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The Dual-Effects of $LaCl_3$ on the Proliferation, Osteogenic Differentiation, and Mineralization of MC3T3-E1 Cells

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Abstract A series of experimental methods including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, alkaline phosphatase (ALP) activity measurement, alizarin red S stain and measurement, quantitative real-time reverse transcriptase polymerase chain reaction, and Western blot analysis were employed to assess the effects of $LaCl_3$ on the proliferation, osteogenic differentiation, and mineralization of a murine preosteoblast cell line MC3T3-E1 at cell and molecular levels. The results indicated that $LaCl_3$ had dual effects on the proliferation, osteogenic differentiation, and mineralization of MC3T3-E1 cells. First, $LaCl_3$ promoted the proliferation, osteogenic differentiation, and mineralization of MC3T3-E1 cells at lower concentrations, then had no effects and further turned to inhibit the proliferation, osteogenic differentiation, and mineralization of MC3T3-E1 cells with increasing concentrations. The expression of runt-related transcription factor 2 (Runx2), bone morphogenetic protein 2 (BMP2), ALP, bone sialoprotein (BSP), collagen I (Col I), and osteocalcin (OCN) genes was upregulated in the presence of 0.0001 and 0.1 μM $LaCl_3$, but these genes were downregulated in the MC3T3-E1 cells treated with 1,000 μM $LaCl_3$. In addition, the expression of BMP2, Runx2, and OCN proteins was promoted by $LaCl_3$ at the concentration of 0.0001 μM, but these proteins were downregulated after 1,000 μM $LaCl_3$ treatment. The results suggest that $LaCl_3$ likely up- or downregulates the expression of Runx2, which subsequently up- or downregulates osteoblasts marker genes Col I and BMP2 at early stages and ALP and OCN at later stages of differentiation, thus causes to promote or inhibit the proliferation, osteogenic differentiation and mineralization of MC3T3-E1 cells. The results will be helpful for understanding the mechanisms of bone metabolism and application of lanthanum-based compounds in the future.

Keywords Lanthanum · Proliferation · Osteogenic differentiation · Mineralization

Abbreviations

ARS	Alizarin red S
ALP	Alkaline phosphatase
α-MEM	Alpha minimum essential medium
BMP2	Bone morphogenetic protein 2
BSP	Bone sialoprotein
Col I	Collagen I
cDNA	Complementary DNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
ECL	Enhanced chemiluminescene
EDTA	Ethylenediaminetetraacetic acid tetrasodium salt
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
La	Lanthanum
OD	Optical density
OBs	Osteoblasts
OCN	Osteocalcin
OS	Osteogenetic induction supplement

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Q-PCR Quantitative real-time reverse transcriptase polymerase chain reaction
Runx2 Runt-related transcription factor 2

Introduction

The biological properties of the lanthanum (La), primarily based on its similarity to calcium, have been the basis for potential therapeutic applications since the early part of the twentieth century. It was reported that oral administration of $LaCl_3$ in rabbits fed an atherogenic diet resulted in inhibition for the development of atherosclerosis [1]. Lanthanum(III) complexes with ligands such as hymecromone, umbelliperone, mendiaxon, warfarin, coumachlor, and niffcoumar have demonstrated cytotoxicity against HL-60 cells. Now, Forsrenol®, as a phosphate binder for the treatment of hyperphosphatemia in renal dialysis patients, has been approved in both the USA and Europe, it provides an alternative approach to control the intake of dietary phosphate without the adverse effects associated with the current aluminum and calcium based phosphate binders [2]. Thus, these extensive applications raised deep concerns regarding their riskiness.

It was reported that La was rapidly cleared from the blood and redistributed to tissues, primarily the liver and bone after intravenous administration. With similar ionic radii to calcium, but a higher charge, the La^{3+} ion has a high affinity for Ca^{2+} sites. Thus, it is likely that La intervenes in bone-remodeling process and affects bone cell function. Li et al. found that long-term use of oral La $(NO)_3$ supplementation to rats caused La accumulation in the bone tissue, reduced Ca/P ratio, decreased bone density, changed microstructure of bone, and increased bone crystalinity [3]. Huang et al. reported that $La(NO_3)_3$ retarded bone maturation of male Wistar rats at the dose of 2.0 mg La $(NO_3)_3 kg^{-1} day^{-1}$ over a 6-month period [4]. We found that La^{3+} inhibited osteoclastic activity in a dose-dependent manner at the concentrations of 1.00×10^{-5}, 1.00×10^{-6}, and 1.00×10^{-7} $mol\cdot L^{-1}$, but the osteoclastic activity was significantly enhanced by La^{3+} at the concentration of 1.00×10^{-8} $mol\cdot L^{-1}$ [5]. Wang et al. reported that La^{3+} exposure enhanced osteoblast (OB) differentiation and the effect depended on extracellular signal-regulated kinase phosphorylation via PTx-sensitive Gi protein signaling [6]. Shi et al. found that $LaCl_3$ suppressed the beta-GP-induced osteoblastic differentiation and calcification in rat vascular smooth muscle cells [7]. Zhang et al. reported that La^{3+} inhibited the proliferation of OBs at the concentrations of 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} $mol\cdot L^{-1}$, significantly increased alkaline phosphatase (ALP) activity of OBs at the concentration of 1×10^{-5} $mol\cdot L^{-1}$, but decreased ALP activity of OBs at the concentrations of 1×10^{-9}, 1×10^{-8}, and 1×10^{-6} $mol\cdot L^{-1}$ [8]. Although the effects of La-based compounds on OBs have been previously reported, the potential effects on bone metabolism are not well-understood.

The MC3T3-E1 cell line, a nontransformed murine preosteoblast cell line, is an excellent cell differentiation model. MC3T3-E1 cells require only serum and ascorbic acid to express a fully differentiated phenotype. So MC3T3-E1 cell line may provide a useful system to study the regulation signals in relation to the different stages from proliferation to mineralization in vitro [9]. In this paper, the effects of $LaCl_3$ on the proliferation, osteogenic differentiation, and mineralization of MC3T3-E1 cells were investigated at cell and molecular levels for the first time.

Materials and Methods

Materials

MC3T3-E1 cell line was purchased from the American Type Culture Collection. Alpha minimum essential medium (α-MEM) and fetal bovine serum (FBS) were purchased from Gibco. (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) (MTT), lanthanum (III) chloride heptahydrate (purity 99.999 %), benzylpenicillin, streptomycin, β-glycerophosphate, dexamethasone, ascorbic acid, and alizarin red S (ARS) were from Sigma. An ALP activity kit was obtained from Nanjing Jiancheng Biological Engineering Institute (Nanjing, China), and a microprotein assay kit was purchased from Beyotime Biotechnology (Haimen, China). Trizol Plus RNA purification kit was obtained from Invitrogen. RT^2 quantitative real-time reverse transcriptase polymerase chain reaction (Q-PCR) Master Mix and SYBR Green/ROX Master Mix were purchased from SABiosciences. Anti-runt-related transcription factor 2 (Runx2), anti-bone morphogenetic protein 2 (BMP2), and anti-osteocalcin (OCN) were from Santa Cruz Biotechnology. Enhanced chemiluminescene (ECL) and western blotting substrate were obtained from Thermo Fisher Scientific Inc. Other reagents were of analytical grade. $LaCl_3$ solution was prepared by dissolving solid lanthanum (III) chloride heptahydrate in a 0.9 % NaCl solution and was diluted to 50 mM $LaCl_3$. The stock solution was stored at −20 °C.

Culture of MC3T3-E1 Cells

MC3T3-E1 cells were cultured in α-MEM supplemented with 10 % (*v*/*v*) FBS, 100 U/ml streptomycin and 100 U/ml penicillin. Incubation was conducted in a CO_2 incubator (5 % CO_2, 95 % air; Sanyo, Model MCO-18AIC) at 37 °C. The cells were subcultured every 3 days in the presence of 0.25 %

(*w*/*v*) trypsin plus 0.02 % (*w*/*v*) ethylenediaminetetraacetic acid tetrasodium salt (EDTA) solution.

Assay for Proliferation

The proliferation of MC3T3-E1 cells was measured according to the MTT assay [10]. In brief, MC3T3-E1 cells were seeded in 96-well tissue culture plates (5,000 cells/well) and incubated overnight. Then, $LaCl_3$ at different concentrations (final concentrations of 0.0001, 0.001, 0.01, 0.1, 1, 10, 100, and 1,000 μM) was added. Cells with NaF and cells without $LaCl_3$ treatment were used as positive control and negative control, respectively, and wells without cells were set as blanks. One-, 2-, and 3-day further incubations were performed, 20 μL of MTT (5.0 $mg \cdot mL^{-1}$) was added and incubated for another 4 h at 37 °C. Then, the supernatant was removed and dimethylsulfoxide was added, and the optical density (OD) at 570 nm was measured on a microplate spectrophotometer (Bio-rad Model 680, USA). The proliferation rate (percent) was calculated according to the formula: $[(OD_{sample} - OD_{blank}) - (OD_{control} - OD_{blank})] / (OD_{control} - OD_{blank}) \times 100$.

Assay for ALP Activity

MC3T3-E1 cells were seeded in 48-well plates (2×10^4 cells/well) with α-MEM medium plus 10 % FBS. After 24 h, the culture medium was changed to α-MEM, 10 % FBS medium and osteogenetic induction supplement (OS) containing 10 $mmol \cdot L^{-1}$ disodium β-glycerophosphate, 0.15 $mmol \cdot L^{-1}$ ascorbic acid and 10^{-8} $mol \cdot L^{-1}$ dexamethasone [11]. A series of dilutions of $LaCl_3$ (final concentrations of 0.0001, 0.001, 0.01, 0.1, 1, 10, 100, and 1,000 μM) were added and incubated for 7, 10, and 14 days. MC3T3-E1 cells treated with only OS were used as the control group. NaF was used as a positive control. After incubation, MC3T3-E1 cells were washed twice with ice-cold D-Hank's and lysed by two cycles of freezing and thawing. ALP activity and protein content were measured by an ALP activity kit and a micro-Bradford assay kit. All results were normalized by protein content.

Assay for Mineralized Matrix Formation

MC3T3-E1 cells were seeded in 24-well tissue culture plates (2×10^4 cells/well) and cultured overnight at 37 °C in a 5 % CO_2 humidified incubator. The medium was then changed to medium containing OS and $LaCl_3$ (final concentrations of 0.0001, 0.001, 0.01, 0.1, 1, 10, 100, and 1,000 μM) for 21 days. The formation of mineralized matrix nodules was determined by ARS staining. Briefly, the cells were fixed in 70 % ethanol for 1 h at room temperature. The fixed cells were washed with D-Hank's and stained with 1 % (*w*/*v*) ARS, pH 4.2, for 30 min at room temperature. Quantitative analysis of ARS staining was performed by elution with 10 % (*w*/*v*) cetylpyridium chloride for 10 min at room temperature and the OD was measured at 570 nm [12]. The mineralization promotion rate (percent) was calculated according to the formula: $(OD_{sample} - OD_{blank}) / (OD_{OS} - OD_{blank}) \times 100$.

Q-PCR Analysis

Total RNA from MC3T3-E1 cells treated with 0.0001, 0.1, and 1,000 μM $LaCl_3$ in the presence of OS for 4 days was extracted with Trizol Plus RNA purification kit and was reverse transcribed to first-strand complementary DNA (cDNA) according to the TaKaRa protocol. Q-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: $2^{-\Delta\Delta CT} = 1$. The primers used for Q-PCR were shown in Table 1.

Western Blot Analysis

Western blots were employed as described previously in detail [13]. In brief, MC3T3-E1 cells were washed with cold phosphate buffer saline and lysed in cold 50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 4.3 M urea, and 1%Triton X-100. Proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis 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 tris-buffered saline and Tween 20 (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 2 h incubation with secondary antibodies conjugated with horseradish peroxidase, and visualized with an ECL kit. The optical densities of bands were quantified by LAS-1000 image analyzer (Fuji-Film) software. Beta-actin protein expression was used as a loading control.

Statistical Analysis

Data were collected from at least three separate experiments and expressed as means±standard deviation. The statistical

Table 1 Q-PCR primers

Gene symbol	Forward primer (5′-3′)	Reverse primer (5′-3′)
Runx2	TTCTCCAACCCACGAATGCAC	CAGGTACGTGTGGTAGTGAGT
BMP2	TGGCCCATTTAGAGGAGAACC	AGGCATGATAGCCCGGAGG
ALP	GTTGCCAAGCTGGGAAGAACAC	CCCACCCCGCTATTCAAAC
OCN	GAACAGACTCCGGCGCTA	AGGGAGGATCAAGTCCCG
BSP	GAATCCACATGCCTATTGC	AGAACCCACTGACCCATT
Col I	AACATGACCAAAAACCAAAAGTG	CATTGTTTCCTGTGTCTTCTGG
GAPDH	GACTTCAACAGCAACTCCCAC	TCCACCACCCTGTTGCTGTA

differences were analyzed by a paired Student's *t* test. *P* values less than 0.05 were considered to indicate statistical significance.

Results

Effect of $LaCl_3$ on the Proliferation of MC3T3-E1 Cells

As shown in Fig. 1, the effect of $LaCl_3$ on the proliferation of MC3T3-E1 cells was similar in a time–course manner, the proliferation rate was decreased with increasing $LaCl_3$ concentrations. After 1, 2, and 3 days of $LaCl_3$ treatment, $LaCl_3$ promoted the proliferation of MC3T3-E1 cells at concentrations of 0.0001, 0.001, and 0.01 μM, had no effect on the proliferation of MC3T3-E1 cells at a concentration of 1 μM, but turned to inhibit the proliferation of MC3T3-E1 cells at concentrations of 10, 100, and 1,000 μM. $LaCl_3$ had no effect on the proliferation of MC3T3-E1 cells at a concentration of 0.1 μM for 1 and 2 days, but promoted the proliferation of MC3T3-E1 cells for 3 days.

Effect of $LaCl_3$ on the Osteogenic Differentiation of MC3T3-E1 Cells

As shown in Fig. 2, the effect of $LaCl_3$ on the osteogenic differentiation of MC3T3-E1 cells was similar in a time–course manner, and showed: promotion–no effect–inhibition. After 7, 10, and 14 days of $LaCl_3$ treatment, $LaCl_3$ increased the ALP activity of MC3T3-E1 cells at concentrations of 0.0001, 0.001, 0.01, 0.1, and 1 μM, but turned to decrease the ALP activity at concentrations of 100 and 1,000 μM. $LaCl_3$ had no effect on the ALP activity of MC3T3-E1 cells at the concentration of 10 μM for 7 and 10 days, but decreased the ALP activity for 14 days.

Effect of $LaCl_3$ on the Formation of Mineralized Matrix Nodules

The effect of $LaCl_3$ on the formation of mineralized matrix nodules showed: promotion–no effect–inhibition. $LaCl_3$ promoted the formation of mineralized matrix nodules of MC3T3-E1 cells at concentrations of 0.0001, 0.001, 0.01,

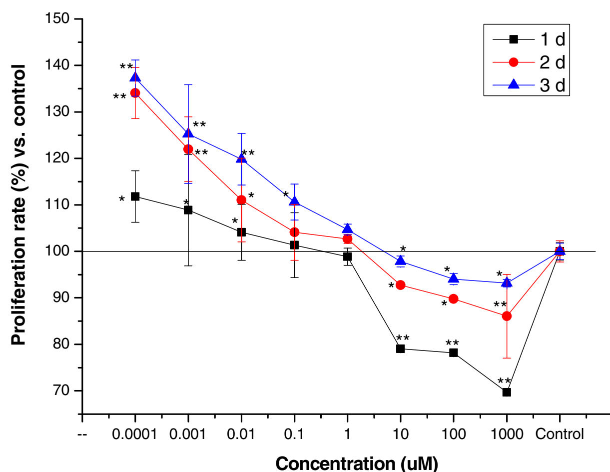


Fig. 1 Effect of $LaCl_3$ on the proliferation of MC3T3-E1 cells ($^{*}P$<0.05, $^{**}P$<0.01 compared with control group, *n*=6)

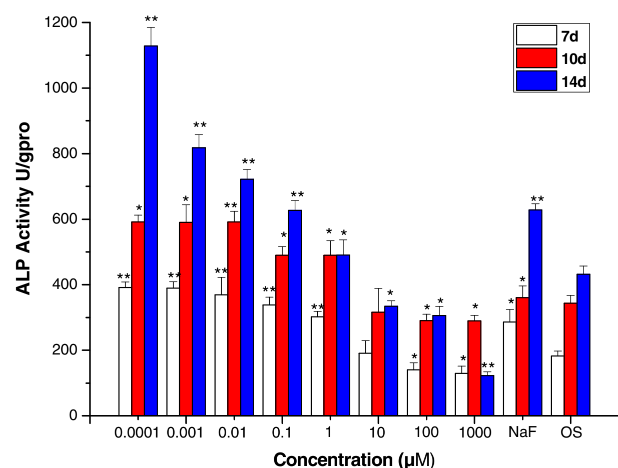


Fig. 2 Effect of $LaCl_3$ on the osteogenic differentiation of MC3T3-E1 cells ($^{*}P$<0.05, $^{**}P$<0.01 compared with OS group, *n*=6)

and 0.1 μM, had no effect on the formation of mineralized matrix nodules at concentrations of 1 and 10 μM, but turned to inhibit the formation of mineralized matrix nodules at concentrations of 100 and 1,000 μM (Fig. 3a). Moreover, the experimental results were in accordance with morphological observations (Fig. 3b).

Q-PCR Analysis of Osteogenic Differentiation Specific Genes

The results showed that several genes (such as Runx2, BMP2, ALP, bone sialoprotein (BSP), collagen I (Col I), and OCN) that were supposed to be activated during the osteogenic differentiation were significantly upregulated in the MC3T3-E1 cells treated with 0.0001 and 0.1 μM $LaCl_3$, but these genes were downregulated in the MC3T3-E1 cells treated with 1,000 μM $LaCl_3$ for 4 days as compared to OS group (Fig. 4).

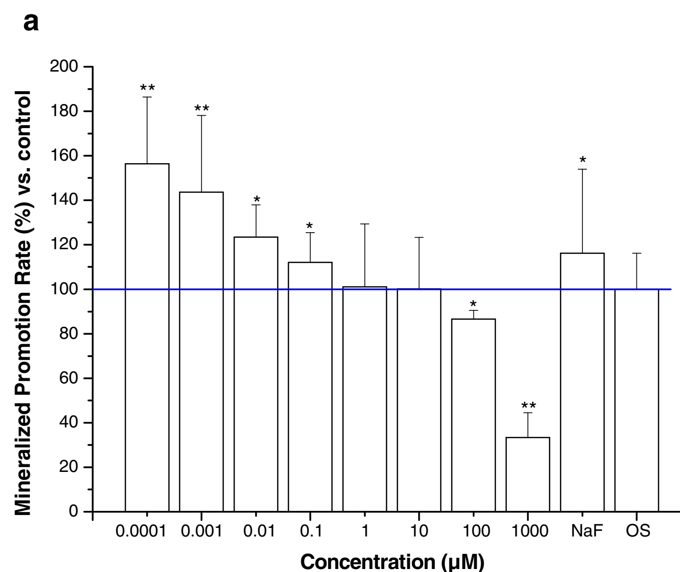
Western Blot Analysis of Osteogenic Differentiation Specific Proteins

As demonstrated in Fig. 5, the expression of BMP2, Runx2, and OCN proteins was upregulated after 0.0001 μM $LaCl_3$ treatment, but the expression of those proteins was downregulated after 1,000 μM $LaCl_3$ treatment.

Discussion

The process of OB differentiation can be subdivided in three subsequent stages: proliferation, extracellular matrix synthesis, and maturation mineralization. The proliferation effect was performed by MTT assay based on the principle that living cells are capable of reducing light color tetrazolium salts into an intense color formazan derivative. ALP is an ectoenzyme which acts as a marker for cells undergoing differentiation to form preosteoblasts and OBs. Generally, ALP activities were increased after in vitro osteogenic induction for 7 days, while later ALP staining was seen after 14 days of osteogenic induction. An essential sign for the osteogenic differentiation of MC3T3-E1 cells is bone matrix maturation and mineralization. After cultured for 2–3 weeks, OB nodes formed and reached peak quantity when OBs started to mineralize. The appearance of ALP activity is an early phenotypic marker for differentiation of OBs, while mineralized nodule formation is a phenotypic marker for the last stage of mature OBs.

In our study, we found that $LaCl_3$ had dual effects on the proliferation, osteogenic differentiation and mineralization of MC3T3-E1 cells. First, $LaCl_3$ promoted the proliferation, osteogenic differentiation, and mineralization of MC3T3-E1 cells at lower concentrations, then had no effects and further turned to inhibit the proliferation, osteogenic differentiation,



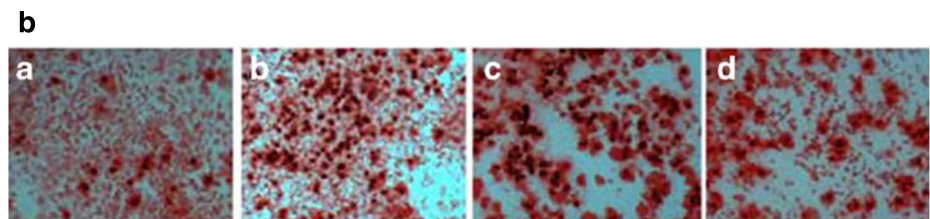


Fig. 3 **a** Effect of $LaCl_3$ on the mineralized nodule formation of MC3T3-E1 cells ($^{*}P<0.05$, $^{**}P<0.01$ compared with OS group, $n=6$). **b** The mineralized nodule formation in the presence of $LaCl_3$ stained by ARS. *a* Cells treated with OS only, *b* cells treated with 0.0001 μM $LaCl_3$+OS, *c* cells treated with 0.01 μM $LaCl_3$+OS, *d* cells treated with 1 μM $LaCl_3$+OS. Original magnification=100

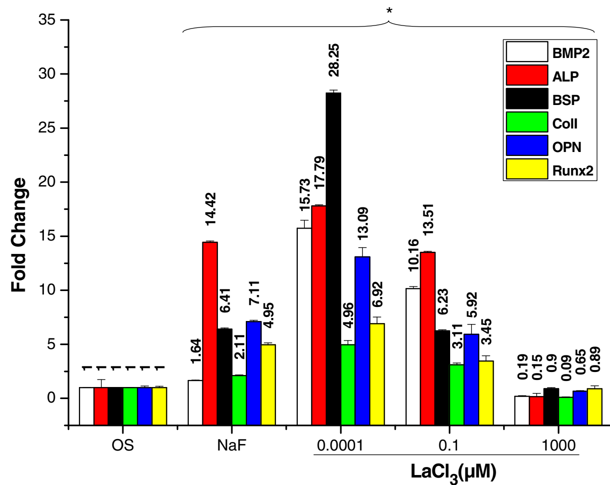
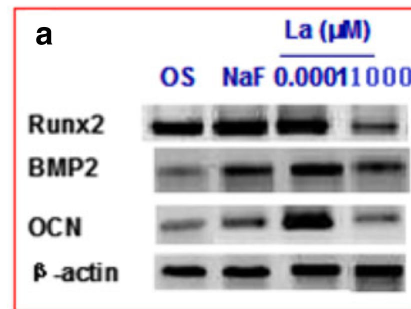


Fig. 4 Osteogenic differentiation specific gene expression was determined by Q-PCR ($^{*}P<0.05$ compared with OS, $n=6$)

and mineralization of MC3T3-E1 cells with increasing concentrations. We previously reported that $LaCl_3$ increased the proliferation of UMR106 cells by promoting the transition of cell cycle from G_0/G_1 phase to S phase, accelerating cells to enter DNA synthesis phase [14]. YCl_3 and $CeCl_3$ (1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, 1×10^{-5}, and 1×10^{-4} mol/L) promoted the proliferation of primary mouse OBs on days 1, 2, and 3. YCl_3 had no effect on the differentiation of OBs at concentrations of 1×10^{-9} and 1×10^{-8} mol/L, promoted the differentiation of OBs at concentration of 1×10^{-7} mol/L, but turned to inhibit the differentiation of OBs at other tested concentrations on day 1. On day 2, it inhibited the differentiation of OBs at all tested concentrations. On day 3, it promoted the differentiation of OBs at lower concentrations of 1×10^{-9} and 1×10^{-8} mol/L, but turned to inhibit the differentiation of OBs at other concentrations. On days 1 and 3, $CeCl_3$ promoted the differentiation of OBs at concentrations of 1×10^{-9}, 1×10^{-7}, and 1×10^{-6} mol/L, but inhibited the differentiation of OBs at higher concentrations. On day 2, it also inhibited the differentiation of OBs at tested concentrations. YCl_3 inhibited the formation of mineralized matrix nodules of OBs at concentrations of 1×10^{-9}, 1×10^{-8}, and 1×10^{-6} mol/L, but turned to promote the formation of mineralized matrix nodules of OBs at other concentrations. $CeCl_3$ inhibited the formation of mineralized matrix nodules of OBs at concentrations of 1×10^{-9}, 1×10^{-8}, and 1×10^{-7} mol/L, and promoted



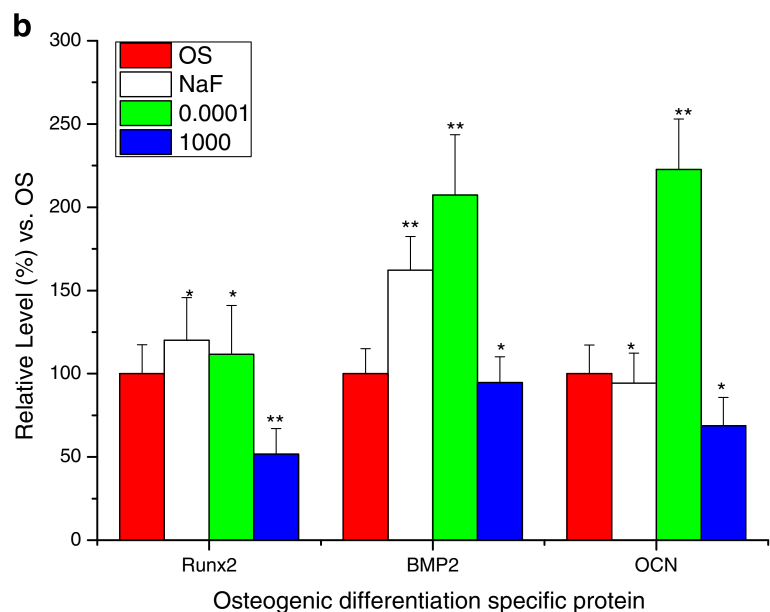


Fig. 5 The expression levels of osteogenic differentiation specific proteins. **a** Western blot analysis for BMP2, Runx2, and OCN proteins in MC3T3-E1 cells treated with $LaCl_3$ after 4-day incubation. **b** Quantification of the blots for BMP2, Runx2, and OCN proteins ($^{*}P<0.05$ compared with OS, $n=6$)

the formation of mineralized matrix nodules of OBs at other concentrations [15, 16]. In summary, the concentration and culture time are key factors for switching the biological effects of rare earth ions from toxicity to activity, or from down-regulation to upregulation. In addition, different rare earth ions may behave differently, which has been recognized in a series of biological effects of lanthanides. These differences may relate to the physicochemical characteristics of the respective cations depending upon features, such as their ionic radii or charge densities [17].

Cells maintain their homeostasis through a comprehensive signaling network. Any perturbation of this network will affect cell function and behavior [18]. A large number of genes which have been associated with bone cells are known to be specifically required for OB differentiation, such as Runx2, BMP-2, ALP, and OCN [19]. Runx2 is a master regulator of osteogenic gene expression and OB differentiation. Runx2 knockout mice exhibited no bone tissues or OBs, indicating that OB differentiation was completely blocked in the absence of Runx2 [20]. ALP is responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. ALP is considered to play an important role in process of mineral formation in tissues like bone, cartilage tooth root cementum, and dentin [21]. 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 [22]. BSP and Col I are significant components of the bone extracellular matrix. BMP-2 is a member of the transforming growth factor-β superfamily and plays a key regulatory role as a cell–cell signaling molecule during bone formation and repair. BMP-2 which is a potent osteogenic protein required for OB differentiation and bone formation, can induce low level expression of osteoblast marker genes such as OCN and ALP in calvarial cells from $Cbfa1^{-/-}$ animals, although these cells are not able to form a mineralized matrix [23]. In our work, $LaCl_3$ displayed the upregulation of these OB specific genes (Runx2, BMP2, ALP, BSP, Col I, and OCN) at concentrations of 0.0001 and 0.1 μM, but these genes were downregulated in the MC3T3-E1 cells treated with 1,000 μM $LaCl_3$ (Fig. 4), the expression of BMP2, Runx2, and OCN proteins was also significantly promoted by $LaCl_3$ at 0.0001 μM, but turned to be downregulated after 1,000 μM $LaCl_3$ treatment (Fig. 5). These experimental results were consistent with the observed effects of $LaCl_3$ on the proliferation, osteogenic differentiation, and mineralized matrix nodule formation of MC3T3-E1 cells at cell level (Figs. 1, 2, and 3). The results suggest that $LaCl_3$ likely up- or downregulates the expression of Runx2, which subsequently up- or downregulates OB marker genes Col I and BMP2 at early stages and ALP and OCN at later stages of differentiation, thus causes to promote or inhibit the proliferation, osteogenic differentiation, and mineralization function of MC3T3-E1 cells. The results will be helpful for understanding the mechanisms of bone metabolism and application of La-based compounds in the future.

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