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# Effect of $La^{3+}$ on osteoblastic differentiation of rat bone marrow stromal cells

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**Abstract** In the present work, the effect of $La^{3+}$ on osteoblastic differentiation of primary rat bone marrow stromal cells (MSCs) as well as the related mechanisms are studied. Differentiation is monitored by detection of alkaline phosphatase (ALP) activity, osteocalcin secretion, the mRNA levels of Type I collagen and osteocalcin, and matrix mineralization. The results show that $La^{3+}$ inhibits osteoblastic differentiation of MSCs in the early and middle stages of culture, as demonstrated by the decrease of ALP activity, osteocalcin secretion, and down-regulation of the mRNA level of osteocalcin. However, $La^{3+}$ does not affect the matrix mineralization in advanced MSCs, because it up-regulates the mRNA levels of Type I collagen, and promotes ALP activity and osteocalcin secretion in MSCs in the late stage of culture. In addition, Western blot analysis exhibits that $La^{3+}$ induces the phosphorylation and activation of mitogen-activated protein kinase (MAPK). Furthermore, MAPK kinase inhibitor PD98059 completely blocks the inhibitory effect of $La^{3+}$ on ALP activity of MSCs in the middle stage of culture. These results suggest that $La^{3+}$ affects MSCs osteoblastic differentiation depending on differentiation stages. $La^{3+}$ inhibits osteoblastic differentiation of MSCs in the early and middle stages by a MAPK-dependent mechanism, but does not affect the matrix mineralization in advanced MSCs.

**Keywords: La3+, marrow stromal cells, differentiation.**

Lanthanides are increasingly used in industry and agriculture in recent years. These applications increase the accumulation of lanthanides in the human body, especially in bone[1]. Thus, people paid extensive attention to the effect of lanthanides on bones. The previous animal experiment showed that La accumulated in rats fed with $La(NO_3)_3$ in low dose, but for a long time, caused the change of bone microstructure, as demonstrated by the slim, sparse, or even broken trabecular bones, and the high crystallinity in bone mineral[2]. However, the underlying cellular and molecular mechanisms still remained unclear.

Osteoblasts, as bone-forming cells, play a pivotal role in the growth, development, injury, repair, remodeling, and maintenance of bones. The reduced osteoblast number and activity cause the declined bone formation, and further osteoporosis. Zhang *et al*.[3] found that $La^{3+}$ promoted the differentiation of osteoblast line UMR106, as characterized by the increased alkaline phosphatase (ALP) activity and calcification. Since osteoblasts become matured from osteoprogenitor cells arising from marrow stromal cells (MSCs)[4], lanthanides might regulate the course of bone formation by affecting the differentiation of MSCs. Early studies showed that MSCs cultured in the medium supplemented with dexamethasone, β-glycerophosphate and ascorbic acid can spontaneously differentiate into osteoblasts[5,6], forming the similar mineral as that *in vivo*. Differentiation of MSCs into osteoblasts can be subdivided into the following phases, i.e. matrix production phase, matrix maturation phase, and matrix mineralization phase[7]. They are associated with the sequential expression of genes involved in the biosynthesis, organization, and mineralization of the bone extracellular matrix. In matrix production phase, genes associated with the biosynthesis of extracellular matrix (e.g., type I collagen) are expressed. In matrix maturation phase, the expression of genes involved in maturation and organization of the bone extracellular matrix (e.g., ALP) is up-regulated, allowing mineralization. In matrix mineralization phase, the expression of genes associated with the orderly deposition of hydroxyapatite (e.g., osteocalcin) occurred. The signaling pathway associated with the osteoblastic differentiation of MSCs has not well documented so far. Some work showed that mitogen-activated protein kinase (MAPK) signaling pathway is essential for osteoblastic differentiation of bone cells[8−10]. In the present work, using primary rat MSCs cultured in the presence of dexamethasone, β-glycerophosphate and ascorbic acid as a model, we studied the effect of $La^{3+}$ on osteoblastic differentiation of MSCs as well as the related mechanisms, with ALP activity, osteocalcin secretion, the

mRNA levels of type I collagen and osteocalcin, and matrix mineralization as indices of differentiation.

## 1 Materials and methods

### *1.1 Materials*

Dulbeccol's Modified Eagle's Medium (DMEM, high glucose, 4.5 g/L) and Trizol were obtained from Gibco BRL. Fetal bovine serum (FBS) was purchased from Hyclone. Ascorbic acid, β-glycerophosphate, dexamethasone, ALP activity assay kit, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT), PMSF, DTT, aprotinin, leupeptin and PD98059 were purchased from Sigma. Osteocalcin [$^{125}$I] radioimmunoassay (RIA) kit was purchased from East Asia Institute of Immune Technology (Beijing, China). *Taq* DNA polymerase and RNase inhibitor were purchased from TaKaRa Biotechnology Co., Ltd (Dalian, China). M-MLV reverse transcriptase was obtained from Promega. Oligo(dT)$_{15}$, the primers for type I collagen, osteocalcin and GAPDH were synthesized by Shanghai Boya Biotechnology Co., Ltd (Shanghai, China). Antibodies against phosphorylated extracellular signal-regulated kinases (pERK1/2), the corresponding secondary antibodies, and enhanced chemiluminescence kit were obtained from Santa Cruz. Other reagents were of analytical grade. $La^{3+}$ (lanthanum chloride) stock solution was prepared by dissolving $La_2O_3$ (purity 99.99%) in concentrated HCl. After the excess HCl was removed by heating, the residue was redissolved in double distilled deionized water. The concentration was determined by EDTA titration. The stock solution of $La^{3+}$ was diluted into the desired concentration with the culture medium immediately before use.

### *1.2 Cell culture*

Rat bone MSCs were prepared and cultured as described by Maniatopoulos *et al*.[6]. Briefly, the femurs were removed aseptically from young adult male SD rats (approximately 150 g) and cleaned of soft tissues. The epiphysis were removed and the marrow was flushed out with a syringe containing DMEM supplemented with 15% FBS and the antibiotic mixture (100 U/mL penicillin; 100 μg/mL streptomycin). A single cell suspension was prepared by repeated pipetting. Cells were cultured in DMEM supplemented with 15% FBS, antibiotics, $10^{-8}$ mol/L dexamethasone, 10 mmol/L β-glycerophosphate and 50 μg/mL of freshly-prepared ascorbic acid. After 4 days, non-adhering hematopoetic cells were discarded from the adherent cells representing bone MSCs. The cell cultures continued to keep up to indicated days, with total renewal of the culture medium every 2 or 3 days.

### *1.3 Cell viability assay*

At day 4 of culture, MSCs were treated with varied concentrations of $La^{3+}$ in the presence of 5% FBS. After 3 days, cell viability was evaluated using the MTT assay[11], in which, cells were incubated with 0.5 mg/mL MTT for 4 h at 37℃; the solution was then removed and formazan salts dissolved with dimethyl sulphoxide, and the absorbance at 570 nm of the resulting solution was measured on a microplate reader (TECAN SUNRISE, Switzerland).

### *1.4 Assay of ALP activity*

At day 4, 11, or 18 of culture, MSCs were treated with varied concentrations of $La^{3+}$ for 3 days. After the cells were washed three times with PBS, cellular proteins were solubilized with 1% Triton X-100 in 0.9% NaCl and centrifuged, and the supernatants were measured for ALP activity as described previously[12] using a commercial ALP activity assay kit. One unit was defined as the activity producing 1 μmol of *p*-nitrophenol within 30 min. ALP activity was normalized to total protein content determined by Lowry method[13].

### *1.5 Osteocalcin secretion detection*

At day 4, 11, or 18 of culture, MSCs were treated with varied concentrations of $La^{3+}$. After 3 days, the cell conditioned media were collected and osteocalcin secretion was measured employing [$^{125}$I] RIA kit. The cell layer were washed three times with PBS and solubilized with 0.1 mol/L NaOH/0.1% SDS. The total protein content was determined with Lowry method[13]. Osteocalcin secretion was normalized to protein content.

### *1.6 mRNA analysis for type I collagen and osteocalcin*

At day 4 culture, MSCs were treated with $10^{-5}$ mol/L $La^{3+}$, and the culture continued up to 14 days. Total RNA was isolated from the cells using Trizol reagent according to the manufacturer's directions. The levels of mRNA for type I collagen and osteocalcin were examined by reverse transcription polymerase chain reaction (RT-PCR) analysis. The synthesis of cDNA from 5 μg of the isolated RNA was carried out with M-MLV reverse transcriptase in the presence of oligo(dT)$_{15}$

primer. Five microliters of this reverse transcribed cDNA was submitted to polymerase chain reaction as previously described[14]. Briefly, PCR was performed after a denaturing step of 5 min at 95℃. Each cycle consisted of 95℃ for 1 min, 60℃ for 1 min, 72℃ for 1 min, for 30 cycles terminating at 72℃ for 10 min. The sequences of the primers for type I collagen, osteocalcin and GAPDH were designed as previously described[14]. Negative control reactions for RT-PCR were performed in each assay using all reagents except RNA. The integrity of RNA isolated from the experimental samples was checked by RT-PCR with primers for GAPDH. The PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

### *1.7 Quantification of calcium deposition*

At day 4 culture, MSCs were treated with varied concentrations of $La^{3+}$, and the culture continued up to 21 days. Calcification was assessed by the method described by Wada *et al.*[15] with modification. Briefly, MSCs were decalcified for 24 h with 0.6 mol/L HCl. The calcium content of HCl supernatant was determined by atomic absorption spectroscopy. After decalcification, the cells were washed three times with PBS and solubilized with 0.1 mol/L NaOH/0.1% SDS. The total protein content was determined with Lowry method[13]. The calcium content of the cell layer was normalized to protein content.

### *1.8 Assay of the phosphorylation of ERK1/2*

At day 14 culture, MSCs were treated with $10^{-5}$ mol/L $La^{3+}$ for 10 min, 20 min, or 2 h. After treatment, the cells were assayed for the phosphorylation of ERK1/2 by Western blot analysis. After being washed twice with cold PBS, cells were lysed in lysis buffer (50 mmol/L HEPES, pH 7.4, containing 150 mmol/L NaCl, 1% Triton X-100, 0.4% SDS, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 1 mmol/L PMSF and 10 mmol/L DTT), and protein concentrations were determined with Lowry method[13]. Proteins (40 μg) were separated by SDS-PAGE, and electrotransferred to nitrate cellulose membrane, followed by probing with the antibody to phosphorylated ERK1/2. Final detection of proteins was by enhanced chemiluminescence.

### *1.9 Statistical analysis*

Results are shown as mean ± SD. Differences between groups were analyzed using the Students' *t*-test and a value of $P<0.05$ was considered significant.

## 2 Results

### *2.1 Effect of $La^{3+}$ on cell viability*

As shown in Fig. 1, $10^{-8}$—$10^{-5}$ mol/L $La^{3+}$ had no significant effect on cell viability after treatment for 3 days, suggesting that $La^{3+}$ in this concentration range promoted neither proliferation nor death in MSCs.

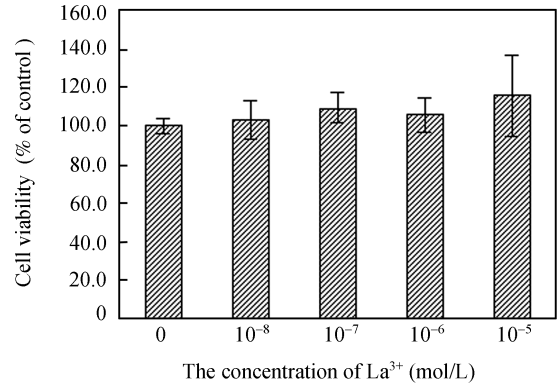


Fig. 1. Effect of different concentrations of $La^{3+}$ on MSCs viability (mean ± SD, $n=4$). At day 4 of culture, MSCs were treated with varied concentrations of $La^{3+}$ in the presence of 5% FBS. After 3 days, cell viability was evaluated using the MTT assay.

### *2.2 Effect of $La^{3+}$ on ALP activity in MSCs*

Induction of ALP activity is an early marker of osteoblastic differentiation and plays an important role in eventual mineralization process[5]. In control MSCs, ALP activity was relatively low at day 7 of culture (matrix production phase), peaked at day 14 of culture (matrix maturation phase), and then declined (matrix mineralization phase) (Fig. 2). This was consistent with the results reported previously[5]. The effect of $La^{3+}$ on ALP activity in MSCs depended on its concentrations and the stage of cell culture (Fig. 2). $La^{3+}$ ($10^{-8}$—$10^{-5}$ mol/L) significantly inhibited ALP activity when applied to MSCs from day 4 through day 7 of culture and from day 11 through 14 of culture; but they significantly enhanced ALP activity when applied to MSCs from day 18 through 21 of culture. In addition, the effect of $La^{3+}$ on ALP activity was concentration-dependent: the effects of $10^{-7}$ and $10^{-6}$ mol/L $La^{3+}$ were always stronger than $10^{-8}$ mol/L and $10^{-5}$ mol/L $La^{3+}$.

### *2.3 Effect of $La^{3+}$ on osteocalcin secretion in MSCs*

Osteocalcin is a more specific and relatively late stage marker of osteoblastic differentiation[5]. As shown in Fig. 3, for MSCs in control, osteocalcin secretion

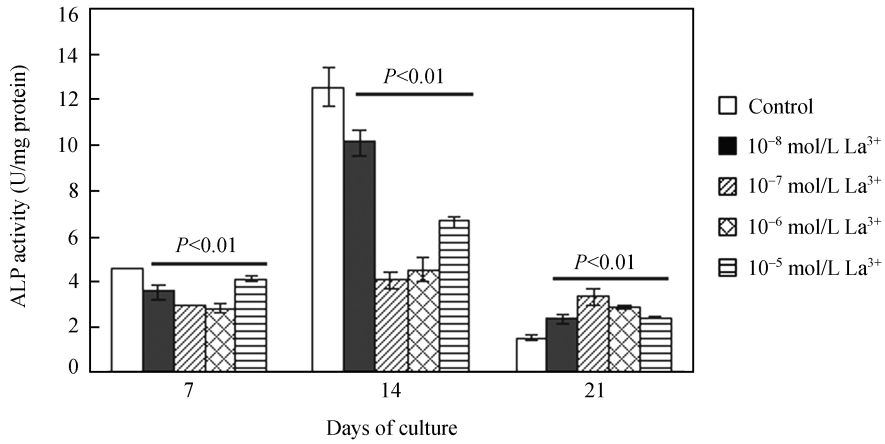


Fig. 2. Effect of $La^{3+}$ on ALP activity in MSCs (mean ± SD, $n$=4). At day 4, 11, or 18 of culture, MSCs were treated with varied concentrations of $La^{3+}$, and ALP activity was measured at day 7, 14 and 21 of culture, respectively.

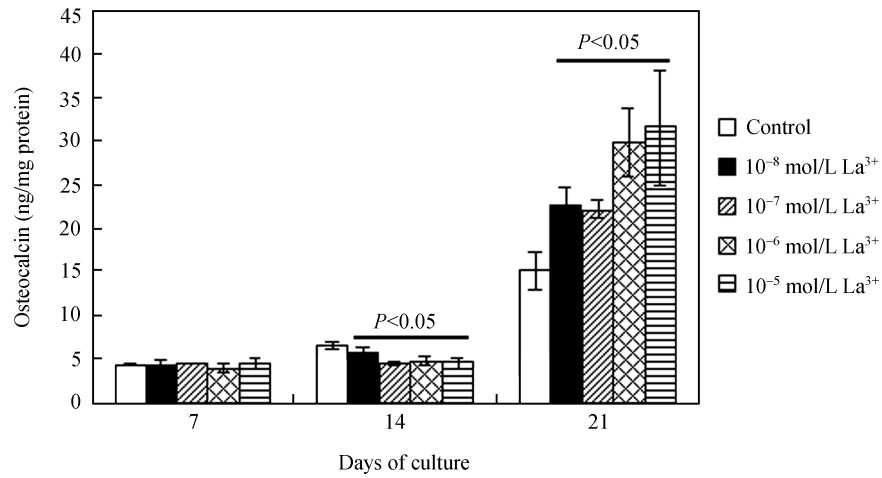


Fig. 3. Effect of $La^{3+}$ on osteocalcin secretion in MSCs (mean ± SD, $n$=4). At day 4, 11, or 18 of culture, MSCs were treated with varied concentrations of $La^{3+}$, and osteocalcin secretion was measured at day 7, 14 and 21 of culture, respectively.

increased gradually to maximum levels at day 21 of culture. Similar to ALP activity, the effect of $La^{3+}$ on osteocalcin serection in MSCs depended on its concentrations and the stage of cell culture (Fig. 3). $La^{3+}$ ($10^{-8}$ —$10^{-5}$ mol/L) had no significant influence on osteocalcin secretion when applied to MSCs from day 4 through 7 of culture, and significantly inhibited osteocalcin secretion when applied to MSCs from day 11 through 14 of culture. However, a significant increase in osteocalcin secretion was observed when $10^{-8}$—$10^{-5}$ mol/L $La^{3+}$ was applied to MSCs from day 18 through 21 of culture.

### *2.4 Effect of $La^{3+}$ on the level of mRNA for type I collagen and osteocalcin*

Type I collagen was known to be necessary for proper differentiation and mineralization of osteoblasts[16]. The effect of $La^{3+}$ on the levels of mRNA for type I collagen and osteocalcin were measured by RT-PCR. The results demonstrated that the level of mRNA for type I collagen increased significantly, while that of osteocalcin decreased significantly after $10^{-5}$ mol/L $La^{3+}$ treatment (Fig. 4).

### *2.5 Effect of $La^{3+}$ on matrix mineralization*

Matrix mineralization, as measured by calcium deposition, was found to be undetectable in control MSCs at day 7 of culture, low (0.51 ± 0.17 μmol/mg protein) at day 14 of culture, and increased to 5.14 ± 1.19 μmol/mg protein at day 21 of culture. $La^{3+}$ ($10^{-8}$—$10^{-5}$ mol/L) treatment exerted no action on the increase of calcium deposition at day 21 of culture (Fig. 5).

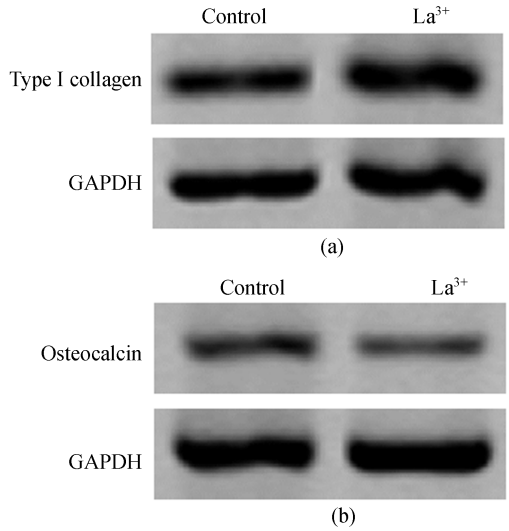


Fig. 4. Effect of $La^{3+}$ on the levels of mRNA for type I collagen (a) and osteocalcin (b). At day 4 culture, MSCs were treated with $10^{-5}$ mol/L $La^{3+}$, and continued to keep up to 14 days. After total RNA was isolated from the cells, the levels of mRNA for type I collagen and osteocalcin were examined by RT-PCR analysis. The PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

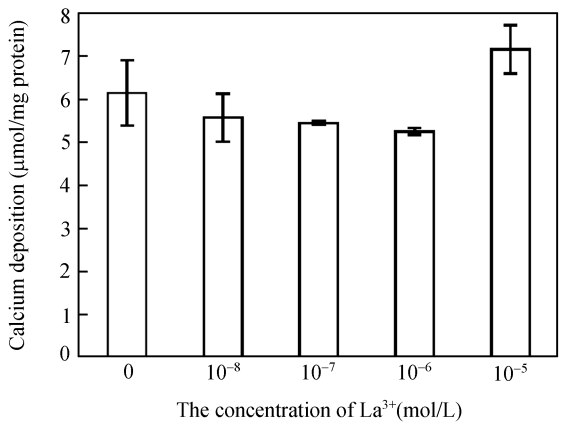


Fig. 5. Effect of $La^{3+}$ on calcium deposition (mean ± SD, $n$=4). At day 4 culture, MSCs were treated with varied concentrations of $La^{3+}$, and calcium deposition was measured at day 21 of culture.

### 2.6 *Role of MAPK signaling pathway*

To assess the role of MAPK in mediating the cell responses to $La^{3+}$, MSCs were pretreated with PD98059 before exposure to $10^{-5}$ mol/L $La^{3+}$. PD98059 is a potent and selective inhibitor of MAPK kinase MEK that blocks MAPK-mediated cellular responses. As shown in Fig. 6, pretreatment of MSCs with PD98059 completely blocked the inhibitory effect of $La^{3+}$ on ALP activity during day 7 to 14 of culture,

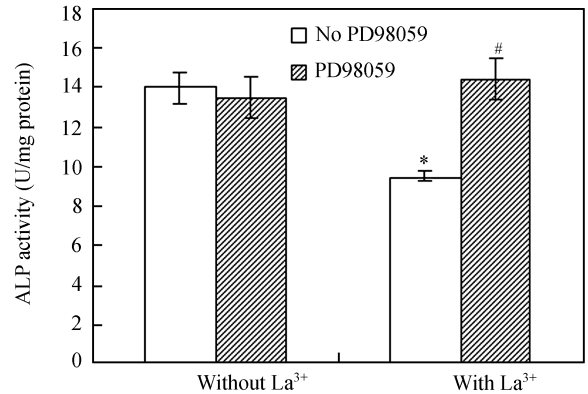


Fig. 6. Effect of MEK inhibitor, PD98059, on ALP activity inhibited by $La^{3+}$ in MSCs in the middle stage of culture (mean ± SD, $n$=4). MSCs at day 7 of culture were pretreated with 2 μmol/L PD98059 for 2 h, then exposed to $10^{-5}$ mol/L $La^{3+}$ until day 14 of culture. * $P < 0.01$, compared with control; # $P$<0.01, compared with cells treated with $La^{3+}$ alone.

suggesting a functional role for the MAPK signaling pathway in the effect of $La^{3+}$ on osteoblastic differentiation of MSCs.

To examine further the role of MAPK pathway in responses of MSCs to $La^{3+}$, cells at day 14 of culture were treated with $10^{-5}$ mol/L $La^{3+}$ for 10 min, 20 min or 2 h, and the cell lysates were subject to Western blot analysis with anti-phosphorylated ERK1/2. The data exhibited that the phosphorylation of ERK1/2 increased markedly by $10^{-5}$ mol/L $La^{3+}$ from 10 min up to 2 h (Fig. 7).

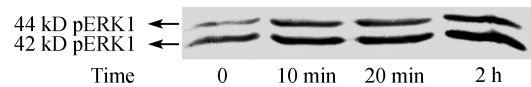


Fig. 7. Effect of $La^{3+}$ on the phosphorylation of ERK1/2 in MSCs at day 14 of culture.

## 3 Discussion

### 3.1 *The effect of $La^{3+}$ on osteoblastic differentiation of MSCs being dependent on differentiation stages*

To evaluate the cytotoxicity and cell compatibility of lanthanum-incorporated hydroxyapatites, Zhang *et al*.[17] investigated the effect of lanthanum-incorporated hydroxyapatites on cell viability, proliferation and differentiation of primary osteoblasts from rat calvaria. Since the lanthanum-incorporated hydroxyapatites did not exhibit any cytotoxic effect, and did not affect ALP activity, this suggested that the differentiation of osteoblasts was not affected. In another report, $La^{3+}$ was

found to promote the differentiation of osteoblast line UMR106 with increased ALP activity and calcification[3]. However, little was known about the effect of $La^{3+}$ on osteoblastic differentiation of primary MSCs. With ALP activity, osteocalcin secretion, the mRNA levels of type I collagen and osteocalcin, and matrix mineralization as indices of differentiation, the effect of different concentrations of $La^{3+}$ on the process of osteoblastic differentiation of MSCs is evaluated in this paper. The data showed that MSCs in different stages of culture had different responses to $La^{3+}$. In the early stage of culture (matrix production phase), $La^{3+}$ treatment caused a significant decrease of ALP activity, but had no effect on osteocalcin secretion. In the middle stage of culture (matrix maturation phase), $La^{3+}$ treatment simultaneously decreased ALP activity and osteocalcin secretion, and down-regulated the mRNA level of osteocalcin, but up-regulated the mRNA level of type I collagen. In the late stage of culture (matrix calcification phase), however, $La^{3+}$ significantly promoted ALP activity and osteocalcin secretion. In addition, $La^{3+}$ ($10^{-8}$—$10^{-5}$ mol/L) treatment for a long time did not affect the matrix mineralization in advanced MSCs. All these results indicated that $La^{3+}$ affected MSCs osteoblastic differentiation depending on differentiation stages. Although $La^{3+}$ had no influence on the matrix mineralization in advanced MSCs, it inhibited differentiation of MSCs in the early and middle stages, and promoted the differentiation of MSCs in the late stage.

$La^{3+}$ up-regulated the mRNA levels of Type I collagen, and promoted ALP activity and osteocalcin secretion in MSCs in the late stage, explaining why $La^{3+}$ exerted no influence on the matrix mineralization. Many studies reported that type I collagen was necessary for proper differentiation and mineralization of osteoblasts[16]. It can be as the carrier of orderly constructed hydroxyapatite crystal, controlling the growth and the size of the crystal. On the other hand, enhanced ALP activity in $La^{3+}$-treated MSCs in the late stage provided more phosphate for hydroxyapatite. In the aspect of bone formation, if the stimulated effect of $La^{3+}$ on the differentiation of MSCs in the late stage could balance the inhibitory effect of $La^{3+}$ on differentiation of MSCs in the early stage, the bone mineral content would not be changed by $La^{3+}$ treatment. The microstructure change of bone induced by $La^{3+}$ observed in the experimental animals[2] might result from the stimulated effect of low concentration of $La^{3+}$ on the activity of osteoclasts, leading to the increased bone resorption[18]. However, further research is needed for the influence of $La^{3+}$ on bone formation, including bone structure, bone mineral density, the constitute and crystal structure of bone mineral.

It was noteworthy that the extent to which $La^{3+}$ affected ALP activity and osteocalcin depended on its concentrations, which deserved further studies.

### *3.2 MAPK signaling pathway playing a role in the inhibitory effect of $La^{3+}$ on osteoblastic differentiation of MSCs in the early and middle stages*

MAPK signaling pathway consists of cascade phosphorylated serine/threonine protein kinases, including MAPK, MAPK kinases (MAPKK), and MAPKK kinases (MAPKKK). It is a membrane-to-nucleus signaling pathway connecting the cytoplasm/nucleus effectors to the cell surface receptor or external stimuli, which is involved in many cellular events such as proliferation, differentiation and death. As a critical factor in MAPK signaling pathway, ERK1/2 connect the upstream MAPKK to the downstream transcription factors, consisting of a signal transduction pathway. Once activated by MAPKK (such as MEK), ERK1/2 can translocate into the nucleus where they phosphorylate transcription factors, thereby regulating their activity. Many studies showed that MAPK signaling pathway, especially ERK1/2, is essential for osteoblastic differentiation of bone cells[8−10]. In the present work, MEK inhibitor, PD98059, completely blocked the inhibitory effect of $La^{3+}$ on ALP activity of MSCs in the middle stage of culture. In addition, Western blot analysis exhibited that $La^{3+}$ induced the phosphorylation and activation of ERK1/2 after short-term treatment. These results suggested that MAPK signaling pathway might play a key role in the inhibitory effect of $La^{3+}$ on osteoblastic differentiation of MSCs in the early and middle stages. Our previous study also demonstrated that $La^{3+}$ could induce a fast increase of ERK1/2 phosphorylation in a concentration-dependent manner in NIH3T3, a murine embryo fibroblast cell line[19]. The activated effect of $La^{3+}$ on ERK1/2 phosphorylation might be similar to the action of growth factors or cytokine. However, as demonstrated by MTT assay, different concentrations of $La^{3+}$ had no influence on MSCs viability, implying that it did not stimulate MSCs proliferation. Thus, the detail of the signal transduction pathway by which $La^{3+}$ affected osteoblastic differentiation of MSCs needs further research.

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