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Effect of Dy^{3+} on osteogenic and adipogenic differentiation of mouse primary bone marrow stromal cells and adipocytic trans-differentiation of mouse primary osteoblasts

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A series of experimental methods including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test, alkaline phosphatase (ALP) activity measurement, mineralized function, Oil Red O stain and measurement were employed to assess the effect of Dy^{3+} on the osteogenic and adipogenic differentiation of mouse primary bone marrow stromal cells (BMSCs) and the adipogenic trans-differentiation of mouse primary osteoblasts (OBs). The results showed that Dy^{3+} had no effect on BMSC proliferation at concentrations of 1×10^{-8} and 1×10^{-5} mol/L, but inhibited BMSC proliferation at other concentrations. Dy^{3+} had no effect on OB proliferation at concentrations of 1×10^{-10} and 1×10^{-9} mol/L, but inhibited OB proliferation at other concentrations. Dy^{3+} had no effect on the osteogenic differentiation of BMSCs at concentrations of 1×10^{-9} and 1×10^{-7} mol/L, and promoted osteogenic differentiation of BMSCs at other concentrations at the 7th day. The osteogenic differentiation of BMSCs was inhibited by Dy^{3+} at concentration of 1×10^{-5} mol/L at the 14th day, but promoted osteogenic differentiation of BMSCs at concentrations of 1×10^{-9}, 1×10^{-8}, 1×10^{-7} and 1×10^{-6} mol/L with the maximal effect at concentration of 10^{-6} mol/L. Dy^{3+} promoted mineralized function of BMSCs at any concentration. Dy^{3+} had no effect on adipogenic differentiation of BMSCs at concentration of 1×10^{-7} mol/L, but inhibited adipogenic differentiation of BMSCs at other concentrations. Dy^{3+} inhibited adipocytic trans-differentiation of OBs at any concentration, suggesting that Dy^{3+} had protective effect on bone and the protective effect on bone may be mediated by modulating differentiation of BMSCs away from the adipocyte and inhibiting adipocytic trans-differentiation of OBs which may promote differentiation and mineralization of OBs. These results may be valuable for better understanding the mechanism of the effect of Dy^{3+} on pathogenesis of osteoporosis.

rare earth ion, bone marrow stromal cells, osteogenic differentiation, adipogenic differentiation, adipocytic trans-differentiation

Osteoporosis is a systemic skeletal disease characterized by low bone mass and the microarchitectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fractures. It is a common disorder with a complex pathophysiology involving endogenous factors[1]. Among them, some essential trace elements were reported to be involved in the pathogenesis of osteoporosis. By clinical trails, zinc supplementation was shown to inhibit postmenopausal bone loss[2]. Moonga et al. reported that zinc ion inhibited osteoclas-

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tic pit formation in a biphasic manner, but had no effect on the number of osteoclasts[3]. Calcium ion also inhibited osteoclastic bone resorption[4]. The similarity between rare earth ions (Ln^{3+}) and Ca^{2+} has been suggested to be responsible for their biochemical behavior[5]. Thus it is likely that the lanthanide ions intervene in bone-remodeling process and affect bone cell function. Jha et al. found that Pr_6O_{11} and Nd_2O_3 promoted bone resorption in mice[6]. Quarles et al. found that Gd^{3+} stimulated DNA synthesis in MC3T3-E1 osteoblasts dose-dependently *in vitro*[7]. Li et al. reported that long-term oral supplementation of La^{3+} to rats caused lanthanum accumulation in the bone tissue, reduced Ca/P ratio, decreased bone density and changed the microstructure of bone at a low dose [8]. Previously, we reported that the effect of Ln^{3+} (La^{3+}, Sm^{3+}, Er^{3+}, Nd^{3+}, Gd^{3+} and Dy^{3+}) on proliferation, differentiation and function expression of osteoclasts and OBs depended on the concentrations, incubation time and species[9−11].

BMSCs are pluripotent cells which have the capacity to become OBs, adipocytes, chondrocytes, myoblasts or fibroblasts[12,13]. Thus, lineage determination between OBs and adipocytes may be a critical component in the regulatory pathways of osteoblastogenesis[14]. Furthermore, there is more and more evidence that suggests a great degree of plasticity exists between OBs and adipocytes and this transdifferentiation is reciprocal[15]. Indeed, it is now hypothesized that an increase in the number of adipocytes occurs at the expense of OBs in osteopenic disorders. It was reported that there was a therapeutic opportunity to either prevent or treat osteopenic disorders by inhibiting marrow adipogenesis[16]. Therefore, it is possible that inhibition of marrow adipogenesis with a concomitant increase in osteoblastogenesis could provide a therapeutic target to either prevent adipocyte formation or divert existing adipocytes to become more OBs with an increase in functional bone cells[17].

So far the osteoclasts and OBs as cell models were used to study the effect of Dy^{3+} on the pathogenesis of osteoporosis. Effects of Dy^{3+} on the osteogenic and adipogenic differentiation of mouse primary BMSCs and the adipogenic trans-differentiation of mouse primary OBs were not reported. In this paper, the effects of Dy^{3+} on the osteogenic and adipogenic differentiation of BMSCs and the adipogenic trans-differentiation of OBs are studied in order to further elucidate the effect of Dy^{3+} on the pathogenesis of osteoporosis.

1 Materials and methods

1.1 Materials

KM mice were obtained from the Guangming Weiwu Biological Product Factory. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Gibco. Benzylpenicillin, streptomycin, MTT, β-glycerophosphate, trypsin, collagenase II, dexamethasone, ascorbic acid, insulin, alizarin red S (ARS), oil red O stain and cetylpyridium chloride were obtained from Sigma. $DyCl_3$ were from www.cre-ol.com. ALP activity kit was obtained from Nanjing Jiancheng Biological Engineering Institute, and micro-protein assay kit was from Beyotime Biotechnology.

1.2 Isolation and culture of primary BMSCs

The mouse BMSCs were obtained from adult KM mice (4−6 weeks) using a modification of the method previously reported[18]. Briefly, mice were sacrificed by decapitation. Femora and tibiae were aseptically harvested, and the whole bone marrow was flushed using DMEM in a 1 mL syringe and a 25-gauge needle. The cells were collected and cultured in a culture flask. After 3 d incubating at 37℃, in 5% CO_2 humidified incubator, the nonadherent cells were removed by gentle aspiration and the medium was replaced with fresh DMEM. Then the medium was changed every 3 d in all experiments.

1.3 Isolation and culture of primary OBs

The mouse OBs were isolated mechanically from newborn mice skull using a modification of the method previously reported[19]. Briefly, skull was dissected from KM mice, endosteum and periosteum were stripped off, and the bone was cut into approximately 1−2 mm^2 pieces and digested with trypsin (2.5 g/L) for 30 min and the digestion was discarded. Then the bone was digested with collagenase II (1.0 g/L) twice with 1 h for each, and the cells were collected and cultured in a culture flask. After incubating overnight at 37℃, in a 5% CO_2 humidified incubator, the DMEM was removed. Then the medium was changed every 3 d in all experiments.

1.4 Cell proliferation assay

The protocol described by Mosmann was followed by some modifications[20]. Briefly, BMSCs (3×10^6 cells per well) or OBs (3×10^4 cells per well) were plated in 96-well culture plates and cultured overnight at 37℃, in a 5% CO_2 humidified incubator. Dy^{3+} was then added to

the wells at final concentrations of 1×10^{-10}, 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6} and 1×10^{-5} mol/L. Control wells were prepared by addition of DMEM. Wells containing DMEM without cells were used as blanks. The plates were incubated at 37℃, in a 5% CO_2 incubator for 44 h. Upon completion of the incubation, MTT dye solution (20 μL, 5 mg/mL) was added to each well. After 4 h incubation, the supernatant was removed and DMSO (100 μL) was added to solubilize the MTT. The optical density (OD) of each well was measured on a microplate spectrophotometer (BioRad Model 3550, USA) at a wavelength of 570 nm. The proliferation rate (%) was calculated according to the formula: $(OD_{treated}/OD_{control}\text{-}1)\times100\%$.

1.5 Measurement of ALP activity

The BMSCs were isolated as above. BMSCs (3×10^6 cells per well) were plated in 48-well culture plates, after being induced by osteogenic supplement (10^{-7} mol/L dexamethasone, 5.0 mmol/L β-glycerophosphate, 50 μg/mL ascorbic acid) and treated with Dy^{3+} at final concentrations of 1×10^{-10}, 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6} and 1×10^{-5} mol/L for 7 d and 14 d respectively. The plates were washed thrice with ice-cold PBS and lysed by two cycles of freezing and thawing. Aliquots of supernatants were subjected to ALP activity and protein measurement using an ALP kit and a micro-protein assay kit respectively. The osteogenic differentiation promotion rate (%) was calculated according to the formula: $(\text{ALP activity}_{treated}/\text{ALP activity}_{control}-1)\times100\%$[21].

1.6 Mineralized matrix formation assay

The BMSCs were isolated as above. BMSCs (2×10^5 cells per well) were plated in 48-well tissue culture plates and cultured overnight at 37℃, in a 5% CO_2 humidified incubator. The medium was then changed to differentiation medium containing 10 mmol/L β-glycero-phosphate and 50 μg/mL ascorbic acid in the presence or absence of the concentrations of 1×10^{-10}, 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6} and 1×10^{-5} mol/L Dy^{3+} for 21 d. The formation of mineralized matrix nodules was determined by ARS stain. Briefly, the cells were fixed in 70% ethanol for 1 h at room temperature. The fixed cells were washed with PBS and stained with 40 mmol/L ARS, pH 4.2, for 30 min at room temperature. Quantitation of ARS staining was performed by elution with 10% (w/v) cetylpyridium chloride for 10 min at room temperature and the absorbance was measured at 570 nm[22]. Results were expressed as moles of ARS/ permilligram of total cellular protein.

1.7 Oil red O stain and measurement

The BMSCs or OBs were isolated as above. BMSCs (3×10^6 cells per well) or OBs (10^4 cells per well) were plated in 48-well culture plates, after being induced by adipogenic supplement (10 μg/mL insulin, 10^{-7} mol/L dexthamethone) and treated with Dy^{3+} at final concentrations of 1×10^{-10}, 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6} and 1×10^{-5} mol/L, and fat droplets within differentiated adipocytes from BMSCs and trans-differentiated adipocytes from OBs were stained using the oil red O described by Ichiro et al. with some modifications[23]. Briefly, cell monolayers were fixed in 4% formaldehyde, washed in water and stained with a 0.6% (w/v) oil red O solution (60% isopropanol, 40% water) for 15 min at room temperature. For quantification, cell monolayers were then washed extensively with water to remove unbound dye, and recorded by inverted phase contrast microscopy (Olympus IX 51), then 1 mL of isopropyl alcohol was added to the culture plates. After 5 min, the absorbance of the extract was measured by a spectrophotometer at 510 nm. The adipogenic differentiation inhibition rate (%) and adipocytic trans-differentiation inhibition rate (%) were calculated according to the formula: $(1\text{-}OD_{treated}/OD_{control})\times100\%$.

1.8 Statistical analysis

Data were collected from at least three separate experiments. The results were expressed as means±standard deviation (SD). The statistical differences were analyzed using SPSS' t-test. *P* values less than 0.05 were considered to indicate statistical differences.

2 Results

2.1 Effect of Dy^{3+} on the BMSC proliferation

As shown in Figure 1, Dy^{3+} had no effect on BMSC proliferation at concentrations of 1×10^{-8} and 1×10^{-5} mol/L, but inhibited BMSC proliferation at other concentrations.

2.2 Effect of Dy^{3+} on the OB proliferation

As shown in Figure 2, Dy^{3+} had no effect on OB proliferation at concentrations of 1×10^{-10} and 1×10^{-9} mol/L, but inhibited OB proliferation at other concentrations.

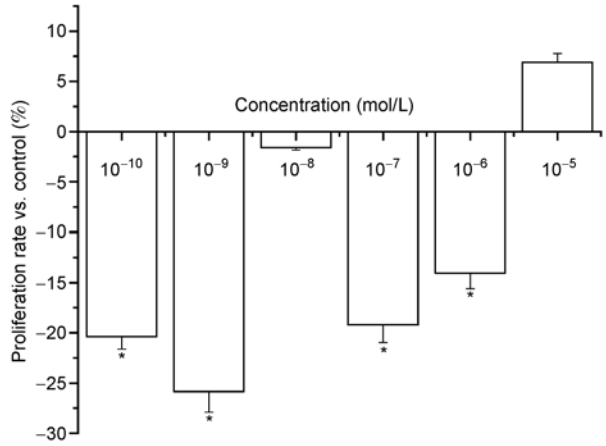

Figure 1 Effect of Dy^{3+} on the proliferation of BMSCs (n = 6, *P<0.05, significant compared to control group).

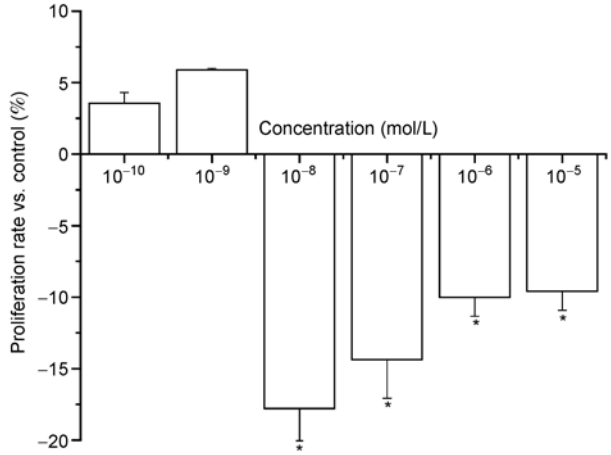

Figure 2 Effect of Dy^{3+} on the proliferation of OBs (n = 6, *P<0.05, significant compared to control group).

2.3 Effect of Dy^{3+} on the osteogenic differentiation of BMSCs

As shown in Figure 3, Dy^{3+} had no effect on the osteogenic differentiation of BMSCs at concentrations of 1×10^{-9} and 1×10^{-7} mol/L, and promoted osteogenic differentiation of BMSCs at other concentrations at the 7th day. The osteogenic differentiation of BMSCs was inhibited by Dy^{3+} at concentration of 1×10^{-5} mol/L at the 14th day, but promoted osteogenic differentiation of BMSCs at concentrations of 1×10^{-9}, 1×10^{-8}, 1×10^{-7} and 1×10^{-6} mol/L with the maximal effect at the concentration of 1×10^{-6} mol/L.

2.4 Effects of Dy^{3+} on the formation of mineralized matrix nodules

As shown in Figure 4, Dy^{3+} promoted the formation of mineralized matrix nodules of BMSCs at any concentration.

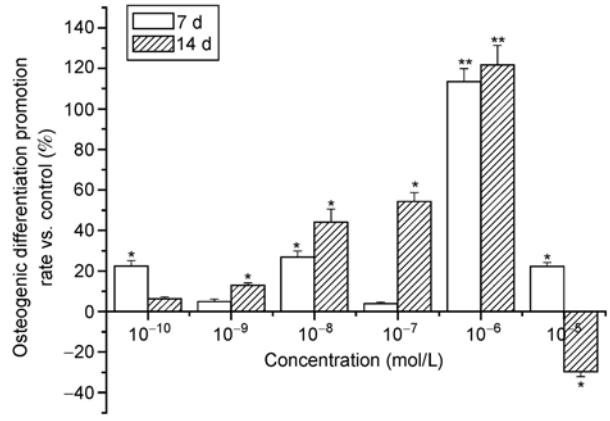

Figure 3 Effect of Dy^{3+} on the osteogenic differentiation of BMSCs (n=6, *P<0.05, **P<0.01, significant compared to control group).

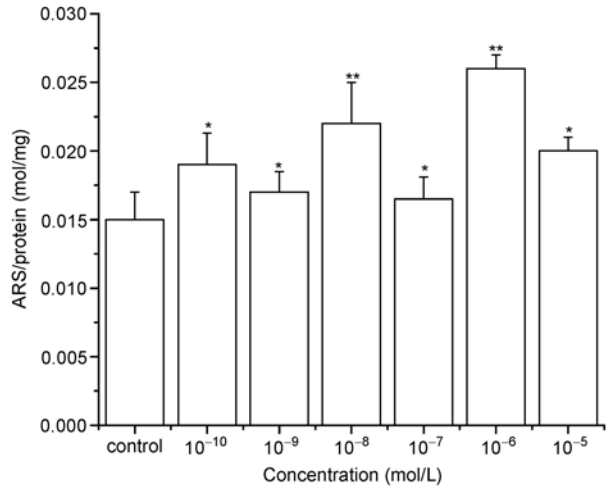

Figure 4 Effect of Dy^{3+} on the mineralized nodule formation of BMSCs (n=6, *P<0.05, **P<0.01, significant compared to control group).

2.5 Effects of Dy^{3+} on the adipogenic differentiation of BMSCs

As shown in Figure 5, Dy^{3+} had no effect on adipogenic differentiation of BMSCs at concentration of 1×10^{-7} mol/L, but inhibited adipogenic differentiation of BMSCs at other concentrations. The morphologic observation accorded with the result of Figure 6.

2.6 Effect of Dy^{3+} on adipocytic trans-differentiation of OBs

As shown in Figure 7, Dy^{3+} inhibited adipocytic trans-differentiation of OBs at any concentration. The morphologic observation accorded with the result of Figure 8.

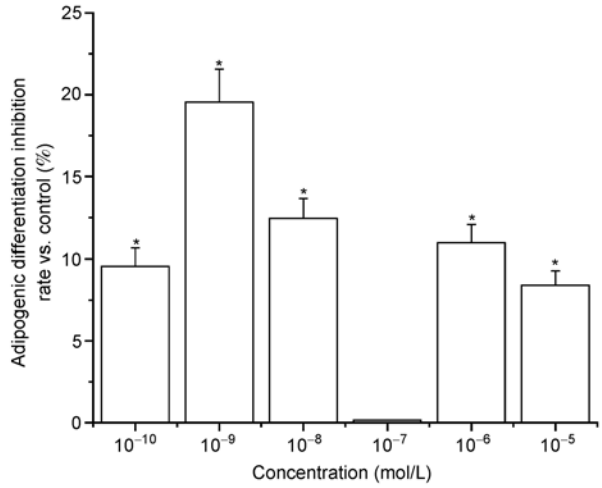

Figure 5 Effect of Dy^{3+} on the adipogenic differentiation of BMSCs (n = 6, *P<0.05, significant compared to control group).

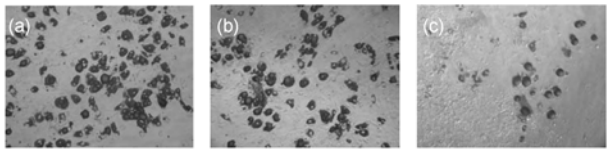

Figure 6 Effect of Dy^{3+} on adipogenic differentiation of BMSCs. (a) adipogenic supplement; (b) adipogenic supplement + 1×10^{-10} mol/L Dy^{3+}; (c) adipogenic supplement+1×10^{-9} mol/L Dy^{3+}.

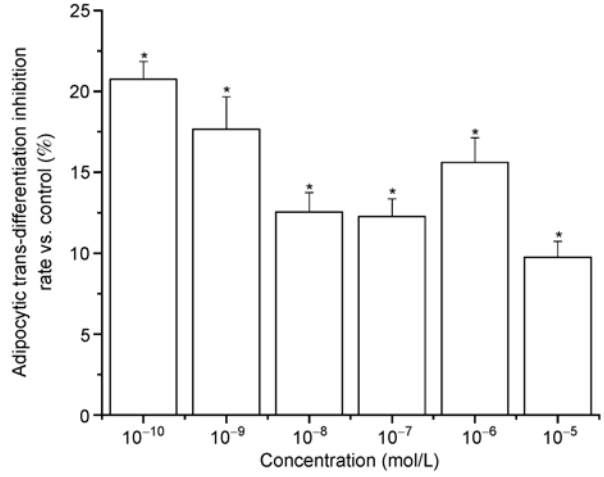

Figure 7 Effect of Dy^{3+} on the adipocytic trans-differentiation of OBs for 10d (n = 6, *P<0.05, significant compared to control group).

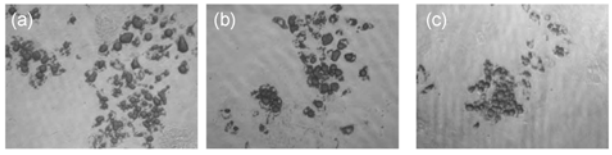

Figure 8 Effect of Dy^{3+} on adipocytic trans-differentiation of OBs. (a) adipogenic supplement; (b) adipogenic supplement + 1×10^{-8} mol/L Dy^{3+}; (c) adipogenic supplement + 1×10^{-10} mol/L Dy^{3+}.

3 Discussion

The precise role of adipocytes in bone marrow is unknown. It has been suggested that they act a purely passive role to fill marrow cavities and play a role in lympho-hematopoiesis. They may also serve an active role in the energy metabolism or participate in the animal's overall metabolism by clearing and storing circulating triglycerides. Adipocytic and osteogenic cells are believed to derive from a multipotential stromal cells in the marrow, and *in vitro* studies have shown an inverse relationship between the differentiation of adipocytic and osteogenic cells[16]. In addition, recent data suggest that medullary adipocytes are secretory cells that may influence hematopoiesis and osteogenesis[24]. It was reported that preadipocytes isolated from mouse marrow may regulate the activity and final differentiation of OBs. The condition medium harvested from mouse stromal preadipocytes decreased the ALP activity of a mouse stromal osteoblastic cell line[25]. Some investigators suggested that adipocytes might be involved in hematopoietic and osteogenic process by supplying the necessary soluble cell surface factors for osteoclast differentiation and function *in vitro*[26]. Sakaguchi et al. have demonstrated that adipocyte-enriched stromal cells support osteoclast formation[27]. Benayahu et al. reported that preadipocytes also have the potential to stimulate osteoclast differentiation[28]. Adipocytes synthesized and released a variety of peptide and nonpeptide compounds or secreted cytokines such as tumor necrosis factor-α (TNF-α) and interleukin (IL-6), and the main effect of these cytokines is a stimulation of bone resorption[24]. So a reversal of adipogenesis will provide an important therapeutic approach to prevent aged-related and steroids-induced osteoporosis.

In this study, we have examined the effects of Dy^{3+} on osteogenic and adipogenic differentiation of BMSCs and the adipogenic trans-differentiation of OBs *in vitro* by employing mouse primary BMSCs and OBs. Our results indicated that: (1) Dy^{3+} inhibited proliferation of BMSCs and OBs at most concentrations; (2) Dy^{3+} promoted osteogenic differentiation of BMSCs except at the concentrations of 1×10^{-9} and 1×10^{-7} mol/L at the 7th day, and promoted osteogenic differentiation of OBs except at the concentration of 1×10^{-5} mol/L at the 14th day; (3) Dy^{3+} promoted mineralized function of BMSCs at tested concentrations; (4) Dy^{3+} inhibited adipogenic differentiation of BMSCs except at the concentration of 1×10^{-7}

mol/L, and inhibited adipocytic trans-differentiation of OBs at any concentration. Previously, we reported that Dy^{3+} at concentrations of 1.00×10^{-5} and 1.00×10^{-6} mol/L inhibited osteoclastic activity as indicated by the dose-dependent reduction in the numbers and surface areas of the lacunae ($P<0.01$). In contrary, the numbers and surface area of lanunae were increased and osteoclastic bone resorbing function was significantly enhanced by Dy^{3+} at concentration of 1.00×10^{-7} mol/L ($P < 0.01$). Dy^{3+} had no effect on osteoclastic bone resorption function at concentration of 1.00×10^{-8} mol/L ($P > 0.05$)[9]. These results indicated that Dy^{3+} might have protective effect on bone. So we deduced that the protective effects of Dy^{3+} on bone may be mediated by decreasing adipocytic cell formation from BMSCs, which may promote OB proliferation, differentiation and mineralization. The mechanism of the effects of Dy^{3+} on the osteogenic and adipogenic differentiation of BMSCs and the adipogenic trans-differentiation of OBs remains to be further studied.

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