mouse BMMSCs

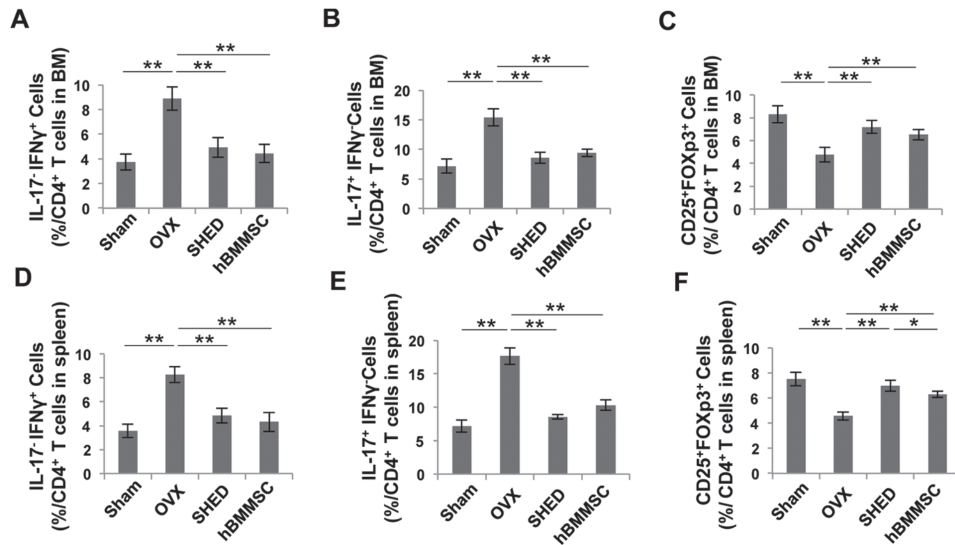
Single-cell suspensions were obtained from bone-marrow-derived all-nuclear cells (ANCs) taken from femurs, and 15×10^6 cells were seeded into 100-mm culture dishes (Corning, Tewksbury, MA, USA) at 37°C under 5% CO_2 conditions. Non-adherent cells were removed after 48 h, and attached cells were maintained for 16 days in alpha minimum essential medium (α-MEM; Invitrogen, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (FBS; Equitech-Bio, Kerrville, TX, USA), 2 mM L-glutamine, 55 μM 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). Colony-forming attached cells were passed once for further experimental use. For colony-forming unit-fibroblastic (CFU-F) assays, 1×10^6 ANCs from bone marrow were seeded into 60-mm culture dishes. After 16 days, the cultures were washed with phosphate buffered saline (PBS) and stained with 1% toluidine blue solution with 2% paraformaldehyde. Clusters with more than 50 cells were counted as colonies under microscopy.

Cell Proliferation Assay

Mouse BMMSCs (10×10^3/well) were seeded on two-well chamber slides (Nunc, Rochester, NY, USA) and cultured for 2 or 3 days. The cultures were incubated with BrdU solution (1:100) (Invitrogen) for 20 h and stained with a BrdU staining kit (Invitrogen) according to the manufacturer's instructions. The samples were then stained with hematoxylin. BrdU-positive and total cell numbers were counted in 10 images *per* subject. The number of BrdU-positive cells was indicated as a percentage of the total cell number. The BrdU assay was repeated on three independent samples for each experimental group.

In vitro Osteogenic Differentiation Assay

Mouse BMMSCs were cultured in osteoinductive medium containing 2 mM β-glycerophosphate (Sigma-Aldrich) and 100 μM L-ascorbic acid 2-phosphate (Wako, Richmond, VA, USA). After 4 wk of induction, the cultures were either stained with Alizarin Red for mineralized nodule formation or subjected to lysis for protein isolation to identify osteogenic gene expression.



Appendix Figure 1. SHED transplantation rescued OVX-induced biphasic changes of T-subtype cells. (**A-C**) Flow cytometric analysis showed that the levels of Th1 and Th17 were significantly increased in the bone marrow of OVX mice compared with the sham-operated mice. However, the levels of Tregs were significantly reduced in the bone marrow of OVX mice. SHED and hBMMSC transplantation reduced the levels of Th1 and Th17, but elevated the levels of Tregs in bone marrow of OVX mice. (**D-E**) Flow cytometric analysis showed that the levels of Th1 and Th17 were significantly increased in the spleens of OVX mice compared with the sham-operated mice. However, the levels of Tregs were significantly reduced in the spleens of OVX mice. SHED and hBMMSC transplantation reduced the levels of Th1 and Th17, but elevated the levels of Tregs in the spleens of OVX mice. n = 5 in each group. $*p < .05$; $**p < .01$; $***p < .005$. Error bars: mean ± SD.

In vitro Adipogenic Culture Conditions

Mouse BMMSCs were cultured under adipogenic culture conditions in medium containing 500 μM isobutylmethylxanthine (Sigma-Aldrich), 60 μM indomethacin (Sigma-Aldrich), 500 nM hydrocortisone (Sigma-Aldrich), 10 μg/mL insulin (Sigma-Aldrich), and 100 μM L-ascorbic acid 2-phosphate. After 7 days, the cultured cells were stained with Oil Red-O (Sigma-Aldrich) and subjected to lysis for protein isolation to identify adipogenic gene expression.

Western Blot Analysis

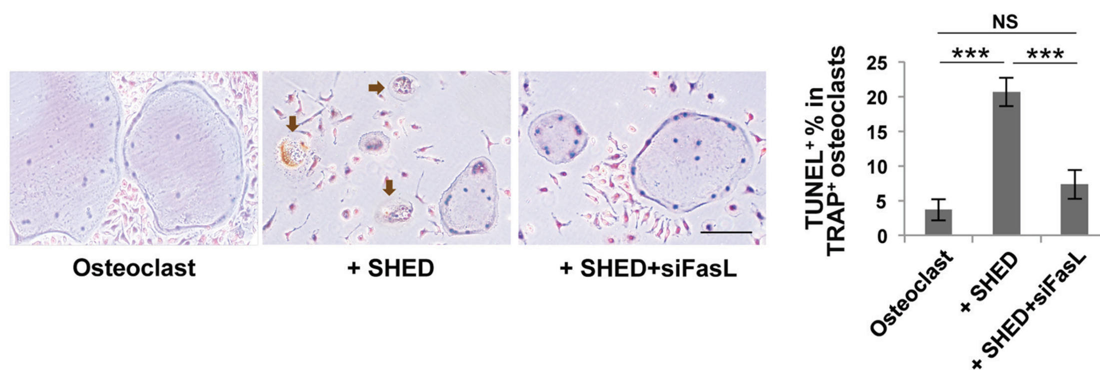
Total protein was extracted with M-PER mammalian protein extraction reagent (Thermo, Rockford, IL, USA). A 20-μg quantity of protein was applied and separated on 4–12% NuPAGE gel (Invitrogen Co.), followed by transfer to nitrocellulose membranes (Millipore Inc., Billerica, MA, USA). Membranes were blocked with 5% non-fat dry milk and 0.1% Tween20 for 1 h, followed by incubation with the primary antibodies at 4°C overnight. HRP-conjugated secondary antibody (Santa Cruz Biotechnology; 1:10,000) was used to treat the membranes for 1 h. Immunoreactive proteins were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo) and BioMax film (Kodak, Rochester, NY, USA). Each membrane was also stripped by means of a stripping buffer (Thermo) and re-probed with anti-β-actin antibody to quantify the amount of loaded protein.

Flow Cytometric Analysis

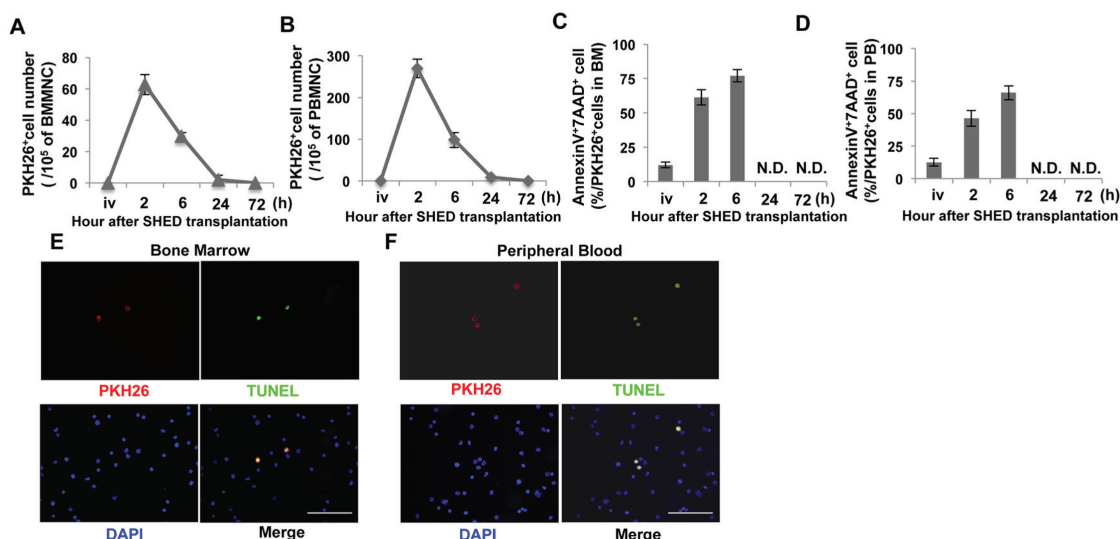
T-cells collected from mouse spleens were treated with ACK lysis buffer (Lonza, Basel, Switzerland) to remove red blood cells. $1\text{-}2\times10^6$ spleen cells were then incubated with 1 μg anti-CD4 antibody for 30 min on ice under dark conditions. For Tregs analysis, 1 μg anti-CD25 was added during the incubation. After cell fixation and permeabilization with a Foxp3 staining buffer kit, cells were stained with 1 μg of anti-Foxp3 for Tregs and anti-IFN-γ/anti-IL17 for Th1 and Th17. After being washed with PBS/0.4% BSA 3 times, cells were analyzed with a FACSCalibur flow cytometer.

T-lymphocyte Apoptosis Assay

SHED or FasL siRNA SHED (0.2×10^6) were seeded on a 24-well culture plate (Corning) containing Dulbecco's Modified Eagle's Medium (DMEM; Lonza, Basel, Switzerland) with 10% heat-inactivated FBS, 50 μM 2-mercaptoethanol, 10 mM HEPES, 1 mM sodium pyruvate (Sigma-Aldrich), 1% non-essential amino acid (Cambrex, East Rutherford, NY, USA), 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. After incubation for 24 h, T-lymphocytes (1×10^6) from the spleen, prestimulated with plate-bound anti-CD3ε (3 μg/mL) and soluble anti-CD28 (2 μg/mL) antibodies, were directly loaded onto BMMSCs and co-cultured for 2 days. Apoptotic T-cells were detected by staining with CD3 antibody, followed by use of the AnnexinV Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA) and then analyzed by use of a FACSCalibur flow cytometer equipped with CellQuest software.



Appendix Figure 2. SHED transplantation induced osteoclast apoptosis *via* FasL/Fas pathway *in vitro*. After co-culture of SHED with matured osteoclasts, deoxynucleotidyl transferase-mediated UTP end labeling (TUNEL)-tartrate resistant acid phosphatase (TRAP) double-staining showed a marked osteoclast apoptosis induced by SHED, but not siFasL-knockdown SHED. Scale bar = 50 μm; *$p < .05$; **$p < .01$; ***$p < .005$. Error bars: mean ± SD.



Appendix Figure 3. Apoptosis of transplanted SHED in peripheral blood and bone marrow. (**A, B**) PKH26-labeled SHED were transplanted into OVX mice *via* the tail vein. Bone marrow and peripheral blood samples were collected at indicated time points for flow cytometric analysis. The number of PKH26-positive transplanted SHED reached a peak at 2 h post-transplantation in bone marrow and peripheral blood and then reduced to undetectable levels at 24 h post-transplantation. (**C, D**) The number of AnnexinV+7AAD+ double-positive apoptotic SHED reached a peak at 6 h post-transplantation in bone marrow and peripheral blood and then reduced to an undetectable level at 24 h post-transplantation. (**E, F**) Immunofluorescent analysis showed TUNEL-PKH26 double-positive apoptotic SHED at 6 h post-transplantation in bone marrow and peripheral blood. n = 5 in each group. Scale bar = 50 μm. Error bars: mean ± SD.

Osteoclast Formation and Co-culture of SHED with Osteoclasts

Bone marrow cells (BMCs) were collected by frequent injection of PBS throughout the entire marrow cavity of the femurs from 10-week-old mice, and 0.5×10^6 BMCs were suspended in αMEM (Invitrogen) containing 15% FBS (Equitech-Bio, Kerrville, TX, USA), L-glutamine (Invitrogen), penicillin and streptomycin (Invitrogen), and 20 ng mL^{-1} macrophage colony-stimulating factor 1 (M-CSF) in a 24-well plate for 48 h. The adherent cells were then harvested and cultured with 20 ng mL^{-1} M- CSF (PeproTech, Rocky Hill, NJ, USA) and 50 ng mL^{-1} sRANKL (PeproTech) for another 4 days. For co-culture assay, 0.1×10^6 SHED or siFasL SHED, in normal media containing M-CSF and sRANKL, were seeded directly in each well of the 24-well plate containing osteoclasts for an additional 2 days, followed by fixation and staining for TRAP and TUNEL according to the manufacturers' protocols.

APPENDIX REFERENCES

Bouxsein ML, Boyd SK, Christiansen BA, Guldberg RE, Jepsen KJ, Müller R (2010). Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. *J Bone Miner Res* 25:1468-1486.

Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, *et al*. (2003). SHED: Stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci USA* 100:5807-5812.

Shi S, Gronthos S, Chen S, Reddi A, Counter CM, Robey PG, *et al*. (2002). Bone formation by human postnatal bone marrow stromal stem cells is enhanced by telomerase expression. *Nat Biotechnol* 20:587-591.