Asian Biomedicine Vol. 7 No. 6 December 2013; 743-752 DOI: 10.5372/1905-7415.0706.236

Original article

Osteogenic differentiation of human umbilical cord-derived mesenchymal stem cells promoted by overexpression of osterix

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Background: Umbilical cord-derived mesenchymal stem cells (UC-MSCs) are considered to be multipotent mesenchymal stem cells that are easily induced to differentiate into functional osteoblasts both in vitro and in vivo. Osterix (Osx), a novel zinc-finger-containing transcription factor of the Sp family, is required for osteoblast differentiation and bone formation.
Objective: We investigated the effect of Osx on the proliferation and osteogenic differentiation of the UC-MSCs.
Method: The primary UC-MSCs were isolated and cultured. An Osx-expressing plasmid (pEGFP-Osx) was constructed and transfected into UC-MSCs. Then expression of bone morphogenesis-related genes, proliferation rate, alkaline phosphatase activity, and mineralization were examined to evaluate the osteogenic potential of the Osx gene-modified UC-MSCs.
Result: UC-MSCs transfected with pEGFP-Osx exhibited apparent osteogenic differentiation as determined by increased activity of alkaline phosphatase, the formation of mineralized nodules and the expression of related osteoblastic genes.
Conclusion: These results confirmed the ability of Osx to enhance osteoblast differentiation of UC-MSCs in vitro, and the Osx gene-modified UC-MSCs are potential as novel cell resources of bone tissue engineering.

Keywords: Osteogenic differentiation, osterix, umbilical cord-derived mesenchymal stem cells

Mesenchymal stem cells (MSCs) have the potential to differentiate along multiple lineages and can be expanded in vitro, making them highly attractive candidates for cell therapy and tissue engineering applications [1]. Among MSCs, bone marrow-derived mesenchymal stem cell (BM-MSCs) have demonstrated in vitro plasticity and a robust expansion and differentiation capacity, making them the most commonly used MSCs source in differentiation studies [2, 3]. However, the acquisition of BM-MSCs from bone marrow is an invasive procedure and can cause surgical complications such as donor-site injury, bleeding, infection, and chronic pain to donors. Moreover, the number and differentiating potential of BM-MSCs in bone marrow and their ability to differentiate decreases with age, limiting the wide application of BM-MSCs in bone tissue engineering and cell-based therapeutics [4,5]. Therefore, the search for possible alternative sources of MSCs is of significant value.

One alternative source is umbilical cord (UC), which can be obtained by a less invasive method without harm for the mother or infant. In the past decade, the studies showed that UC-derived mesenchymal stem cells (UC-MSCs) are similar to BM-MSCs with respect to cellular properties and multilineage differentiation potential [6, 7]. Baksh et al. and Wang et al. reported that under appropriate inductive conditions, human UC-MSCs can be directed toward the osteogenic lineage [8, 9]. Chang et al. reported that human UC-MSCs have a significantly stronger capacity for osteogenic differentiation than BM-MSCs in vitro, showing that UC-MSCs may be a potential cell source for bone tissue engineering [10]. Moreover, UC-MSCs are

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simply expanded to large numbers in vitro, when compared with BM-MSCs. Therefore, it is suggested that UC-MSCs would be a promising alternative for MSCs used in the research and application of bone tissue engineering.

Osterix (Osx) is a recently identified zinc-finger-containing transcription factor encoded by the Sp7 gene, which regulates gene expression in committed osteoblastic precursor cells [11-13]. There is growing evidence indicating that Osx plays an essential role in the differentiation of osteoblasts and bone formation. In Osx knockout mice, no bone or bone trabeculae were formed either in intramembranous or endochondral ossification [12, 14]. Osx regulates the expression of related osteoblastic genes such as osteocalcin (OCN), osteopontin (OPN), collagen type I (COL I) and bone sialoprotein (BSP) [11, 15]. It was also reported that, overexpression of Osx promote the proliferation and osteogenic differentiation and mineralization of murine embryonic stem cells and BM-MSCs [16].

However, no reports have been available to focus on the functions of Osx in the osteogenic differentiation of UC-MSCs in vitro. This prompted our attempt to investigate the effect of Osx on the osteogenic differentiation of cultured UC-MSCs population and its application in genetic modified tissue engineering.

Materials and methods

Isolation and culture of UC-MSCs

The UC isolation procedure was approved by the institutional ethical review committee of Provincial Hospital, Affiliated to Shandong University. The collection and expansion of UC-MSCs was described previously [17, 18]. Briefly, human umbilical cords from both sexes were collected from full-term births with informed consent of the mother after stored at 4°C in sterile saline until processing. To isolate UC-MSCs, the cord was rinsed several times with sterile phosphate-buffered saline (PBS) and cut into 2–4 cm lengths. Next, the vessels were stripped manually from cord segments, the wall of the cord was opened and the tissue immersed in an enzyme cocktail (including hyaluronidase, trypsin, and collagenase) for 45–60 min at 37°C. This tissue was then crushed with forceps to release individual UC-MSCs cells and large pieces of tissue were removed. The cells were pelleted by low speed centrifugation (250*g* for 5 minutes), suspended in low-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Gaithersburg, MD, USA) supplemented with 15% fetal bovine serum (FBS, Gibco BRL), 100 U/mL penicillin, 100 U/mL streptomycin (Gibco BRL), then plated in 25 cm^2 plastic cell culture flasks (Corning, Acton, MA, USA). The cells were maintained at 37°C in 5% CO_2 in fully humidified atmosphere. Culture medium was changed twice weekly. When cells grew to 95% confluence, the primary culture cells were treated with 0.25% trypsin, and replated the cells at a ratio of 1:3. The successive passage was cultured for about 8 days and cells of passage 3 were used for the gene transfection.

Plasmid transfection

The recombinant plasmid pEGFP-Osx was constructed by directed cloning technique by Shanghai Yuanxiang Biotech (Shanghai, China). For plasmid transfection, UC-MSCs were plated in six-well plates at a density of 3×10^5 cells/well. Transfection of UC-MSCs was performed using lipofectamine TM2000 (Invitrogen, USA) according to the protocol provided by the manufacturer. Briefly, 2 μg purified pEGFP-Osx diluted in 100 μl of serum-free medium was mixed with 4 μg lipofectamine TM2000 diluted in 100 μl of serum-free medium. The DNA-Lipofectamine TM2000 mixture was incubated for 25 minutes at room temperature. Then 800 μl α-MEM of without FBS was added into the mixed solution. Four hours later, the medium was changed and the cells were cultured in standard α-MEM with no additions, containing 15% FBS. The cells were observed under fluorescent microscopy (Leica, Tokyo, Japan) to determine the transfection efficiency. After 24 hours, the cells were passaged and subjected to G418 (500 μg/ml) selection for 2 weeks (pOsx-UC-MSCs). Control cells were mock transfected with "empty" vector (mock-UC-MSCs). The expression of Osx was detected by Western blotting and real time reverse-transcription polymerase chain reaction (RT-PCR).

Western blotting

Samples of pOsx-UC-MSCs, mock-UC-MSCs and control UC-MSCs were homogenized on ice-cold Tris lysis buffer (pH 7.6) containing protease inhibitors. Lysates were then centrifuged for 10 min at 12,000*g* and supernatant was collected. Protein samples were resolved by 10%sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following gel electrophoresis, proteins were transferred to a poly(vinylidene difluoride) (PVDF) membrane (Schleicher and Schuell, Keene, NH). The membranes

were blocked with 5% (v/v) skimmed milk in Tris-buffered saline Tween-20 (TBS-T) at 37°C for 2 hours and then and incubated with primary antibodies overnight at 4°C. Primary anti-Osx monoclonal antibody (Sigma, St. Louis, MO, USA), and β-actin monoclonal antibody (Sigma) were applied at dilutions of 1:1000. Membranes were washed three times for 10 min in PBST before 1h room temperature incubation with HRPs and conjugated secondary antibodies (goat anti-rabbit IgG, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunoreactive bands were observed using enhanced chemiluminescence (ECL) (Amersham Biosciences) for 1 minute before exposure to Kodak X-Omit AR film (Eastman Kodak, Rochester, NY, USA). Membranes were reprobed with □-actin as a loading control.

Cell proliferation assay

Three different group cells were seeded into 96-well microplates at a density of 1×10^3 cells/well. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay was performed to measure the proliferation of UC-MSCs with plasmid transfection and control group [19]. Briefly, 20 μl MTT (5 mg/ml) was added to each well and the microplate was further incubated at 37 °C for 4 hours. After the supernatant was removed, 200 μl dimethyl sulfoxide (DMSO) was added into each well and the absorbance was examined by using an HTS 7000 Plus Bio Assay Reader (PerkinElmer Life Sciences, USA) at the wavelength of 570 nm.

ALP activity assay

At days 3, 6, 9, 14, and 21, a set of cultured cells were washed with PBS, scraped, lysed by 0.1% Triton100 (Sigma), and sonicated to destroy cell membranes. After centrifugation at 15,000 rpm for 10 minutes at 4°C, 100 μL supernatant samples were extracted from each sample and assayed for ALP activity and DNA content measurements. To determine the protein concentration, the prepared cell lysates were measured with a bicinchoninic acid (BCA) Protein Assay Kit (Jiancheng Biotech, Nanjing, China). Subsequently, to assess the quantitative and kinetic determination of ALP activity, 20 μl supernatant of each sample was added to 100 μl freshly prepared substrate solution (para-nitrophenyl phosphate solution) and incubated at 37°C for 1 hour. The enzymatic reaction was stopped by adding 100 μl of 0.2 mol/l NaOH. The optical density of the yellow product para-nitrophenol was determined by a HTS 7000 Plus Bio Assay reader (Perkin Elmer Life Sciences, USA) at a wavelength of 405 nm.

Alizarin red S staining

The mineralized nodules of cultured cells after plasmid transfection at 21 days were determined by staining of 1% Alizarin red S (AR). Brie□y, the cells on slides were rinsed in PBS and incubated with 40 mmol/L AR for 30 minutes. Then they were rinsed 5 times with water followed by a 15 minutes washing with PBS by rotation to reduce nonspecific AR staining. The stained nodules were observed through a microscope.

Quantitative real-time RT-PCR analysis

Total RNA was isolated from cultured UC-MSCs using Trizol reagent (Tiangen Biotech, Shanghai, China). Quantity and quality of RNA was assessed by absorbance at 260 nm and agarose gel electrophoresis. Total RNA was reversely transcribed into first-strand cDNA by using the TaKaRa reverse transcriptase (RT)-PCR Kit (TaKaRa, Shiga, Japan). Sybr green-based real-time PCR analysis was conducted with an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA). Each reaction contained 12.5 μl 2× SYBR Premix Ex Taq, 1 μl forward primer, 1 μl reverse primer, 2 μl cDNA template, and ddH_2O to a final volume of 25 μl per reaction. The primers used for ampli□cation are shown in Table 1. The real-time PCR protocol was as follows: 10 minutes of initial denaturation at 95°C; 45 cycles comprising 10 seconds of denaturation at 95°C and 10 seconds of annealing at 55°C (Osx, BSP, OCN, ALP, OPN, and Col I); and 5 seconds of extension at 72°C, with a single □uorescence detection point at the end of the annealing step in each cycle. Gene expression was normalized to the expression of the house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same sample. All real-time PCR processes were performed three times.

Statistical analysis

Results were expressed as mean ± SD and analyzed by a paired analysis of variance. Differences between the experimental and control groups were regarded to be statistically signi□cant when $p \leq 0.05$.

Results

Culture of UC-MSCs

After primary culture, human UC-MSCs exhibited a morphologically homogeneous spindle-like population as shown in **Figure 1**. The mean population doubling time of UC-MSCs was 34.2–38.2 hours during the culture time tested. During the culture period, primary or passaged UC-MSCs displayed fibroblast-like morphological features, without visible morphologic alteration.

Expression of Osx in UC-MSCs by DNA transfection

The UC-MSCs grew well after pEGFP-Osx or pEGFP transfection by uorescent microscopy observation. The initial transfection ef ciency was about 46% at 48 hours (**Figure 2**). Strong green uorescence was continually detected at 14 days after G418 selection. Real-time RT-PCR and Western blotting analysis confirmed that pOsx-UC-MSCs overexpressed Osx when compared with mock-UC-MSCs and normal control cells (**Figure 3A**). The expression of Osx mRNA was about 4-fold higher than those of mock-UC-MSCs and normal control. Western blotting analysis revealed expression of Osx protein in pOsx-UC-MSCs. However, the expression of Osx peptide was not detectable in mock-UC-MSCs and normal control cells (**Figure 3B**). It was suggested that Osx was expressed effectively in the UC-MSCs transfected with pEGFP-Osx.

Proliferation ability of UC-MSCs promoted by Osx

To functionally characterize the proliferation ability of pOsx-UC-MSCs, mock-UC-MSCs and normal control cells, the proliferation rate was evaluated by MTT assay. As shown in **Figure 4**, proliferation rate of pOsx-UC-MSCs was significantly higher than those of mock-transfected cells and normal control cells ($p < 0.05$).

ALP activity in UC-MSCs

Osteogenic differentiation of UC-MSCs was investigated after Osx transfection at days 3, 6, 9, 14, and 21. ALP activity, a marker for early osteogenic differentiation, was increased after pEGFP-Osx transfected UC-MSCs compared with the control group and mock group. ALP activity reached its peak levels at day 14 after pEGFP-Osx transfection (**Figure 5**).

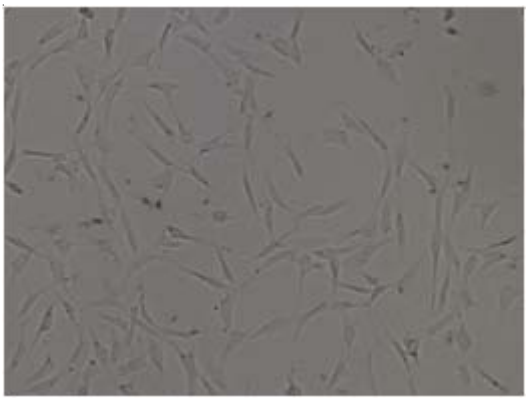

Figure 1. Morphology of primary cultured human UC-MSCs. Adhesive primary human UC-MSCs show spindle-like shape on day 3 (×100).

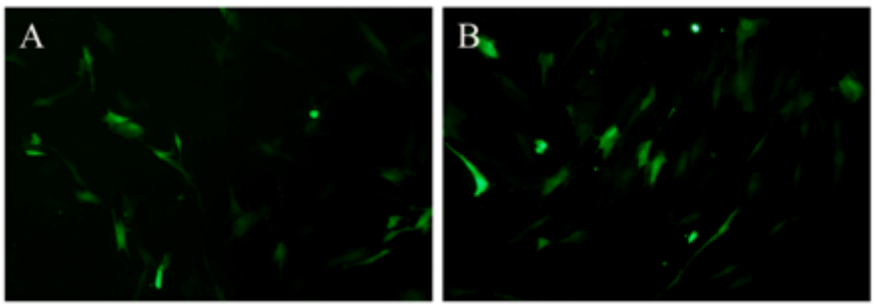

Figure 2. UC-MSCs emitted bright and intensive green fluorescence after transfected with pEGFP (A) and pEGFP-Osx (B) by fluorescence microscopy observation (×200).

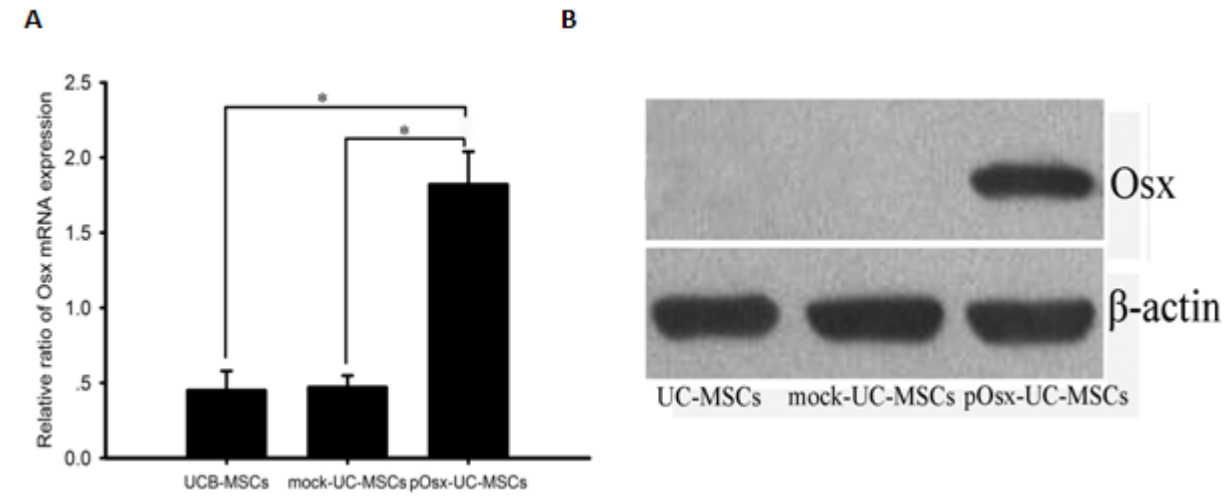

Figure 3. Expression of Osx in UC-MSCs detected by real-time RT-PCR and Western blotting. **A:** The expression of Osx mRNA of pOsx-UC-MSCs, mock-UC-MSCs, and normal control cells. Each value was expressed as the mean ± SEM (n = 3). *$p < 0.05$. **B:** Western blotting showed a speci□c Osx protein band is present in Osx transfected UC-MSCs.

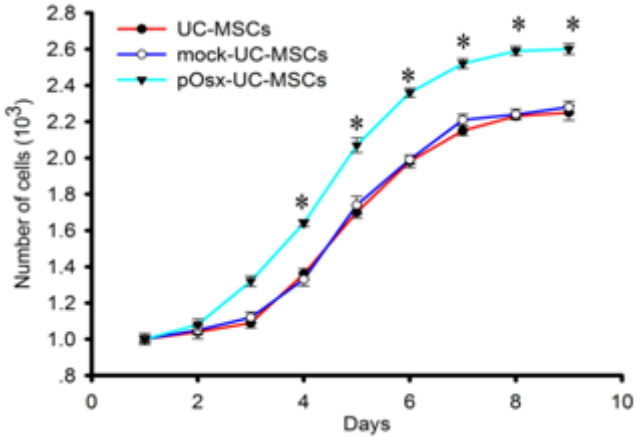

Figure 4. Proliferation rates of normal control UC-MSCs, mock-UC-MSCs, and pOsx-UC-MSCs. * $p < 0.05$, compared with mock-transfected cells and normal control cells

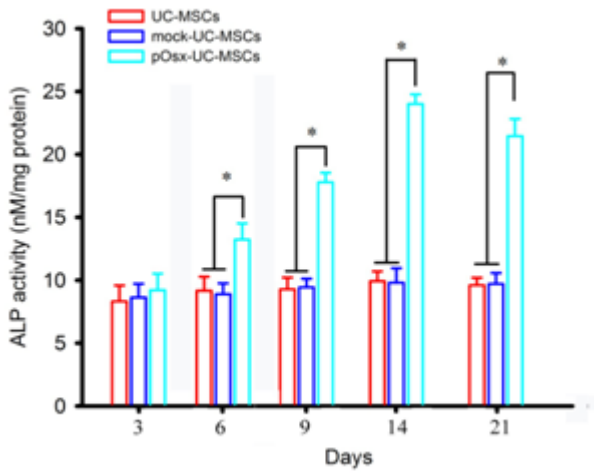

Figure 5. ALP activity (nmol/mg protein) was measured at days 3, 6, 9, 14, and 21 after pEGFP-Osx and pEGFP transfection. ALP activity of pOsx-UC-MSCs markedly increased at days 6 with a peak at days 14 post transduction, and remained at a higher level than the two control groups. ALP activity in control UC-MSCs and mock-UC-MSCs group remained unchanged (mean ± SEM, n = 6, * $p < 0.05$, compared with mock-transfected cells and normal control cells).

Mineralized nodule formation

Alizarin red staining was performed to investigate whether the expression of bone-related genes resulted in a mineralized nodule formation. As shown in **Figure 6,** the mineralized nodules positive for alizarin red staining formed at 21 days after con□uence in pOsx-UC-MSCs. On the other hand, mock-UC-MSCs and normal control UC-MSCs could not form any mineralized nodules at 21 days after con□uence.

Expression of osteoblastic genes

Expression of osteogenic genes was assessed at 7 and 14 days post transfection by real-time RT-PCR. These genes included OCN, cbf□1, OPN, COL I, ALP, and BSP. **Figure 7** showed that overexpression of Osx resulted in upregulated mRNA expression of all six osteogenic genes that were corresponding to the terminally differentiated stage of osteoblasts.

Discussion

Over the past few years, UC-MSCs are considered as promising alternative for MSCs used in the research and application of bone tissue engineering because they are easy to be isolated and expanded in vitro and be attained by a less invasive method without harm for the mother or infant [6]. However, it is still a challenge to enhance the MSCs proliferation and differentiation in the application of bone tissue engineering, and this problem may be solved by gene therapy approaches [20-22]. Recent studies have showed that MSCs osteogenesis was controlled by a number of signaling molecules. These signaling molecules include bone morphogenetic proteins (BMPs), secreted polypeptides of the Hedgehog, WNT, and FGF families, as well as transcription factors of Runx2, Osx and sox9 [23,24]. Among these molecules that pattern skeletal elements, the zinc □nger transcription factor Osterix has been shown to be a speci□c regulator of osteoblast differentiation [11]. However, no reports have been available to focus on the functions of Osx in the osteogenic differentiation of UC-MSCs in vitro.

In present study, we demonstrate that the constitutive overexpression of Osx enhanced the osteoblastic lineage differentiation of UC-MSCs by real time RT-PCR and western blotting. MTT assays indicated that pEGFP-Osx transfection promote the proliferation rate of UC-MSCs. Our data also indicted that these osteoblasts-like cells derived from UC-MSCs showed high levels of ALP activity, and formed well-de□ned mineralized nodules. Finally, real-time RT-PCR results showed an upregulation for the osteogenic related gene expression in pEGFP-Osx transfected UC-MSCs.

Genetic modi□cation to stem cells is an important development in bone engineering. Ideally, a stem cell for regenerative medicinal applications should meet the following criteria: (1) be abundantly available (millions to billions of cells); (2) be able to be harvested by a minimally invasive procedure; (3) be able to be differentiated reliably along various cell lineage pathways in a way that can be regulated and reproduced; (4) be safely and efficaciously transplantable to either an autologous or allogeneic host; (5) be able to be manufactured in accordance with current [25]. According the previous studies, US-MSCs are competent cells sources in tissue engineering and regenerative medicine, with the advantages of easy isolation and expansion [6, 7]. The different effects of Osx on cells of osteoblastic lineage may reflect the fact that different mechanisms may be operational in the various stem cells. It has been reported that overexpression of BMP-2 inhibited the proliferation of a cell line derived from rat

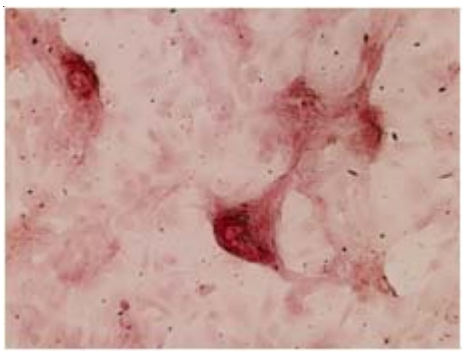

Figure 6. Alizarin red S staining of calcium nodules at 21 days after transfection with pEGFP-Osx (×100).

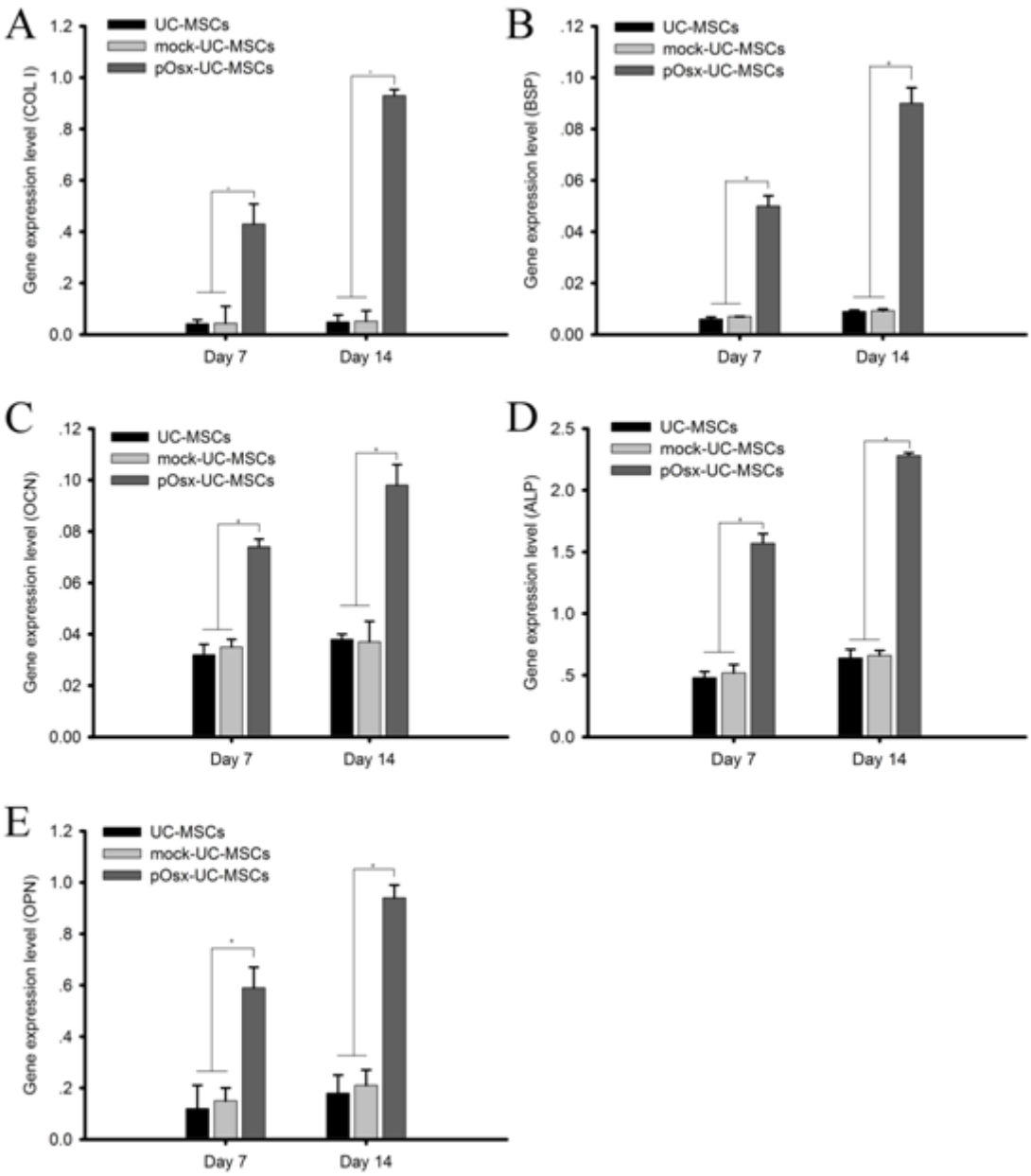

Figure 7. Expression of osteogenic differentiation–related genes. **A:** Type I collagen (COL I), **B:** bone sialoprotein (BSP), **C:** osteocalcin (OCN), **D:** ALP, and **E:** osteopontin (OPN) were evaluated by real-time reverse-transcription polymerase chain reaction at 7 and 14 days. Each value was expressed as the mean ± SEM (n = 3). *$p < 0.05$.

osteosarcoma, though it promoted their osteogenic differentiation [26]. Cho et al. and Igarashi et al. demonstrated that proliferative abilities of osteoblasts and mesenchymal stem cells were in□uenced by osteogenic induction [27, 28]. Therefore, it was believed that osteogenic induction on pluripotent cells with gene therapy approaches or chemical inducement affected their proliferation rate. However, our present studies revealed that Osx overexpression obviously increased the proliferation rate of UC-MSCs, as significant difference between Osx-transfected UC-MSCs and mock-UC-MSCs was found. This □nding is profound in the possible application of bone tissue engineering with genetically modi□ed UC-MSCs.

The ALP activity was used as a marker of osteoblastic differentiation in an early state or premature osteoblasts [29]. The results show that the ALP activity was upregulated in Osx transfected UC-MSCs. In addition, the ALP activity reached its peak levels at day 14 in pOsx-UC-MSCs group. In vitro

mineralization was considered to be a parameter of osteoblastic differentiation in the mature stage [30]. It is obvious in this study that Osx is the strongest osteoinductive factor, because it induced the highest amount of matrix mineralization without any osteogenic factors in the culture media. Similarly to our results, overexpression of Osx in muscle derived stem cells had been proved to enhance bone nodule formation in vitro [31]. By contrast, Osx overexpression did not promote osteoblastic differentiation or mineralization in fibroblasts NIH3T3 cell line [32], which suggested that the promotion of osteogenic differentiation was specific in multipotent stem cells or cells of osteoblastic lineage.

To further explore the effect of Osx overexpression in osteogenic differentiation of UC-MSCs, the mRNA expression of several osteogenic related genes were detected using real time RT-PCR. Genetically, Osx modified US-MSCs had the ability to express ALP, BSP, OCN, OPN, and Col I, which were responsible for the deposition and maturation of bone extracellular matrix. ALP is the most often used early marker of osteoblast differentiation [33]. As demonstrated in this study, ALP activity was upregulated in Osx-transfected UC-MSCs. OPN, also known as secreted phosphoprotein-1 (SPP1), urinary stone phosphoprotein, uropontin, and early T-cell activator (ETA-1), is involved in the regulation of bone mineralization as its affinity for calcium and its distribution in regions of mineralization [34,35]. BSP is a major noncollagenous protein in mineralizing connective tissues such as dentin, cementum and calcified cartilage tissues. As a member of the small integrin-binding ligand, N-linked glycoprotein (SIBLING) gene family of glycoproteins, BSP is involved in regulating hydroxyapatite crystal formation in bones and teeth, and has long been used as a marker gene for osteogenic differentiation [36]. On the other hand, OCN is produced by mature osteoblasts and odontoblasts during mineralization, and the message level is elevated during both cell proliferation and differentiation [37]. Taken together, the coexpression of these genes, together with the characteristic morphological and polarization changes, suggested the differentiation of the Osx transfected UC-MSCs into osteoblasts.

Although the exact mechanism by which Osx is involved in regulating the commitment of multipotent MSCs toward the osteoblastic lineage is yet well understood, growing evidence shows the Sp family plays an important regulatory role during proliferation and differentiation in musculoskeletal cells. Gersbach et al. demonstrated that Runx2 is an essential transcriptional regulator of chondrocyte hypertrophy, osteoblast differentiation, and bone formation [38]. Many studies have demonstrated that overexpression of Runx2 upregulates osteoblast-specific gene expression and induces mineralization in a cell-type-dependent manner in vitro, and promote bone formation in heterotopic (subcutaneous and intramuscular) and orthotopic (bone defect) sites [39, 40]. Nakashima et al. showed that Osx acts downstream of Runx2 to induce the differentiation of osteoprogenitors into mature osteoblasts and overexpression of OSX did not increase Cbfa1/Runx2 expression, but promoted bone regeneration [11]. Therefore, the MSCs transfected with OSX will probably provide a better gene therapy approach than Cbfa1/Runx2 in bone regeneration and wound repairing. In the present study, overexpression of Osx induced primary UC-MSCs into a mineralizing osteoblastic phenotype. These results further confirmed that Osx plays an important and effective role either directly or indirectly in the osteoblast differentiation of nonosteoblastic cells.

Conclusion

Osx overexpression can enhance the osteogenic differentiation of UC-MSCs in vitro. Concerning their simple isolation and proliferation, it is believed that genetically engineered UC-MSCs would play important roles in the study and application of bone tissue engineering.

The authors have no conflict of interest to declare. The first two authors contributed equally to this study.

References

1. Feldmann RE Jr, Bieback K, Maurer MH, Kalenka A, Burgers HF, Gross B, et al. Stem cell proteomes: a profile of human mesenchymal stem cells derived from umbilical cord blood. Electrophoresis. 2005; 26: 2749-58.
2. Chen TL, Shen WJ, Kraemer FB. Human BMP-7/OP-1 induces the growth and differentiation of adipocytes and osteoblasts in bone marrow stromal cell cultures. J Cell Biochem. 2001; 2:187-99.
3. Allan EH, Ho PW, Umezawa A, Hata J, Makishima F, Gillespie MT, et al. Differentiation potential of a mouse bone marrow stromal cell line. J Cell Biochem. 2003; 1: 158-69.

4. Mueller SM, Glowacki J. Age-related decline in the osteogenic potential of human bone marrow cells cultured in three-dimensional collagen sponges. J Cell Biochem. 2001; 82:583-90.
5. Stenderup K, Justesen J, Clausen C, Kassem M. Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. Bone. 2003:33:919-26.
6. Lu LL, Liu YJ, Yang SG, Zhao QJ, Wang X, Gong W, et al. Isolation and characterization of human umbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials. Haematologica. 2006; 91:1017-26.
7. Romanov YA, Svintsitskaya VA, Smirnov VN. Searching for alternative sources of postnatal human mesenchymal stem cells: Candidate MSC-like cells from umbilical cord. Stem Cells. 2003; 21:105-10.
8. Baksh D, Yao R, Tuan RS. Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. Stem Cells. 2007; 25:1384-92.
9. Wang HS, Hung SC, Peng ST, Huang CC, Wei HM, Guo YJ, et al. Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. Stem Cells. 2004; 22: 1330-7.
10. Chang YJ, Shih DT, Tseng CP, Hsieh TB, Lee DC, Hwang SM. Disparate mesenchyme-lineage tendencies in mesenchymal stem cells from human bone marrow and umbilical cord blood. Stem Cells. 2006; 24: 679-85.
11. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, et al. The novel zinc □nger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell. 2002; 108: 17-29.
12. Omoteyama K, Takagi M. The effects of Sp7/Osterix gene silencing in the chondroprogenitor cell line, ATDC5. Biochem Biophys Res Commun. 2010; 403: 242-6.
13. Lee JS, Lee JM, Im GI. Electroporation-mediated transfer of Runx2 and Osterix genes to enhance osteogenesis of adipose stem cells. Biomaterials. 2011; 32:760-8.
14. Yoshida CA, Furuichi T, Fujita T, Fukuyama R, Kanatani N, Kobayashi S, et al. Core-binding factor beta interacts with Runx2 and is required for skeletal development. Nat Genet. 2002; 32:633-8.
15. Cao Y, Zhou Z, de Crombrugghe B, Nakashima K, Guan H, Duan X, et al. Osterix, a transcription factor for osteoblast differentiation, mediates antitumor activity in murine osteosarcoma. Cancer Res. 2005; 65: 1124-8.
16. Tu Q, Valverde P, Chen J. Osterix enhances proliferation and osteogenic potential of bone marrow stromal cells. Biochem Biophys Res Commun. 2006; 341: 1257-65.
17. Ishige I, Nagamura-Inoue T, Honda MJ, Harnprasopwat R, Kido M, Sugimoto M, et al. Comparison of mesenchymal stem cells derived from arterial, venous, and Wharton's jelly explants of human umbilical cord. Int J Hematol. 2009; 90:261-9.
18. Xu J, Liao W, Gu D, Liang L, Liu M, Du W, et al. Neural ganglioside GD2 identifies a subpopulation of mesenchymal stem cells in umbilical cord. Cell Physiol Biochem. 2009; 23:415-24.
19. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods.1983; 65: 55-63.
20. Betz VM, Betz OB, Harris MB, Vrahas MS, Evans CH. Bone tissue engineering and repair by gene therapy. Front Biosci. 2008; 13:833-41.
21. Gamradt SC, Lieberman JR. Genetic modification of stem cells to enhance bone repair. Ann Biomed Eng. 2004; 32:136-47.
22. Kimelman N, Pelled G, Helm GA, Huard J, Schwarz EM, Gazit D. Review: gene- and stem cell-based therapeutics for bone regeneration and repair. Tissue Eng. 2007; 13:1135-50.
23. Harada S, Rodan GA. Control of osteoblast function and regulation of bone mass. Nature. 2003; 423:349-55.
24. Olsen BR, Reginato AM, Wang W. Bone development. Annu Rev Cell Dev Biol. 2000; 16:191-220.
25. Wu X, Wang S, Chen B, An X. Muscle-derived stem cells: isolation, characterization, differentiation, and application in cell and gene therapy. Cell Tissue Res. 2010; 340:549-67.
26. Huang W, Rudkin GH, Carlsen B, Ishida K, Ghasri P, Anvar B, et al. Overexpression of BMP-2 modulates morphology, growth, and gene expression in osteoblastic cells. Exp Cell Res. 2000; 2274:226-34.
27. Cho HH, Park HT, Kim YJ, Bae YC, Suh KT, Jung JS. Induction of osteogenic differentiation of human mesenchymal stem cells by histone deacetylase inhibitors. J Cell Biochem. 2005; 96:533-42.
28. Igarashi M, Kamiya N, Hasegawa M, Kasuya T, Takahashi T, Takagi M. Inductive effects of dexamethasone on the gene expression of Cbfa1, Osterix and bone matrix proteins during differentiation of cultured primary rat osteoblasts. J Mol Histol. 2004;

35:3-10.
29. Aubin JE. Advances in the osteoblast lineage. Biochem Cell Biol. 1998; 76:899-910.
30. Beck GR Jr, Sullivan EC, Moran E, Zerler B. Relationship between alkaline phosphatase levels, osteopontin expression, and mineralization in differentiating MC3T3-E1 osteoblasts. J Cell Biochem. 1998; 68: 269-80.
31. Sun S, Wang Z, Hao Y. Osterix overexpression enhances osteoblast differentiation of muscle satellite cells in vitro.Int J Oral Maxillofac Surg. 2008; 37:350-6.
32. Kim YJ, Kim HN, Park EK, Lee BH, Ryoo HM, Kim SY, et al. The bone-related Zn □nger transcription factor Osterix promotes proliferation of mesenchymal cells. Gene. 2006; 366:145-51.
33. Huang S, Wang Z. Influence of platelet-rich plasma on proliferation and osteogenic differentiation of skeletal muscle satellite cells: an in vitro study. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2010; 110:453-62.
34. Standal T, Borset M, Sundan A. Role of osteopontin in adhesion, migration, cell survival and bone remodeling. Exp Oncol. 2004; 26:179-84.
35. Gericke A, Qin C, Spevak L, Fujimoto Y, Butler WT, Sorensen ES, et al. Importance of phosphorylation for osteopontin regulation of biomineralization. Calcif Tissue Int. 2005; 77:45-54.
36. Golub EE. Role of Matrix Vesicles in Biomineralization. Biochim Biophys Acta. 2009; 1790: 1592-8.
37. Allori AC, Sailon AM, Warren SM. Biological basis of bone formation, remodeling, and repair-part II: extracellular matrix. Tissue Eng Part B Rev. 2008; 14: 275-83.
38. Gersbach CA, Byers BA, Pavlath GK, Garcia AJ. Runx2/Cbfa1 stimulates transdifferentiation of primary skeletal myoblasts into a mineralizing osteoblastic phenotype. Exp Cell Res. 2004; 300:406-17.
39. Yang S, Wei D, Wang D, Phimphilai M, Krebsbach PH, Franceschi RT. In vitro and in vivo synergistic interactions between the Runx2/Cbfa1 transcription factor and bone morphogenetic protein-2 in stimulating osteoblast differentiation. J Bone Miner Res. 2003; 18: 705-15.
40. Byers BA, Pavlath GK, Murphy TJ, Karsenty G, Garcia AJ. Cell-type-dependent up-regulation of in vitro mineralization after overexpression of the osteoblast-speci□c transcription factor Runx2/Cbfal. J Bone Miner Res. 2002; 17:1931-44.