ARTICLE

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# Mesenchymal Stem Cells Repress Osteoblast Differentiation Under Osteogenic-Inducing Conditions

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## ABSTRACT

This study was designed to investigate the influence of mesenchymal stem cells (MSCs) on osteoblast (OB) differentiation. Rat bone marrow MSCs were cultured either in growth medium that maintained a MSC phenotype or in osteogenic medium that induced differentiation into OBs. Then, cells were grown in two different culture conditions: indirect co-culture of MSCs and OBs and OBs cultured in MSC-conditioned medium. As a control culture condition, OBs were grown in osteogenic medium without the influence of MSCs. We evaluated cell proliferation, the gene expression of key bone markers, alkaline phosphatase (ALP) activity, bone sialoprotein (BSP) expression, and extracellular matrix mineralization. The results showed that, regardless of whether OBs were indirectly co-cultured with MSCs or cultured in MSC-conditioned medium, MSCs repressed OB differentiation, as evidenced by the downregulation of all evaluated bone marker genes, decreased ALP activity, inhibition of BSP protein expression, and reduced extracellular matrix mineralization. Taken together, these results indicate that despite the key role of both MSCs and OBs in the osteogenic process, the repressive effect of MSCs on OB differentiation in an osteogenic environment may represent a barrier to the strategy of using them together in cell-based therapies to induce bone repair. J. Cell. Biochem. 116: 2896–2902, 2015. © 2015 Wiley Periodicals, Inc.

**KEY WORDS:** BONE MARROW; CELL CULTURE; DIFFERENTIATION; MESENCHYMAL STEM CELL; OSTEOBLAST

Cell therapy and tissue engineering are treatments based on using cells that have been widely investigated in dentistry and medicine with the aim of repairing bone defects. Tissue engineering has been defined as an interdisciplinary field that applies the principles of engineering and biological sciences to develop substitutes to repair, maintain, or improve tissue function using scaffolds, growth factors, and cells [Langer and Vacanti, 1993; Rosa et al., 2008]. The American Society of Gene and Cell Therapy named cell therapy "the transplant of cells to treat a genetic or acquired disease."

Mesenchymal stem cells (MSCs) have been considered a promising therapeutic alternative for many diseases because they have the capacity to self-renew and to differentiate into cells of distinct tissues, such as cartilage, fat, muscle, and bone [Prockop, 1997; Pino et al., 2012; Fernández Vallone et al., 2013]. Osteoblasts (OBs) are derived from MSCs, and both cells exist in intimate contact and interact during bone formation and repair [Liao et al., 2011]. Recently, we have suggested that the combination of MSCs and OBs could be an attractive strategy to drive bone repair [Beloti et al., 2012].

Cell interactions can occur directly through cell–cell contact and indirectly through the diffusion of factors secreted by one cell that act on the specific receptors of another cell [Grellier et al., 2009]. To assess these interactions, co-culture models in which two or more distinct cell populations are grown in the same environment have been used [Kanazawa and Hosick, 1992]. This approach allows for a partial mimicking of the in vivo environment during in vitro evaluations, keeping co-cultured cells in direct contact with secreted cytokines and autocrine and paracrine factors [Malekshah et al., 2006; Bigdeli et al., 2009].

Several studies have focused on OB differentiation of MSCs both in vivo and in vitro and on the effects of OBs on MSCs [Gerstenfeld et al., 2002; Heino et al., 2004; Csaki et al., 2009; Ilmer et al., 2009; Birmingham et al., 2012; Stockmann et al., 2012; Tsai et al., 2012;

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Sun et al., 2013; Yu et al., 2014]. On the other hand, until now, the influence of MSCs on OBs, which might impact bone repair, has been underexplored and not fully understood [Kim et al., 2003; Sun et al., 2012]. Little synergism was observed between rabbit MSCs and rat OBs in the promotion of in vitro osteogenesis under direct co-culture conditions [Kim et al., 2003]. Additionally, it has been shown that conditioned medium from rat MSCs retards rat OB differentiation [Sun et al., 2012]. In addition to conditioned medium and direct co-culture, indirect co-culture is a valuable tool to study cell–cell interactions. In this context, our work was designed to investigate the effects of MSCs on OB differentiation in an osteogenic environment using two different approaches: indirect co-culture of MSCs and OBs and culture of OBs in MSC-conditioned medium.

## MATERIALS AND METHODS

### MSCs AND OBs

All animal procedures were performed under the approval of the Committee of Ethics in Animal Research of the University of São Paulo. Bone marrow MSCs were obtained from the femurs of male Wistar rats weighting 140–150 g, as previously described [Maniatopoulos et al., 1988]. The cells were cultured in growth medium consisting of α-MEM (Invitrogen-Gibco, Grand Island, NY) supplemented with 15% fetal calf serum (Gibco), 50 μg/ml gentamycin (Gibco), 50 μg/ml vancomycin (Acros Organics, Geel, Belgium) and 0.3 μg/ml fungizone (Gibco) until reaching subconfluence. Some MSCs were cultured in growth medium to maintain MSC characteristics, while others were cultured in osteogenic medium containing growth medium plus 5 μg/ml ascorbic acid (Gibco), 7 mM β-glycerophosphate (Sigma–Aldrich, St. Louis, MO) and $10^{-7}$ M dexamethasone (Sigma) to stimulate differentiation into OBs. After 4 days, MSCs and OBs were used in the experiments described below. During the culture period, cells were incubated at 37°C in a humidified atmosphere of 5% $CO_2$, and the medium was changed every 48 h.

### INDIRECT CO-CULTURE

The indirect co-culture was established using transwell inserts with 0.4-μm filters (Corning, Corning, NY). MSCs and OBs were co-cultured (MSCs→OBs) at a ratio of 1:2 with the insert containing MSCs and the OBs cultured on the bottom of well in osteogenic medium. OBs cultured without the influence of MSCs in osteogenic medium were used as a control.

### CONDITIONED MEDIUM

The conditioned medium was the growth medium collected from MSC cultures during medium changes, excluding the medium from the first change. Then, the medium was centrifuged at 2000 rpm for 5 min and stored at −20°C. The conditioned medium was mixed with osteogenic medium at a ratio of 1:1 and used to culture OBs. A mixture of osteogenic medium and fresh growth medium at ratio of 1:1 (non-conditioned medium) was used to culture OBs as a control.

### CELL PROLIFERATION AND DIFFERENTIATION PARAMETERS

**Cell proliferation.** Cell proliferation was evaluated at days 3, 7, and 10 with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma–Aldrich). Cells were incubated with 2 ml of MTT (5 mg/ml) in phosphate-buffered saline at 37°C. After 4 h, the solution was aspirated and 1 ml of acid isopropanol (0.04 N HCl in isopropanol) was added. After shaking for 5 min, 150 μl of this solution was collected, and the optical density was read at 570 nm (μQuant, Bio-Tek, Winooski, VT). The data were obtained in quintuplicate ($n = 5$) and expressed as absorbance, which is directly proportional to the number of proliferating and living cells.

**Alkaline phosphatase (ALP) activity.** At day 10, cells were lysed in 0.1% sodium lauryl sulfate (Sigma–Aldrich) for 30 min, and the release of thymolphthalein from thymolphthalein monophosphate was determined to measure the ALP activity using a commercial kit (Labtest Diagnostica SA, Lagoa Santa MG, Brazil). Briefly, 50 μl of thymolphthalein monophosphate was mixed with 0.5 ml of 0.3 M diethanolamine buffer, pH 10.1, and kept for 2 min at 37°C before the addition of 50 μl of cell lysate. After 10 min at 37°C, 2 ml of $Na_2CO_3$ (0.09 mmol/ml) and NaOH (0.25 mmol/ml) solution was added to stop the reaction, and the optical density was measured at 590 nm (μQuant, Bio-Tek). The data were obtained in quintuplicate ($n = 5$) and expressed as ALP activity normalized by total protein content, which was determined by the Lowry method [Lowry et al., 1951].

**Extracellular matrix mineralization.** Extracellular matrix mineralization was detected at day 17 by alizarin red staining (Sigma–Aldrich). Cells were fixed in 10% formalin for 2 h at room temperature, dehydrated through a graded series of alcohol, and stained with 2% alizarin red pH 4.2 (Sigma–Aldrich) for 10 min. For qualitative analysis, culture images were captured with a high-resolution digital camera (Canon EOS Digital Rebel Camera, Canon, Lake Success, NY). Then, calcium content was evaluated using a colorimetric method. Briefly, 280 μl of 10% acetic acid was added to each well stained with alizarin red, and the plate was incubated at room temperature for 30 min with shaking. This solution was transferred to a microcentrifuge tube and vortexed for 1 min. The slurry was overlaid with 100 μl of mineral oil (Sigma–Aldrich), heated to 85°C for 10 min and transferred to ice for 5 min. The slurry was then centrifuged at 20,000*g* for 15 min, and 100 μl of supernatant was transferred to a new microcentrifuge tube. Then, 40 μl of 10% ammonium hydroxide was added to neutralize the acid and the optical density was read at 405 nm (μQuant, Bio-Tek). The data were obtained in quintuplicate ($n = 5$) and expressed as absorbance.

**Gene expression of key OB markers.** Quantitative real-time polymerase chain reaction (PCR) was performed on day 10 to evaluate the gene expression of runt-related transcription factor 2 (RUNX2), osterix (OSX), ALP, bone sialoprotein (BSP), and osteocalcin (OC). Total RNA was extracted with Trizol reagent (Life-Technologies), and the concentration was determined by reading the optical density at the following different wavelengths: 260, 280, 230, and 320 nm (GE Healthcare, Milwaukee, WI). Complementary DNA (cDNA) was synthesized using 1 μg of RNA through a reverse transcription reaction (Life Technologies-Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. Real-time PCR was performed in a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Philadelphia, PA) using TaqMan (Applied Biosystems) probes for the target genes. The standard PCR conditions were 50°C (2 min), 95°C (10 min), 40 cycles

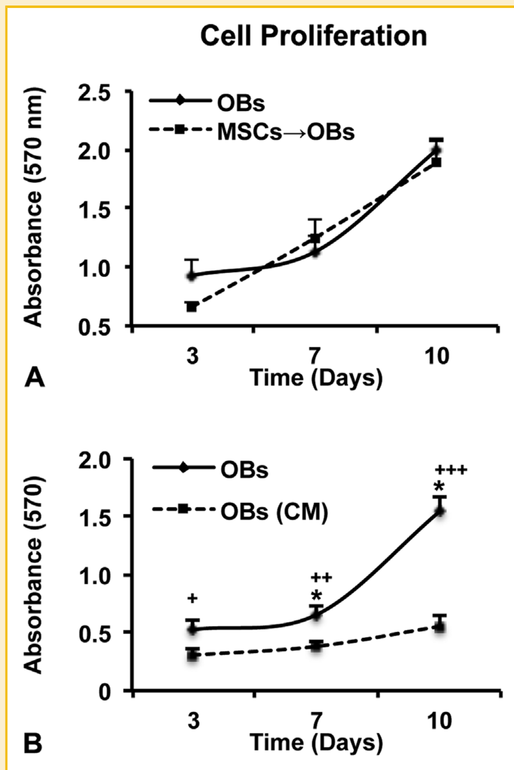


Fig. 1. Cell proliferation at days 3, 7, and 10 under two culture conditions: co-culture (A) and culture with conditioned medium (B). OBs, osteoblasts; MSCs→OBs, co-culture of osteoblasts with mesenchymal stem cells on the insert; OBs (CM), osteoblasts cultured in MSC-conditioned medium. The data are presented as the mean ± standard deviation (n = 5), the asterisks indicate significant differences between groups at the same time-point and the crosses indicate significant differences among time-points for OBs ($P \leq 0.05$).

of 95°C (15 s) and 60°C (1 min). The relative gene expression was calculated in reference to both β-actin and glyceraldehyde-3-phosphate dehydrogenase (GADPH) expression and its respective control using the cycle threshold (Ct) method [Livak and Schmittgen, 2001]. This assay was performed in quadruplicate (n = 4).

**Immunolocalization of BSP.** At day 10, cells were fixed for 10 min at room temperature using 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.2, permeabilized with 0.5% Triton X-100 (Sigma–Aldrich) in PB for 10 min and blocked with 5% nonfat powdered milk (Bio-Rad Laboratories) in PB for 30 min. Then, the cells were processed for immunofluorescence labeling as previously described [de Oliveira et al., 2007]. Cells were incubated with an anti-BSP monoclonal primary antibody (1:200, WVID1-9C5; Developmental Studies Hybridoma Bank, Iowa City, IA), followed by incubation with a mixture of an Alexa Fluor 594 (red fluorescence)-conjugated goat-anti-mouse secondary antibody (1:200; Molecular Probes-Life Technologies, Eugene, OR) and Alexa Fluor 488 (green fluorescence)-conjugated phalloidin (1:200; Molecular Probes-Life Technologies) to label the actin cytoskeleton for 1 h at room temperature. Cell nuclei were stained with 300 nM 40,6-diamidino-2-phenylindole, dihydrochloride (DAPI; Molecular Probes-Life Technologies) for 5 min. A glass coverslip was mounted with an antifade kit (Vectashield, Vector Laboratories, Burlingame, CA) on the thermanox (Nalge Nunc Intl., Penfield, NY) surface containing the cells. The samples were then examined using an Axio Imager M2 fluorescence microscope (Carl Zeiss, Göttingen, GO, Germany) outfitted with an Axiocam MRm digital camera (Carl Zeiss) under epifluorescence, and the acquired images were processed using Adobe Photoshop software (Adobe Systems, San Jose, CA).

### STATISTICAL ANALYSES

Statistical analyses were performed using SigmaPlot 12.5 software (Systat Software, Witzenhausen, HE, Germany). The data of cell proliferation were compared with a Kruskal–Wallis test and if significant differences were detected, the Mann–Whitney test was used. The data of ALP activity, extracellular matrix mineralization, and gene expression were compared with a Mann–Whitney test. For all experiments the level of significance was established at $P \leq 0.05$.

## RESULTS

### CELL PROLIFERATION

Cell proliferation was not affected by co-culture ($P = 0.66$; Fig. 1A). The MSC-conditioned medium reduced OB proliferation compared with non-conditioned medium at all evaluated time points

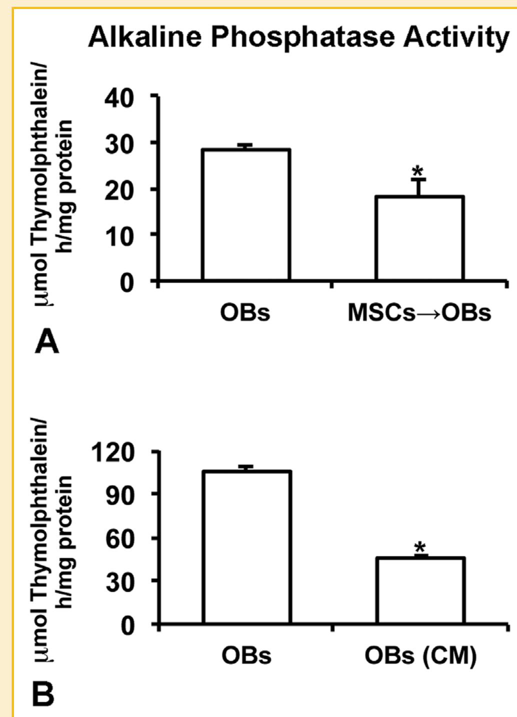


Fig. 2. Alkaline phosphatase (ALP) activity at day 10 under two culture conditions: co-culture (A) and culture with conditioned medium (B). OBs, osteoblasts; MSCs→OBs, co-culture of osteoblasts with mesenchymal stem cells on the insert; OBs (CM), osteoblasts cultured in MSC-conditioned medium. The data are presented as the mean ± standard deviation (n = 5), and the asterisks indicate significant differences ($P \leq 0.05$).

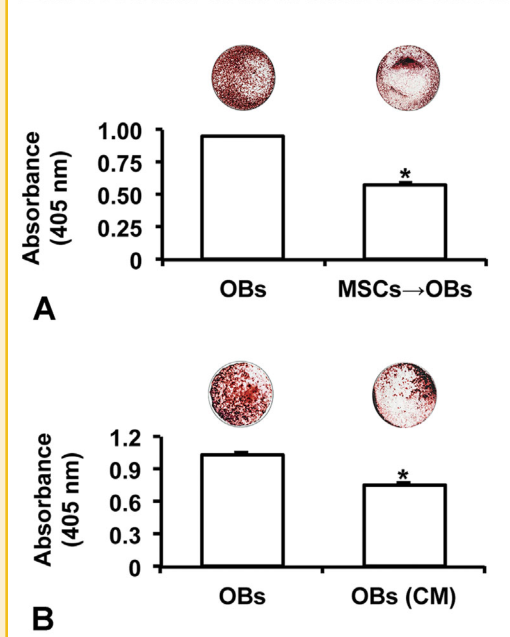


Fig. 3. Extracellular matrix mineralization at day 17 under two culture conditions: co-culture (A) and culture with conditioned medium (B). OBs, osteoblasts; MSCs→OBs, co-culture of osteoblasts with mesenchymal stem cells on the insert; OBs (CM), osteoblasts cultured in MSC-conditioned medium. The data are presented as the mean ± standard deviation (n = 5), and the asterisks indicate significant differences ($P \leq 0.05$).

($P < 0.001$ for all time points; Fig. 1B). Moreover, the cell number increased ($P < 0.001$) over time regardless of the culture conditions (Fig. 1A and B).

## ALP ACTIVITY

MSCs reduced the ALP activity of OBs under co-culture compared with OBs grown under the non-co-culture condition ($P = 0.03$; Fig. 2A). In agreement with this, the MSC-conditioned medium also reduced ALP activity in OBs compared with OBs grown in non-conditioned medium ($P = 0.008$; Fig. 2B).

## EXTRACELLULAR MATRIX MINERALIZATION

Mineralized extracellular matrix was detected under both culture conditions, with OBs producing the most dense and regularly distributed matrix (Fig. 3A and B). MSCs reduced the extracellular matrix mineralization of OBs under co-culture compared with OBs grown under non-co-culture conditions ($P = 0.03$; Fig. 3A). The MSC-conditioned medium also significantly reduced the extracellular matrix mineralization in OBs compared with OBs grown in non-conditioned medium ($P = 0.008$; Fig. 3B).

## GENE EXPRESSION OF KEY OB MARKERS

OBs co-cultured with MSCs or in MSC-conditioned medium displayed lower gene expression ($P = 0.03$ for all) of RUNX2 (Fig. 4A and F), OSX (Fig. 4B and G), ALP (Fig. 4C and H), BSP (Fig. 4D and I) and OC (Fig. 4E and J).

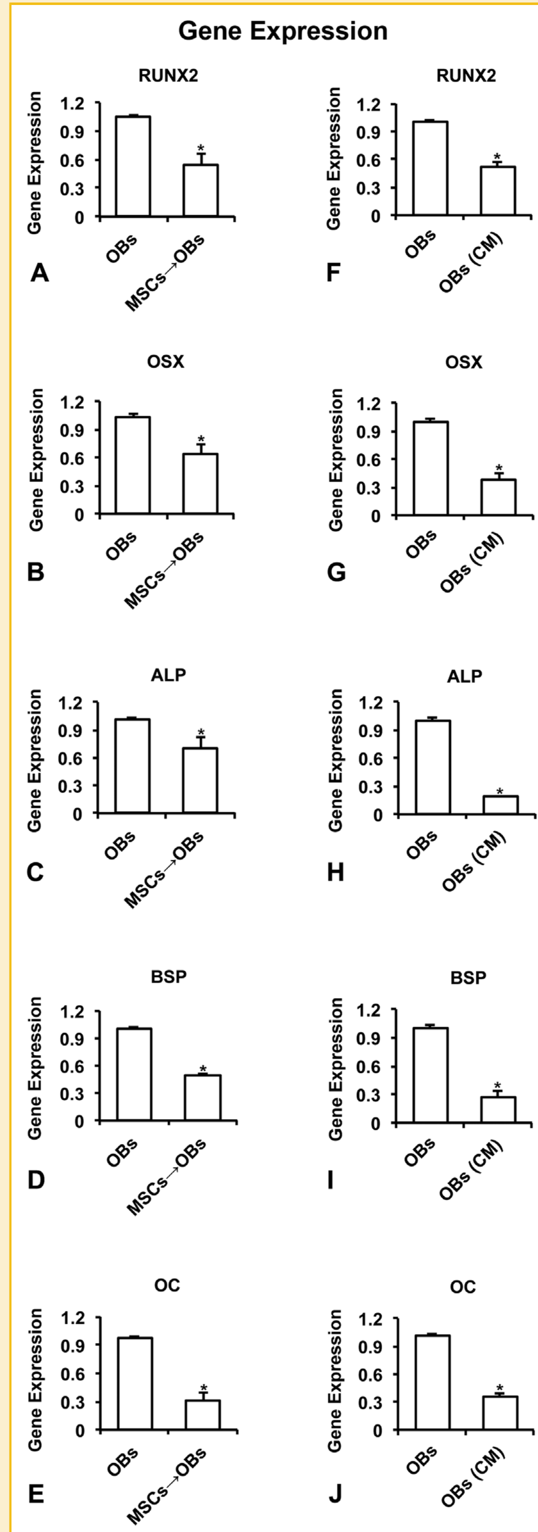


Fig. 4. Osteoblast marker gene expression at day 10 under two culture conditions: co-culture (A–E) and culture with conditioned medium (F–J). Gene expression of runt related transcription factor 2 (A, F), osterix (B, G), alkaline phosphatase (C, H), bone sialoprotein (D, I), and osteocalcin (E, J). OBs, osteoblasts; MSCs→OBs, co-culture of osteoblasts with mesenchymal stem cells on the insert; OBs (CM), osteoblasts cultured in MSC-conditioned medium. The data are presented as the mean ± standard deviation (n = 4), and the asterisks indicate significant differences ($P \leq 0.05$).

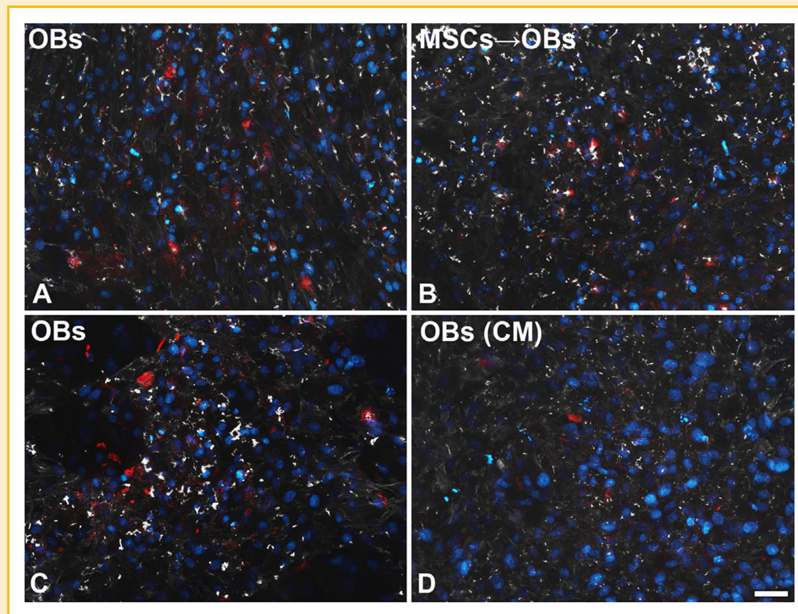


**Fig. 5.** Immunolocalization of bone sialoprotein at day 10 under two culture conditions: co-culture (A, B) and culture with conditioned medium (C, D). OBs, osteoblasts; MSCs→OBs, co-culture of osteoblasts with mesenchymal stem cells on the insert; OBs (CM), osteoblasts cultured in MSC-conditioned medium. Red fluorescence indicates immunolocalization of bone sialoprotein, actin cytoskeleton (marked with gray) and cell nuclei (marked with blue). Scale bar, 100 μm.

### IMMUNOLOCALIZATION OF BSP

BSP was predominantly expressed in the perinuclear region of the cytoplasm (Fig. 5A–D) and was more evident in OBs (Fig. 5A–C). OBs co-cultured with MSCs (Fig. 5B) and OBs grown with MSC-conditioned medium (Fig. 5D) exhibited reduced BSP expression.

## DISCUSSION

The present study was designed to evaluate the influence of MSCs on OBs in an osteogenic environment using different culture conditions. To reproduce the paracrine effects, two approaches were used: (1) indirect co-culture of MSCs and OBs, where both cells were cultured under the same conditions but separated by a filter preventing cell migration; and (2) OBs cultured in MSC-conditioned medium. Our results showed that, irrespective of culture conditions, MSCs repressed OB differentiation.

It is well known that several differentiated cells, such as adipocytes, chondrocytes, osteocytes, and OBs, affect MSC behavior [Gerstenfeld et al., 2002; Heino et al., 2004; Birmingham et al., 2012; Sadie-Van Gijsen et al., 2013]. In particular, OB differentiation of MSCs under the influence of distinct cells, such as OBs, osteocytes, and hematopoietic cells, has been investigated [Heino et al., 2004; Ilmer et al., 2009; Liao et al., 2011; Birmingham et al., 2012]. Conversely, few studies have evaluated the effect of MSCs on OBs using either direct co-culture or conditioned medium, and in both situations, it was observed that MSCs did not favor OB differentiation [Kim et al., 2003; Sun et al., 2012]. Corroborating these findings, in addition to direct co-culture and conditioned medium, we also observed the inhibitory effect of MSCs on OB differentiation under indirect co-culture conditions. As cells were grown in osteogenic medium during co-culture, such effect was observed while MSCs were in their own process of OB differentiation, suggesting that OBs at early stages of differentiation may also repress more differentiated OBs.

In the present study, only MSC-conditioned medium inhibited OB proliferation, whereas there was no effect of MSCs co-cultured with OBs on OB proliferation. Similarly, a more pronounced reduction in the proliferation of OBs induced by MSCs was observed when conditioned medium was used at a ratio of 1:1 [Sun et al., 2012]. Furthermore, other studies have also reported an inhibitory effect of MSCs on the proliferation of immune system-, liver-, and prostate cancer-derived cells [Spaggiari et al., 2009; Qin et al., 2012; Cuerquis et al., 2014; Takahara et al., 2014]. The suppression of OB differentiation by MSCs is evidenced by the reduction of ALP activity and gene expression, an early marker of the OB phenotype, as well as by the reduction of BSP gene and protein expressions, OC gene expression, and mineralized matrix formation, which is the final stage of osteogenesis. Suppression of OB differentiation was likely due to the inhibition of the transcription factors RUNX2 and OSX, both of which are essential for the acquisition of an OB phenotype and subsequent bone formation [Komori et al., 1997; Ducy et al., 1999; Nakashima et al., 2002; Tai et al., 2004]. Several mechanisms may be involved in this inhibitory effect including some factors secreted by MSCs such as tumor necrosis factor-α, which negatively affect OB phenotype expression [Li et al., 2010a,b].

The inhibitory effect of MSCs on OBs derived from MSCs observed here persisted for the entire culture period, resulting in a reduced extracellular matrix mineralization in both evaluated conditions.

Additionally, MSC-conditioned medium reduced ALP activity and extracellular matrix mineralization of OBs derived from rat calvariae (Fig. S1). In contrast with our results, it was reported that MSC-conditioned medium only transiently retard OB differentiation of cells derived from rat calvariae as the expression of some bone markers was recovery from day 3 to day 7 [Sun et al., 2012]. However, while transiency could be attributed to physiological fluctuations of the gene and protein expression of osteoblasts during culture time-course [Lian et al., 2003], we observed inhibition of the mineralized matrix formation, the final event of in vitro OB differentiation, suggesting that the negative effect of MSCs on OB phenotype expression is permanent and independent of the OB differentiation stage.

In conclusion, our results indicate that despite the fact that both MSCs and OBs are essential for the promotion of osteogenesis, the inhibitory effect of MSCs on OB differentiation in an osteogenic environment observed in two distinct culture conditions may represent a barrier to the strategy of using them together in cell-based therapies that aim to induce bone repair.


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## SUPPORTING INFORMATION

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