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Participation of TNF-α in Inhibitory Effects of Adipocytes on Osteoblast Differentiation

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Mesenchymal stem cells from bone marrow (BM-MSCs) and adipose tissue (AT-MSCs) are attractive tools for cell-based therapies to repair bone tissue. In this study, we investigated the osteogenic and adipogenic potential of BM-MSCs and AT-MSCs as well as the effect of crosstalk between osteoblasts and adipocytes on cell phenotype expression. Rat BM-MSCs and AT-MSCs were cultured either in growth, osteogenic, or adipogenic medium to evaluate osteoblast and adipocyte differentiation. Additionally, osteoblasts and adipocytes were indirectly co-cultured to investigate the effect of adipocytes on osteoblast differentiation and vice versa. BM-MSCs and AT-MSCs exhibit osteogenic and adipogenic potential under non-differentiation-inducing conditions. When exposed to osteogenic medium, BM-MSCs exhibited higher expression of bone markers compared with AT-MSCs. Conversely, under adipogenic conditions, AT-MSCs displayed higher expression of adipose tissue markers compared with BM-MSCs. The presence of adipocytes as indirect co-culture repressed the expression of the osteoblast phenotype, whereas osteoblasts did not exert remarkable effect on adipocytes. The inhibitory effect of adipocytes on osteoblasts was due to the release of tumor necrosis factor alpha (TNF-α) in culture medium by adipocytes. Indeed, the addition of exogenous TNF-α in culture medium repressed the differentiation of BM-MSCs into osteoblasts mimicking the indirect co-culture effect. In conclusion, our study showed that BM-MSCs are more osteogenic while AT-MSCs are more adipogenic. Additionally, we demonstrated the key role of TNF-α secreted by adipocytes on the inhibition of osteoblast differentiation. Thus, we postulate that the higher osteogenic potential of BM-MSCs makes them the first choice for inducing bone repair in cell-based therapies.

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Adult stem cells represent great promise for the repair and regeneration of tissues and organs through cell therapy and tissue engineering approaches. Initially, mesenchymal stem cells (MSCs) were defined as non-hematopoietic and plastic adherent progenitors isolated from the bone marrow, which were capable of self-renewing and differentiating into osteogenic, chondrogenic and adipogenic lineages both in vitro and in vivo (Friedenstein et al., 1968; Prockop, 1997). However, multiple studies have shown that in addition to bone marrow, MSCs can be obtained from several tissues, such as skeletal muscle, epithelium, and fat (Vasioukhin et al., 1999; Bosch et al., 2000; Hicok et al., 2004).

To use MSCs therapeutically, the potential for differentiation of MSCs into cells of a specific tissue is of major importance (Zhang et al., 2012). Regarding bone tissue repair and considering the interrelationship between bone and fat, the osteoblast and adipocyte differentiation of MSCs derived from bone marrow (BM-MSCs) and adipose tissue (AT-MSCs) has been extensively investigated and compared (Beresford et al., 1992; Kern et al., 2006; Yoshimura et al., 2007; Peng et al., 2008). It has been shown that BM-MSCs and AT-MSCs are able to differentiate into osteoblasts and adipocytes; however, results from different studies are inconsistent, as osteogenic and adipogenic potentials are not directly correlated with the cell source; for example, BM-MSCs may present higher, lower or the same osteogenic potential compared with AT-MSCs (Hattori et al., 2004; Sakaguchi et al., 2005; Rebelatto et al., 2008). Additionally, it has been suggested that MSCs exhibit some commitment with their tissue of origin as periosteum-derived MSCs are more osteogenic compared with AT-MSCs, which are more adipogenic (Sakaguchi et al., 2005; Pachón-Peña et al., 2011).

In addition to differentiation ability, the crosstalk between osteoblasts and adipocytes is of relevance to determine the osteogenic and/or adipogenic potential of a cell population. Several adipocyte-derived factors, such as estrogens and adipokines, have effects on osteoblast differentiation. It has been shown that estradiol induces osteogenic/adipogenic bipotential in a pre-osteoblastic cell population whereas adiponectin inhibits osteoblast differentiation (Okazaki et al., 2002; Shinoda et al., 2006). Regarding factors secreted by osteoblasts, it was observed that osteocalcin increases glucose transport and adiponectin release in adipocyte cell cultures (Hill et al., 2014).

Because differentiation of MSCs into either osteoblasts or adipocytes is governed by a complex interplay among several distinct intracellular signaling pathways and phenotype-specific transcription factors, for example, runt-related transcription factor (RUNX2) in osteoblasts and peroxisome proliferator-activated receptor gamma (PPARγ) in adipocytes, the aim of this study was to investigate the osteogenic and adipogenic potentials of BM-MSCs and AT-MSCs as well as the effect of crosstalk between osteoblasts and adipocytes on cell phenotype expression.

Conflict of interest: None.

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Materials and Methods

Isolation and Culture of MSCs

MSCs were harvested from eight male Wistar rats weighing 250–300 g, and all experiments were carried out following the protocols approved by the Committee of Ethics in Research from the University of São Paulo.

BM-MSCs were obtained by flushing the femur medullary canals and were cultured in growth medium; that is, minimum essential medium alpha (α-MEM—Gibco-Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco-Invitrogen), 50 μg/ml gentamycin (Gibco-Invitrogen), and 0.3 μg/ml fungizone (Gibco-Invitrogen). AT-MSCs were obtained from inguinal adipose tissue, which was digested using 0.075% collagenase type II (Gibco-Invitrogen) at 37°C for 40 min. Cells were centrifuged, the floating adipocytes were removed, and the pellet resuspended in growth medium, that is, Dulbecco's modified eagle medium (D-MEM—Gibco) supplemented with 10% fetal calf serum (Gibco), 50 μg/ml gentamycin (Gibco), and 0.3 μg/ml fungisone (Gibco). MSCs were selected by adherence to polystyrene and expanded in the same medium until subconfluence.

After reaching subconfluence, BM-MSCs and AT-MSCs were cultured in 24-well culture plates in three different conditions: (1) growth medium (non-differentiation-inducing medium), plating 1×10^3 cells/well; (2) osteogenic medium, which was growth medium supplemented with 5 μg/ml ascorbic acid (Gibco-Invitrogen), 7 mM β-glycerophosphate (Sigma–Aldrich, Saint Louis, MO) and 10^{-7} M dexamethasone (Sigma–Aldrich), plating 2×10^4 cells/well; and (3) adipogenic medium, which was growth medium supplemented with 10^{-6} M dexamethasone (Sigma–Aldrich), 0.5 μM 3–isobutil-1-metilxantine (Sigma–Aldrich), 10 μg/ml of insulin (Sigma–Aldrich), and 0.1 M indomethacin (Sigma–Aldrich), plating 2×10^4 cells/well. Only for the colony forming unit (CFU) assay, cells were cultured in 6-well culture plates at a cell density of 200 cells/well. During the entire culture time, cells were kept at 37°C in a humidified atmosphere of 5% CO_2 and 95% air and the medium was changed 3 times a week.

CFU

At day 7, cells grown on the three different media were fixed in 10% formalin for 2 h and washed and stained with 0.5% crystal violet (Sigma–Aldrich) in methanol for 10 min at room temperature. Then, the number of CFUs containing more than 30 cells was counted under light microscopy at 10× magnification and expressed as CFU/well.

Cell Proliferation

At days 4, 10, and 17, cells grown on the three different media were enzymatically detached from polystyrene using 1 mM EDTA, 1.3 mg/ml collagenase and 0.25% trypsin solution (Gibco-Life Technologies). The total number of cells was counted after Trypan blue (Sigma–Aldrich) staining using an automated cell counter (Invitrogen-Life Technologies) and expressed as the number of cells $\times 10^4$/well.

Gene Expression of Key Osteoblast and Adipocyte Markers

Quantitative real-time PCR was carried out at days 4, 10, and 17 to evaluate the gene expression of three osteoblast markers [RUNX2, alkaline phosphatase (ALP) and osteocalcin] and three adipocyte markers [PPARγ, adipocyte protein 2 (AP2) and resistin] in both BM-MSCs and AT-MSCs cultured in growth, osteogenic and adipogenic media. The total RNA was extracted with Trizol reagent (Invitrogen-Life Technologies) according to the manufacturer's instructions. The concentration and purity of RNA samples was determined by optical density at a wavelength of 260 nm and 260:280 nm, respectively, and only samples presenting 260:280 ratios higher than 1.8 were analyzed. Complementary DNA (cDNA) was synthesized using 1 μg of RNA through a reverse transcription reaction (M–MLV reverse transcriptase, Promega Corporation, Madison, WI). Real-time PCR was carried out in a Step One Plus Real-Time PCR (Life-Technologies) using Taqman PCR Master Mix (Applied Biosystems, Foster City, CA). The relative gene expressions were normalized to β-actin expression, and the real changes were expressed relative to BM-MSCs using the comparative threshold method ($2^{-\Delta\Delta Ct}$).

RUNX2 and PPARγ Protein Detection

At day 10, Western blotting was carried out to detect RUNX2 and PPARγ protein expression in both BM-MSCs and AT-MSCs grown in osteogenic and adipogenic medium, respectively. Cells were lysed in 500 μl of lysis buffer containing 1X protease inhibitor mixture (Roche Applied Science, Indianapolis, IN) and 25 μM MG132 proteasome inhibitor (Roche Applied Science) and then boiled for 5 min. Equal amounts of total protein (20 μg) for each sample were subjected to electrophoresis in a denaturing 8.5% polyacrylamide gel and transferred to a Hybond C-Extra membrane (GE Healthcare Life Science, Piscataway, NJ) using a semidry transfer apparatus (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked for 1 h in Tris-buffered saline, 0.1% Tween 20 (TBS-T, Sigma–Aldrich) containing 5% non-fat powdered milk (Bio-Rad Laboratories). RUNX2 and PPARγ protein were detected by incubating the membrane with rabbit polyclonal antibody anti-RUNX2 (1:2,000) and anti-PPARγ (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) followed by goat anti-rabbit IgG HRP secondary antibody (1:2,000, Santa Cruz Biotechnology). Both primary and secondary antibodies were diluted in TBS-T containing 2.5% non-fat powdered milk (Bio-Rad Laboratories). Mouse monoclonal anti-GAPDH (1:8000, Sigma–Aldrich) was used as a control followed by goat anti-mouse IgG HRP secondary antibody (1:2,000, Santa Cruz Biotechnology). Secondary antibodies were detected using western lightning chemiluminescence reagent (Perkin Elmer Life Sciences, Waltham, MA), and the images were acquired using G-Box gel imaging (Syngene, Cambridge, UK). The RUNX2 and PPARγ expressions were quantified by counting pixels and normalized by GAPDH.

ALP Activity

At days 4, 10, and 17, both BM-MSCs and AT-MSCs grown in osteogenic medium were lysed in 0.1% sodium lauryl sulphate (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, MG, Brazil). Briefly, 50 μl of thymolphthalein monophosphate was mixed with 0.5 ml of 0.3 M diethanolamine buffer, pH 10.1, and left for 2 min at 37°C. Then, 50 μl of the cell lysates from each well were added, and after 10 min at 37°C, 2 ml of a solution of Na_2CO_3 (0.09 mmol/ml) and NaOH (0.25 mmol/ml) were used to stop the reaction. The absorbance was measured at 590 nm in the plate reader μQuant (BioTek, Winooski, VT), and ALP activity was calculated from a standard curve using thymolphthalein to give a range from 0.012 to 0.4 μmol thymolphthalein/h/ml. Data are expressed as ALP activity normalized by total protein content, quantified in the same cell lysates, at the respective time-point.

Extracellular Matrix Mineralization

At day 21, both BM-MSCs and AT-MSCs grown in osteogenic medium were fixed in 10% formalin for 2 h at room temperature, dehydrated and stained with 2% alizarin red S (Sigma–Aldrich), pH 4.2, for 10 min. The calcium content was detected using a colorimetric assay. Briefly, 280 μl of 10% acetic acid were added to each well, and the plate was incubated at room temperature for

30 min under shaking. This solution was vortexed for 1 min, heated at 85°C for 10 min, and transferred to ice for 5 min. The slurry was centrifuged at 13,000 g for 15 min, 100 μl of the supernatant was mixed with 40 μl of 10% ammonium hydroxide, this solution was read at 405 nm in a plate reader μQuant (BioTek), and the data were expressed as absorbance.

Lipid Accumulation

At day 17, both BM-MSCs and AT-MSCs grown in adipogenic medium were fixed in 10% formalin for 2 h at room temperature, washed with isopropanol 60% (Merck-Germany) and stained with 0.3% oil red O (Sigma–Aldrich) for 10 min. The lipid accumulation was measured using a colorimetric assay. The incorporated oil red O was extracted by incubation with 100% isopropanol for 10 min under shaking at room temperature. After appropriate dilution, this solution was read at 500 nm in a plate reader μQuant (BioTek), and the data were expressed as absorbance.

Co-Cultures

BM-MSCs and AT-MSCs were cultured in growth medium for 4 days. Then, the medium was replaced by osteogenic medium to induce osteoblast differentiation of BM-MSCs and by adipogenic medium to induce adipocyte differentiation of AT-MSCs. Cells were grown for another 4 days. After that, cells were indirectly co-cultured (1:1) using transwell porous filters (Corning Incorporated, Tewksbury, MA) in two different conditions: (1) osteoblasts under the influence of adipocytes in osteogenic medium and (2) adipocytes under the influence of osteoblasts in adipogenic medium. Non-co-cultured osteoblasts and adipocytes were used as controls. At day 3, cells were collected for evaluating gene and protein expression as well as extracellular matrix mineralization and lipid accumulation, as described above. Additionally, the concentration of tumor necrosis factor alpha (TNF-α) and adiponectin in the culture medium was evaluated as described below.

TNF-α and Adiponectin Protein Detection

The concentrations of TNF-α and adiponectin in serum-free culture medium of osteoblasts grown under the influence of adipocytes in osteogenic conditions were detected at 36 h. Culture media of non-co-cultured osteoblasts and adipocytes grown under osteogenic and adipogenic conditions were used as controls. The samples were assayed using two commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions, and the data were expressed as pg/ml.

Addition of TNF-α and Adiponectin to the Osteogenic Medium

To evaluate the effect of TNF-α and adiponectin on differentiation of BM-MSCs into osteoblasts, cells were cultured in osteogenic medium supplemented with adiponectin 10^{-7} M, TNF-α 3×10^{-7} M or the association of both (Gilbert et al., 2002; Pacheco-Pantoja et al., 2014). Cells cultured in osteogenic medium without adiponectin or TNF-α were used as controls. Cells were collected

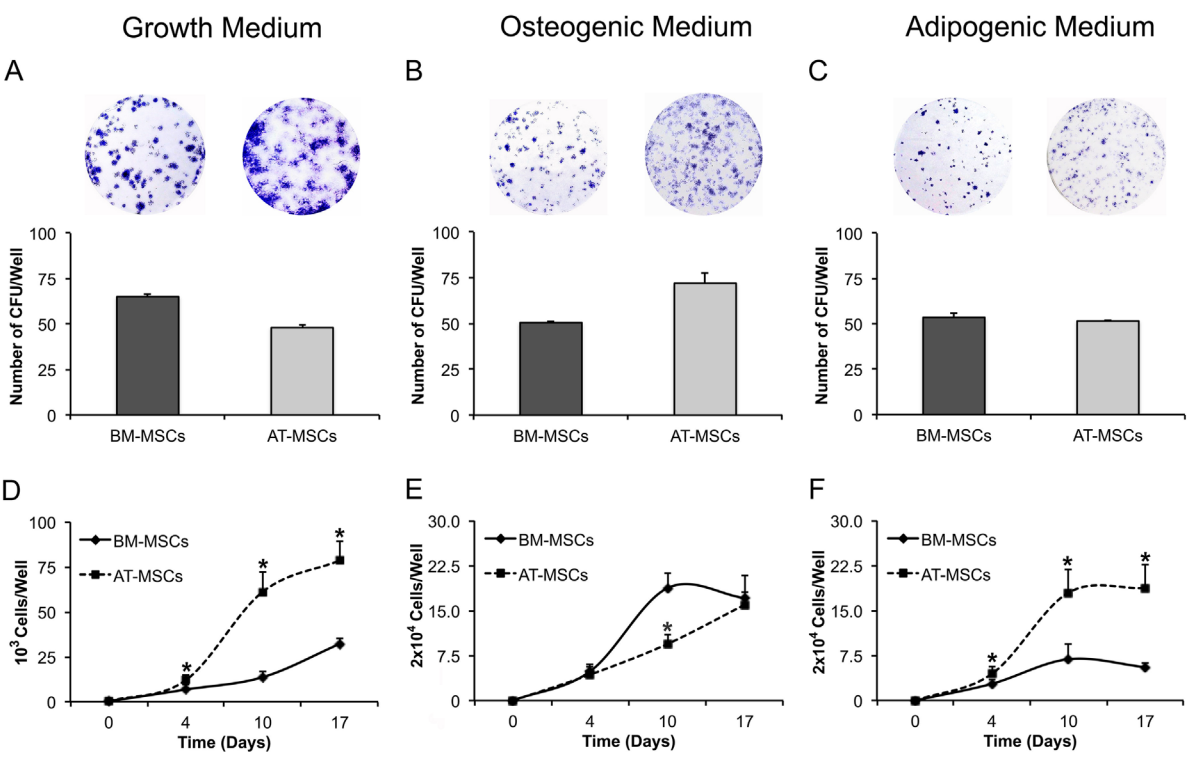

Fig. 1. Colony-forming units (CFU) (A–C) and proliferation (D–F) of mesenchymal stem cells derived from bone marrow (BM-MSCs) and adipose tissue (AT-MSCs) cultured in growth (A, D), osteogenic (B, E) and adipogenic (C, F) media. The number of cells was lower in cultures of BM-MSCs compared with AT-MSCs grown in growth (D) and adipogenic (F) media at 4 days, 10 days and 17 days ($P < 0.05$). The number of cells was higher in cultures of BM-MSCs compared with AT-MSCs grown in osteogenic medium (E) at 10 days ($P < 0.05$). All data are presented as mean ± standard deviation (n = 5). *indicates statistically significant difference between BM-MSCs and AT-MSCs at the same time point.

for evaluating gene expression of RUNX2, ALP and osteocalcin, RUNX2 protein expression and ALP activity at day 10, and extracellular matrix mineralization at day 21, as described above.

Statistical Analysis

The data presented in this work are the representative results of four independent experiments using four sets of cultures established from four different pools of rats. For each experiment, CFU, cell proliferation, ALP activity, extracellular matrix mineralization and lipid accumulation were carried out in quintuplicate (n=5), and gene and protein expression, in triplicate (n = 3). The data were analyzed using either a Kruskal–Wallis test followed by a Student Newman Keuls post-test or a Mann–Whitney U-test when appropriate. For all experiments the level of significance was established at $P \leq 0.05$.

Results

CFU and Cell Proliferation

The number of CFUs at day 7 was not affected by the cell source, irrespective of culture medium; that is, either

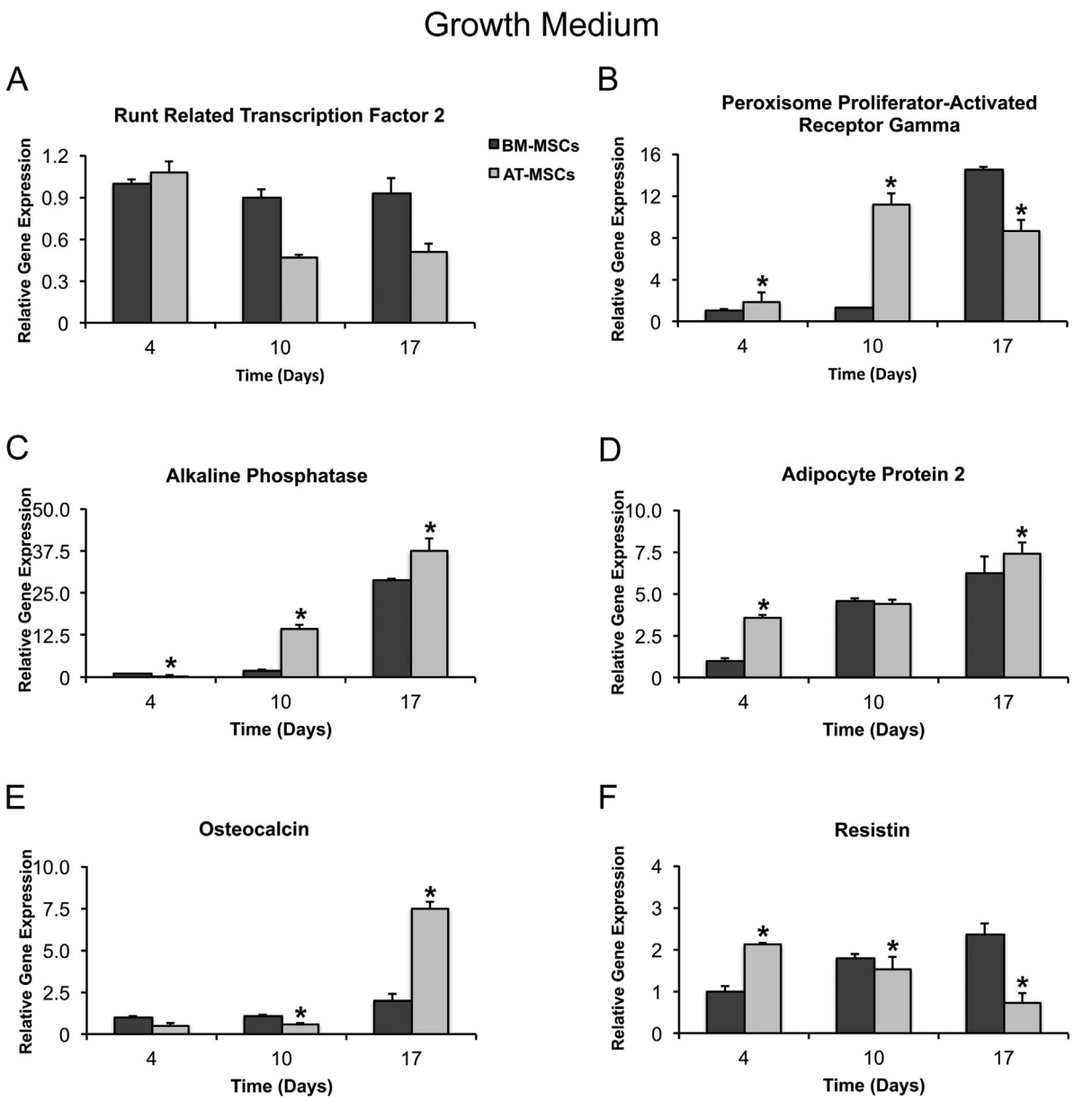

Fig. 2. Gene expression of bone (A, C, E) and adipose (B, D, F) markers of mesenchymal stem cells derived from bone marrow (BM-MSCs) and adipose tissue (AT-MSCs) cultured in growth medium. A lower expression of alkaline phosphatase (C) was observed in cultures of BM-MSCs compared with AT-MSCs at 4 , 10, and 17 days ($P < 0.05$). The expression of osteocalcin (E) was higher at 10 days and lower at 17 days in cultures of BM-MSCs compared with AT-MSCs ($P < 0.05$). The expression of peroxime proliferator-activated receptor gamma (B) was lower at 4 days and 10 days and higher at 17 days in cultures of BM-MSCs compared with AT-MSCs ($P < 0.05$). A lower expression of adipocyte protein 2 (D) was observed in cultures of BM-MSCs compared with AT-MSCs at 4 days and 17 days ($P < 0.05$). The expression of resistin (F) was lower at 4 days and higher at 10 days and 17 days in cultures of BM-MSCs compared with AT-MSCs ($P < 0.05$). All data are presented as mean ± standard deviation (n = 3). *indicates statistically significant difference between BM-MSCs and AT-MSCs at the same time point.

growth medium ($P = 1.0$; Fig. 1A), osteogenic medium ($P = 1.0$; Fig. 1B), or adipogenic medium ($P = 1.0$; Fig. 1C). The number of cells was lower in cultures of BM-MSCs compared with AT-MSCs grown in growth (Fig. 1D) and adipogenic (Fig. 1F) media at days 4, 10, and 17 ($P < 0.05$). The number of cells was higher in cultures of BM-MSCs compared with AT-MSCs grown in osteogenic medium at day 10 ($P < 0.05$), without statistically significant differences at days 4 and 17 ($P > 0.05$; Fig. 1E).

Osteogenic and Adipogenic Potential of BM-MSCs and AT-MSCs

In cells cultured in growth medium, that is, in non-differentiation-inducing conditions, the gene expression of RUNX2 was not affected by the cell source at all evaluated time-points ($P > 0.05$; Fig. 2A). A lower gene expression of ALP was observed in cultures of BM-MSCs compared with AT-MSCs at days 4, 10, and 17 ($P < 0.05$; Fig. 2C). The gene expression of osteocalcin was higher at day 10 and lower at day 17 ($P < 0.05$) in cultures of BM-MSCs compared with AT-MSCs, without a statistically significant difference at day 4 ($P > 0.05$; Fig. 2E). The gene expression of PPARγ was lower at days 4 and 10 and higher at day 17 in cultures of BM-MSCs compared with AT-MSCs ($P < 0.05$; Fig. 2B). A lower gene expression of AP2 was observed in cultures of BM-MSCs compared with AT-MSCs at days 4 and 17 ($P < 0.05$), without a statistically significant difference at day 10 ($P > 0.05$; Fig. 2D). The expression of resistin was lower at day 4 and higher at days 10 and 17 ($P < 0.05$) in cultures of BM-MSCs compared with AT-MSCs (Fig. 2F).

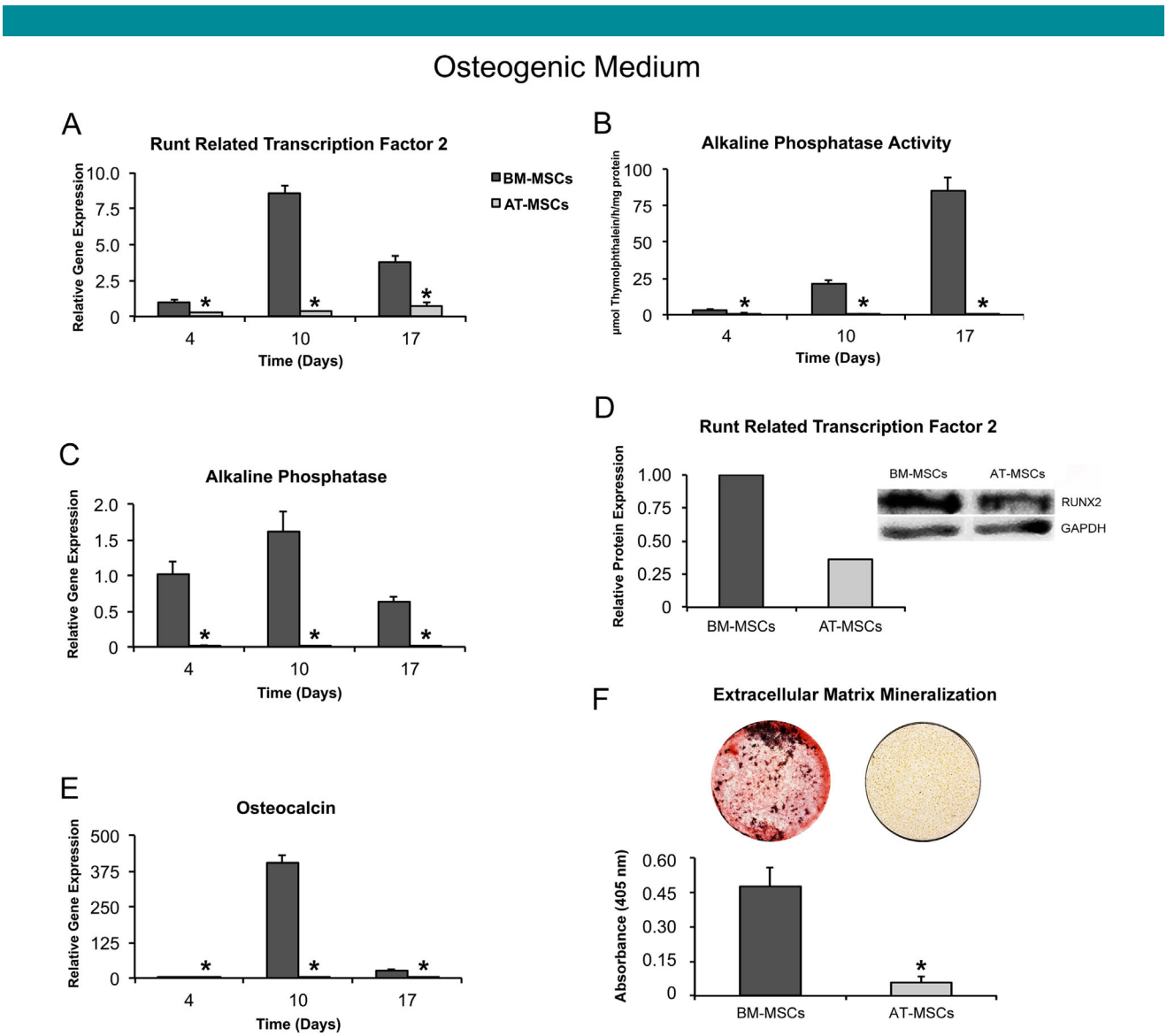

Fig. 3. Osteoblast differentiation of mesenchymal stem cells derived from bone marrow (BM-MSCs) and adipose tissue (AT-MSCs) cultured in osteogenic medium. Gene expression levels of the bone markers runt related transcription factor 2 (Runx2) (A), alkaline phosphatase (C) and osteocalcin (E) were higher in cultures of BM-MSCs compared with AT-MSCs at 4, 10, and 17 days ($P < 0.05$). Alkaline phosphatase (ALP) activity (B) at days 4, 10, and 17 ($P < 0.05$), protein expression of Runx2 (D) at day 10 and extracellular matrix mineralization (F) at day 21 ($P = 0.029$) were higher in cultures of BM-MSCs compared with AT-MSCs. Data from gene expression (n = 3), ALP activity (n = 5), and extracellular matrix mineralization (n = 5) are presented as mean ± standard deviation. *indicates statistically significant difference between BM-MSCs and AT-MSCs at the same time point.

Under osteogenic culture conditions, the gene expression of RUNX2 (Fig. 3A), ALP (Fig. 3C) and osteocalcin (Fig. 3E) was higher in cultures of BM-MSCs compared with AT-MSCs at 4, 10, and 17 days ($P < 0.05$). Additionally, ALP activity at days 4, 10, and 17 ($P < 0.05$; Fig. 3B), protein expression of Runx2 at day 10 (Fig. 3D) and extracellular matrix mineralization at day 21 ($P = 0.029$; Fig. 3F) were higher in cultures of BM-MSCs compared with AT-MSCs.

Under adipogenic culture conditions, the gene expression of PPAR-γ was lower in cultures of BM-MSCs compared with AT-MSCs at days 4, 10, and 17 ($P < 0.05$; Fig. 4A). The gene expression of AP2 at days 4 and 10 was lower and at day 17 was higher ($P < 0.05$) in cultures of BM-MSCs compared with AT-MSCs (Fig. 4C). At days 10 and 17, a lower gene expression of resistin was observed in cultures of BM-MSCs compared with AT-MSCs ($P < 0.05$), without a statistically significant difference at day 4 ($P > 0.05$; Fig. 4E). Protein expression of PPAR-γ at day 10 (Fig. 4B) and lipid accumulation at day 21 (Fig. 4D) were lower in cultures of BM-MSCs compared with AT-MSCs ($P = 0.002$).

Crosstalk Between Osteoblasts and Adipocytes

Under osteogenic culture conditions, the presence of adipocytes, as indirect co-culture, repressed the expression of osteoblast markers as revealed by downregulation of gene expression of RUNX2 ($P = 0.029$; Fig. 5A), ALP ($P = 0.029$; Fig. 5C), and osteocalcin ($P = 0.029$; Fig. 5E) and RUNX2 protein expression (Fig. 5B) at day 10, as well as reduced extracellular matrix mineralization at day 21 ($P = 0.029$; Fig. 5D) compared with osteoblasts grown in the absence of adipocytes.

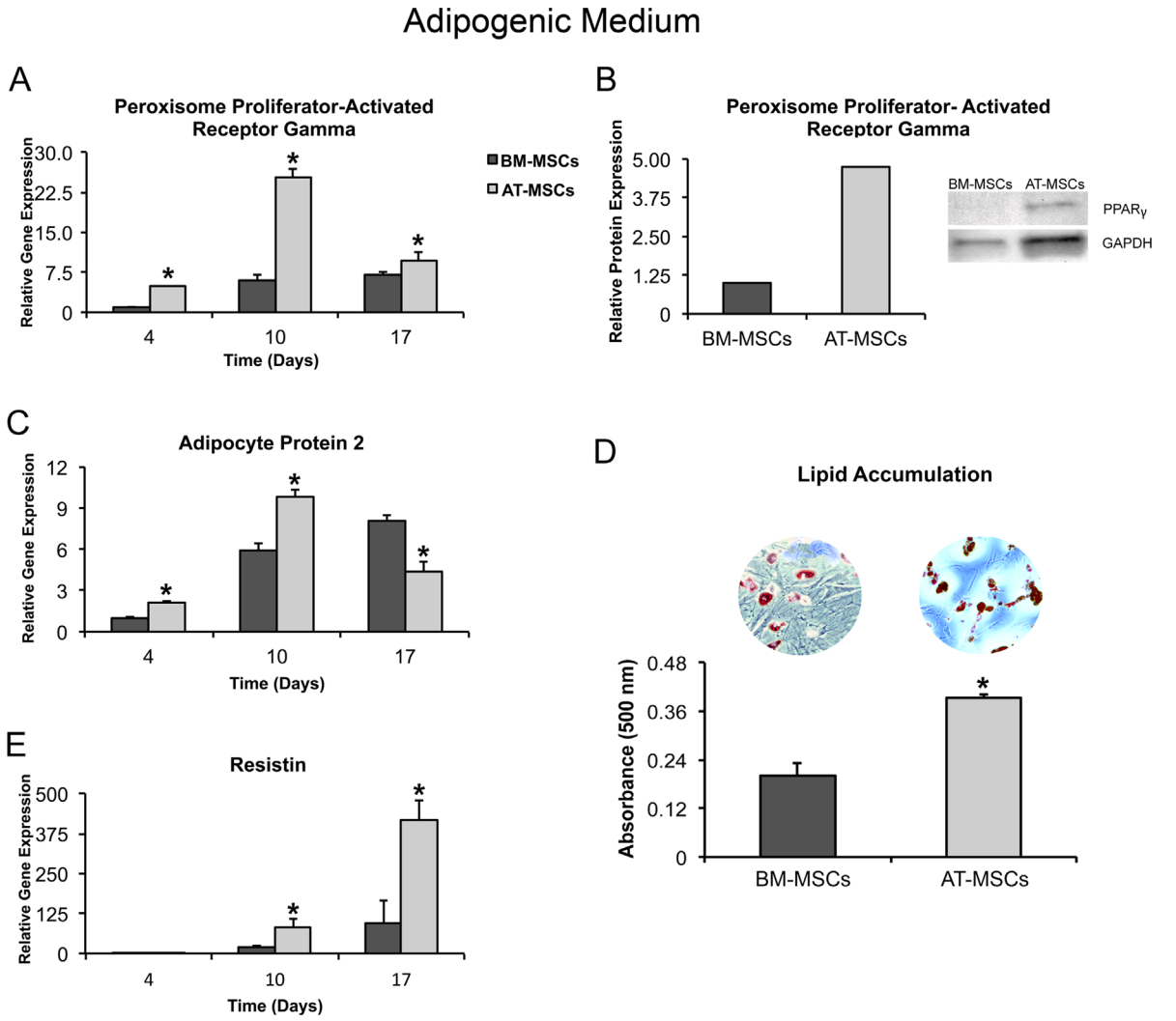

Fig. 4. Adipocyte differentiation of mesenchymal stem cells derived from bone marrow (BM-MSCs) and adipose tissue (AT-MSCs) cultured in adipogenic medium. Gene expression levels of the adipose tissue markers peroxime proliferator-activated receptor gamma (PPAR-γ) (A) at days 4, 10, and 17, adipocyte protein 2 (AP2) (C) at days 4 and 10 and resistin (E) at days 10 and 17 were lower in cultures of BM-MSCs compared with AT-MSCs ($P < 0.05$). The expression of AP2 (C) was higher in cultures of BM-MSCs compared with AT-MSCs at day 17 ($P < 0.05$). Protein expression of PPAR-γ (B) at day 10 and lipid accumulation (E) at day 21 ($P = 0.002$) were lower in cultures of BM-MSCs compared with AT-MSCs. Data from gene expression (n = 3) and lipid accumulation (n = 5) are presented as mean ± standard deviation. *indicates statistically significant difference between BM-MSCs and AT-MSCs at the same time point.

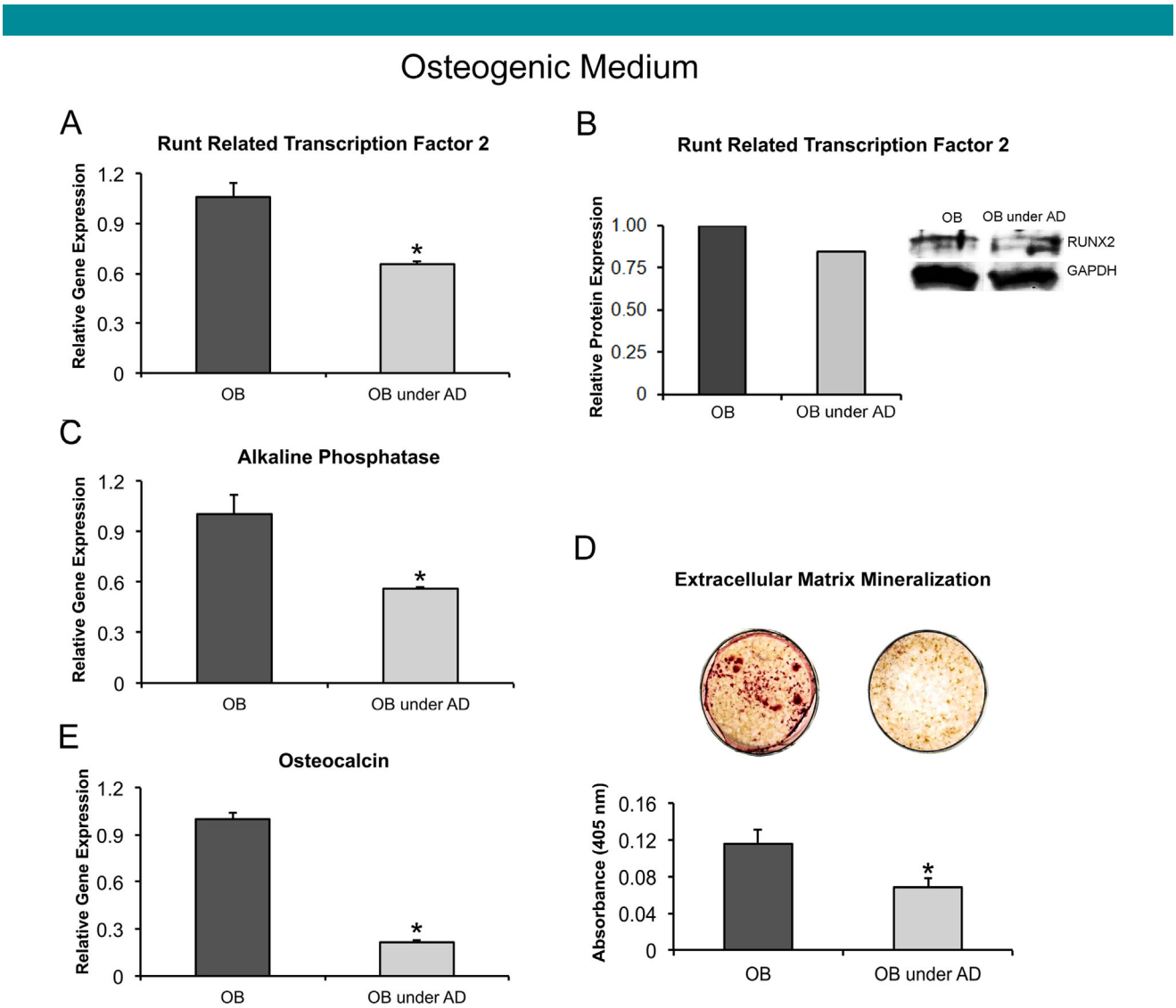

Fig. 5. Effect of adipocytes differentiated from adipose tissue-derived mesenchymal stem cells (AT-MSCs) on osteoblasts differentiated from bone marrow-derived mesenchymal stem cells (BM-MSCs) cultured in osteogenic medium. Gene expression levels of the bone markers runt related transcription factor 2 (RUNX2) (A), alkaline phosphatase (C) and osteocalcin (E) and RUNX2 protein expression (B), at day 10, and extracellular matrix mineralization (D) at day 21 were reduced by the presence of adipocytes ($P = 0.029$). Data from gene expression ($n = 3$) and extracellular matrix mineralization ($n = 5$) are presented as mean ± standard deviation. *indicates statistically significant difference.

Under adipogenic culture conditions, the presence of osteoblasts as indirect co-culture exhibited a more complex effect on the expression of adipocyte markers, since at day 10, the gene expression of PPAR-γ ($P = 0.029$; Fig. 6A) and resistin ($P = 0.029$; Fig. 6E) was upregulated while AP2 ($P = 0.029$; Fig. 6C) was downregulated compared with adipocytes grown in the absence of osteoblasts. Additionally, PPAR-γ protein expression was reduced at day 10 (Fig. 6B), and adipocyte lipid accumulation at day 17 was not affected by the presence of osteoblasts ($P = 0.114$; Fig. 6D).

Participation of TNF-α and Adiponectin on the Inhibitory Effect of Adipocytes on Osteoblasts

To elucidate the mechanism involved in the inhibitory effect of adipocytes on osteoblasts and based on data from the literature, we selected two proteins synthesized by adipocytes (TNF-α and adiponectin) and investigated their effects on osteoblasts (Clabaut et al., 2010; Li et al., 2010; Shinoda et al., 2006). At 36 h, the concentrations of TNF-α (Fig. 7A) and adiponectin (Fig. 7B) were higher in the conditioned medium of adipocytes, followed by co-culture of adipocytes and osteoblasts, and osteoblasts ($P = 0.001$ for all).

The addition of exogenous TNF-α or the association of TNF-α and adiponectin repressed the expression of osteoblast markers compared with controls, without a statistically significant difference between them ($P > 0.05$). This effect was revealed by downregulation of the gene expression of RUNX2 ($P = 0.001$; Fig. 8A), ALP ($P = 0.001$; Fig. 8C), and osteocalcin ($P = 0.001$; Fig. 8E), RUNX2 protein expression (Fig. 8B) and ALP activity ($P = 0.001$; Fig. 8D) as well as reduced extracellular matrix mineralization ($P = 0.029$; Fig. 8F). However, the addition of exogenous adiponectin alone upregulated the gene expression of RUNX2 ($P = 0.001$; Fig. 8A), ALP ($P = 0.001$; Fig. 8C) and osteocalcin ($P = 0.001$; Fig. 8E), and RUNX2 protein

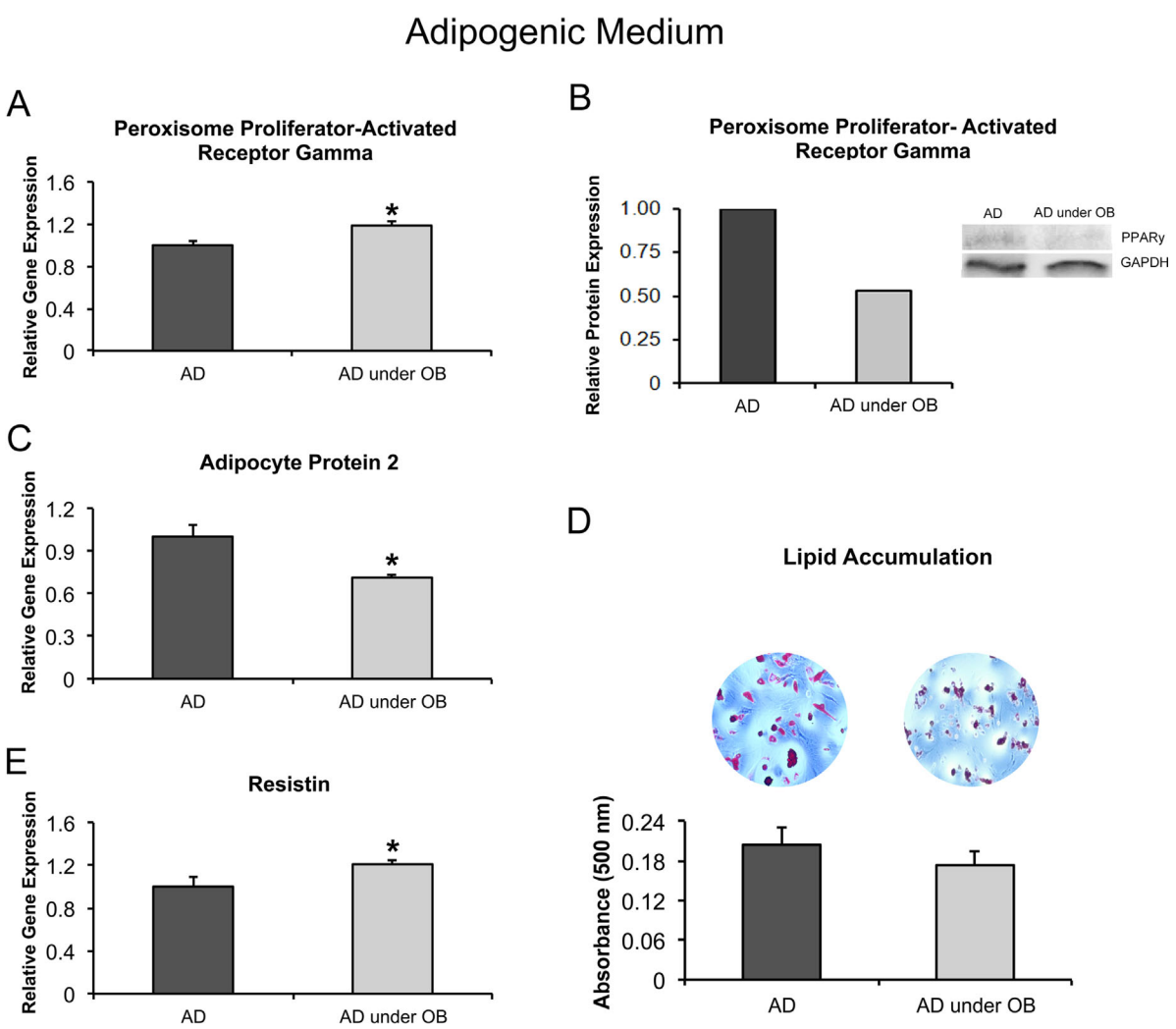

Fig. 6. Effect of osteoblasts differentiated from bone marrow-derived mesenchymal stem cells (BM-MSCs) on adipocytes differentiated from adipose tissue-derived mesenchymal stem cells (AT-MSCs) cultured in adipogenic medium. Gene expression levels of the adipose tissue markers peroxime proliferator-activated receptor gamma (A) and resistin (E) were increased ($P = 0.029$), and adipocyte protein 2 (B) was reduced by the presence of osteoblasts at day 10. Data from gene expression (n = 3) and lipid accumulation (n = 5) are presented as mean ± standard deviation. *indicates statistically significant difference.

expression (Fig. 8B), while it reduced ALP activity ($P = 0.03$) (Fig. 8D) but did not affect extracellular matrix mineralization ($P = 0.690$; Fig. 8F) compared with controls.

Discussion

To date, there has been some controversy regarding the osteogenic and adipogenic potentials of MSCs from distinct cell sources as contrasting results from studies carried out with cells from different sites have been reported. Here, we showed that, despite both BM-MSCs and AT-MSCs being able to differentiate into osteoblasts and adipocytes, BM-MSCs exhibited a higher osteogenic potential while AT-MSCs were more adipogenic. Additionally, our results indicated that the crosstalk between adipocytes and osteoblasts repressed osteoblast phenotype expression, at least in part, through the TNF-α signaling pathway. On the other hand, osteoblasts had no detectable effect on adipocytes.

The ability to form colonies and to expand under in vitro conditions is a key feature of MSCs. It has been demonstrated that the colony number in cultures of AT-MSCs was more than 100-fold higher, but the number of cells per colony was lower than in BM-MSCs (Sakaguchi et al., 2005). Additionally, no relevant differences in terms of cell proliferation were reported when BM-MSCs and AT-MSCs were cultured under both non-differentiation-inducing and osteogenic conditions (Hattori et al., 2004). In this study, the amount of CFUs was not affected by cell source, but the number of cells per colony, as well as the total number of cells, was higher in cultures growth under non-differentiation-inducing conditions. In addition, BM-MSCs are more proliferative when exposed to an osteogenic medium but not under both non-differentiation-inducing and adipogenic conditions compared with AT-MSCs. Together, these results indicated that for cell-based therapies to repair bone tissue, prior to osteoblast differentiation induction, cells should be expanded

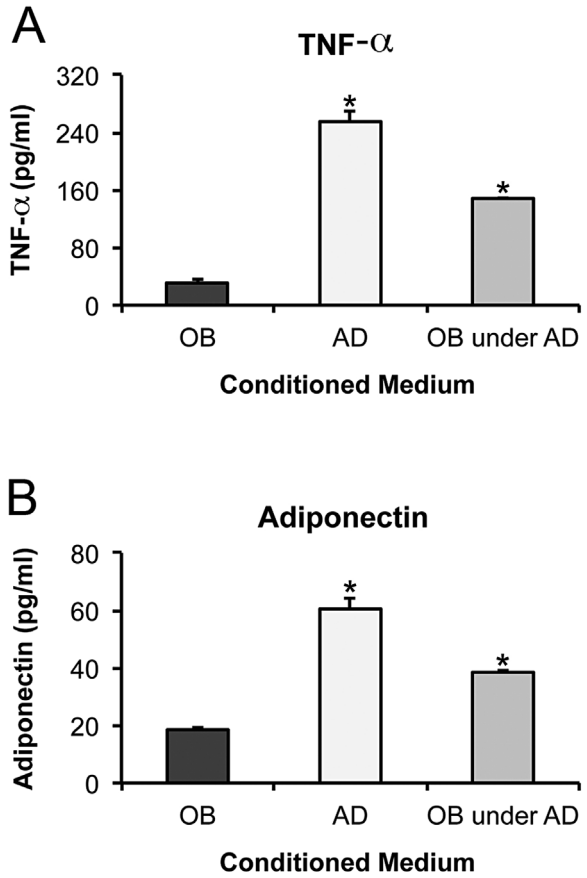

Fig. 7. Detection of tumor necrosis factor alpha (TNF-α) and adiponectin in conditioned media of osteoblasts differentiated from bone marrow-derived mesenchymal stem cells (BM-MSCs), of osteoblasts differentiated from BM-MSCs cocultured with adipocytes differentiated from adipose tissue-derived mesenchymal stem cells (AT-MSCs) and of adipocytes differentiated from AT-MSCs at 36 h. The concentration of TNF-α (A) and adiponectin (B) was higher in conditioned medium of adipocytes followed by osteoblasts co-cultured with adipocytes and osteoblasts ($P = 0.001$). Data ($n = 3$) are presented as mean ± standard deviation *indicates statistically significant difference compared with OB.

under non-differentiation-inducing conditions to generate a suitable number of cells. As we have used cells from rats, and other studies were carried out with cells from humans, the unmatched results may be attributed to species-specific features.

Regarding the differentiation capacity, discrepant results have been reported in the literature. It has been shown that BM-MSCs and AT-MSCs, under identical culture conditions, were able to differentiate into osteoblasts and adipocytes (Pachón-Peña et al., 2011). Corroborating this finding, we demonstrated that, in non-differentiation-inducing medium, BM-MSCs and AT-MSCs exhibited osteogenic and adipogenic potentials as revealed by gene expression of osteoblast and adipocyte markers. However, when exposed to osteogenic medium, BM-MSCs exhibited higher osteogenic potential compared with AT-MSCs as noticed by higher ALP activity, gene and protein expression of osteoblast markers and extracellular matrix mineralization. Conversely, under adipogenic conditions, AT-MSCs displayed higher gene and protein expression of adipocyte markers as well as more lipid accumulation compared with BM-MSCs. Based on similar results showing higher osteoblast and adipocyte differentiation of BM-MSCs and AT-MSCs, respectively, the hypothesis that cells may preserve some features of their tissue of origin has been proposed, which is supported by our findings (Sakaguchi et al., 2005; Post et al., 2008; Pachón-Peña et al., 2011).

In terms of bone repair, the crosstalk between osteoblasts and adipocytes is as important as the cell differentiation potential, since they coexist and interact in bone marrow (Abdallah and Kassem, 2012; Sadie-Van Gijsen et al., 2013). Such interactions should be taken into consideration in clinical situations since the increased adipogenesis in bone marrow is correlated to the decreased bone mineral density in animal models and elderly and osteoporotic populations (Duque et al., 2004; Duque, 2008; Liu et al., 2011). By using a co-culture model that prevents direct contact between osteoblasts and adipocytes, we observed that adipocytes suppress osteoblast differentiation as revealed by the downregulation of gene and protein expression of osteoblast markers and reduced extracellular matrix mineralization. This finding can be, at least in part, attributed to some factors secreted by adipocytes that could affect osteoblast phenotype expression, such as TNF-α and adiponectin (Clabaut et al., 2010; Li et al., 2010; Shinoda et al., 2006). While TNF-α inhibits osteoblast differentiation, adiponectin may either suppress or stimulate osteoblast phenotype expression (Zhan et al., 2014; Chen et al., 2015; Jang et al., 2015). Indeed, we detected higher concentrations of these proteins in conditioned medium of both adipocytes and co-culture of adipocytes and osteoblasts compared with the conditioned medium of osteoblasts without contact with adipocytes, indicating that adipocytes may increase the availability of TNF-α and adiponectin to osteoblasts in a co-culture condition. To investigate if these factors could be related to the repression of osteoblast differentiation induced by adipocytes, we cultured osteoblasts in the presence of exogenous TNF-α and adiponectin. Our results showed that TNF-α but not adiponectin dramatically represses osteoblast differentiation, demonstrating the involvement of TNF-α in the negative effect of adipocytes on osteoblast phenotype development in this culture model. Additionally, it has been shown that mice with a selective knock-out of PPAR-γ in white adipose tissue exhibited increased osteoblast activity and bone formation, suggesting that adipocytes may inhibit osteoblast functions even in physiological conditions (Akune et al., 2004; Cock et al., 2004). Regarding the influence of osteoblasts on adipocytes, and despite it being shown that osteocalcin can modulate some adipocyte activities, we did not observe relevant effects on adipocyte differentiation (Hill et al., 2014).

In conclusion, the present study showed that both BM-MSCs and AT-MSCs are able to differentiate into osteoblasts and adipocytes. Additionally, under appropriate inducing conditions BM-MSCs exhibited higher osteogenic potential while AT-MSCs were more adipogenic. Finally, we demonstrated the key role of TNF-α secretion by adipocytes on the inhibition of osteoblast differentiation. Thus, we postulate that the pronounced capability of cells from adipose tissue to produce TNF-α together with the higher osteogenic potential of BM-MSCs makes the latter a good choice for inducing bone repair in cell-based therapies.

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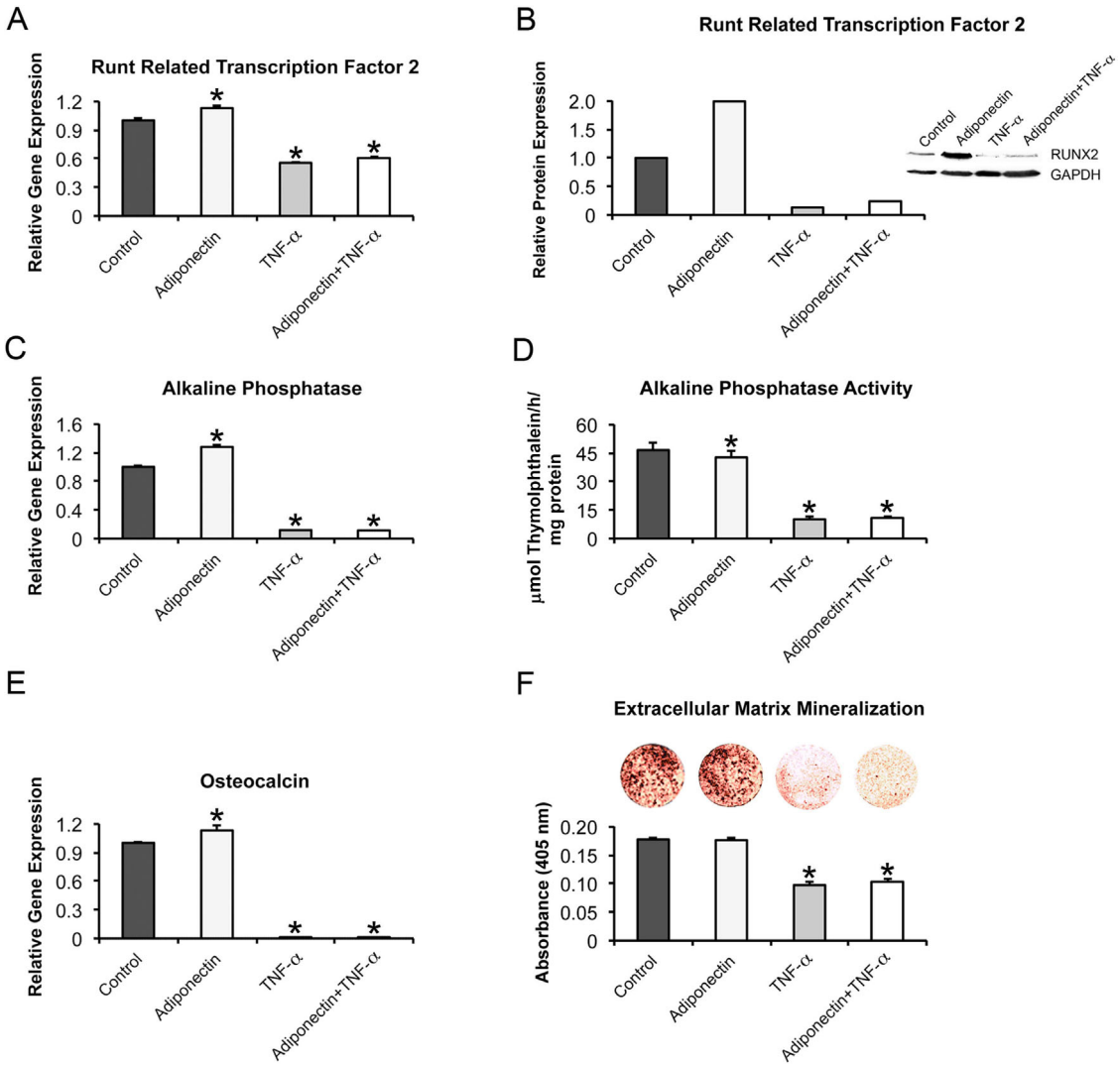

Fig. 8. Effect of tumor necrosis factor alpha (TNF-α) and adiponectin on osteoblast differentiation of bone marrow-derived mesenchymal stem cells (BM-MSCs) cultured in osteogenic medium. Gene expression levels of the bone markers runt related transcription factor 2 (RUNX2) (A), alkaline phosphatase (ALP) (C) and osteocalcin (E), RUNX2 protein expression (B) and ALP activity (D) ($P = 0.001$), at day 10, and extracellular matrix mineralization (F) ($P = 0.029$) at day 21 were reduced by TNF-α or the association of TNF-α compared with controls. At day 10, gene expression of RUNX2 (A), ALP (C) and osteocalcin (E) ($P = 0.001$) and RUNX2 protein expression (B) were increased and ALP activity ($P = 0.03$) (D) was reduced by TNF-α or the association of TNF-α compared with controls. Data from gene expression (n = 3), ALP activity (n = 5) and extracellular matrix mineralization (n = 5) are presented as mean ± standard deviation. *indicates statistically significant difference compared with controls.

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