Differentiation of Human Mesenchymal Stem Cells: The Potential Mechanism for Estrogen-Induced Preferential Osteoblast Versus Adipocyte Differentiation

Jian-Wu Zhao, MD, PhD, Zhong-Li Gao, MD, Hong Mei, MD, PhD, Yu-Lin Li, MD, PhD and Yan Wang, MD, PhD

Abstract: *Introduction:* Bone marrow-derived mesenchymal stem cells (MSCs) are capable of differentiating into osteoblasts and adipocytes. This critical balance between osteoblast and adipocyte differentiation plays a significant role in maintaining normal bone homeostasis. In osteoporosis, a metabolic bone disease seen mainly in postmenopausal women because of estrogen deficiency, the concomitant occurrence of increased bone marrow adipocyte production with diminished production of osteoblasts, points to the potential role of estrogen in shifting the balance of MSC differentiation. *Methods:* We established an *in vitro* differentiation model of isolated human MSCs (hMSCs) and examined the role of distinct estrogen signaling pathways in regulating the differentiation of hMSCs. *Results:* Estrogen promoted the differentiation of hMSCs to osteoblasts in contrast to adipocytes, the former of which was mediated through the PI3K/SSH1L but not the mitogen-activated protein kinase pathway. *Conclusion:* This study provides a novel mechanistic understanding of estrogen-related osteoporosis and identifies potential targets for antiosteoporosis therapies.

Key Indexing Terms: Mesenchymal stem cells; Osteoporosis; Estrogen; Osteoblasts; Adipocytes. **[Am J Med Sci 2011;341(6):460–468.]**

Osteoporosis is a systemic skeletal metabolic disease characterized by the degeneration of bone microstructure, loss of bone mass and increased susceptibility to fracture.[1] It is a growing public health problem, affecting approximately 200 million people worldwide, with a tendency to worsen invariably as the mean age of the population increases in the forthcoming years.[2] In postmenopausal women, estrogen deficiency has been cited as the major cause of osteoporosis.[3] Studies comparing women undergoing spontaneous and surgically induced menopause have revealed that the lumbar bone mineral density and bone metabolism for the 2 groups are not significantly different,[4] suggesting that reduced estrogen but not aging is mainly responsible for the pathogenesis of osteoporosis. However, the underlying mechanism for estrogen-related osteoporosis remains poorly elucidated.

Estrogens are a group of steroid hormones with important roles during the estrous cycle. There are 3 main types of naturally occurring estrogens in women: estrone (E1), estradiol (E2) and estriol (E3), with E2 possessing the most potent bioactivity.[5] Estrogens signal mainly through 2 classical estrogen receptors (ERs), ERα and ERβ, which normally reside in the cytosol. On ligand binding, ERs adopt conformational changes, translocate into the nucleus and regulate the transcription of target genes.[6] Several signaling pathways have been demonstrated to be triggered by estrogen, such as growth factor and cAMP/calcium signaling, the mitogen-activated protein kinase (MAPK) pathway and the PI3K/Akt pathway,[6] However, the ER-mediated molecular signaling during the differentiation of human mesenchymal stem cells (hMSCs) remain largely elusive.

Bone is a dynamic organ composed of 3 main types of bone cells—osteoblasts, osteoclasts and osteocytes—the communication and balance between which ensure the homeostasis and normal physiological functions of the skeletal system.[7] Throughout life, bone undergoes constant remodeling through bone formation by osteoblasts and bone resorption by osteoclasts. Osteoclasts are derived from hematopoietic stem cells of the monocyte/macrophage lineage, whereas osteoblasts are derived from bone marrow MSCs and undergo terminal differentiation to form osteocytes.[8] Besides osteoblasts, bone marrow MSCs give rise to a range of cell types, including adipocytes, chondrocytes and myoblasts.[9] During osteoporosis, a shift in the differentiation of MSCs to favor the adipocyte lineage over the osteoblast lineage has been correlated with the imbalance between bone formation/resorption and ultimately bone loss.[10] In support of this correlation, *in vitro* studies demonstrated that the differentiation of MSCs to 1 lineage suppresses the differentiation of another lineage.[11,12] Further mechanistic studies have identified several signaling players in modifying adipogenic or osteoblastogenic differentiation, including PPARγ, bone morphogenetic proteins, Wnt signaling and transcriptional coactivator with PDZ-binding motif.[13]

In the current study, we examined the effects of estrogen on the differentiation of hMSCs isolated from healthy adults and explored the involvement of several estrogen targets downstream in the signaling process. Our results suggest that E2 (17 β-estradiol) promoted the osteoblastogenic differentiation and inhibited the adipogenic differentiation of hMSCs. Mechanistically, we identified a novel signaling mediator for this effect, SSH1L, and demonstrated that the responsible signaling was through the ER-PI3K/Akt-SSH1L axis but not the MAPK pathway.

MATERIALS AND METHODS

Isolation and *In Vitro* Culture of hMSCs

This study was approved by the institutional review board of Jilin University. Human bone marrow was aspirated from the iliac crest of 5 nonosteoporosic healthy donors (3 women and 2 men, age range 30–50 years) with written consent obtained. The isolation and culture of hMSCs were

From the Department of Orthopaedics (J-WZ, Z-LG) and Research Center for Gene Therapy (YW), China-Japan Union Hospital of Jilin University, Changchun, China; and The Key Laboratory of Pathobiology (HM, Y-LL), Ministry of Education, Jilin University, Changchun, China.

This study was supported by Projects of International Cooperation of Jilin Provincial Science & Technology Department (No. 20080723), and NSFC (No. 30772488).

Submitted August 23, 2010; accepted in revised form November 22, 2010.

Correspondence: Yan Wang, MD, PhD, Research Center for Gene Therapy, China-Japan Union Hospital, Jilin University, Changchun 130033, China (E-mail: wangy01@jlu.edu.cn).

performed as previously described[14] with minor modifications. Briefly, bone marrow cells were resuspended in phenol-free Dulbecco's modified Eagle's medium (DMEM; AppliChem, Darmstadt, Germany) containing 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel), centrifuged briefly to remove the supernatant that contained lipids and carefully loaded onto Percoll (density 1.073 g/mL; GE Healthcare, Piscataway, NJ). After centrifugation at 900*g* for 30 minutes with the brake off, the middle layer, containing mononuclear cells, was collected and transferred to a tissue culture dish containing DMEM with 10% FBS (complete growth medium). Twenty-four to 48 hours later, the nonadherent cells were removed, and adherent cells were maintained in complete growth medium, with the medium changed every 3 days. From the initially isolated cells that were sparsely distributed on the plate, single-cell clones gradually expanded, which were picked at a stage containing approximately 40 to 100 cells, transferred to a new 24-well plate (passage 1) and further passaged at a 1:3 ratio. At least 3 individual cells clones of isolated hMSCs from each donor were tested independently for each experiment as described below.

For treating the cells with different inhibitors, hMSCs were preincubated with ICI182, 780, a specific steroidal estrogen antagonist (working concentration 10^{-6} M; Tocris Bioscience, Bristol, United Kingdom), LY294002, a potent inhibitor for PI3K (working concentration 10 μM) (Sigma, St. Louis, MO), or PD98059, a selective MEK1 inhibitor (working concentration 20 μM) (Sigma) for 1 hour before the addition of the optimal concentration of estrogen.

Transmission Electron Microscopy

The cells were fixed in 4% glutaraldehyde, followed by postfixation in 1% osmium tetroxide solution and dehydration in a series of ethanol baths. The cells were then embedded in Epon812 resin, cut into 70-nm sections, stained with uranyl acetate and lead citrate and viewed with a JEM-1200EX transmission electron microscope.

Cell Cycle Analysis

For cell cycle distribution, the cells were fixed in 70% ethanol at 4°C overnight, washed with phosphate-buffered saline (PBS) and digested with RNase at 37°C for 30 minutes. The genomic DNA was then stained with propidium iodide (PI) at 4°C for 30 minutes and analyzed by flow cytometry.

Mapping of Growth Curve and Determination of Cell Doubling Time

To map the growth curve, 6×10^3 isolated hMSCs, growing in log phase, were split into a 24-well plate and cultured in complete growth medium at 37°C, 5% CO_2 for a total of 7 days. Every 24 hours, cells from 3 wells were stained with trypan blue and counted, and the average number was calculated. The cell doubling time was then calculated using the Patterson's formula, $\mathrm{Td} = T\log 2/\log(N_t/N_0)$, where Td is the doubling time in h; N_t and N_0 are the cell number at time t and time 0, respectively; and T is the time by which the cell number has increased from N_0 to N_t.

Flow Cytometry Analysis

Cultured adherent hMSCs were harvested with 0.25% trypsin/ethylenediamine tetraacetic acid, washed with PBS once and incubated with the relevant primary antibodies (1:50 for all antibodies used; NeoMarker, Fremont, CA) at room temperature for 30 minutes. After washing with PBS 2 more times, the cells were incubated with their corresponding secondary antibodies with fluorescein isothiocyanat (conjugated goat antimouse; 1:50; Santa Cruz, CA) at 4°C in the dark for 30 minutes. Labeled cells were washed with PBS twice and analyzed by fluorescence-activated cell sorting (BD Biosciences, San Jose, CA).

Differentiation of hMSCs

The isolated hMSCs at passage 6 were seeded into a 10-cm tissue culture plate at a density of 3×10^3 in complete growth medium for 24 hours. The cells were then washed with PBS and cultured in complete growth medium with either the basal osteoblastogenic differentiation medium (complete hMSC growth medium with 10^{-9} M dexamethasone, 10 mM sodium β-glycerophosphate and 0.05 mM ascorbic acid) or the basal adipogenic differentiation medium (complete hMSC growth medium with 0.01 mg/mL insulin, 0.2 mM indomethacin, 0.5 mM 3-isobutyl-1-methylxanthine and 0.2 μM dexamethasone), with the medium changed every 4 days and a total induction period of 24 days for osteoblasts and 14 days for adipocyte differentiations.

To determine the optimal estrogen concentration for osteoblastogenic differentiation, 17 β-estradiol at different concentrations (0, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M) was added to the cells during the differentiation and replenished every 2 days and 7 days later. The alkaline phosphatase (ALP) level in the supernatant was measured using the ALP assay kit following the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute Nanjing, China).

Cell Proliferation Assay

The hMSCs (10^3 cells/well) were seeded into a 96-well plate (5 replicates for each condition) in complete growth medium until the cells attached; then, the medium was replaced with serum-free, phenol-free DMEM for another 24 hours, followed by phenol-free DMEM with 1% FBS for a further 24 hours. The optical density of the wells was read at 450 nm. Ten microliters of cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) reagent was added into each well and incubated at 37°C for 2 hours, with the optical density read again at 450 nm. The cell proliferation was calculated as instructed by the CCK-8 kit manufacturer.

Transfection of hMSCs

The siRNA-expressing plasmids, including 1 containing a wild-type human SSH1L-targeting sequence (TCGTCACCCAAGAAAGATA, pSuper-SSH1) and 1 containing a mutant targeting sequence [TCTTCCCCCAAGAAAGATA, pSuper-SSH1L(mt)], were constructed as previously described.[15,16] The transfection of siRNA-expressing plasmids was performed with lipofectamine 2000 (Invitrogen, Carlsbad, CA), following the manufacturer's instructions.

Immunocytochemical Staining

Cells were fixed in 4% paraformaldehyde at room temperature for 20 minutes and stained with anti-ALP antibody (1:100) at room temperature for 1 hour, followed by anti-rabbit secondary antibody (1:200; Wuhan Biosynthesis Boster, Wuhan, China). The signals were developed with diaminobenzidine chromogen (Wuhan Biosynthesis Boster).

Alizarin Red S Staining for Calcium Salts

Cells were fixed with neutral buffered formalin at room temperature for 30 minutes, washed with distilled H_2O 3 times and stained with alizarin red S solution (GENMED Scientifics, Arlington, MA) at room temperature for 20 minutes. Excess dye was removed with distilled H_2O, dehydrated in acetone-