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Temporal expression of estrogen receptor alpha in rat bone marrow mesenchymal stem cells

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Abstract

Estrogen responsiveness of bone formation is mediated by the estrogen receptor alpha (ERα) in osteoblastic lineage. As osteoblasts arise from the multipotent bone marrow stromal (mesenchymal) cells, this study was undertaken to observe the ERα in primary female adult rat bone marrow mesenchymal stem cells (BMSCs). The ERα was localized using immunocytochemical analysis in identified primary BMSCs. Then, using real-time PCR analysis, we measured the expression of ERα messenger RNA (mRNA) in BMSCs. ERα transcripts showed different trends between untreated cultures (control group) and osteogenic-induced cultures (treated group). In the control group, ERα mRNA climbed at peak levels at a confluence stage and decreased until day 20, whereas, in the treated group, the ERα mRNA kept climbing from a low level until day 20. Thus, the observed developmental expression of ERα mRNA correlates with progressive BMSCs growth and osteogenic differentiation and BMSCs may be a primary target cell for estrogen in maintaining bone formation.

Keywords: Estrogen receptor alpha; Stem cells; Bone marrow derived mesenchymal stem cells; Osteogenesis; Differentiation; Rat

It is known that estrogens play a role in the function of osteoblasts [4]. These hormones prevent postmenopausal bone loss in part by directly enhancing osteoblastic activity and bone formation by transcriptional regulation via ligand-dependent estrogen receptors (ERs). The two main E_2-binding ER isoforms are ERα and ERβ. They belong to the nuclear hormone receptor super-family and mediate a variety of physiological signals [1,2] by directly activating and/or repressing gene transcription [2]. ERs have been identified in cells of the osteoblast lineage [3].

Because estrogens are one of the key factors in the regulation of bone remodeling, and the ERα has been demonstrated in osteoblasts [4,5], it is likely that ERα plays an important role in the differentiation of the osteoblast lineage. Several model systems have been developed to explore the effect of estrogen on osteoblastlike cells. Recently, analysis of genes expressed in osteoblasts has revealed that sex steroid receptor expression profiles did not exhibit the same patterns of regulation if osteoblast cultures were grown without ascorbic acid in medium that did not support extracellular matrix deposition [6]. Osteoblastic activities

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are regulated by hormones, growth factors, and cytokines. ERα mRNA in cultured rat calvarial-derived osteoblasts shows developmental expression correlating with progressive osteoblast differentiation and may be a contributing factor to differential regulation of gene expression by 17β-E_2 in bone [3]. E_2 also favored early osteogenic commitment of mouse ST_2 cells over-expressing ERα [7].

Bone marrow-derived mesenchymal stem cells (BMSCs) are multipotent progenitor cells with a capacity to form bone tissue *in vivo* [8], and are able to differentiate into the osteoblastic lineage *in vitro* [9–11]. It has been demonstrated that stromal cells isolated from bone marrow can be induced by glucocorticoids to differentiate into cells possessing the characteristics of mature osteoblasts [12]. During differentiation *in vitro*, osteoblast phenotypic markers appear in the following order: accumulation of collagenous matrix, expression of alkaline phosphatase, secretion of osteocalcin, and finally mineralization of bone nodules [13].

However, considering osteoblastic differentiation caused by estrogen, few studies have been conducted in primary BMSCs. ERs, occurring at low levels, have been detected in osteoblastic lineage *in vitro* but have not been detected in primary BMSCs. In this study, we asked whether ERα expression was related to the maturational stage of BMSCs and chose primary cultured BMSCs as our model. By examining mRNA levels of the ERα during development of the BMSCs phenotype from the proliferating cells to the mature stage, the present study was designed to answer the above question.

Materials and methods

Cell isolation and culture. All procedures were carried out according to the guidelines of the Animal Care Committee of the Fourth Military Medical University, Xi'an, China. Bone marrow cells were obtained from female adult Sprague–Dawley (SD) rats as previously described [14]. Briefly, the femurs and tibias were dissected, the ends of the bones were cut, and marrow was flushed out with 2 ml of ice-cold DMEM by using a needle and syringe. A suspension of bone marrow cells was obtained by repeated aspiration of the cell preparation through a 22-gauge needle, and nucleated cells were counted with a hemocytometer. Cells were seeded into 12-well plates or 60 mm plates at a density of 1×10^7 cells/ml and cultured for 5 days in DMEM (phenol-red free) with 15% FCS (charcoal stripped, heat-inactivated), 100 U/ml penicillin, and 100 mg/ml streptomycin, in a humidified incubator with 5% CO_2 and 95% air at 37 °C. On day 5, all non-adherent cells were removed with the first medium change and then the adherent cells (representing BMSCs) were grown for additional periods of up to 12 days. In the subsequent experiments, the beginning day of culture in the differentiation medium was defined as day 0. Treatment reagents as indicated were, respectively, added every other day and the medium was replaced every 4 days thereafter.

Flow cytometry (FCM) analysis. Early-passage BMSCs were subjected to flow cytometry to determine purity as described by Woodbury et al. [15]. Cells were harvested by treatment with 0.05% trypsin and 0.53 mM ethylenediaminetetraacetic acid, and were re-suspended in PBS containing 0.1% BSA and 0.01% sodium azide. Cell aliquots (10^5 to 5×10^5 cells/100 μl) were incubated on ice with conjugated mAbs against CD11b, CD29, CD44, and CD45 (BD Pharmingen, Le Pont de Claix, France) or conjugated isotypic controls. Flow cytometry was performed on a fluorescence-activated cell sorter (FACS Scan, BD Biosciences, Le Pont de Claix, France), and data were analyzed with the Cellquest software (BD Pharmingen).

Adipogenic stimulation and Oil Red O staining. The adipogenic differentiation potential for BM-derived MSC was determined according to a previously described protocol [16]. Cells were plated in an 8-well Lab-Tek chamber slide (Nalge Nunc International, Naperville, IL) at the initial density of 2500 cells/well, and cultured in phenol red-free MEM supplemented with 10% charcoal-treated FBS. Twenty-four hours later, the medium was changed and test agents (10^{-8} M insulin and 10^{-7} M dexamethasone) were added to the culture. After 3 days, the medium was changed again and the cells were cultured 3 more days in the presence of test agents. At the end of the culture, fat droplets within differentiated adipocytes were observed at day 21 using the Oil Red O staining method as previously described [17]. Briefly, cells were fixed with 60% isopropanol, and, after washing with 1 × PBS, a dilution of 0.25% (wt/vol) Oil Red O (Sigma) in 60% isopropanol was added for 20 min. Photomicrographs were taken with a digital IX 50 microscope (Olympus, Tokyo, Japan).

Osteogenic induction. Osteogenesis was induced by culturing the cells at low density (1.5×10^4 cells in 100-mm-diameter culture dishes) for 21 days in an osteogenic medium. The osteogenic medium consisted of DMEM supplemented with 10% FCS, 10 mM β-glycerophosphate (Sigma), 0.1 μM dexamethasone (Sigma), and 0.05 mM ascorbic acid (Sigma). Osteogenic differentiation was performed using a described protocol [8]. BMSCs were maintained in osteogenic-induction media for 21 days, with fresh media added every other day.

Von Kossa staining for mineralized nodule formation. BMSCs were plated at a density of 5×10^3 cells/ml in 60 mm dishes and were cultured. When nodule formation was detected at day 14, the samples were fixed with 4% polyoxymethylene for 2 h and Von Kossa staining was performed as reported [18] with some modifications. First, cells were washed twice with ddH_2O and incubated with 5% silver nitrate in the dark for 30 min at room temperature. Second, silver nitrate was removed, and the cells were rinsed twice with ddH_2O. After being air-dried, cells were exposed to ultraviolet light for 1 h until color development was complete and then the cells were immersed in a solution of 5% sodium thiosulfate in distilled water for 2 min. Finally, BMSCs were counterstained with nuclear fast-red solution before visualization by microscopy. Calcium salts stain in dark brown/black with this technique. All figures for mineralized nodules are typical results of three separate experiments, unless otherwise specified.

MTT assay. After 24 h of seeding(10^3/well) with/without osteogenic induction media, 20 μl (5 mg/ml) of MTT was added to each well of a 96-well plate for incubation 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. The optical density (OD) was determined with an enzyme-linked immunosorbent assay (ELISA) detector at a wavelength of 490 nm.

ALP activity assay. BMSCs were seeded at a density of 1×10^3 cells/well in 96-well plates. After 1, 3, 5, 8, 11, 14, 17, and 21 days of culture, the ALP activity of BMSCs was determined using an ALP assay kit (JianCheng Co., Nanjing, China) according to the manufacturer's instructions. The results were measured spectrophotometrically at 520 nm and described in King and Armstrong units [19].

Immunocytochemical analysis. BMSCs in control medium were seeded on coverslips at a density of 2×10^5 cells/ml, maintained for another 2 days, and then fixed with 4% polyoxymethylene. Immunocytochemical analyses were performed using the streptavidin–biotin complex (SABC) method according to the manufacturer's recommended protocol. Antibodies included: polyclonal rabbit anti-rat ERα at 1:150 dilution, polyclonal rabbit anti-rat STRO-1 at 1:500 dilution. The antibodies were obtained from the Santa Cruz Biotechnology Inc. (Santa Cruz, CA). All samples were counterstained with hematoxylin and examined with an Olympus compound microscope (Olympus Optical Co., Ltd., Japan) and Nikon digital camera (Nikon Corp., Japan).

Quantitative real-time PCR. For quantification of gene expression levels, we used a real-time PCR assay based on the multiplex method as previously described [20]. For biochemical and mRNA analyses, cells with/without osteogenic induction were harvested at timed intervals (1, 4, 8, 12, 16, 18, and 20 days).

Briefly, total RNA was isolated using the RNeasy minikit with an on-column DNase I digest (Qiagen, Basel, Switzerland). First-strand cDNA

was synthesized in a 20-μl reaction from 5 μg total RNA by reverse transcription with SuperScript II (Invitrogen) using 250 ng random hexamers (Microsynth, Balgach, Switzerland). The conditions for the cDNA synthesis were: 10 min for random primer annealing at 70 °C followed by a 2-min cooling step and then the reverse transcription reaction (25 °C for 10 min, 42 °C for 30 min, 48 °C for 20 min, and 70 °C for 15 min). The cDNA was subsequently diluted to 500 μl in diethylpyrocarbonate-treated water. In a 50-μl PCR, 10 μl cDNA (corresponding to 100 ng total RNA input) was amplified in a 7700 sequence detector (Applied Biosystems), using the 2× Universal master mix (Applied Biosystems), 25 nM primers, and 100 nM probe (VIC-TAMRA labeled) for the 18S rRNA internal control, and 300 nM primers and 200 nM probe (FAM-TAMRA labeled) for the gene of interest. All probe and primer sets were designed with the Primer Express program version 1.0 (Applied Biosystems) and initially tested to have comparable (>90%) efficiency in multiplex assays using 18S rRNA as internal control. The primers were based on the cDNA sequence of rat ERα (GenBank Accession No. NM_012689) and GAPDH (GenBank Accession No. NM_017008). Primer oligonucleotide sequences were as follows: ERα: forward primer 5′-AGGAGACTCGCTACTGTGC TG-3′, reverse primer 5′-ATCATGCCCACTTCGTAACAC-3′; GAPDH: forward primer 5′-AAGAAGGTGGTGAAGCAGGC-3′, reverse primer 5′-TCCACCACCCTGTTGCTGTA-3′. Content of cDNA samples was normalized by subtracting the number of copies of the endogenous GAPDH reference gene from the number of copies of the target gene ($\Delta C_t = C_t$ of target gene $- C_t$ of GAPDH). Expression of the specific gene was calculated using the formula $2^{-(\Delta C_t)}$.

Statistical analysis. All experiments were performed at least three times. Results of representative experiments are presented except where otherwise indicated. Values are expressed as means ± SEM. Statistical analysis was performed by Kruskal–Wallis (non-parametric) one-way analysis of variance (ANOVA) with the Dunn multiple comparison test and by Student's *t* test.

Results

Phenotypic characterization of BMSCs identification

Adherent cells isolated from bone marrow were first characterized according to the expression of surface markers known to be expressed or absent on BMSCs. By FACS analysis, we showed that more than 95% of these cells were negative for the expression of CD11b, CD29 and were positive for CD44, CD45 (Fig. 1). Indeed, using various markers, the adherent cell populations displayed a phenotype commonly observed with MSC-containing cell populations derived from bone marrow.

Differentiation capacities of BMSCs

The STRO-1 antigen is a cell-surface glycoprotein on subsets of bone marrow stromal (mesenchymal) cells. Here, the cells were STRO-1 positive (Fig. 2A). BMSCs have

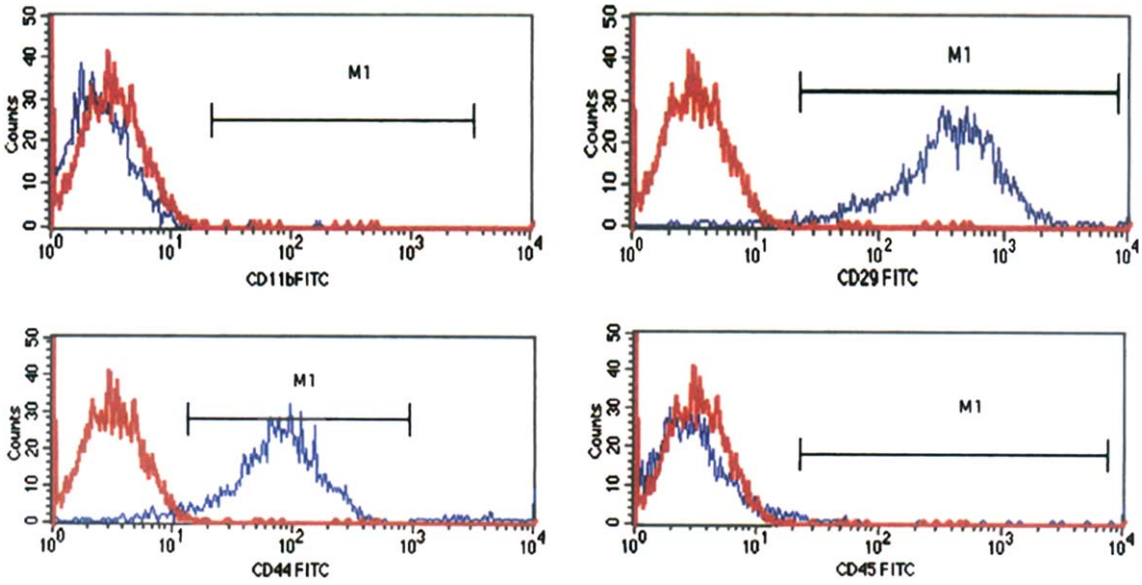

Fig. 1. Phenotypic characterization of BMSCs. Fluorescent-activated cell sorting analysis revealed cells were negative for CD11b and CD29 expression and positive for CD44, CD45, phenotypes currently known to be characteristic of BMSCs.

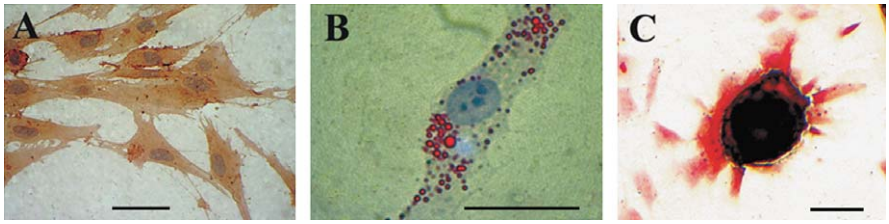

Fig. 2. Cytochemical analysis of the primary BMSCs for multipotential capacity identification (A) STRO-1 positive staining in bone marrow cultures before differentiation shows the undifferentiated features (bar: 50 μm). (B) Adipogenesis of BMSCs after adipogenic induction. Lipid droplets were visualized using Oil Red O staining (bar: 50 μm). (C) Von Kossa staining of MSCs maintained in osteogenic media for 13 days. Dark staining of the nodules demonstrates deposition of mineralized matrix characteristic of osteogenesis (bar: 50 μm).

been reported to be mesenchymal precursor cells, which are multipotent cells that give rise to adipocytes, osteocytes, smooth myocytes, fibroblasts, and chondrocytes. After adipogenetic induction, Oil Red stained positive cells were identified (Fig. 2B). Von Kossa staining of BMSCs was maintained in the osteogenic media for 14 days. Dark staining of the nodules demonstrated deposition of mineralized matrix characteristic of bone formation (Fig. 2C).

Growth curve and ALP activity

Proliferation of induced and untreated BMSCs was described with a growth curve using the MTT method. At the first day, both cells were at the same low level. Then, with a short period (2 days) delay, exponential growth began. The untreated BMSCs reached the peak at day 5, whereas the induced group reached the peak at day 6 (Fig. 3). The peak levels were significantly different between the two groups ($p < 0.01$) being higher in the untreated group (Fig. 3).

It is well known that ALP is a marker for osteoblast differentiation, because osteoblasts show much higher ALP activity than BMSCs. Our data clearly demonstrated that there was significantly ($p < 0.01$) more ALP activity in the treated group compared to that of the control group at almost every time point except for day 1 (Fig. 4). ALP activity in induced BMSCs quickly increased as they differentiated and reached a peak level at day 11, then declined gradually when mineralized nodules from differentiated BMSCs were apparent at day 14 (Fig. 4). The ALP activity in the control group steadily increased with culture time as the cells grew more confluent (Fig. 4).

Mineralization of the cell matrix is a hallmark of functional and fully differentiated osteoblasts. However, BMSCs could not spontaneously form the mineralized nodules until 21-day routine culture, while the treated BMSCs were induced to mineralize at day 14, as indicated by the Von Kossa staining (Fig. 2C). Our study thus indicates that osteogenic media can up-regulate the ALP activity and promote the secretion of extracellular matrix of BMSCs to form the mineralized nodules.

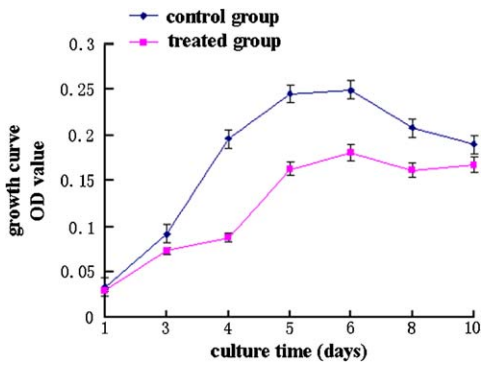

Fig. 3. The growth curves of untreated and osteogenic-induced BMSCs. The proliferation rate in the treated group was less than the untreated control. Each bar represents the mean ± SD with culture time ($p < 0.01$).

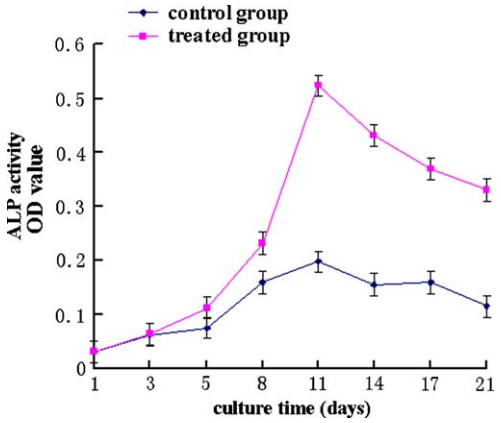

Fig. 4. Alkaline phosphatase activity in treated and untreated BMSCs. Both groups reached a peak at day 11. There was significantly ($p < 0.01$) more ALP activity in the treated group compared to that of the control group. Each bar represents the mean ± SD with culture time.

Immunocytochemical assay for ERα

ERα antigen is a nuclear membrane receptor. The assayed primary BMSCs were positively stained on the nucleus, which was in contrast to the negative controls (Fig. 5).

ERα gene temporal expression

Gene expression profiling revealed a change in ERα during differentiation. We profiled the mRNA expression levels of the ERα to characterize estrogenic signaling during growth (at days 1, 4, 8, 12, 16, and 20) of the BMSCs. To obtain the relative gene expression values of the receptor, we applied quantitative real-time RT-PCR. ERα mRNA levels at both induced and untreated groups were first detected at day 1 and significantly increased during confluence. ERα was found to be expressed at lowest levels at day 1. The highest expression levels occurred at day 8 in untreated BMSCs. However, the increase was maintained during osteogenesis in the treated group. In the undifferentiated stromal cells, ERα mRNA was the abundant ER transcript but decreased to low levels during the differentiation processes. We also observed that ERα mRNA expression levels were substantially up-regulated during osteogenesis. During osteogenic differentiation, we found a steady increase until day 20 (Fig. 6).

Discussion

Many factors affect bone formation [21,22]. Estrogens, which are regarded as one of the most important environmental factors for osteogenic lineages, function via estrogen receptors (ERs), the crucial intrinsic factors [23]. Recently, ERs have been found in osteoblasts and osteocytes, correlating with osteogenesis [24]. Although BMSCs originate osteoblast lineage [10], few studies have focused on their estrogen receptors.

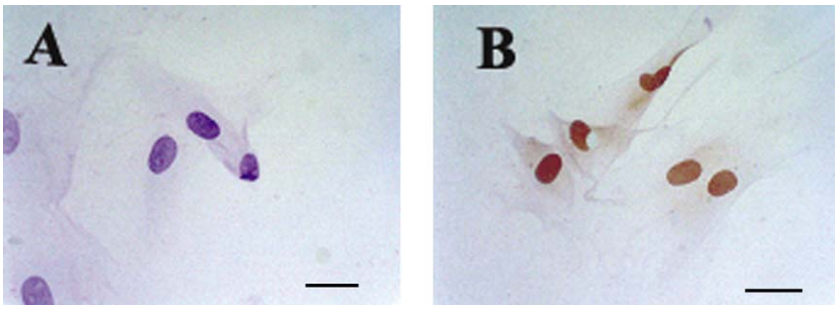

Fig. 5. The results of immunocytochemical staining in BMSCs. (A) Negative control. (B) BMSCs were immunopositive with antibodies for ERα (bar: 50 μm).

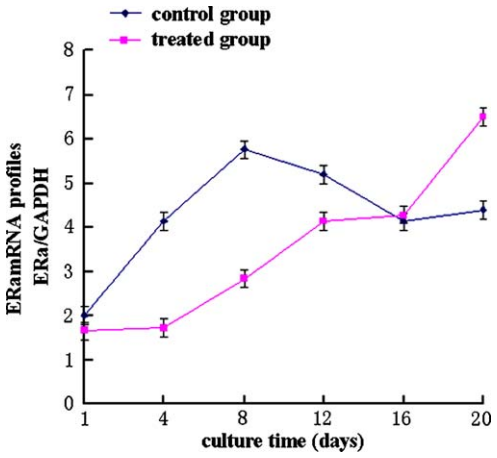

Fig. 6. The different ERα mRNA patterns of untreated and osteogenic-induced BMSCs. The level in untreated BMSCs increased at first with cell proliferation, and then dropped down after confluence, whereas in osteogenic-induced BMSCs, it steadily increased. Each bar represents the mean ± SD with culture time.

We approached this problem using female adult rat bone marrow stromal (mesenchymal) cell cultures. These cells have been shown to contain multipotent mesenchymal progenitor cells that can give rise to several lineages such as osteoblasts, adipocytes, chondrocytes, and myoblasts [25–27]. The phenotype aided to verify that the adherent cells were mesenchymal stem cells derived from bone marrow. The surface antigens expressed differentially between mesenchymal and hemopoietic cells, which are the two major cell components in bone marrow [25]. Here, we selected four representative surface antigens to test the isolated cells. They were CD29, CD44, CD11b, and CD45. CD29 and CD44 are surface antigens on mesenchymal cells, whereas CD11b and CD45 are on the hemopoietic cells in bone marrow [28]. Our results showed that among the four surface antigens only the markers of the mesenchymal cells expressed positively. The percentages of the CD29 and CD44 positive cells were high (>95%), meaning that the mesenchymal cells were pure.

The STRO-1 antigen is a marker for undifferentiated cells, which aided in this study to characterize the mesenchymal cells, verified by the above tests, to be stem cells [29,30]. The results showed that the primary cultured and isolated bone marrow cells were STRO-1 positive.

Multipotential differentiation is also a characteristic of BMSCs. Through adipogenetic induction or osteogenetic induction, the cells were positive with the specific marker for adipocytes (Oil Red O positive staining) or osteocytes (more ALP activities and mineralized nodule formation with Von Kossa stain), respectively. These represented differentiation and supported the conclusion that the cells were primary cultured BMSCs. This is in line with previous studies [31,32].

In this study, we focused on the ERα protein and gene expression during primary BMSC growth *in vitro*. Using immunocytochemical analysis, we observed that most of the isolated BMSCs were positively stained for the ERα. Then, we determined the mRNA profiles for ERα throughout all the stages of basal media-cultured and the osteogenic-induced primary BMSCs. These expression patterns are of interest because they reveal possible regulators of estrogen signaling during the differentiation states.

BMSCs undergoing osteogenic commitment can be divided into three different stages: (1) proliferation and commitment (day 0–7), which is accompanied by an increase in cell numbers; (2) the differentiation phase (day 7–11), which goes along with an up-regulation of ALP expression; and (3) the maturation stage (beginning at day 15), which is characterized by induction of mineralized nodule formation [33].

Studies of estrogen responses have shown that, ERα plays a more important role in bone formation among ERs [34]. ERα has been reported to be the isoform predominantly expressed in mature osteoblasts [35]. This implies a dominant role for ERα-mediated signaling in the cell type. In primary BMSCs without differentiation induction, our results showed that the increase of the ERα mRNA mainly occurred before cell confluence during proliferation. After the peak at the confluence stage, the ERα mRNA decreased with differentiation. However, the ERα mRNA level increased steadily in osteogenic-induced BMSCs. It altered more significantly at the differentiation point. The profile from the ER isoforms in our

differentiating BMSC culture model revealed a stage-dependent up-regulation of ERα mRNA.

Overall, these results led us to conclude that mesenchymal stem cells, like the mature osteoblasts, are the major targets for local estrogen action in bone. However, additional studies will be needed to resolve the exact role of ER in the *in vivo* regulation of bone metabolism.

In summary, this report describes a new cell model for observing the developmental expression of ERα. Moreover, to our knowledge it is the first report on primary cultured BMSCs as the osteoblastic precursors to study ERα biology. The BMSCs possess the capacity to differentiate into multiple mesenchymal lineages, including bone and adipose tissue. With osteogenic induction, the alteration of the ERα mRNA with time within a passage of the primary cultured BMSCs is up-regulated. Our research suggests further studies on other factors impacting ERα mRNA functional expression.

Acknowledgment

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References

[1] J.M. Hall, J.F. Couse, K.S. Korach, The multifaceted mechanisms of estradiol and estrogen receptor signaling, J. Biol. Chem. 276 (2001) 36869–36872.
[2] C.J. Gruber, W. Tschugguel, C. Schneeberger, J.C. Huber, Production and actions of estrogens, N. Engl. J. Med. 346 (2002) 340–352.
[3] P.V. Bodine, R.A. Henderson, J. Green, M. Aronow, T. Owen, G.S. Stein, Estrogen receptor-alpha is developmentally regulated during osteoblast differentiation and contributes to selective responsiveness of gene expression, Endocrinology 139 (1998) 2048–2057.
[4] E.F. Eriksen, D.S. Colvard, N.J. Berg, M.L. Graham, K.G. Mann, T.C. Spelsberg, Evidence of estrogen receptors in normal human osteoblast-like cells, Science 241 (1988) 84–86.
[5] H. Yoshikubo, N. Suzuki, K. Takemura, M. Hoso, S. Yashima, S. Iwamuro, Osteoblastic activity and estrogenic response in the regenerating scale of goldfish, a good model of osteogenesis, Life Sci. 76 (2005) 2699–2709.
[6] K.M. Wiren, E.A. Chapman, X.W. Zhang, Osteoblast differentiation influences androgen and estrogen receptor-alpha and -beta expression, J. Endocrinol. 175 (2002) 683–694.
[7] R. Okazaki, D. Inoue, M. Shibata, M. Saika, S. Kido, H. Ooka, Estrogen promotes early osteoblast differentiation and inhibits adipocyte differentiation in mouse bone marrow stromal cell lines that express estrogen receptor (ER) alpha or beta, Endocrinology 143 (2002) 2349–2356.
[8] H. Kim, H. Suh, S.A. Jo, H.W. Kim, J.M. Lee, E.H. Kim, In vivo bone formation by human marrow stromal cells in biodegradable scaffolds that release dexamethasone and ascorbate-2-phosphate, Biochem. Biophys. Res. Commun. 332 (2005) 1053–1060.
[9] M.F. ittenger, A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, Multilineage potential of adult human mesenchymal stem cells, Science 284 (1999) 143–147.
[10] F.P. Barry, J.M. Murphy, Mesenchymal stem cells: clinical applications and biological characterization, Int. J. Biochem. Cell Biol. 36 (2004) 568–584.
[11] J.E. Aubin, F. Liu, L. Malaval, A.K. Gupta, Osteoblast and chondroblast differentiation, Bone 17 (1995) 77S–83S.
[12] D. Benayahu, Y. Kletter, D. Zipori, S. Wientroub, Bone marrow-derived stromal cell line expressing osteoblastic phenotype in vitro and osteogenic capacity in vivo, J. Cell. Physiol. 140 (1989) 1–7.
[13] G.S. Stein, J.B. Lian, Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype, Endocr. Rev. 14 (1993) 424–442.
[14] Z.S. Xiao, L.D. Quarles, Q.Q. Chen, Y.H. Yu, X.P. Qu, C.H. Jiang, Effect of asymmetric dimethylarginine on osteoblastic differentiation, Kidney Int. 60 (2001) 1699–1704.
[15] D. Woodbury, E.J. Schwarz, D.J. Prockop, I.B. Black, Adult rat and human bone marrow stromal cells differentiate into neurons, J. Neurosci. Res. 61 (2000) 364–370.
[16] A. Peister, J.A. Mellad, B.L. Larson, B.M. Hall, L.F. Gibson, D.J. Prockop, Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential, Blood 103 (2004) 1662–1668.
[17] M. Heim, J. Johnson, F. Boess, I. Bendik, P. Weber, W. Hunziker, Phytanic acid, a natural peroxisome proliferator-activated receptor (PPAR) agonist, regulates glucose metabolism in rat primary hepatocytes, FASEB J. 16 (2002) 718–720.
[18] M.J. Deng, Y. Jin, J.N. Shi, H.B. Lu, Y. Liu, D.W. He, Multilineage differentiation of ectomesenchymal cells isolated from the first branchial arch, Tissue Eng. 10 (2004) 1597–1606.
[19] E.J. King, A.R. Armstrong, Convenient method for determining serum and bile phosphatase activity, Can. Med. Assoc. J. 31 (1934) 376.
[20] M. Heim, O. Frank, G. Kampmann, N. Sochocky, T. Pennimpede, P. Fuchs, The phytoestrogen genistein enhances osteogenesis and represses adipogenic differentiation of human primary bone marrow stromal cells, Endocrinology 145 (2004) 848–859.
[21] C. Makiishi-Shimobayashi, T. Tsujimura, T. Iwasaki, N. Yamada, A. Sugihara, H. Okamura, Interleukin-18 up-regulates osteoprotegerin expression in stromal/osteoblastic cells, Biochem. Biophys. Res. Commun. 281 (2001) 361–366.
[22] S. Takeda, Central control of bone remodeling, Biochem. Biophys. Res. Commun. 328 (2005) 697–699.
[23] F. Syed, S. Khosla, Mechanisms of sex steroid effects on bone, Biochem. Biophys. Res. Commun. 328 (2005) 688–696.
[24] O. Vidal, M. Lindberg, L. Savendahl, D.B. Lubahn, E.M. Ritzen, J.A. Gustafsson, Disproportional body growth in female estrogen receptor-alpha-inactivated mice, Biochem. Biophys. Res. Commun. 265 (1999) 569–571.
[25] D.J. Prockop, Marrow stromal cells as stem cells for nonhematopoietic tissues, Science 276 (1997) 71–74.
[26] M. Miura, X.D. Chen, M.R. Allen, Y. Bi, S. Gronthos, B.M. Seo, A crucial role of caspase-3 in osteogenic differentiation of bone marrow stromal stem cells, J. Clin. Invest. 114 (2004) 1704–1713.
[27] P. Bianco, G.P. Robey, Marrow stromal stem cells, J. Clin. Invest. 105 (2000) 1663–1668.
[28] M. Koda, S. Okada, T. Nakayama, S. Koshizuka, T. Kamada, Y. Nishio, Hematopoietic stem cell and marrow stromal cell for spinal cord injury in mice, Neuroreport 16 (2005) 1763–1767.
[29] S. Gronthos, A.C. Zannettino, S.J. Hay, S. Shi, S.E. Graves, A. Kortesidis, Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow, J. Cell Sci. 116 (2003) 1827–1835.
[30] J.E. Dennis, J.P. Carbillet, A.I. Caplan, P. Charbord, The STRO-1+ marrow cell population is multipotential, Cells Tissues Org. 170 (2002) 73–82.
[31] L. Song, R.S. Tuan, Transdifferentiation potential of human mesenchymal stem cells derived from bone marrow, FASEB J. 18 (2004) 980–982.
[32] I. Sekiya, B.L. Larson, J.T. Vuoristo, J.G. Cui, D.J. Prockop, Adipogenic differentiation of human adult stem cells from bone marrow stroma (MSCs), J. Bone Miner. Res. 19 (2004) 256–264.
[33] B. Lecka-Czernik, I. Gubrij, E.J. Moerman, O. Kajkenova, D.A. Lipschitz, S.C. Manolagas, Inhibition of Osf2/Cbfa1 expression and

terminal osteoblast differentiation by PPARgamma2, J. Cell. Biochem. 74 (1999) 357–371.
[34] M.K. Lindberg, M. Erlandsson, S.L. Alatalo, S. Windahl, G. Andersson, J.M. Halleen, Estrogen receptor alpha, but not estrogen receptor beta, is involved in the regulation of the OPG/RANKL (osteoprotegerin/receptor activator of NF-kappa B ligand) ratio and serum interleukin-6 in male mice, J. Endocrinol. 171 (2001) 425–433.
[35] L.K. McCauley, T.F. Tozum, T.J. Rosol, Estrogen receptors in skeletal metabolism: lessons from genetically modified models of receptor function, Crit. Rev. Eukaryot. Gene Expr. 12 (2002) 89–100.