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Stimulatory effect of 17β-estradiol on osteogenic differentiation potential of rat adipose tissue-derived stem cells

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Abstract. Adipose tissue-derived stem cells (ADSCs) are considered as a potential cell source for regenerative medicine and tissue engineering. Although ADSCs have greater proliferation capacity than bone marrow stem cells (BMSCs), lower differentiation ability of these cells limits their utility in experimental and clinical studies. The purpose of this study was to investigate whether 17β-estradiol (E_2) has a stimulatory effect on osteogenic differentiation potential of ADSCs *in vitro*. ADSCs were isolated from visceral adipose tissues of rats and treated with different concentrations of E_2 in osteogenic medium (OM) for 21 days. The differences in osteogenic differentiation potential of the cultures were assessed by von Kossa staining, measurement of alkaline phosphatase (ALP) activity and calcium levels. ADSCs cultured in OM supplemented with E_2 showed greater bone-like nodule formation and mineral deposition in comparing with the cells grown in OM. In addition, ALP activity and calcium levels also were significantly higher in the cultures exposed to E_2 than the cells treated only with OM ($p < 0.005$, $n = 5$). Our results suggest that E_2 may stimulate the osteogenic differentiation of ADSCs and therefore, can be used as an inducing agent to improve the efficiency of these cells in *in vitro* and *in vivo* studies.

Key words: Adipose tissue — Stem cell — Osteogenesis — Rat — Estradiol — Differentiation

Abbreviations: AA, ascorbic acid; $ADSC_S$, adipose-tissue derived stem cells; ALP, alkaline phosphatase; βGP, β-glycerophosphate; BMSCs, bone marrow stem cells; Dex, dexamethasone; DMEM, Dulbecco's Modified Eagle medium; E_2, 17β-estradiol; FBS, fetal bovine serum; OM, osteogenic medium PBS, phosphate-buffered saline.

Introduction

Recently, stem cells have been recognized as an attractive source for regenerative medicine and tissue engineering. Although embryonic stem cells have enormous self-renewal capacity and multilineage potential, their use in experimental and clinical studies are limited due to many ethical and political issues. Therefore, adult stem cells from stromal compartment of mesenchymal tissues have been proposed as an alternative source for cell-based therapies (Alhadlaq and Mao 2004; Krampera et al. 2006; Caplan 2007). To date, stromal cells or often called mesenchymal stem cells have been isolated from bone marrow, muscles, skin and adipose tissue and characterized widely (Krampera et al. 2006; Caplan 2007; Peng et al. 2008). Mesenchymal stem cells include three major characteristics: the ability to adhere to plastic surfaces to form fibroblastic colonies, extensive proliferative capacity and the ability to differentiate into several cell lineages.

Bone marrow is the main reserve of the stem cells and contains two types of stem cell populations: hematopoetic stem cells and mesenchymal stem cells. Although mesenchymal stem cells represent a very small portion of the nucleated cells in marrow they can be isolated and expanded with high efficiency under well-defined culture conditions. Previous studies have reported that bone marrow stem cells (BMSCs) can selectively form osteoblasts, chondrocytes, adipocytes *in vitro* in response to appropriate growth media (De Ugarte et al. 2003; Romanov et al. 2005; Peng et al. 2008). Adipose tissue is also derived from mesenchyme, and adipose tissue-derived stem cells (ADSCs) can proliferate extensively and give rise to several lineages such as osteoblasts, adipocytes and chondrocytes similar to BMSCs (Zuk et al. 2002; De

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Ugarte et al. 2003; Romanov et al. 2005; Peng et al. 2008). Compare with bone marrow, adipose tissue is a practical and alternative mesenchymal stem cell source for stem cell and regenerative medicine studies due to the simplicity of extracting cells in a large volume with minimal morbidity. Although differentiation capacity of ADSCs is generally lower than BMSCs, recent evidences suggest that effective modulation of ADSCs *in vitro* may improve their proliferation and differentiation potential (Zhu et al. 2008).

Bone formation is a complex process including proliferation of osteoprogenitor cells and the differentiation of pre-osteoblasts into mature osteoblasts which produce and mineralize bone matrix during pre- and postnatal life. Several hormones and local factors are essential to stimulate osteogenic differentiation *in vitro* and *in vivo*. Adult stem cells typically require dexamethasone (Dex), ascorbic acid (AA) and β-glycerophosphate (βGP) to differentiate into osteoblasts and produce a mineralized matrix *in vitro*. Recent studies mention a positive effect of Dex on *in vitro* bone nodule formation and mineralization, whereas βGP is generally used as a source of organic phosphate for the formation of hydroxyapatite in culture systems (Jaiswal et al. 1997; Huang et al. 2002).

It is well recognized that estrogen has modulatory effects on cell growth and differentiation *via* its intracellular receptors and stimulation-related genes (Zhou et al. 2001; Hong et al. 2004; Ray et al. 2008). Recent studies have demonstrated the presence of estrogen receptors on embryonic stem cells, supporting that estrogen may alter the functions of those cells (Hong et al. 2004; Han et al. 2006). In a previous study, 17β-estradiol (E_2) has been reported to enhance the expression of genes for alkaline phosphatase (ALP) activity, collagen I and TGF-β1 by mesenchymal stem cells in osteoporotic mice (Zhou et al. 2001). Although data are reported regarding the role of estrogens on osteogenic differentiation of BMSCs both in animals and human, there is limited information about the effect of estrogens on osteogenic potential of ADSCs. The purpose of this study was to investigate whether E_2 treatment has a stimulatory effect on osteogenic differentiation potential of ADSCs isolated from rats. For this reason, we exposed rat ADSCs to different concentrations of E_2 in the culture medium and measured osteogenic capacity of these cells with histological and biochemical methods.

Materials and Methods

Isolation of ADSCs

Eight adult Sprague-Dawley rats weighing approximately 200–250 g were used in the study. The protocols employed in the study were approved by the Institutional Animal Ethics Committee of Ege University, Izmir. All chemicals and culture media were obtained from Sigma Chemicals. Rats were euthanized with an overdose of sodium pentobarbital before the experiments. ADSCs were isolated from the visceral adipose tissue of the rats. Briefly, a midline incision was made in the epigastric area of the rat and abdominal cavity and its contents were exposed. Visceral adipose tissues around the stomach, intestines and bilateral inguinal region were carefully dissected away from the body and put in ice-cold sterile phosphate-buffered saline solution (PBS) (Huang et al. 2002; Tholpady et al. 2003). The adipose tissue was rinsed three times with PBS, finely minced with scissors and then digested with 0.075% collagenase I with vigorous shaking at 37°C for 1 hour. After the collagenase I was neutralized by adding equal volume of Dulbecco's Modified Eagle medium (DMEM), the cells were filtered by using a cell strainer to remove the debris, rinsed twice with DMEM with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic and centrifuged at 500 × *g* for 5 minutes. Finally, the cells were re-suspended in 1 ml of serum-containing medium and cell count and cell viability were determined by trypan blue staining. The nucleated cells (2.5×10^5) were plated in 100 mm dishes in medium consisting of DMEM with 10% FBS and 1% antibiotic/antimycotic. The cells were incubated in an incubator at 37°C with 5% CO_2 and culture medium was refreshed every 2–3 days. When the large fibroblast-like cell colonies developed and reached 80% confluence, cultures were harvested with 0.25% trypsin/EDTA for 5–10 min at 37°C and re-plated on 100 mm dish. The resulting cultures were referred to as first passage cultures. The primary and passage cultures were examined and photographed using a phase-contrast microscope. Passage 5 cells were used to evaluate both osteogenic and chondogenic potentiality.

Osteogenic differentiation

ADSCs at passage 5 were seeded on to 24-well plates at a density 1×10^4/well in DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic. Medium was changed the day after seeding and then every 3 days. When the cells reached 70–80% confluency, cultures were treated with osteogenic medium (OM) consisting of 50 μM AA, 100 nM Dex and 10 mM βGP for 21 days *in vitro* (Jaiswal et al. 1997; Tholpady et al. 2003). In addition, to evaluate the effect of βGP and Dex on osteogenic capacity of ADSCs, some of the cultures were incubated in different doses of Dex (10 and 1000 nM) and βGP (5 and 50 mM). To examine the effect of E_2 on osteogenic potential of ADSCs, the cells were exposed to different concentrations of E_2 (10^{-12}, 10^{-10} and 10^{-8} M) in OM (50 μM AA + 100 nM Dex + 10 mM βGP in DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic). Control group was treated with only control medium containing DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic. The cells were cultured for 21 days and osteoblastic activity in the cultures was assessed by von Kossa staining, calcium and ALP activity measurement.

Chondrogenic differentiation

To evaluate the multipotency of ADSCs, cells were also induced with chondrogenic medium using pellet culture system. For the preparation of each pellet, passage 5 cells were trypsinized, counted and resuspended in a chondrogenic basal media consisting of high glucose-DMEM supplemented by 10 ng/ml transforming growth factor-ß1, 100 nM Dex, 6.25 µg/ml insulin transferin, selenium + premix (ITS), 50 µg/ml ascorbate-2-phosphate, 40 µg/ml proline, 100 µg/ml pyruvate and 1% antibiotic/antimycotic. Briefly, aliquots of 1×10^6 cells were spun down at 800 × *g* in 15 ml conical tubes and cultured at 37°C, 5% CO_2 for 21 days by changing the medium every 2–3 days. Finally, the pellets were collected and proteoglycan production in the extracellular matrix was visualized by toluidine blue staining (Shirasawa et al. 2006).

von Kossa staining

The secretion of calcified extracellular matrix was evaluated with von Kossa staining (Huang et al. 2002). The cells were rinsed with deionized water and fixed with 10% formalin solution for 30 minutes. After rinsing three times with deionized water, wells were incubated in 5% silver nitrate solution (w/v) for 1 h in the dark, rinsed with deionized water and exposed to UV light for 30 minutes. After adding 5% sodium thiosulfate for the neutralization of residual silver nitrate, cells were rinsed with deionized water and dried for light microscopic examination. von Kossa positive deposits were visualized as dark brown or black precipitates.

Total calcium assay

Cell layers were rinsed with PBS and extracted with 1 ml of 0.5 N HCl for 1 h with continuous shaking at room temperature. After centrifugation at 1000 × *g* for 5 min, calcium levels in the supernatants were determined spectrophotometrically using *o*-cresolphthalein complexon method (Jäger et al. 2005). In this method, calcium forms a purple-colored complex with *o*-cresolphthalein complexon in alkaline AMP buffer. The intensity of the color was measured at 540 nm and compared with a standard solution.

ALP activity assay

The ALP activity was determined spectrophotometrically by measuring the release of *p*-nitrophenol from *p*-nitrophenyl phosphate as substrate (Jaiswal et al. 1997). Briefly, the cells were rinsed with distilled water and incubated with the substrate prepared in substrate buffer (50 mM glycine, 1 mM $MgCl_2$, pH 10.5) for 15 minutes. After the reaction was stopped with 1 M NaOH, the absorbance of the samples were read at 405 nm and compared with a *p*-nitrophenol standard solution. Enzyme activity was expressed as nmol of *p*-nitrophenol/µg protein.

Total protein assay

Total protein concentration in the cell lysates was determined according to Bradford's method using bovine serum albumin as standard (Bradford 1976).

Statistical analysis

All quantitative data were analyzed by using non-parametric (Mann-Whitney U) test. Student's *t*-test was used to evaluate the differences between the groups. Data are presented as mean values ± standard error of the mean (SEM). A two tailed *p*-value smaller than 0.05 was considered statistically significant.

Results

Morphological evaluation of the cultures

In the present study, rat ADSCs were used to assess the effects of estrogen on osteogenic differentiation. The morphology of the cells was evaluated daily during the primary and passage cultures. The initially adherent cells grew into spindle-shaped cells and colonies became visible around the 3rd day of the cultures. Primary cultures were passaged when large colonies developed and reached 80% confluence (Fig. 1). The fibroblast-like morphology became more homogenous and confluent by days 7–8 of the passage cultures. While the cultures in control

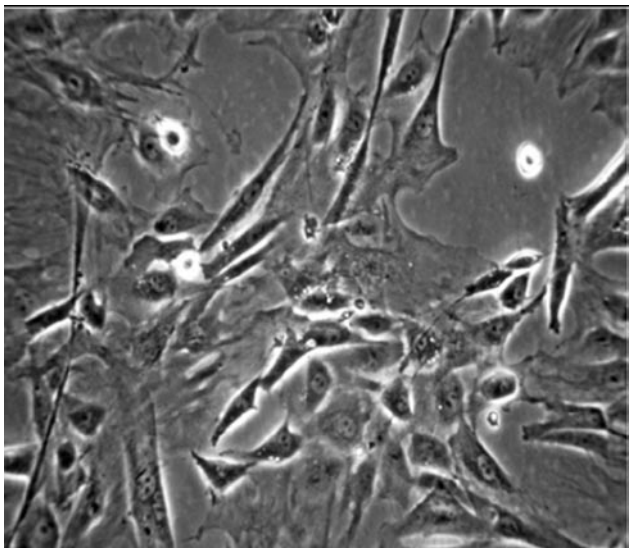

Figure 1. Morphology of rat ADSCs cultured in monolayer. The cells exhibited fibroblast-like shape. Original magnification ×40.

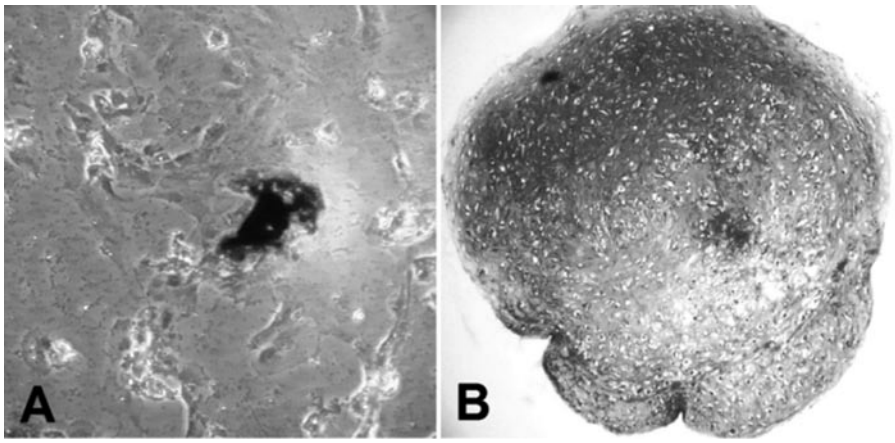

Figure 2. Osteogenic and chondrogenic differentiation of ADSCs. **A.** Cells placed in OM lost their characteristic shape and began to form cell aggregates. The mineralization of the bone-like nodules was observed as black deposits with von Kossa staining. **B.** Chondrogenic potential of ADSCs. Histological analysis of pellets by toluidine blue staining demonstrated proteoglycan synthesis in the matrix. Original magnification ×40.

medium proliferate uniformly and demonstrate fibroblast-like morphology, ADSCs placed in OM (containing 50 μM AA + 100 nM Dex + 10 mM βGP) lost their characteristic shapes and became mostly polygonal or cuboidal. Typically, OM-induced cells began to form cell aggregates and bone-like nodules by day 8 of the cultures. Mineralization of the nodules was observed during the second week of the cultures (Fig. 2A) while control cultures never formed nodule and mineralization. To evaluate the chondrogenic potential of ADSCs, pellet cultures were prepared from Passage 5 cells and cultured in chondrogenic medium for 21 days. Histological evaluation of pellet cultures demonstrated proteoglycan synthesis in the matrix (Fig. 2B).

Effect of βGP and Dex on osteogenic differentiation

The osteogenic potential of ADSCs was also studied by measuring ALP activity and calcium levels in cell lysates. Table 1 compares the effect of OM and different doses of βGP and Dex on ALP activity and calcium content in the cultures. Statistical analysis of the data demonstrated significant differences between the groups for both ALP activity and calcium deposition measured in the cultures (ANOVA, $p < 0.0005$). The incubation of ADSCs with OM for 21 days caused a significant increase in ALP activity and calcium levels compared with the control group ($p < 0.0005$). In order to evaluate the effect of βGP on osteogenic differentiation of ADSCs, cultures were incubated with different concentrations of βGP in OM containing 50 μM AA and 100 nM Dex. The ALP activity and calcium levels were significantly higher in 5 mM and 50 mM βGP added to cultures compare with the control group ($p < 0.05$ and $p < 0.005$, respectively) (Table 1). A similar response was produced by the exposure to different doses of Dex. In comparison with controls, both 10 nM and 1000 nM Dex enhanced the ALP activity in the cultures ($p < 0.05$ and $p < 0.0005$, respectively), but no difference was observed between OM (which contains 100 nM Dex) and 1000 nM Dex treated cells (Table 1).

Table 1. Effect of different concentrations of βGP and Dex on osteogenic differentiation of ADSCs *in vitro*

		ALP activity (p-nitrophenol nmol/ μg protein)	Ca^{2+} levels (μg/ml)
Control (basal medium)		27.18 ± 5.88	63.5 ± 5.1
OM (10 mM βGP + 100 nM Dex)		$100.99 \pm 8.13^{a,c,e}$	$93.2 \pm 2.0^{a,c}$
βGP	5 mM	60.96 ± 9.18^{b}	76.1 ± 3.9^{b}
	50 mM	$112.06 \pm 6.76^{a,d}$	$98.4 \pm 1.4^{a,c}$
Dex	10 nM	68.19 ± 4.54^{b}	83.5 ± 3.5^{b}
	1000 nM	$129.04 \pm 20.07^{a,e}$	$96.9 \pm 2.6^{a,e}$

βGP and Dex showed dose dependent effect on ALP activity and calcium deposition. Values represent the mean ± SEM, $n = 5$. [a] $p < 0.0005$ and [b] $p < 0.05$ *vs.* control; [c] $p < 0.05$ and [d] $p < 0.005$ *vs.* 5mM βGP; [e] $p < 0.05$ *vs.* 10 nM Dex.

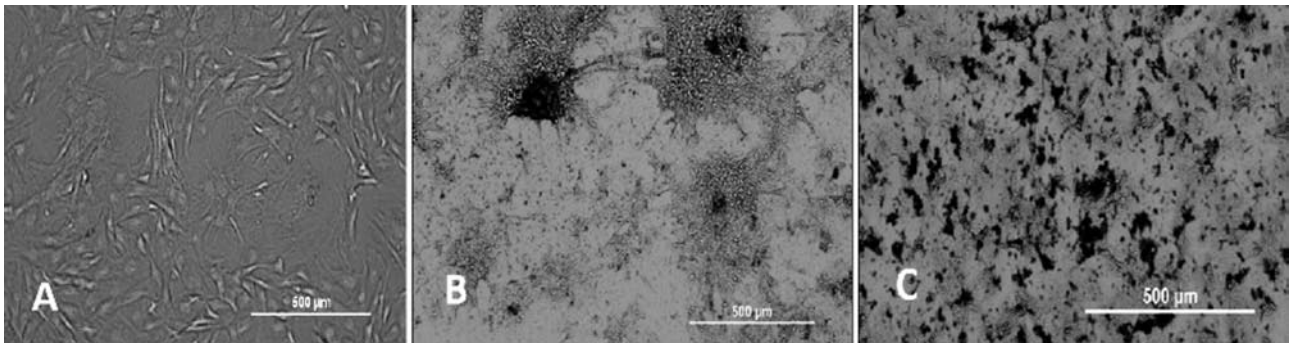

Figure 3. The effect of E_2 on osteogenic differentiation of ADSCs. **A.** Control cells exhibited fibroblast-like morphology and proliferated uniformly until the day 21 of the cultures. **B.** OM-treated cultures. Nodule formation and mineralization were clearly visualized at the end of the 3rd week. **C.** Treatment of the cultures with E_2 (10^{-8} M) caused extensive mineralization and nodule formation compared to cells grown in OM. Bars indicate 500 µm.

Effect of 17β-estradiol on osteogenic potential of ADSCs

In order to test the effect of E_2 on osteogenic potential of ADSCs *in vitro*, the cells were grown in OM supplemented with different doses of E_2 (10^{-12}, 10^{-10} and 10^{-8} M) for 21 days. The effect of E_2 on the formation of bone-like nodules and mineralization of the matrix was assessed by von Kossa staining of the cultures. The treatment of ADSCs with OM for 21 days caused typically mineralized nodules stained as black precipitates. In addition, the cultures grown in OM supplemented with 10^{-8} M E_2 formed mineralized nodules widely diffused on the culture plate (Fig. 3).

All cultures treated with different doses of E_2 demonstrated higher ALP activity than control cells ($p < 0.0005$). The stimulating effect of E_2 on ALP activity was dose-dependent whereas the highest ALP activity was detected in the cultures with E_2 supplement at concentration of 10^{-8} M. Moreover, a significant difference was observed between the OM (without E_2) and OM with E_2 concentrations of 10^{-10} and 10^{-8} M indicating a further stimulatory effect of E_2 on osteogenesis *in vitro* ($p < 0.005$, Fig. 4).

Similarly, ADSCs grown in OM supplemented with different doses of E_2 displayed higher levels of calcium compare with control cells ($p < 0.0005$), but no difference was detected between the OM (without E_2) and OM with E_2 concentrations of 10^{-12} and 10^{-10} M. However, the treatment of the cells with 10^{-8} M E_2 caused a significant increase in calcium content compare with OM group ($p < 0.005$, Fig. 5).

Discussion

The present study revealed that E_2 may have a stimulatory effect on osteoblastic differentiation of ADSCs isolated from rats. Supplementation of E_2 significantly enhanced ALP activity, calcium levels and mineral deposition in the cultures.

As indicated in earlier studies, steroid hormones strongly contribute in bone metabolism and have modulatory effects in many cell types including BMSCs. Stem cells require glucocorticoids in the culture medium for osteogenic, chondrogenic and adipogenic differentiation (Jaiswal et al. 1997; Zuk et al. 2002; Alhadlaq et al. 2004; Ray et al. 2008). Glucocorticoids, including Dex, promote osteogenic differentiation and induce the formation of bone-like nodules in embryonic and adult stem cells cultures. Although Dex is a potent inducer of osteogenic differentiation, it also posseses a dose-related inhibitory effect on proliferation of bone marrow cells *in vitro*

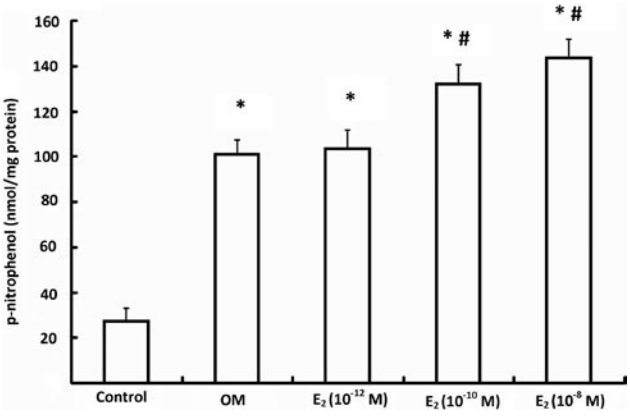

Figure 4. ALP activities in ADSCs cultures. ALP activity was significantly higher in all the groups than in the control. The addition of E_2 (both 10^{-10} M and 10^{-8} M) into OM significantly increased the enzyme activity compared to OM-treated group. Values represent the mean ± SEM, $n = 5$. * $p < 0.0005$ *vs.* control and # $p < 0.05$ *vs.* OM-treated group.

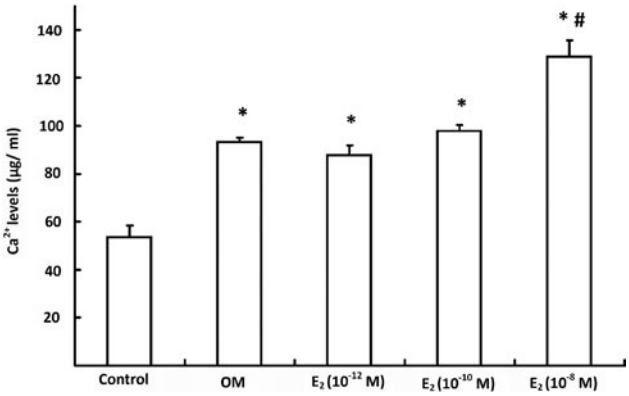

Figure 5. The amount of calcium deposition in cell lysates of ADSCs cultures. All groups showed higher levels of calcium than in the control. The highest calcium level was found in the OM supplemented with E_2 (10^{-8} M) group. Values represent the mean ± SEM, $n = 5$. * $p < 0.0005$ *vs.* control; # $p < 0.05$ *vs.* OM and OM with E_2 (both 10^{-12} M and 10^{-10} M) treated groups.

(Walsh et al. 2001). In an effort to identify the optimal culture conditions for osteogenic differentiation, we cultured these cells in OM with three different doses of Dex (10 nM, 100 nM and 1 μM) for 21 days. Consistent with the previous studies, we observed a dose-dependent effect of Dex on ALP activities and calcium levels compared with control. The highest ALP activity and calcium level were measured in the cultures treated with 1000 nM Dex, however, this dose typically resulted in detachment of the cells from the surface of the plate during the culture period. We also investigated the effect of of βGP on osteogenic potential of ADSCs by adding different doses (5, 10 and 50 mM) of βGP into OM. Although there was a significant increase in ALP activity and calcium levels with all doses of βGP compared with control, no difference was observed between the cultures exposed to 10 and 50 mM βGP. In the present study, both histological and biochemical results clearly demonstrated that the addition of osteogenic supplements including 100 nM Dex, 10 mM βGP and 50 μM AA is sufficient for a rapid osteogenesis in ADSCs cultures.

Estrogens possess an important role in the regulation of osteogenesis both in human and animals. Experimental studies have demonstrated that E_2 not only inhibits bone resorption but also stimulates bone formation (Chow et. 1992). E_2 exerts its physiological effects on target tissues *via* intracellular estrogen receptors ER-α and ER-β, which are discovered on many cell types. Bodine et al. (1998) reported increased levels of estrogen receptor during osteoblast differentiation in primary cultures of rat calvarial osteoblasts. More recently, it has been suggested that E_2 may also effectively enhance the differentiation capacity of both embryonic stem cells and BMSCs into ostoeblasts *in vitro* (Qu et al. 1998; Hong et al. 2006; Tielens et al. 2008).

In osteogenesis studies using osteoblast or stem cell cultures, ALP measurement is considered as an early marker of osteogenic differentiation whereas calcium deposition in the cultures usually reflect the endpoint of osteogenesis *in vitro*. Enhanced ALP expression in response to E_2 by osteoblasts and stem cells was demonstrated in some recent studies (Qu et al. 1998; Holzer et al. 2002; Tielens et al. 2008; Hoemann et al. 2009). Furthermore, E_2 probably acts not only to control the ALP activity of cells expressing the enzyme, but also the proliferation rate of ALP-expressing cells in the marrow stromal cell population (Holzer et al. 2002). In contrast, no stimulatory effect on ALP expression and proliferation of human marrow stromal cells was described by other investigators (Kim and Cheng 1994).

In the present study, we cultured rat ADSCs in OM supplemented with different doses of E_2 to investigate whether E_2 has a stimulatory effect on osteogenic differentiation potential. Our results showed a dose-dependent enhancement in ALP activities in the cultures treated with E_2, whereas the calcium level was significantly elevated only at concentration of 10^{-8} M E_2 compared with the cells treated with only OM. Furthermore, as confirmed by histological evaluation of the cultures with von Kossa staining, E_2-supplemented cultures demonstrated more extensive nodule formation and calcium deposition compare with the cells treated with OM.

Consistent with our findings, Hong et al. (2007) have reported that estrogen may have a modulatory role on the osteogenic and adipogenic differentiation of human ADSCs isolated from a healthy female donor. Similarly, they observed an increase in ALP activities, calcium levels and matrix calcification in the cultures treated with E_2 at concentrations of 10^{-9} and 10^{-8} M. In a recent paper, the same researchers indicated that E_2 and Dex interactively stimulate the proliferation and osteogenic differentiation of human BMSCs isolated from both male and female donors, but optimal concentration of E_2 may differ with the gender (Hong et al. 2009). In another study, Aksu et al. (2008) have reported the effects of some variables such as gender, fat depot and optimal duration on osteogenic potential of human ADSCs. According to their findings, whereas male ADSCs isolated from superficial adipose layer have greater osteogenic capacity than deep layer cells, no significant difference was observed between the cultures isolated from female donors. In contrast to our and previous results, Ng et al. (2009) found that both donor reproductive status (pregnancy, premenopause and postmenopause) and *in vitro* E_2 treatment have no significant effect on proliferation rate of human ADSCs. Overall, these conflicting findings may reflect the fact that proliferation rate and differentiation potential of stem cells including ADSCs can be influenced by several factors, such as donor age, gender, cell isolation and culture techniques, tissue sources. Stem cells themselves may also produce

autocrine and paracrine factors during proliferation and differentiation depending on different culture conditions. In addition, regulatory effects of E_2 on cell proliferation and osteoblastic differentiation could be mediated by not only its specific receptors but also some osteogenic growth factors, including BMP-6 and TGF-β1 (Qu et al. 1998; Plant and Tobias 2002).

In summary, our present results suggest that *in vitro* osteogenic potential of ADSCs can be influenced by E_2 treatment in a dose-dependent manner. However, further *in vitro* and *in vivo* studies are required to improve the effectiveness of ADSCs in tissue engineering and regenerative medicine.

References

Aksu A. E., Rubin J. P., Dudas J. R., Marra K. G. (2008): Role of gender and anatomical region on induction of osteogenic differentiation of human adipose-derived stem cells. Ann. Plast. Surg. **60,** 306–322
doi:10.1097/SAP.0b013e3180621ff0

Alhadlaq A., Mao J. J. (2004): Mesenchymal stem cells: Isolation and therapeutics. Stem Cells Dev. **13,** 436–448
doi:10.1089/scd.2004.13.436

Bodine P. V., Henderson R. A., Green J., Aronow M., Owen T., Stein G. S., Lian J. B., Komm B. S. (1998): Estrogen receptor-alpha is developmentally regulated during osteoblast differentiation and contributes to selective responsiveness of gene expression. Endocrinology **139,** 2048–2057
doi:10.1210/en.139.4.2048

Bradford M. M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248–254
doi:10.1016/0003-2697(76)90527-3

Caplan A. I. (2007): Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. J. Cell. Physiol. **213,** 341–347
doi:10.1002/jcp.21200

Chow J., Tobias J. H., Colston K. W., Chambers T. J. (1992): Estrogen maintains trabecular bone volume in rats not only by suppression of bone resorption but also by stimulation of bone formation. J. Clin. Invest. **89,** 74–78
doi:10.1172/JCI115588

De Ugarte D. A., Morizono K., Elbarbary A., Alfonso Z., Zuk P. A., Zhu M., Dragoo J. L., Ashjian P., Thomas B., Benhaim P., Chen I., Fraser J., Hedrick M. H. (2003): Comparison of multi-lineage cells from human adipose tissue and bone marrow. Cells Tissues Organs **174,** 101–109
doi:10.1159/000071150

Han H. J., Heo J. S., Lee Y. J. (2006): Estradiol-17beta stimulates proliferation of mouse embryonic stem cells: involvement of MAPKs and CDKs as well as protooncogenes. Am. Physiol. Cell Physiol. **290,** 1067–1075
doi:10.1152/ajpcell.00222.2005

Hoemann C. D., El-Gabalawy H., McKee M. D. (2009): In vitro osteogenesis assays: Influence of the primary cell source on alkaline phosphatase activity and mineralization. Pathol. Biol. **57,** 318–323
doi:10.1016/j.patbio.2008.06.004

Holzer G., Einhorn T. A., Majeska R. J. (2002): Estrogen regulation of growth and alkaline phosphatase expression by cultured human bone marrow stromal cells. J. Orthop. Res. **20,** 281–288

Hong S. H., Nah H. Y., Lee Y. J., Lee J. W., Park J. H., Kim S. J., Lee J. B., Yoon H. S., Kim C. H. (2004): Expression of estrogen receptor-alpha and -beta, glucocorticoid receptor, and progesterone receptor genes in human embryonic stem cells and embryoid bodies. Mol. Cells **18,** 320–325

Hong L., Colpan A., Peptan I. A. (2006): Modulations of 17-beta estradiol on osteogenic and adipogenic differentiations of human mesenchymal stem cells. Tissue Eng. **12,** 2747–2753
doi:10.1089/ten.2006.12.2747

Hong L., Colpan A., Peptan I. A., Daw J., George A., Evans C. A. (2007): 17-Beta estradiol enhances osteogenic and adipogenic differentiation of human adipose-derived stromal cells. Tissue Eng. **13,** 1197–1203
doi:10.1089/ten.2006.0317

Hong L., Sultana H., Paulius K., Zhang G. (2009): Steroid regulation of proliferation and osteogenic differentiation of bone marrow stromal cells: a gender difference. J. Steroid Biochem. Mol. Biol. **114,** 180–185
doi:10.1016/j.jsbmb.2009.02.001

Huang J. I., Beanes S. R., Zhu M., Lorenz H. P., Hedrick M. H., Benhaim P. (2002): Rat extramedullary adipose tissue as a source of osteochondrogenic progenitor cells. Plast. Reconstr. Surg. **109,** 1033–1041
doi:10.1097/00006534-200203000-00037

Jäger M., Feser T., Denck H., Krauspe R. (2005): Proliferation and osteogenic differentiation of mesenchymal stem cells cultured onto three different polymers in vitro. Ann. Biomed. Eng. **33,** 1319–1332
doi:10.1007/s10439-005-5889-2

Jaiswal N., Haynesworth S. E., Caplan A. I., Bruder S. P. (1997): Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J. Cell. Biochem. **64,** 295–312
doi:10.1002/(SICI)1097-4644(199702)64:2<295::AID-JCB12>3.0.CO;2-I

Kim G. S., Cheng S. L. (1994): Absence of a direct anabolic effect of 17beta-estradiol on normal human bone marrow stromal cells. Korean J. Intern. Med. **9,** 14–19

Krampera M., Pizzolo G., Aprili G., Franchini M. (2006): Mesenchymal stem cells for bone, cartilage, tendon and skeletal muscle repair. Bone **39,** 678–683
doi:10.1016/j.bone.2006.04.020

Ng L. W., Yip S. K., Wong H. K., Yam G. H., Liu Y. M., Lui W. T., Wang C. C., Choy K. W. (2009): Adipose-derived stem cells from pregnant women show higher proliferation rate unrelated to estrogen. Hum. Reprod. **24,** 1164–1170
doi:10.1093/humrep/dep001

Peng L., Jia Z., Yin X., Zhang X., Liu Y., Chen P. (2008): Comparative analysis of mesenchymal stem cells from bone marrow, cartilage, and adipose tissue. Stem Cells Dev. **17,** 761–773
doi:10.1089/scd.2007.0217

Plant A., Tobias J. H. (2002): Increased bone morphogenetic protein- 6 expression in mouse long bones after estrogen administration. J. Bone Miner. Res. **17,** 782–790
doi:10.1359/jbmr.2002.17.5.782

Qu Q., Perälä-Heape M., Kapanen A., Dahllund J., Salo J., Väänänen H. K., Härkönen P. (1998): Estrogen enhances differentiation of osteoblasts in mouse bone marrow culture. Bone **22,** 201–209
doi:10.1016/S8756-3282(97)00276-7

Ray R., Novotny N. M., Crisostomo P. R., Lahm T., Abarbanell A., Meldrum D. R. (2008): Sex steroids and stem cell function. Mol. Med. **14,** 493–501
doi:10.2119/2008-00004.Ray

Romanov Y. A., Darevskaya A. N., Merzlikina N. V., Buravkova L. B. (2005): Mesenchymal stem cells from human bone marrow and adipose tissue: isolation, characterization, and differentiation potentialities. Bull. Exp. Biol. Med. **140,** 138–143
doi:10.1007/s10517-005-0430-z

Shirasawa S., Sekiya I., Sakaguchi Y., Yagishita K., Ichinose S., Muneta T. (2006): In vitro chondrogenesis of human synovium-derived mesenchymal stem cells: optimal condition and comparison with bone marrow-derived cells. J. Cell. Biochem. **97,** 84–97
doi:10.1002/jcb.20546

Tholpady S. S., Katz A. J., Ogle R. C. (2003): Mesenchymal stem cells from rat visceral fat exhibit multipotential differentiation in vitro. Anat. Rec. A. Discov. Mol. Cell. Evol. Biol. **272,** 398–402
doi:10.1002/ar.a.10039

Tielens S., Wymeersch F., Declercq H., Cornelissen M. (2008): Effect of 17beta-estradiol on the in vitro differentiation of murine embryonic stem cells into the osteogenic lineage. In Vitro Cell. Dev. Biol. Anim. **44,** 368–378
doi:10.1007/s11626-008-9126-9

Walsh S., Jordan G. R., Jefferiss C., Stewart K., Beresford J. N. (2001): High concentrations of dexamethasone suppress the proliferation but not the differentiation or further maturation of human osteoblast precursors in vitro: relevance to glucocorticoid-induced osteoporosis. Rheumatology **40,** 74–83
doi:10.1093/rheumatology/40.1.74

Zhou S., Zilberman Y., Wassermann K., Bain S. D., Sadovsky Y., Gazit D. (2001): Estrogen modulates estrogen receptor alpha and beta expression, osteogenic activity, and apoptosis in mesenchymal stem cells (MSCs) of osteoporotic mice. J. Cell. Biochem. Suppl. **36,** 144–155
doi:10.1002/jcb.1096

Zhu Y., Liu T., Song K., Fan X., Ma X., Cui Z. (2008): Adipose-derived stem cell: a better stem cell than BMSC. Cell Biochem. Funct. **26,** 664–675
doi:10.1002/cbf.1488

Zuk P. A., Zhu M., Ashjian P., De Ugarte D. A., Huang J. I., Mizuno H., Alfonso Z. C., Fraser J. K., Benhaim P., Hedrick M. H. (2002): Human adipose tissue is a source of multipotent stem cells. Mol. Biol. Cell. **13,** 4279–4295
doi:10.1091/mbc.E02-02-0105

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