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TGF-β/BMP Signaling Pathway Is Involved in Cerium-Promoted Osteogenic Differentiation of Mesenchymal Stem Cells

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ABSTRACT

The extensive applications of cerium (Ce) increased the chance of human exposure to Ce and its compounds. It was reported that Ce was mainly deposited in the bone after administration. However, the potential effect and mechanism of Ce on bone metabolism are not well understood. In this study, we investigated the cellular effects of Ce on the differentiation of mesenchymal stem cells (MSCs) and the associated molecular mechanisms. The results indicated that Ce promoted the osteogenic differentiation and inhibited the adipogenic differentiation of MSCs at cell level. Genes involved in transforming growth factor-β/bone morphogenetic proteins (TGF-β/BMP) signaling pathway were significantly changed when the MSCs were exposed to 0.0001 μM Ce by RT^{2}Profiler^{TM} PCR Array analysis. The expression of genes and proteins related to pathways, osteogenic, and adipogenic biomarkers of MSCs upon interaction with Ce was further confirmed by quantitative real-time reverse transcriptase polymerase chain reaction (Q-PCR) and Western blot analysis. The results suggest that Ce exerts the effects by interacting with bone morphogenetic protein receptor (BMPR) and activates TGF-β/BMP signaling pathway, leads to the up-regulation of the osteogenic master transcription factor, runt-related transcription factor 2 (Runx 2), and the down-regulation of the adipocytic master transcription factor, peroxisome proliferator-activated receptor gamma 2 (PPARγ2). Runx2, which subsequently up-regulates osteoblast (OB) marker genes collagen I (Col I) and BMP2 at early stages, alkaline phosphatase (ALP), and osteocalcin (OCN) at later stages of differentiation, thus driving MSCs to differentiate into OBs. The results provide novel evidence to elucidate the mechanisms of bone metabolism by Ce. J. Cell. Biochem. 114: 1105–1114, 2013.

KEY WORDS: CERIUM; MESENCHYMAL STEM CELLS; OSTEOGENIC DIFFERENTIATION; ADIPOGENIC DIFFERENTIATION; SIGNALING PATHWAY

Cerium, one of the lanthanides (Ln), exhibits unusual physical and chemical properties. It also displays a variety of biochemical and physiological effects primarily based on its similarity to calcium. For example, the use of cerium oxalate as an anti-emetic was first described in the mid-nineteenth century. Several cerium(III) salts including acetate, stearate, chloride, and nitrate were reported to have antibacterial activity. Systematic studies have confirmed that cerium nitrate had broad-spectrum antibacterial activity against a range of bacteria including *Pseudomonas aeruginosa* and *Staphylococcus aureus* [Havanur et al., 2011]. It was reported that cerium(III) iodide had activity against solid tumors, cerium(III) complexes with coumarin demonstrated cytotoxicity against HL-60 cell line [Fricker, 2006]. In China, lanthanides have been used to promote the growth of crops for many years [Wang et al., 1999, 2003]. In addition, cerium oxide has also been used in emission control systems in gasoline engines as a diesel fuel-born catalyst to reduce particulate matter emissions [Limbach et al., 2008; Park et al., 2008a; Xu et al., 2008]. However, these extensive applications increased the chance of human exposure to Ce and its compounds and thus raised deep concerns regarding their riskiness.

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It was reported that cerium was rapidly cleared from the blood and redistributed to tissues, primarily bone after administration [Fricker, 2006]. The similarity between Ln^{3+} and Ca^{2+} has been suggested to be responsible for some of their biochemical behaviors. Thus it is likely that Ce intervenes in bone-remodeling process and affects bone cell function. We previously reported that the effects of Ce on the proliferation, differentiation, and mineralization function of primary OBs depended on the concentration and culture time at cell level [Zhang et al., 2010]. However, the potential effect and the mechanism of Ce on bone metabolism are not well understood.

MSCs are pluripotent cells which have the capacity to become OBs and adipocytes [Owen and Friedenstein, 1988; Prockop, 1997]. Thus, lineage determination between OBs and adipocytes may be a critical component in the regulatory pathways of osteoblastogenesis. Furthermore, there is more and more evidence that suggests a great degree of plasticity exists between OBs and adipocytes and this transdifferentiation is reciprocal. Indeed, it is now hypothesized that an increase in the number of adipocytes occurs at the expense of OBs in osteopenic disorders [Nuttall and Gimble, 2000]. In addition, the experimental data suggest that medullary adipocytes are secretory cells that may affect hematopoiesis and osteogenesis. It was reported that the condition medium harvested from mouse stromal preadipocytes decreased the ALP activity of a mouse stromal osteoblastic cell line [Benayahu et al., 1993]. Ailhaud et al. [1992] reported that adipocytes could synthesize and release a variety of peptide and nonpeptide compounds or secrete cytokines such as tumor necrosis factor-α (TNF-α), the main effect of these cytokines is a stimulation of bone resorption. So it was reported that there might be a therapeutic opportunity to either prevent or treat osteopenic disorders by inhibiting marrow adipogenesis with a concomitant increase in osteoblastogenesis [Tamama et al., 2008]. Until now, whether and how Ce has the potential effect on bone metabolism by modulating osteogenic differentiation and adipocytic differentiation of MSCs have not been reported.

In this study, we investigated the effects of Ce on the osteogenic and adipogenic differentiation of MSCs and the associated molecular mechanisms. The cellular effects of Ce on the proliferation, osteogenic differentiation, adipogenic differentiation, and mineralization of MSCs were evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H tetrazolium bromide (MTT), ALP activity, Oil Red O staining and alizarin red staining (ARS) staining assays, respectively. On the basis of the cellular effects, Q-PCR and Western blot analysis were designed to elucidate the interactional mechanism between Ce and MSCs by determining the expression of genes and proteins related to pathways, the osteogenic and adipogenic biomarkers of MSCs upon interaction with Ce. The results suggest that the interaction of MSCs with Ce may lead to the activation of the TGF-β/BMP signaling pathway and the up-regulation of osteogenic genes and the down-regulation of adipogenesis specific genes.

MATERIALS AND METHODS

MATERIALS

Four to 6 weeks specific pathogen free (SPF) kunming (KM) female mice were obtained from Medical Laboratory Animal Center of Hebei Province (for all studies involving the use of animals, we have received permission from the Institutional Animal Care and Use Committee). Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco. MTT, cerium(III) chloride heptahydrate (purity 99.999%), benzylpenicillin, streptomycin, β-glycerophosphate, dexamethasone, ascorbic acid, collagen II, insulin, Oil Red O stain, and ARS were from Sigma. An ALP activity kit was obtained from Nanjing Jiancheng Biological Engineering Institute (Nanjing city, China), and a micro-protein assay kit was purchased from Beyotime Biotechnology (Haimen city, China). Trizol Plus RNA purification kit was obtained from Invitrogen. RT^2 qPCR Master Mix, SYBR Green/ROX Master Mix, and Mouse Mesenchymal Stem Cell PCR Arrays were purchased from SABiosciences. Anti-Runx2, anti-BMP2, anti-OCN, and anti-PPARγ2 were from Santa Cruz Biotechnology. Anti-CCAAT enhancer binding protein α (anti-C/EBPα), anti-CCAAT enhancer binding protein β (anti-C/EBPβ), and anti-CCAAT enhancer binding protein δ (anti-C/EBPδ) were from Bioworld Technology. Pierce enhanced chemiluminescene (ECL) Western blot substrate was obtained from Thermo Fisher Scientific Inc. Other reagents were of analytical grade. $CeCl_3$ solution was prepared by dissolving solid cerium(III) chloride heptahydrate in 0.9% NaCl solution and was diluted to 50 mM $CeCl_3$. The stock solution was stored at −20°C.

ISOLATION AND CULTURE OF MSCs

MSCs were prepared from 6-week-old SPF KM female mice as described in detail previously [Liu et al., 2010]. In brief, 12 mice were executed by cervical dislocation. Femora and tibiae were aseptically harvested, and the whole bone marrow was flushed using supplemented DMEM in a 10 ml syringe and a 25 gauge needle. Suspended whole bone marrow was washed by DMEM. The cells were collected and cultured in DMEM with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin for 3 days in a humidified atmosphere of 5% CO_2 in air at 37°C, then replaced with fresh medium. The culture medium was changed every 3 days during the experiments.

CELL VIABILITY ASSAY

The MSCs viability was determined by MTT assay as described in detail previously [Liu et al., 2010]. In brief, MSCs were seeded in 96-well tissue culture plates at the density of 4×10^6 cells/well and incubated for 72 h. After the addition of $CeCl_3$ at different concentrations (final concentration 0.0001, 0.001, 0.01, 0.1, 1, 10, and 100 μM), 24, 48, and 72 h further incubations were performed. Cells without $CeCl_3$ treatment were used as control. Then, the adherent cells were harvested for MTT assay. Briefly, 20 μl of MTT (5.0 mg/ml in 1× phosphate buffered saline (PBS)) was added and incubated for another 4 h at 37°C. The supernatant was removed, and dimethyl sulfoxide (DMSO) was added to each well for 10 min to dissolve formazan crystals. The optical density (OD) was measured at 550 nm using a microplate spectrophotometer (Bioradmodel680, USA). Each sample was assayed in quadruplicate, with each experiment repeated at three times independently. The MSCs viability (%) was expressed as a percentage of $[OD_{sample} - OD_{blank}]/[OD_{control} - OD_{blank}] \times 100$.

ALP ACTIVITY ASSAY FOR OSTEOGENIC DIFFERENTIATION OF MSCs

MSCs were seeded in 48-well tissue culture plates at the density of 5×10^6 cells/well with the osteogenic induction supplement (OS) containing 5.0 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and 0.1 μM dexamethasone. A series of dilutions of $CeCl_3$ (final concentration 0.0001, 0.001, 0.01, 0.1, 1, 10, and 100 μM) were added to the culture medium with OS in 48-well plates for 7, 10, and 14 days. MSCs treated with only OS were used as control group. NaF was used as a positive control. After incubation, MSCs were washed twice with ice-cold PBS and lysed by two cycles of freezing and thaw. Aliquots of supernatants were subjected to ALP activity and protein content measurement by an ALP activity kit and a micro-Bradford assay kit [Zhang et al., 2007]. Each sample was assayed in quadruplicate, with each experiment repeated at three times independently. All results were normalized by protein content.

ASSAY FOR MINERALIZED MATRIX FORMATION

ARS staining assay was used to evaluate mineralized matrix formation as described in detail previously [Liu et al., 2010]. MSCs (5×10^6 cells/well) were seeded in 48-well tissue culture plates and cultured for 3 days. Then, the medium was changed to medium containing OS and $CeCl_3$ (final concentration 0.0001, 0.001, 0.01, 0.1, 1, 10, and 100 μM) or 1.0 μM NaF for 14, 18, and 22 days. The formation of mineralized matrix nodules was determined by ARS staining. In brief, MSCs were fixed in 70% ethanol for 1 h and then stained with 40 mM ARS (pH 4.2) for 30 min at room temperature. Quantification of ARS staining was performed by elution with 10% (w/v) cetylpyridiumchloride for 10 min and measurement of the OD value at 570 nm. Each sample was assayed in quadruplicate, with each experiment repeated at three times independently. The mineralization promotion rate (%) was expressed as a percentage of $[OD_{sample} - OD_{blank}]/[OD_{control} - OD_{blank}] \times 100$.

OIL RED O STAINING ASSAY FOR ADIPOGENIC DIFFERENTIATION OF MSCs

The adipocytic differentiation rates of MSCs in the absence and presence of $CeCl_3$ were determined by specifically staining intracytoplasmic lipids with Oil Red O [Sekiya et al., 2002]. MSCs (1×10^7 cells/well) were seeded in 48-well tissue culture plates and cultured for 15, 18, and 21 days. The adipogenic induction supplement (AS; 10 mg/L insulin, 1.00×10^{-7} mol/L dexamethasone) and $CeCl_3$ (final concentration 0.0001, 0.001, 0.01, 0.1, 1, 10, and 100 μM) were added to the culture medium. After incubation, MSCs were washed by PBS twice, and then stained by 0.6% (w/v) Oil Red O solution (60% isopropyl alcohol, 40% water) for 15 min at room temperature. For quantification of Oil Red O content, MSCs were washed with dH_2O three times to remove background staining, and isopropyl alcohol was added to resolve Oil Red O. The OD value at 510 nm was measured on a microplate spectrophotometer (Bio-rad Model 680, USA). Each sample was assayed in quadruplicate, with each experiment repeated at three times independently. The adipogenic differentiation rate (%) was expressed as a percentage of $[OD_{sample} - OD_{blank}]/[OD_{control} - OD_{blank}] \times 100$.

Q-PCR ANALYSIS

Total RNA from MSCs which were treated with 0.0001, 0.01, and 1 μM $CeCl_3$ in the presence of OS or AS for 4 days was extracted using Trizol Plus RNA purification kit and was reverse transcribed to first-strand cDNA according to the TaKaRa protocol. An RT^2ProfilerPCR Array System containing 84 relevant pathway focused genes of differentiation of MSCs was performed using ABI 7300 Sequence Detection System (Applied Biosystems, USA) according to the manufacturer's protocol. A 25 μl portion of the experiment cocktail containing 13 μl of SABiosciences RT^2 qPCR Master Mix, 1 μl of cDNA, and 11 μl of ddH_2O was added to each well where the gene-specific primer used for RT-PCR was immobilized. PCR products were analyzed with PCR array data analysis web portal from the following address: http://www.SABiosciences.com/pcrarraydataanalysis.php. RT-PCR was performed in a total volume of 25 μl with 1 μl of cDNA, 1 μl of gene-specific 10 μM PCR primer pair stock, and 12.5 μl of SYBR Green/ROX Master Mix using ABI 7000 Sequence Detection System. The PCR profile began with 10 min at 95°C to activate TaqDNA polymerase, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C, and later followed by the melting curve test. The relative amount of mRNA expression normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was expressed as fold change, which was calculated by the comparative CT ($2^{-\Delta\Delta Ct}$) relative to control group as a reference: $2^{-\Delta\Delta Ct} = 1$ [Liu et al., 2010]. Each sample was assayed in quadruplicate, with each experiment repeated at three times independently. The primers used for qRT-PCR were shown in Table I.

WESTERN BLOT ANALYSIS

Western blots were employed to study protein expression of MSCs upon their interactions with Ce, as described in detailed previously [Liu et al., 2010]. In brief, MSCs were washed with cold PBS and

TABLE I. qRT-PCR Primers

Gene symbol	Forward primer (5′–3′)	Reverse primer (5′–3′)
Runx-2	TTCTCCAACCCACGAATGCAC	CAGGTACGTGTGGTAGTGAGT
BMP-2	TGGCCCATTTAGAGGAGAACC	AGGCATGATAGCCCGGAGG
ALP	GTTGCCAAGCTGGGAAGAACAC	CCCACCCCGCTATTCCAAAC
BSP	GAATCCACATGCCTATTGC	AGAACCCACTGACCCATT
OCN	GAACAGACTCCGGCGCTA	AGGGAGGATCAAGTCCCG
Col I	AACATGACCAAAAACCAAAAGTG	CATTGTTTCCTGTGTCTTCTGG
ERα-1	GAATCCACATGCCTATTGC	AGAACCCACTGACCCATT
GAPDH	GACTTCAACAGCAACTCCCAC	TCCACCACCCTGTTGCTGTA
PPARγ2	TGTGGGGATAAAGCATCAGGC	CCGGCAGTTAAGATCACACCTAT
C/EBPα	GTGCTTCATGGAGCAAGCCAA	TGTCGATGGAGTGCTCGTTCT
C/EBPβ	GCGCGAGCGCAACAACATCG	CAGCACAGGCTGTTGACCATCATA
C/EBPδ	GAGCGTCCTACGCGCCAGTAC	GATCACGGAGCTGTGCCGGTC

lysed in cold 50 mM Tris–HCl (pH 7.4), 10 mM ethylene diamine tetraacetie acid (EDTA), 4.3 M Urea, and 1% Triton X-100. Proteins were subjected to sodium dodecylsulfonate–polyacrylate gel electrophoresis (SDS–PAGE) using 10% gel and transferred onto a nitrocellulose membrane. The membrane was blocked for 2 h at room temperature with 5% bovine serum albumin in TBST solution (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20). Then, the blots were incubated with corresponding primary antibodies in the TBST solution overnight at 4°C, followed by 1 h incubation with secondary antibodies conjugated with horseradish peroxidase, and visualized with an enhanced chemiluminescene (ECL) kit. The OD of bands was quantified by LAS-1000 image analyzer (Fuji-Film) software. β-Actin protein expression was used as a loading control. Each sample was assayed in quadruplicate, with each experiment repeated at three times independently.

STATISTICAL ANALYSIS

Data were collected from three separate experiments and expressed as mean ± standard deviation (SD). The statistical differences were analyzed by Student's *t*-test. *P* values less than 0.05 were considered to indicate statistical differences.

RESULTS

EFFECT OF Ce ON THE VIABILITY OF MSCs

MTT assay was employed to measure the metabolic activity of the mitochondria of cells based on the principle that the living cells are capable of reducing light color tetrazolium salts into an intense color formazan derivative. As shown in Figure 1A, the results revealed that Ce displayed a slight positive effect on the MSC viability at concentrations of 0.0001, 0.001, 0.01, and 0.1 μM, had no effect on the MSC viability at a concentration of 1 μM, turned to decrease the viability of MSCs at concentrations of 10 and 100 μM for 1 and 2 days. On day 3, Ce increased the viability of MSCs at concentrations of 0.0001, 0.001, 0.01, 0.1, 1, and 10 μM, but decreased the viability of MSCs at a concentration of 100 μM. In summary, the effect of Ce on the viability of MSCs was similar, the viability of MSCs at 3 days was found to be higher than 1 and 2 days, moreover, the viability of MSCs was decreased with increasing Ce concentrations.

EFFECT OF Ce ON THE OSTEOGENIC DIFFERENTIATION OF MSCs

Differentiation of pluripotent progenitor MSCs into OBs is a crucial step of osteogenesis. The appearance of ALP activity is an early phenotypic marker for osteogenic differentiation of MSCs. Generally, ALP activities were expressed after in vitro osteogenic induction for 7 days, while later positive ALP staining was seen after 14 days of osteogenic induction. The effects of Ce on osteogenic differentiation of MSCs cultured for 7, 10, and 14 days in the presence of OS were assessed by measuring ALP activity normalized to total protein content. As shown in Figure 1B, the results showed that ALP activity of MSCs treated by all concentrations of Ce was increased compared with that of OS on Day 7 and 10. On day 14, Ce increased the ALP activity at concentrations of 0.0001, 0.001, 0.01, 0.1, and 1 μM, but decreased ALP activity at concentrations of 10 and 100 μM. These results indicated a substantial increase of ALP activity in MSCs upon treatment of Ce in time-dependent manners at most concentrations.

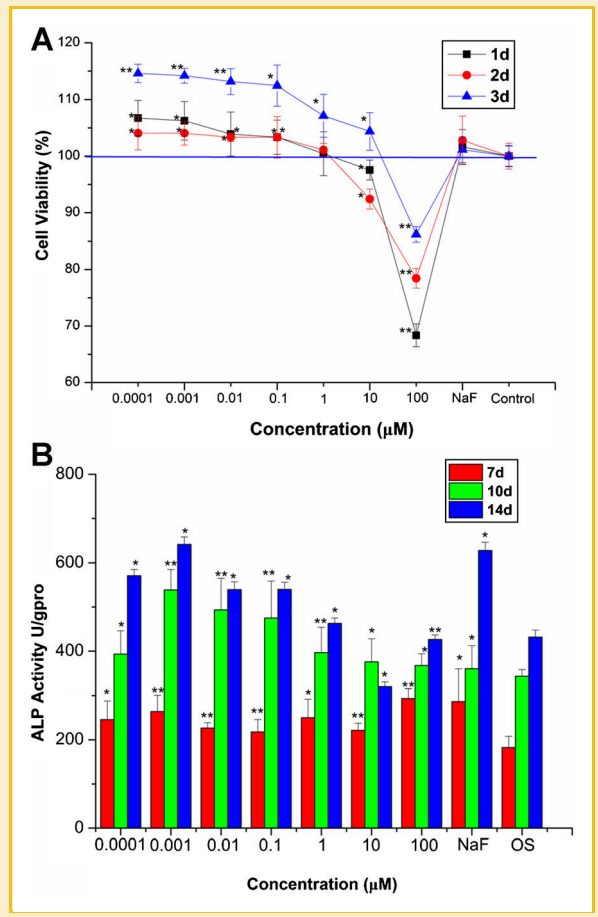

Fig. 1. Effects of Ce on the viability and ALP activity of MSCs. A: the viability: MSCs were treated with various concentrations of Ce for 1, 2, and 3 days and analyzed by MTT assay. B: the ALP activity: MSCs were treated with various concentrations of Ce for 7, 10, and 14 days and assessed by measuring the ALP activity normalized to total protein content. Data are presented as mean ± SD from a representative of three separate experiments performed in quadruplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control.

EFFECT OF Ce ON THE FORMATION OF MINERALIZED MATRIX NODULES

Mineralized nodule formation is a phenotypic marker for the last stage of mature OBs. An essential sign for the osteogenic differentiation of MSCs is bone matrix maturation and mineralization. After cultured for 2–3 weeks, OB nodes formed and reached peak quantity when OBs started to mineralize. MSCs from bone marrow exhibited mineralization upon treatment with Ce for 14, 18, and 22 days. The mineralized nodules were counted by staining with ARS (Fig. 2A–D), and the amount of mineralization was quantitated by elution of ARS from stained mineral deposits. As shown in Figure 2E, MSCs treated with OS were used as normal control, and 1.0 μM NaF was used as positive control, which promoted mineralization of MSCs by 34%, 70%, and 84% on day 14, 18,

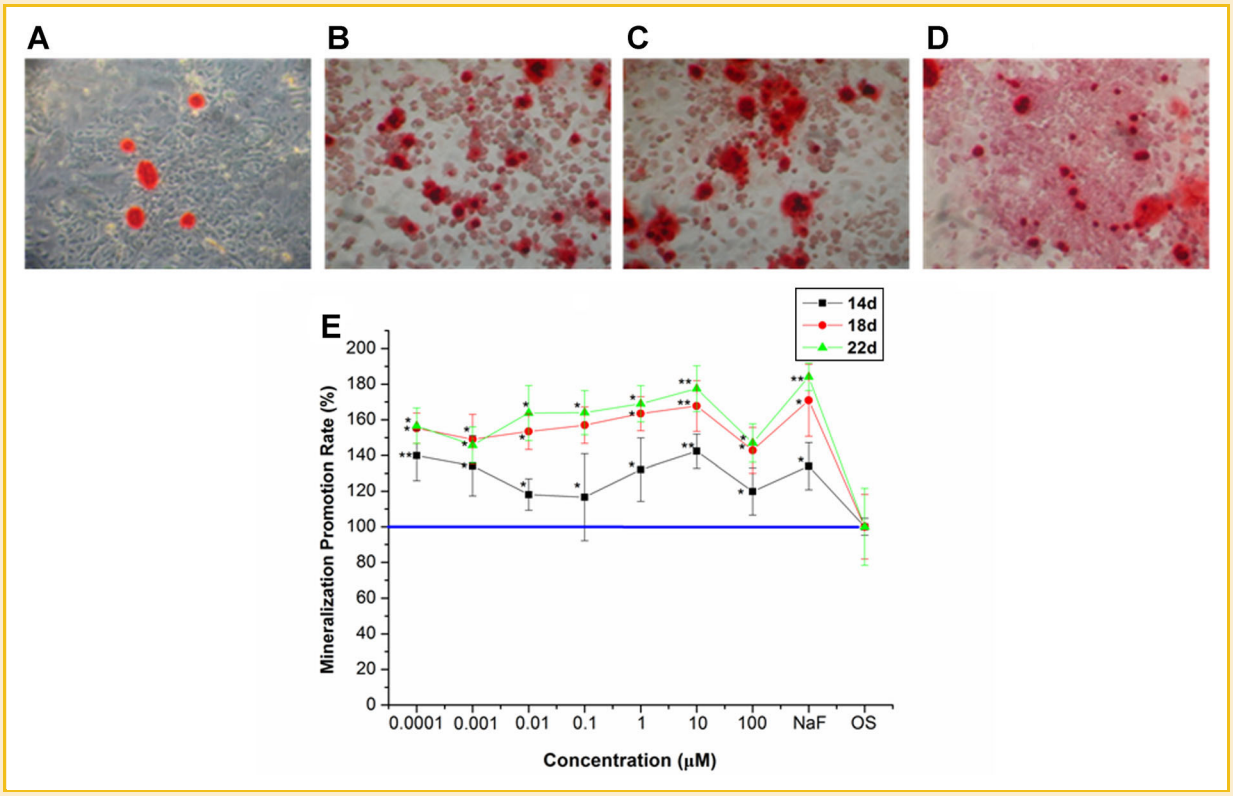

Fig. 2. Effect of Ce on the formation of mineralized matrix nodules. A–D: Photographs of mineralized nodules stained by ARS. A: Cells treated with OS only. B: Cells treated with 0.01 μM Ce + OS. C: Cells treated with 1 μM Ce + OS. D: Cells treated with 10 μM Ce + OS. Original magnification = 100×. E: The mineralization promotion rate was performed by quantification of ARS staining. Data are presented as mean ± SD from a representative of three separate experiments performed in quadruplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the OS group.

and 22, respectively. Coupling the number count with quantitation of ARS deposition revealed that Ce promoted the formation of mineralized matrix nodules of MSCs in a time-dependent manner without evident dose-dependence at most concentrations.

EFFECT OF Ce ON THE ADIPOGENIC DIFFERENTIATION OF MSCs

Both adipocytes and OBs originate from MSCs and have an inversely proportional relationship. Therefore, it is also important to investigate the effects of Ce on the adipocytic differentiation of MSCs. The adipocytic differentiation inhibition rates of MSCs in the absence and presence of Ce were determined by specifically staining intracytoplasmic lipids with Oil Red O. Figure 3A–C shows adipocytic differentiation of MSCs in the absence and presence of Ce stained by Oil Red O. As shown in Figure 3D, on the whole, Ce inhibited adipogenic differentiation of MSCs at tested concentrations, but had no obvious dose- or time-dependent tendency.

Q-PCR ANALYSIS OF OSTEOGENIC AND ADIPOGENIC DIFFERENTIATION SPECIFIC GENES

Q-PCR analysis was used to measure the expression of mRNA transcripts of tissue specific molecules in order to reveal the involvement of specific signaling pathways in Ce-modulated differentiation of MSCs. The GAPDH gene was used as the calibrator gene (control). Parts of results from RT^{2}Profiler^{TM} PCR Array were shown in Figure 4, those genes expression were significantly changed when the MSCs were exposed to 0.0001 μM Ce. Interestingly, most of these genes were involved in TGF-β/BMP signaling pathway, such as Tgfb3, Tgfb1, Smad4, Bmp7, Bmp6, Bmp4, and Bmp2 were up-regulated. On the other hand, the expression of Smurf1, Smurf2, Gdf7, Gdf6, Gdf5, and Gdf15 was down-regulated (see Supporting Information, Fig. S1, Tables S1, S2, and S3 for descriptions of all genes and their functions).

As shown in Figure 5A, the results showed that several genes (such as Runx2, BMP2, ALP, BSP, Col I, OCN, and ERα) that were supposed to be activated during osteogenic differentiation were significantly up-regulated in the MSCs treated with Ce (0.0001, 0.01, and 1 μM) for 4 days as compared to OS group.

Because of the reciprocal relationship between osteogenic and adipogenic differentiation of MSCs, it is possible that the promotion of osteogenesis occurs concurrently with the inhibition of adipogenesis. As shown in Figure 5B, the results also showed that several genes (such as C/EBPα, C/EBPβ, C/EBPδ, and PPARγ2) were significantly down-regulated in the MSCs treated with Ce (0.0001, 0.01, and 1 μM) for 4 days as compared to AS group.

WESTERN BLOT ANALYSIS OF KEY PROTEINS INVOLVED IN THE BMP SIGNALING PATHWAY

Western blot can be used to detect the expression of a specific protein in a given sample. To demonstrate the activation of the BMP signaling pathway and subsequent promotion osteogenic differen-

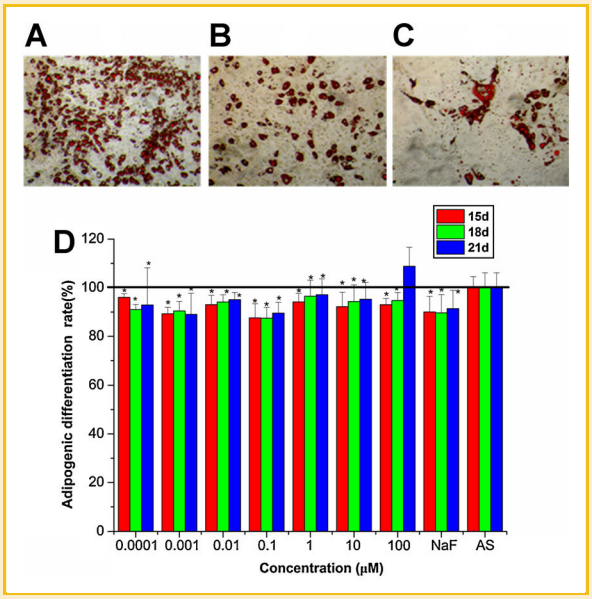

Fig. 3. Effect of Ce on the adipogenic differentiation of MSCs. A–D: Photographs of adipocytes stained by Oil Red O. A: Cells treated with AS only. B: Cells treated with AS + 0.01 μM Ce. C: Cells treated with AS + 10 μM Ce. D: The adipogenic differentiation rate was performed by quantification of Oil Red O content. Data are presented as mean ± SD from a representative of three separate experiments performed in quadruplicate. $^{*}P < 0.05$ compared with the AS group.

tiation of MSCs, the expression levels of key proteins involved in the BMP signaling pathway, such as Smad protein and phosphorylated Smad proteins (pSmad1/5/8), and the master transcription factors proteins, such as Runx2 and PPARγ proteins, were measured. Western blotting results (Fig. 6A,B) showed that the expression of p-Smad1/5/8 was significantly promoted by Ce at 0.0001 μM. In

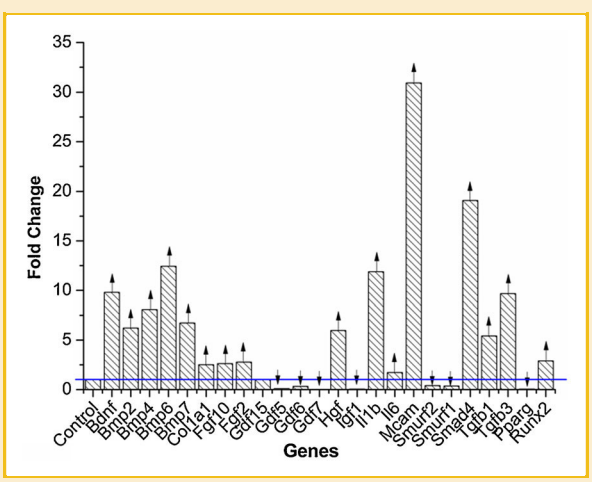

Fig. 4. TGF-β/BMP signaling genes up-regulated in SuperArray gene profiles. An RT^{2}ProfilerPCR Array System containing 84 relevant pathway focused genes of differentiation of MSCs was performed using ABI 7300 Sequence Detection System according to the manufacturer's protocol.

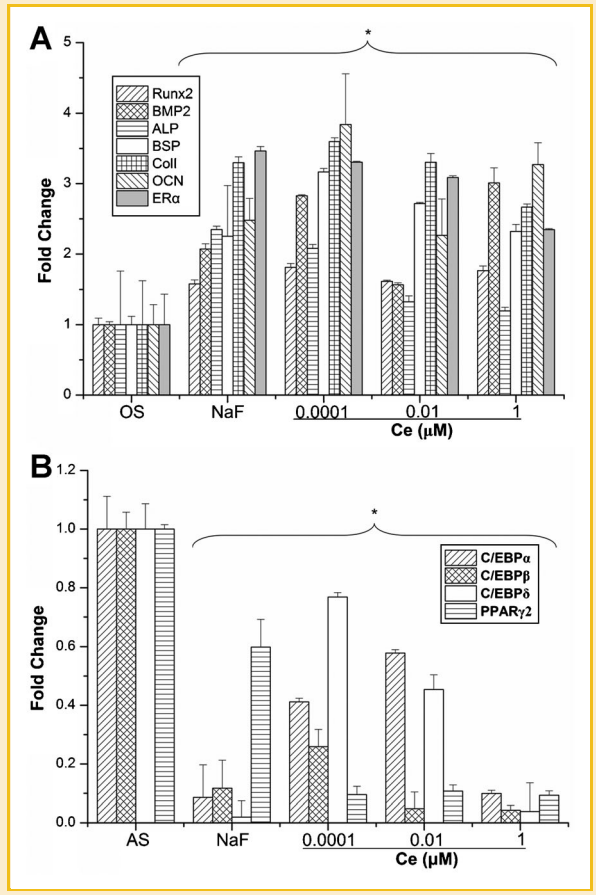

Fig. 5. Q-PCR analysis for the expression of osteogenic and adipogenic differentiation specific genes. A: The expression of osteogenic differentiation specific genes was examined in the presence or absence of 0.0001, 0.01, and 1 μM Ce for 4 days. Data are presented as mean ± SD from a representative of three separate experiments performed in quadruplicate. $^{*}P < 0.05$ compared with the OS group. B: The expression of adipogenic differentiation specific genes was examined in the presence or absence of 0.0001, 0.01, and 1 μM Ce for 4 days. Data are presented as mean ± SD from a representative of three separate experiments performed in quadruplicate. $^{*}P < 0.05$ compared with the AS group.

addition, the protein expression of osteogenesis in MSCs upon osteogenic induction was showed in Figure 6C,D. The expression of BMP2, Runx2, and OCN was up-regulated after Ce treatment. To be in conformity with the PCR results, the expression of adipogenic differentiation related proteins was down-regulated by Ce (Fig. 7).

DISCUSSION

Cells maintain their homeostasis through a comprehensive signaling network. Any perturbation of this system by Ce will influence cell function and behavior [Mu et al., 2009]. The results from viability assay suggested that Ce increased the cell viability at most concentrations (Fig. 1A). A substantial increase of ALP activity in MSCs upon treatment of Ce in time-dependent manner was observed at most concentrations (Fig. 1B). Coupling the number count with

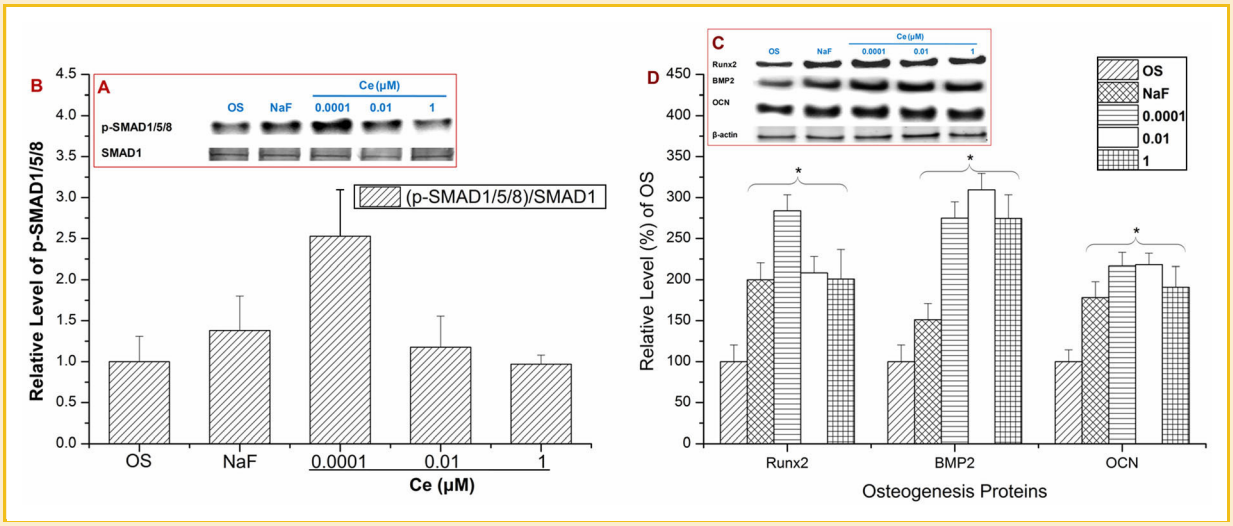

Fig. 6. The expression levels of key proteins involved in the BMP signaling pathway. The expression levels of key proteins involved in the BMP signaling pathway were examined in the presence or absence of 0.0001, 0.01, and 1 μM Ce under the OS conditions for 4 h. A: Western blot analysis for Smad1 and pSmad1/5/8 proteins. B: Quantification of the blots for p-Smad1/5/8/Smad1 protein. C: Western blot analysis for BMP2, Runx2, and OCN proteins. D: Quantification of the blots for BMP2, Runx2, and OCN proteins. Data are presented as mean ± SD from a representative of three separate experiments performed in quadruplicate. $^{*}P < 0.05$ compared with the OS group.

quantization of ARS deposition revealed Ce promoted the formation of mineralized matrix nodules of MSCs at all tested concentrations (Fig. 2). Ce inhibited adipogenic differentiation of MSCs at all tested concentrations, but had no obvious dose- or time-dependent tendency (Fig. 3). These data strongly suggested that Ce promoted the osteogenic differentiation and inhibited the adipogenic differentiation of MSCs.

But does which biologically active species regulate the osteogenic and adipogenic differentiation of MSCs upon treatment of Ce? It is

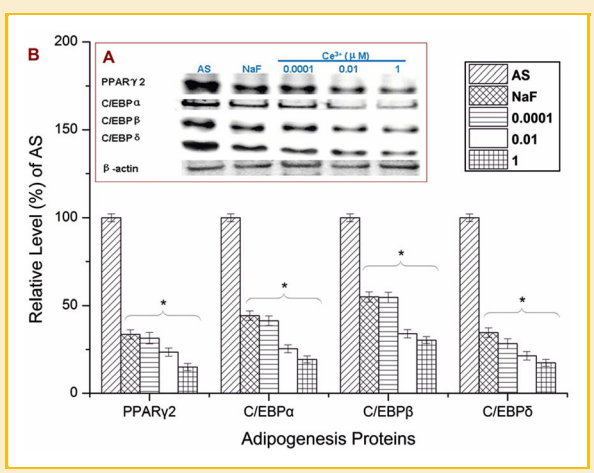

Fig. 7. The expression levels of adipogenic differentiation related proteins. The expression levels of adipogenic differentiation related proteins were examined in the presence or absence of 0.0001, 0.01, and 1 μM Ce under the AS conditions for 4 days. A: Western blot analysis for PPARγ2, C/EBPα, C/EBPβ, and C/EBPδ proteins. B: Quantification of the blots for PPARγ2, C/EBPα, C/EBPβ, and C/EBPδ proteins. Data are presented as mean ± SD from a representative of three separate experiments performed in quadruplicate. $^{*}P < 0.05$ compared with the AS group.

well known that pH 7.4 is fundamental for cell culture in vitro and the regular cell culture medium usually includes 0.91 mM PO_4^{3-}, which is similar to the concentration of phosphate in blood. Numerous pieces of evidence showed that "precipitation problem" was involved in cellular studies of the biological effects of multivalent metal cations [Berthon, 2002]. Li et al. found that gadolinium ions formed gadolinium and phosphorous-containing nanoparticles or microparticles and deposited on the cell surface in cell culture medium. The particles may have acted as a biologically active entity to mediate cell cycle progression in NIH3T3 cells. The proliferation-promoting effect might be attributed to a nonspecific ROS-associated mechanism. The smaller gadolinium particles exhibited a stronger cell-cycle-promoting effect than the larger ones, but they shared the common signaling pathways. Furthermore, the results showed that the particles may act as a gadolinium "buffer pool," from which Gd^{3+} ions were released to promote cell cycle progression in NIH3T3 cells [Li et al., 2010]. As stated above, we cannot also exclude the possibility that Ce at higher concentrations would form precipitates under physiological conditions. In fact, it has reported that Ce ions have been used as the capture agent for inorganic phosphate released, cerium phosphate reaction product appears as nanoparticles and deposited on the cell surface [Robinson and Karnovsky, 1983]. It was found that cerium in the cardiac tissue and serum of patients was accumulated, the biologically active species might be the precipitate of hydroxide or insoluble salts according to their chemical properties [Rzigalinski, 2005]. In addition, studies also showed that free radicals might form a biological intermediate in cerium-stimulated cardiac fibroblast proliferation [Preeta and Nair, 1999]. Actually, oxidative stress was generally pronounced in the cellular study of cerium oxide nanoparticles [Park et al., 2008b]. So we deduce that cerium ions may form cerium-containing nanoparticles or microparticles and deposited on the cell surface in cell culture medium, the particles

may have acted as a cerium "buffer pool," from which Ce^{3+} ions were released to modulate the osteogenic and adipogenic differentiation of MSCs. The difference between cerium and gadolinium may be due to the unique properties of cerium such as its higher redox capacity.

It is well known that the Smad-dependent BMP signaling pathway plays an important role in osteogenic differentiation of MSCs [Li, 2008; Zhang et al., 2009; Luo et al., 2010]. Therefore, we hypothesized that the promotion of osteogenic differentiation of MSCs was modulated through a Smad-dependent BMP signaling pathway. In our article, the results from RT^{2}Profiler^{TM} PCR Array were shown in Figure 4, the up-regulation of 16 marker genes, which controlled the osteogenic differentiation of MSCs, was observed in MSCs treated with Ce. Interestingly, most of these genes were involved in TGF-β/BMP signaling pathway (see Supporting Information, Fig. S1, Tables S1, S2, and S3 for descriptions of all genes and their functions).

BMPs are responsible for enhancing osteogenic differentiation, including stimulation of the expression of bone structural proteins such as Col-I and OCN, and the mineralization of bone matrix. BMPs belong to TGF-β superfamily, which bind to the BMPR2 to regulate cellular functions including cell differentiation and growth via the phosphorylation of Smad1/5/8. P-Smad1/5/8 proteins translocate to the nucleus, bind to target genes, and regulate the transcription. The data shown in Figure 6A,B indicated that the expression of p-Smad1/5/8 was promoted after treating with Ce, leading to subsequent up-regulation of the expression of related osteogenic differentiation genes. In parallel, it has reported that the over-expressed E3 ubiquitin ligases (Smurf1 and Smurf2) decreased the expression of Smads and BMPs. Because Runx2 interacts with Smad1, whose degradation is mediated by Smurf1, the effect of Smurf1 on Runx2 degradation in MSCs was examined. However, we found that the expression of Smurf1 and Smurf2 was significant down-regulated by Ce and subsequent proteosomal degradation was inhibited (Fig. 4). Mcam, an adhesion molecule, belongs to the Ig superfamily. It has been widely accepted that Mcam as a marker for identifying MSCs isolated from bone marrow, and its higher expression on the cell surface may be linked to greater multipotency and differentiation potential [Covas et al., 2008; Russell et al., 2010]. As shown in Figure 4, the expression of Mcam showed an approximately 30-fold increase when MSCs were treated with Ce. Hgf has potent anti-apoptotic effects on cells, Rodrigues et al. [2010] showed that short-term exposure of Hgf could promote the proliferation of MSCs. Besides the Hgf, Bdnf was another positive regulatory of MSCs proliferation [Tauber et al., 2005; Wang et al., 2009]. As shown in Figure 4, the expression of Hgf and Bdnf showed 7- and 10-fold increase when MSCs were treated with Ce. These data provide the evidence for the fact that Ce promots proliferation of MSCs, which is consistent with the observed promotion effects of Ce on the proliferation of MSCs (Fig. 1A). It has reported that the proliferation of MSCs is controlled by a wide range of bioactive compounds, as well as by mechanical signals. In particular, it was shown that TGF-β promoted DNA replication and active proliferation [Erlebacher et al., 2004; Tsai et al., 2009]. Gdfs are members of the TGF-β superfamily which regulate many aspects of development, including osteogenesis, chondrogenesis, and adipogenesis. It has been reported that the expression of Gdf5, Gdf6, and Gdf7 controls the chondrogenesis and increases proliferation of chondrocytes [Francis-West et al., 1999; Skolnick et al., 2000; Byeong et al., 2006], Gdf15 plays an important role in regulating tenogenesis [Snjezana et al., 2004]. In our study, the down-regulated expression of Gdf5, Gdf6, Gdf7, and Gdf15 suggested that MSCs treated with Ce failed to differentiate into chondrocytes and tenocytes (Fig. 4).

BMP2 is a member of the TGF-β superfamily and plays a key regulatory role as a cell–cell signaling molecule during bone formation and repair. The lineage commitment gene Runx2 belongs to the Runx family and plays a vital role in determining the OB lineage from the pluripotent MSCs. It has been demonstrated that no OB differentiation can be observed in the absence of Runx2. Runx2 cooperates with BMP-activated Smads to stimulate the osteogenic differentiation of MSCs [Ducy et al., 2000]. BSP and Col I are significant components of the bone extracellular matrix. ALP is responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. ALP is considered to be important in producing bone mineral and is a marker for cells that are undergoing differentiation forming preosteoblasts and OBs. OCN is the most specific gene for the OB differentiation and mineralization. OCN is expressed during the postproliferative period and reaches its maximum expression during mineralization and accumulates in the mineralized bone. It has been demonstrated that MSC treated with La exhibited the higher expression levels of ALP, OCN, and ColI during the activation of osteogenic differentiation [Liu et al., 2006]. In our work, Ce exhibited the up-regulation of all these OB specific genes (Fig. 5A), which was consistent with the observed promotion effects of Ce on ALP activity (Fig. 1B) and mineralized matrix nodule formation of MSCs (Fig. 2).

Because of the reciprocal relationship between the differentiation of adipogenic and osteogenic cells in the MSC culture, it is expected that the promotion of osteogenic differentiation of MSCs may result in the inhibition of adipogenic differentiation of MSCs. In fact, our results also indicated that Ce inhibited adipogenic differentiation of MSCs at all tested concentrations, but had no obvious dose- or time-dependent tendency. It has been reported that Runx2 determines the osteogenesis in MSCs [Komori, 2003], while PPARγ contributes to adipogenesis [Zhang et al., 2008]. Specific activation of PPARγ2 leads to suppression of the main transcription factors of osteogenesis, and also to increase conversion of MSCs into adipocytes [Lecka-Czernik et al., 2002]. Adipogenic differentiation of MSCs starts with the transient expression of C/EBPβ and C/EBPδ, which activates C/EBPα and PPARγ. C/EBPα and PPARγ together coordinate the expression of adipogenic genes underlying the phenotype of terminally differentiated adipocytes [Yi et al., 2010]. The expression of adipogenic specific genes and proteins is expected to be attenuated in MSCs upon Ce treatment. In our work, the expression of adipogenic differentiation specific genes and protein was also down-regulated when MSCs were cultured in the presence of AS and Ce (Figs. 5B and 7), which is consistent with the observed inhibitory effects of Ce on adipogenic differentiation of MSCs (Fig. 3).

On the basis of the above findings, a schematic model was proposed to describe the modulation of osteogenic and adipogenic differentiation of MSCs by Ce through the TGF-β/BMP signaling

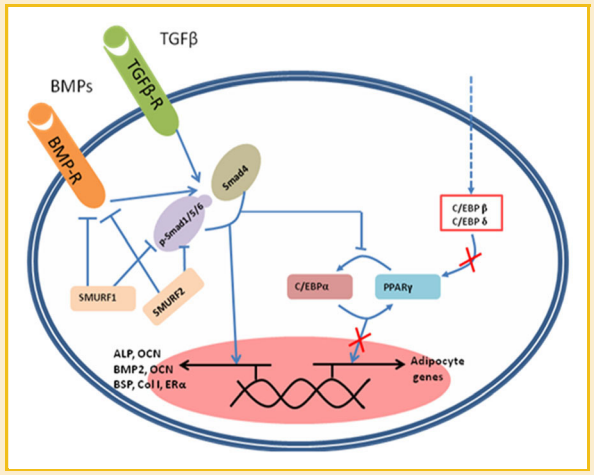

Fig. 8. Plausible molecular mechanism of the modulation of osteogenic and adipogenic differentiation of MSCs by Ce through TGF-β/BMP signaling pathway.

pathway (Fig. 8). Ce likely activates the BMP signaling pathway, which interacts with BMPR and further activates the expression of p-Smad1/5/8. P-Smad1/5/8 combining with Smad4 leads to up-regulation of the osteogenic master transcription factor, Runx2 [Lee et al., 2003], and the down-regulation of the adipocytic master transcription factor, PPARγ2 [Evan and Ormond, 2006]. Runx2, which subsequently induces OB marker genes Col I and BMP2 at early stages and ALP and OCN at later stages of differentiation [Sowa et al., 2003; Lee et al., 2010]. At the same time, the expression of p-Smad1/5/8 inhibits the adipogenesis in MSCs by suppressing the expression of C/EBPα and PPARγ2, thus driving MSCs to differentiate into OBs.

In conclusion, the results showed that Ce promoted the proliferation and osteogenic differentiation and inhibited the adipogenic differentiation of MSCs for the first time. The expression of a panel of osteogenic differentiation specific genes was significantly up-regulated during the differentiation of MSCs in the presence of Ce. Q-PCR results and Western blot analysis together confirmed that the promotion of proliferation and osteogenic differentiation of MSCs was related to the TGF-β/BMP signaling pathway. On the other hand, the expression of a panel of adipogenic differentiation specific genes was found to be significantly down-regulated. The results provide novel evidence to elucidate the mechanisms of bone metabolism by Ce and may be helpful for more rational application of Ce-based compounds in the future.

REFERENCES

Ailhaud G, Grimaldi P, Negrel R. 1992. Cellular and molecular aspects of adipose tissue development. Annu Rev Nutr 12:207–233.

Benayahu D, Zipori D, Wientroub S. 1993. Marrow adipocytes regulate growth and differentiation of osteoblasts. Biochem Biophys Res Commun 197:1245–1252.

Berthon G. 2002. Aluminum speciation in relation to aluminum bioavailability, metabolism and toxicity. Coord Chem Rev 228:319–341.

Byeong SY, Robert P, Dmitri AO, Isaac Y, Yuji M, Richard RB, Karen ML. 2006. BMPs regulate multiple aspects of growth-plate chondrogenesis through opposing actions on FGF pathways. Development 133:4667–4678.

Covas DT, Panepucci RA, Fontes AM, Silva WA, Orellana MD, Freitas MCC, Neder L, Santos ARD, Peres LC, Jamur MC, Zago MA. 2008. Multipotent mesenchymal stromal cells obtained from diverse human tissues share functional properties and gene-expression profile with $CD146^{+}$ perivascular cells and fibroblasts. Exp Hematol 36:642–654.

Ducy P, Schinke T, Karsenty G. 2000. The osteoblast: A sophisticated fibroblast under central surveillance. Science 289:1501–1504.

Erlebacher A, Price KA, Glimcher LH. 2004. Maintenance of mouse trophoblast stem cell proliferation by TGF-beta/activin. Dev Biol 275: 158–169.

Evan DR, Ormond AM. 2006. Adipocyte differentiation from the inside out. Nat Rev Mol Cell Biol 7:885–896.

Francis-West PH, Abdelfattah A, Chen P, Allen C, Parish J, Ladher R, Allen S, MacPherson S, Luyten FP, Archer CW. 1999. Mechanisms of GDF-5 action during skeletal development. Development 126:1305–1315.

Fricker SP. 2006. The therapeutic application of lanthanides. Chem Soc Rev 35:524–533.

Havanur VC, Badiger DS, Ligade SG, Gudasi KB. 2011. Synthesis, characterization and antimicrobial study of lanthanide(III) complexes of 2-anilino-N_1-[pyridine-2-ylethylidene]acetohydrazide. Der Pharma Chemica 3:292–304.

Komori T. 2003. Requisite roles of Runx2 and Cbfb in skeletal development. J Bone Miner Metab 21:193–197.

Lecka-Czernik B, Moerman EJ, Grant DF, Lehmann JM, Manolagas SC, Jilka RL. 2002. Divergent effects of selective peroxisome proliferator-activated receptor-gamma 2 ligands on adipocyte versus osteoblast differentiation. Endocrinology 143:2376–2384.

Lee MH, Kim YJ, Kim HJ, Park HD, Kang AR, Kyung HM, Sung JH, Wozney JM, Kim HJ, Ryoo HM. 2003. BMP-2-induced Runx2 expression is mediated by Dlx5, and TGF-β1 opposes the BMP-2-induced osteoblast differentiation by suppression of Dlx5 differentiation. J Biol Chem 278: 34387–34394.

Lee SJ, Kang SW, Do HJ, Han I, Shin DA, Kim JH, Lee SH. 2010. Enhancement of bone regeneration by gene delivery of BMP2/Runx2 bicistronic veBTor into adipose-derived stromal cells. Biomaterials 31:5652–5659.

Li BJ. 2008. Bone morphogenetic protein-smad pathway as drug targets for osteoporosis and cancer therapy. Endocr Metab Immune Disord Drug Targets 8:208–219.

Li JX, Liu JC, Wang K, Yang XG. 2010. Gadolinium-containing bioparticles as an active entity to promote cell cycle progression in mouse embryo fibroblast NIH3T3 cells. J Biol Inorg Chem 15:547–557.

Limbach LK, Bereiter R, Muller E, Krebs R, Galli R, Stark WJ. 2008. Removal of oxide nanoparticles in a model wastewater treatment plant: Influence of agglomeration and surfactants on clearing efficiency. Environ Sci Technol 42:5828–5833.

Liu HM, Zhang TL, Xu SJ, Wang K. 2006. Effect of La^{3+} on osteoblastic differentiation of rat bone marrow stromal cells. Chin Sci Bull 51:31–37.

Liu DD, Yi CQ, Zhang DW, Zhang JC, Yang MS. 2010. Inhibition of proliferation and differentiation of mesenchymal stem cells by carboxylated carbon nanotubes. ACS Nano 4:2185–2195.

Luo J, Tang M, Huang JY, He BC, Gao JL, Chen L, Guo WZ, Zhang WL, Luo Q, Shi Q, Zhang Q, Yang B, Luo XJ, Jian W, Su YX, Shen JK, Kim SH, Huang EY, Gao YH, Zhou JZ, Yang K, Luu HH, Pan XC, Haydon RC, Deng ZL, He TC. 2010. TGFβ/BMP type I receptors ALK1 and ALK2 are essential for BMP9-induced osteogenic signaling in mesenchymal stem cells. J Biol Chem 285:29588–29598.

Mu QX, Du GQ, Chen TS, Zhang B, Yan B. 2009. Suppression of human bone mophorgenetic protein (BMP) signaling by carboxylated single-walled carbon nanotubes. ACS Nano 3:1139–1144.

Nuttall ME, Gimble JM. 2000. Is there a therapeutic opportunity to either prevent or treat osteopenic disorders by inhibiting marrow adipogenesis? Bone 27:177–184.

Owen M, Friedenstein AJ. 1988. Stromal stem cells: Marrow-derived osteogenic precursors. Ciba Found Symp 136:42–60.

Park B, Donaldson K, Duffin R, Tran L, Kelly F, Mudway I, Morin JP, Guest R, Jenkinson P, Samaras Z, Giannouli M, Kouridis H, Martin P. 2008a. Hazard and risk assessment of a nanoparticulate cerium oxide-based diesel fuel additive—A case study. Inhal Toxicol 20:547–566.

Park EJ, Choi J, Park YK, Park K. 2008b. Oxidative stress induced by cerium oxide nanoparticles in cultured BEAS-2B cells. Toxicology 245:90–100.

Preeta R, Nair RR. 1999. Stimulation of cardiac fibroblast proliferation by cerium: A superoxide anion-mediated response. J Mol Cell Cardiol 31:1573–1580.

Prockop DJ. 1997. Marrow stromal cells as stem cells for non-hematopoietic tissues. Science 276:71–74.

Robinson JM, Karnovsky MJ. 1983. Ultrastructural localization of several phosphatases with cerium. J Histochem Cytochem 31:1191–1208.

Rodrigues M, Griffith LG, Wells A. 2010. Growth factor regulation of proliferation and survival of multipotential stromal cells. Stem Cell Ther 1:32–37.

Russell KC, Phinney DG, Lacey MR, Barrilleaux BL, Meyertholen KE, O'Connor KC. 2010. In vitro high-capacity assay to quantify the clonal heterogeniety in tri-lineage potential of mesenchymal stem cells reveals a complex hierarchy of lineage committment. Stem Cells 28:788–798.

Rzigalinski BA. 2005. Nanoparticles and cell longevity. Technol Cancer Res Treat 4:651–659.

Sekiya I, Larson BL, Smith JR, Pochampally R, Cui JG, Prockop DJ. 2002. Expansion of human adult stem cells from bone marrow stroma: Conditions that maximize the yields of early progentitors and evaluate their quality. Stem Cells 20:530–541.

Skolnick J, Fetrow JS, Kolinski A. 2000. Structural genomics and its importance for gene function analysis. Nat Biotechnol 18:283–287.

Snjezana M, Sanja M, Veronika K, Nikolina B, Jasminka JR, Fran B, Drago B, Petra S, Lovorka G, Boris L, Slobodan V. 2004. Expression of bone morphogenetic proteins in stromal cells from human bone marrow long-term culture. J Histochem Cytochem 52:1159–1167.

Sowa H, Kaji H, Canaff L, Hendy GN, Tsukamoto T, Yamaguchi T, Miyazono K, Sugimoto T, Chihara K. 2003. Inactivation of menin, the product of the multiple endocrine neoplasia type 1 gene, inhibits the commitment of multipotential mesenchymal stem cells into the osteoblast lineage. J Biol Chem 278:21058–21069.

Tamama K, Sen CK, Wells A. 2008. Differentiation of bone marrow mesenchymal stem cells into the smooth muscle lineage by blocking ERK/MAPK signaling pathway. Stem Cells Dev 17:897–908.

Tauber SC, Stadelmann C, Spreer A, Brück W, Nau R, Gerber J. 2005. Increased expression of BDNF and proliferation of dentate granule cells after bacterial meningitis. J Neuropathol Exp Neurol 64: 806–815.

Tsai S, Hollenbeck ST, Ryer EJ, Edlin R, Yamanouchi D, Kundi R, Wang CJ, Liu B, Craig KK. 2009. TGF-β through Smad3 signaling stimulates vascular smooth muscle cell proliferation and neointimal formation. Am J Physiol Heart Circ Physiol 297:H540–H549.

Wang K, Li RC, Cheng Y, Zhu B. 1999. Lanthanides—The future drugs? Coord Chem Rev 190–192:297–308.

Wang K, Cheng Y, Yang XD, Li RC. 2003. Cell responses to lanthanides and potential pharmmacological actions of lanthanides. Met Ions Biol Syst 40:707–751.

Wang J, Ding F, Gu Y, Liu J, Gu XS. 2009. Bone marrow mesenchymal stem cells promote cell proliferation and neurotrophic function of Schwann cells in vitro and in vivo. Brain Res 1262:7–15.

Xu JX, Li GS, Li LP. 2008. CeO_2 nanocrystals: Seed-mediated synthesis and size control. Mater Res Bull 43:990–995.

Yi CQ, Liu DD, Fong CC, Zhang JC, Yang MS. 2010. Gold nanoparticles promote osteogenic differentiation of mesenchymal stem cells through p38 MAPK pathway. ACS Nano 4:6439–6448.

Zhang DW, Yi CQ, Zhang JC, Chen Y, Yao XS, Yang MS. 2007. The effects of carbon nanotubes on the proliferation and differentiation of primary osteoblasts. Nanotechnology 18:475102–475111.

Zhang WX, Yang NL, Shi XM. 2008. Regulation of mesenchymal stem cell osteogenic differentiation by glucocorticoid-induced leucine zipper (GILZ). J Biol Chem 283:4723–4729.

Zhang F, Qiu T, Wu X, Wan C, Shi W, Wang Y, Chen JG, Wan M, Clemens TL, Cao X. 2009. Sustained BMP signaling in osteoblasts stimulates bone formation by promoting angiogenesis and osteoblast differentiation. J Bone Miner Res 24:1224–1233.

Zhang JC, Liu CL, Li YP, Sun J, Wang P, Di KQ, Zhao YY. 2010. Effect of cerium ion on the proliferation, differentiation and mineralization function of primary mouse osteoblasts in vitro. J Rare Earth 28:138–142.