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Research paper

Cilomilast enhances osteoblast differentiation of mesenchymal stem cells and bone formation induced by bone morphogenetic protein 2

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ABSTRACT

A rapid and efficient method to stimulate bone regeneration would be useful in orthopaedic stem cell therapies. Rolipram is an inhibitor of phosphodiesterase 4 (PDE4), which mediates cyclic adenosine monophosphate (cAMP) degradation. Systemic injection of rolipram enhances osteogenesis induced by bone morphogenetic protein 2 (BMP-2) in mice. However, there is little data on the precise mechanism, by which the PDE4 inhibitor regulates osteoblast gene expression. In this study, we investigated the combined ability of BMP-2 and cilomilast, a second-generation PDE4 inhibitor, to enhance the osteoblastic differentiation of mesenchymal stem cells (MSCs). The alkaline phosphatase (ALP) activity of MSCs treated with PDE4 inhibitor (cilomilast or rolipram), BMP-2, and/or H89 was compared with the ALP activity of MSCs differentiated only by osteogenic medium (OM). Moreover, expression of *Runx2*, *osterix*, and *osteocalcin* was quantified using real-time polymerase chain reaction (RT-PCR). It was found that cilomilast enhances the osteoblastic differentiation of MSCs equally well as rolipram in primary cultured MSCs. Moreover, according to the H89 inhibition experiments, Smad pathway was found to be an important signal transduction pathway in mediating the osteogenic effect of BMP-2, and this effect is intensified by an increase in cAMP levels induced by PDE4 inhibitor.

1. Introduction

Bone regeneration is one of the most important issues in regenerative medicine. Since the effect of drugs that inhibit bone resorption is not satisfactory, the development of bone anabolic molecules is necessary in patients who have suffered substantial bone loss. Therefore, the enhancement of bone formation is a technology of utmost importance in scaffold-based tissue engineering.

Bone morphogenetic proteins (BMPs) play critical roles in osteoblast differentiation of mesenchymal stem cells (MSCs). In numerous clinical studies, BMP-2 has been used to stimulate the differentiation of osteoprogenitor cells and has been employed as an effective alternative to autogenous bone graft [1–4]. In fact, BMP-2 controls the expression and function of *Runx2* through Smad signalling [5], which directs multipotent MSCs to an osteoblastic lineage and inhibits them from differentiating into adipocytic and chondrocytic lineages. Although several studies have reported that the possibility to use agents capable of increasing the effect of BMPs, the mechanisms on these effects are uncertain [6–9]. In different experimental osteopaenia models, therapeutic effects were achieved by using specific or nonspecific phosphodiesterase (PDE) inhibitors [10,11]. Sugama et al. [12] studied the combinative effect of PeTx, a nonspecific PDE inhibitor, and BMP-4 on ST2 cells, and suggested that cyclic adenosine monophosphate (cAMP) may be involved in the intensification of the BMPs signalling. However, Rawadi et al. [13] showed that the osteoblast differentiation promoted by PeTx is protein kinase A (PKA) independent. These contrasting observations may be resulted from the different model cell features used in these works [12,13]. Hence, we investigated the significant role in the rapid mineralization of the combination of BMP-2 and a specific PDE4 inhibitor using primary cultured MSCs. We hypothesised that a specific PDE4 inhibitor might increase the osteogenetic effect of BMP-2, in a dose-dependent manner, through the enhancement of the cAMP levels. Thus, we tested the combined effect of BMP-2 and two different PDE4 inhibitors (the first-generation PDE4 inhibitor rolipram or the second-generation PDE4 inhibitor cilomilast) on primary MSC differentiation, with or without H89, a PKA inhibitor. Moreover, by evaluating alkaline phosphatise (ALP) activity levels and *Runx2*, *osterix* (*Osx*), and

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osteocalcin (*OCN)* gene expression, we investigated the differentiation mechanism underlying that the key factor in the enanhced bone formation of primary MSCs, is PKA. In particular, our study showed that only the combination of BMP-2 and a specific PDE4 inhibitor activates both the Smad and cAMP pathways, and thereby enhances the mineralization.

2. Materials and methods

2.1. Reagents

Standard medium (SM) consisted of alpha minimal essential medium (α-MEM) supplemented with antibiotics/antimycotics (50 U/mL and 50 mg/mL, respectively; Gibco-Invitrogen, Carlsbad, CA, USA) and 15% foetal bovine serum (FBS). Osteogenic medium (OM) consisted of standard medium supplemented with 50 μM ascorbate, 1 μM dexamethasone, 2 mM glutamine, and 10 mM β-glycerophosphate. Recombinant human BMP-2 (1 mg/300 μL in acetic acid; Creative BioMart, Shirley, NY, USA) was stored at −20 °C. Standard medium containing 30 or 300 ng/mL BMP-2 was freshly prepared. Cilomilast (Ariflo®/SB-207499, S1455; Selleckchem, Houston, TX, USA) and rolipram (R6520; Sigma-Aldrich, Japan) were dissolved in 1 mL of dimethylsulphoxide (DMSO) to obtain 4 mM solutions. Subsequently, the aliquots of the solutions were added to standard medium, with or without BMP-2, for a final concentration of 10 μM or 40 μM.

2.2. Preparation of bone marrow cells and cell cultures

Rat stromal cells were isolated according to the protocol of Kopen et al. [14]. Bone marrow was obtained from the tibias and femurs of male Wistar rats (4 weeks old) by flushing femurs and tibias with α-MEM medium and penicillin/streptomycin (50 U/mL and 50 mg/mL, respectively; Invitrogen) supplemented with heparin. Cells were then washed in medium without heparin, centrifuged, and plated in a dish coated with fibronectin. One day later, non-adherent cells were removed by 3 washes with PBS. Adherent cells were further cultured in standard medium for 3 days. MSCs were grown in SM at 37 °C in a 5% CO_2 atmosphere. The medium was change twice a week, and a subculture was performed every week. At passage 3, the cells were trypsinised and plated at a density of 38,000/cm^2 for use in experiments. After 3 days, medium, with and without reagents, was added. The treatment was performed for 11 days (or 21 days for Alizarin Red staining), changing the medium every 3 days.

2.3. RNA preparation and RT-PCR

Total RNA was isolated from cultured cells in the presence of PDE4 inhibitors, BMP-2, standard and osteogenic medium by using QuickGene-Mini80 (Fujifilm Co., Tokyo, Japan), according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed using the SuperScript™ III CellsDirect cDNA Synthesis system (Invitrogen). The reaction program was 10 min at 25 °C, 2 h at 37 °C, and 5 s at 85 °C. Aliquots of the cDNA were subjected to PCR and amplified in a 20-μL reaction mixture using SYBR® Green Real-time PCR Master Mix (Toyobo Co., Tokyo, Japan). Amplifications were performed in StepOne Plus (Applied Biosystems, Foster, CA, USA) with an initial denaturation step at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing for 60 s at the specified temperature, and extension at 72 °C for 60 s. The PCR primer sequences and annealing temperatures are presented in Table 1.

Table 1
Primers used in this study.

	Primer sequence (5′–3′)	Annealing temperature (°C)
Runx2	F: CAGTTCCCAGGCATTTCATC	60
	R: CAGCGTCAACACCATCATTC	
Osterix (*Osx*)	F: CACTGGCTCCTGGTTCTCTC	60
	R: CCACTCCTCCTCTTCGTGAG	
Osteocalcin (*OCN*)	F: TCTCTGCTCACTCTGCTGGC	57
	R: TCCAGGGCAACACATGCCCTA	

2.4. Assay for alkaline phosphatase activity

Cell cultures were processed for ALP activity after 11 days of treatment with the reagents. Intracellular ALP activity was measured using the TRACP & ALP Assay Kit (MK301 colorimetric kit; Takara Bio Inc., Tokyo, Japan). Cells were washed 3 times with PBS, and then, lysed with 500 μL of lysis buffer containing physiological saline and 1% NP-40. Lysates were also prepared from cells grown in DMSO, standard and osteogenic medium as controls. The cells lysates were then mixed with an assay mixture containing *p*-nitrophenyl phosphate and incubated at 37 °C for 30 min, at which time the reaction was stopped by the addition of 0.4 M NaOH. After incubation, the amount of *p*-nitrophenol released by the reaction was measured with a spectrophotometer at 405 nm. All values were normalised against cell number. Protein in the cell lysates was determined using the micro Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA). Data were expressed as a ratio of ALP activity per milligram of protein.

2.5. Alizarin Red staining

Cells were washed twice with PBS and fixed in 10% formalin for 10 min. The cells were then stained with 1% Alizarin Red solution for 10 min at 37 °C and washed 3 times with PBS.

2.6. Statistical analysis

Data are expressed as the mean (SD) for each group. Statistical differences among treatment groups were evaluated by Student's *t*-test. A p value of <0.05 was considered significant.

3. Results and discussion

Since osteoblasts can be differentiated from pluripotent MSCs, a bone marrow stromal progenitor cells, in our study, we modelled this process of bone formation using primary MSCs. In fact, the osteogenic induction of MSCs by BMPs has been extensively studied. BMP-2 binds to a heterotetrameric complex of type I and type II transmembrane serine/threonine kinase receptor proteins. Upon activation by BMP-2, these receptors phosphorylate the intracellular signalling molecule receptor-regulated Smad (R-Smad). After activation, R-Smad associates with Smad4 (C-Smad), and the R-Smad/C-Smad complex accumulates in the nucleus where it interacts with the transcription factor *Runx2* and upregulates osteoblastic gene expression (Fig. 1). This differentiation process can be divided into 3 stages (proliferation, matrix development and, maturation and mineralisation), which are characterized by several markers. The markers most frequently used are ALP, *Runx2*, *Osx*, and *OCN*. In general, ALP, an early marker of the osteoblastic phenotype, is upregulated at the early stages and decreases as the cell differentiation progresses, while *OCN* is considered an advanced marker for maturation and a link to bone mineralisation. Therefore, since pre-osteoblasts are histologically similar to osteoblasts, but do not acquire all the characteristics of

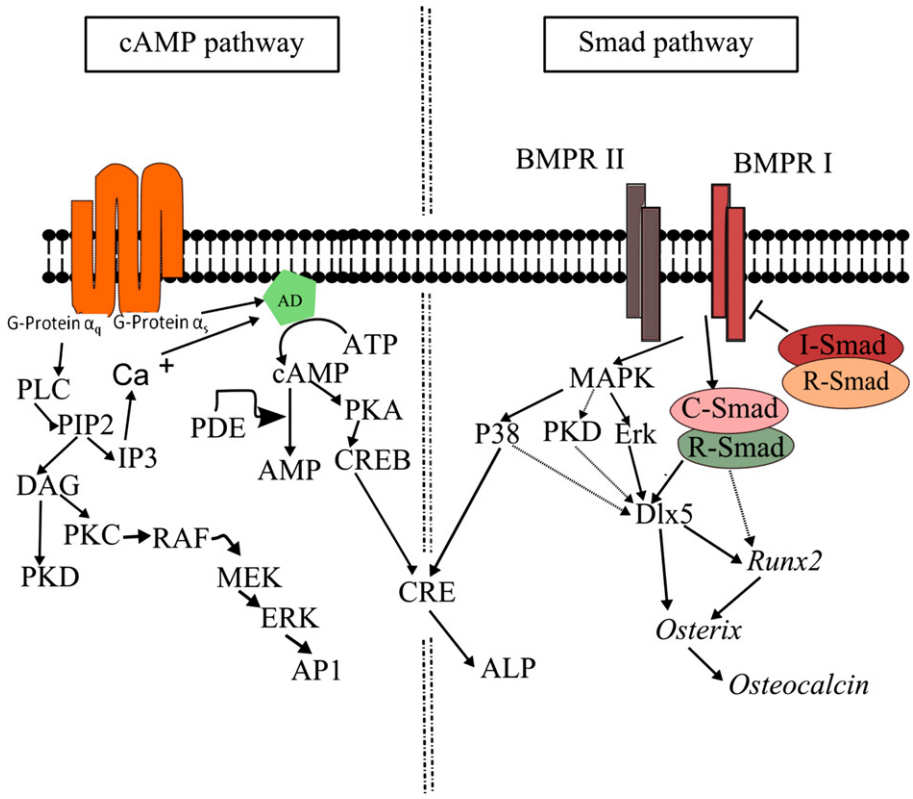

Fig. 1. Schematic representation of the BMP-2 and cAMP pathways. Several mechanisms are involved in the osteogenetic pathway, including (1) a signaling from BMP receptor I and BMP receptor II, which activates the R-Smad pathway (regulated by I-Smad binding proteins); (2) a signaling from BMPR I and BMPR II, which results in the activation of a MAPK pathway; and (3) an intracellular cAMP signalling regulated by the balance between adenylate cyclase (AD) and PDE.

a fully mature osteoblast, the mature osteoblast can be identified through the characterization of the ability to synthesise ALP, bone matrix proteins (including collagen type-I), and several non-collagenous proteins (including *OCN*).

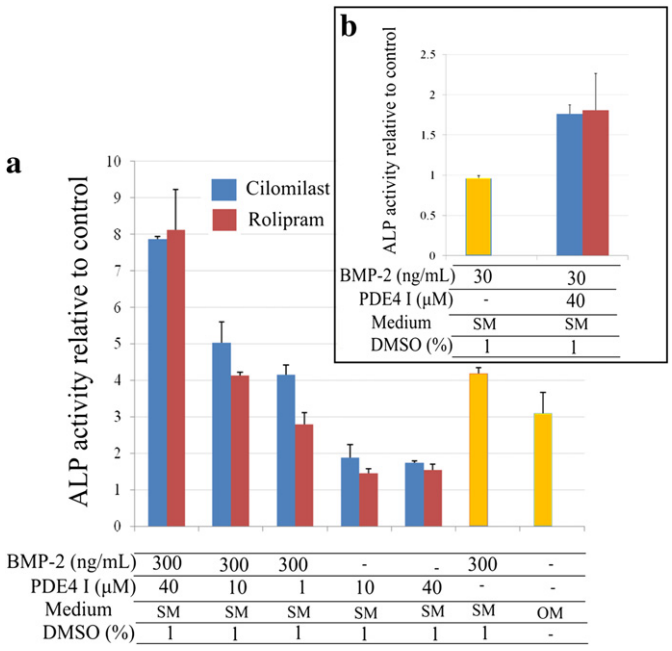

Fig. 2. ALP activity levels relative to control: (a) samples treated with 300 ng/mL of BMP-2; (b) samples treated with 30 ng/mL of BMP-2. Cells cultured with 1% DMSO for 11 days were used as control cells. Bar represent mean ± SD for 8 experiments. PDE4 I = PDE4 inhibitor, SM = standard medium, OM = osteogenic medium.

3.1. Alkaline phosphatase assay

Our results (Fig. 2a) demonstrate that inhibition of PDE enhances ALP expression in MSCs via the cAMP pathway. The increase in the level of ALP activity is dependent on the dose of PDE4 inhibitor. Cells treated with only PDE4 inhibitors show a slight increase in ALP levels, which is, probably, an effect of DMSO.

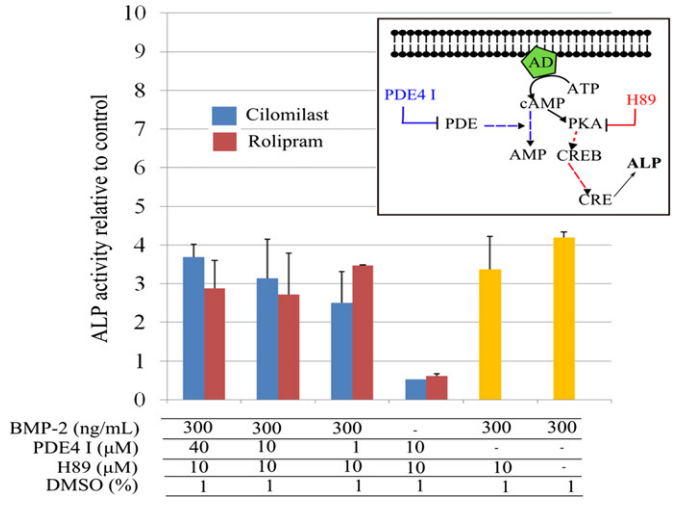

Fig. 3. ALP activity levels relative to samples treated with DMSO. Cells cultured with 1% DMSO for 11 days were used as control cells. The inset shows a schematic representation of the PDE4 inhibitor (PDE4 I) and H89 pathways. PDE4 I blocks the degradation of the intracellular cAMP signalling by PDE, and increases the PKA levels. By the use of an efficient PKA inhibitor, H89, the pathway activated downstream by PKA is completely blocked. Bar represent mean ± SD for 8 experiments.

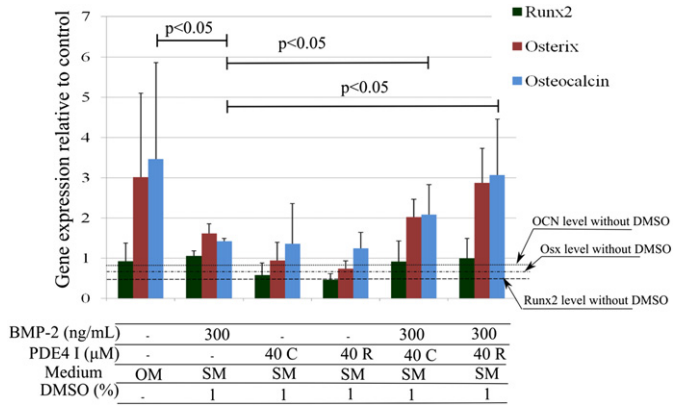

Fig. 4. *Runx2*, *Osterix* and Osteocalcin gene expression relative to samples treated with 1% DMSO. PDE4 I = PDE4 inhibitor, C = cilomilast, R = rolipram. Dotted lines represent Osteocalcin, *Osx* and *Runx2* expression levels when cultured with only standard medium without DMSO. Bar represent mean ± SD for 3 experiments

DMSO is a cell-differentiating agent [15]. Exposure to DMSO increases cAMP levels, and induces noticeable morphological changes and formation of mineralised bone-like nodules [16]. According to Cheung et al. [16], the osteoblastogenic effect of DMSO is mediated by *Runx2* and *Osx*, and depends upon the activation of protein kinase D (PKD) via protein kinase C (PKC) pathways. Furthermore, Stephens et a.l [17] reported that in primary human osteoblasts, DMSO enhances ALP expression and induces extracellular matrix mineralisation [17] in a dose-dependent manner. In our experiments, we confirmed this trend: we detected an increase in ALP activity and a decrease in the cell proliferation rate with increasing DMSO levels (data not shown). Therefore, to study the effect of the inducers on MSC differentiation at similar proliferation rates, we treated MCSs, except those cultured in osteogenic medium, with 1% DMSO. We compared MSCs cultured for 11 days in the presence of different inducers with MSCs cultured in osteogenic medium in order to quantify the osteogenetic effects of the inducers. We found that the ALP activity levels of MCSs treated with a combination of PDE4 inhibitor (40 μM) and BMP-2 (300 ng/mL) were almost double the ALP activity level of MSCs treated with osteogenic medium, suggesting that the mineralisation process is more rapid.

In Fig. 2b, the relative activity of ALP in cells treated with 30 ng/mL BMP-2 was 1.0; even in combination with PDE4 inhibitors, the results are comparable to those for PDE4 inhibitors alone. Thus, the effect of 30 ng/mL BMP-2 is negligible.

3.2. Effect of the PKA inhibitor H89

To verify the involvement of PKA in the increase of ALP activity, a PKA inhibitor, H89, at the concentration of 10 μM was used (Fig. 3, see inset) [13]. The PKA inhibition by H89 would annihilate the

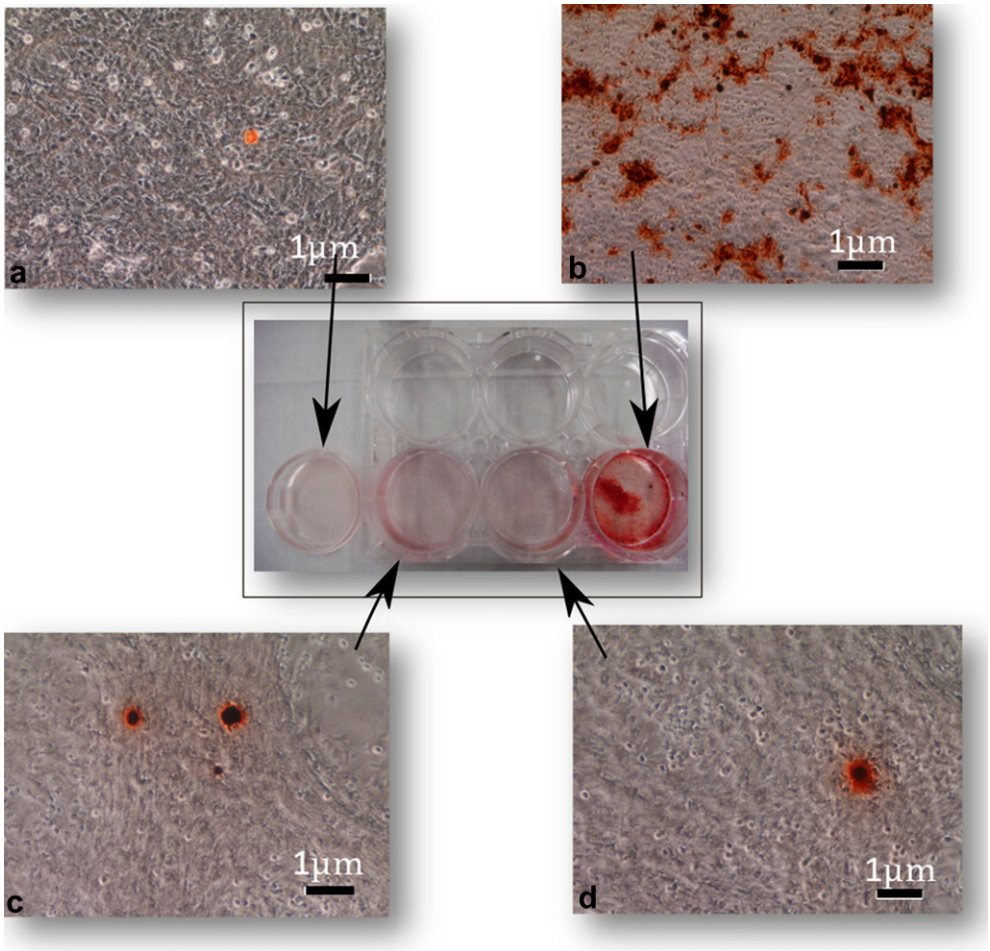

Fig. 5. Alizarin Red staining results: (a) MSCs treated with osteogenic medium for 11 days, (b) MSCs treated with osteogenic medium for 21 days, (c) MSCs treated with BMP-2 (300 ng/mL) and cilomilast (10 μM) for 21 days, and (d) MSCs treated with BMP-2 (300 ng/mL) and rolipram (10 μM) for 21 days.

cAMP increment, but no effect on the enhancement of ALP activity induced by BMP-2 was observed. This suggests that BMP-2 induced differentiation is cAMP independent. The enhancement of ALP in samples treated with BMP-2 and PDE4 inhibitor (Fig. 2) was completely cancelled by addition of 10 μM H89, irrespective of PDE4 inhibitor concentrations. The resulting ALP levels were comparable to the cells treated with only BMP-2. This observation confirms that PKA is the key factor for the ALP enhancement induced by the PDE4 inhibitor. Therefore, we can conclude that the specific PDE4 inhibitors enhance ALP levels mainly through the cAMP pathway.

3.3. Expression of Runx2, Osx and OCN

In the RT-PCR results (Fig. 4), a statistically significant difference in the expression of *Runx2* after 11 days of treatment was not found. This is in agreement with the observation that *Runx2* levels are maximal in the early stage of osteogenesis, and then, gradually decrease [18]. The level of *Osx* and *OCN* were higher in MSCs treated with the combination of PDE4 inhibitor and BMP-2, than in cells treated with PDE4 inhibitor or BMP-2 alone. In addition, gene expression obtained by the combination of BMP-2 and PDE4 inhibitor was comparable to the expression in MSCs treated with osteogenic medium. These results suggest that this combinated treatment is able to induce osteogenesis equally well as osteogenic medium. A significant difference between the two PDE4 inhibitors was not found.

In the study of Kinoshita et al. [9], the treatment of mice with rolipram for 5 weeks, induced an increase of bone mass. This was possible because, in BMP-responding cells, the use of PDE4 inhibitor amplifies the effect of natural BMPs and stimulates the differentiation. Considering our results (Fig. 4), it is possible to hypothesize that a specific PDE4 inhibitors might have on primary MSCs an osteogenetic effect in the presence of DMSO (Section 3.1 Refs. [19–22]). The PDE4 inhibitors might promote the differentiation, which do not results in the bone formation at 11 days of treatment. In fact, the primary MSCs treated with only PDE4 inhibitor (Fig. 4) did not show a statistically significant increase in the *Osx* and *OCN* expressions, although they exhibited higher ALP activity levels than the control (Fig. 2a). Thus, those cells are not fully mature and functional osteoblasts. Moreover, our data demonstrate that only the primary MSCs treated with or ostegenic medium, or with the combination of BMP-2 and PDE4 inhibitor, shown characteristics of fully differentiated osteoblasts.

3.4. Alizarin Red staining

Alizarin Red staining was used to detect calcium deposits. For osteogenesis studies, MSCs were cultured for 21 days with BMP-2 (300 ng/mL) and PDE4 inhibitor (10 μM) or with osteogenic medium for 11 and 21 days. Mineralisation in cells treated with only 10 μM of both PDE4 inhibitors (Fig. 5c and d) was superior to that in cells treated for 11 days with osteogenic medium (Fig. 5a), but inferior to that in cells treated for 21 days with osteogenic medium (Fig. 5b). This could be due to a combination of low concentration of PDE4 inhibitor and the presence of 1% DMSO. The cell proliferation is not significantly affected by 0.5% (or lower) DMSO, although it is significantly reduced by 1% DMSO [16]. Based on Fig. 2a, we might expect mineralisation to be similar in MSCs treated with osteogenic medium and in MSCs treated with the combination of BMP-2 and PDE4 inhibitor. In all likelihood, the decrease in cell proliferation influenced the differentiation process, slowing the reduced efficacy of the treatment. Therefore, it may be possible to induce bone formation, but DMSO could affect the efficacy of the treatment in the absence of a suitable quantity of PDE4 inhibitor (40 μM).

4. Conclusion

In this study, we investigated osteoblast differentiation in the combined presence of BMP-2 and PDE4 inhibitors. Our results support the notions that signal transduction via the cAMP pathway is necessary but not sufficient to induce MSC differentiation. In fact, PDE4 inhibitor alone did not induce a complete differentiation of MSCs, even though the PDE4 inhibitor could enhance the effect of BMP-2. Moreover, we demonstrated that the cAMP pathway is not involved in the differentiation of primary MSC induced by BMP-2 alone but is critical in the enhancement of bone formation in the presence of PDE4 inhibitors and BMP-2. We have shown that cilomilast, a second-generation PDE4 inhibitor, could be a useful new tool in orthopaedic stem cell therapies, because it is able to induce rapid mineralisation in the presence of BMP-2.

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