S100B suppresses the differentiation of C3H/10T1/2 murine embryonic mesenchymal cells into osteoblasts

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Abstract. S100 calcium-binding protein B (S100B) is expressed and released by adipocytes, and is positively correlated with body mass index, however, the direct effects of S100B on adipocytes remain unclear. Bone marrow-derived mesenchymal stem cells have the capacity to differentiate into osteoblasts and adipocytes, which is important for bone metabolism. The current study aimed to determine the effect of S100B on adipogenesis and osteogenesis. The mouse embryo cell line C3H/10T1/2 was used to build cell models with varying levels of S100B protein expression. Western blot analysis was performed to assess the expression of various marker proteins. Oil red O staining and alizarin red S staining were used to detect adipogenesis and osteogenesis, respectively. S100B overexpression was associated with a significant increase in oil red O staining and a significant reduction in alizarin red S staining. Runt-related transcription factor-2 and bone morphogenetic protein 2 expression levels were significantly increased in the S100B underexpression group, however not in the S100B overexpression group. By contrast, the expression levels of the adipogenesis markers peroxisome proliferator-activated receptor γ and CCAAT-enhancer-binding protein α was significantly increased in the S100B overexpression group, however not in the S100B underexpression group. Osteogenesis stimulation increased extracellular signal-regulated kinase (ERK) phosphorylation, and adipogenesis stimulation increased c-Jun N-terminal kinase (JNK) phosphorylation. The results suggest that S100B inhibits osteogenesis, however stimulates adipogenesis. The ERK pathway is involved in the regulation of osteogenesis, whereas the JNK pathway is involved in the regulation of adipogenesis.

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Introduction

Osteoporosis and obesity are two of the most common chronic conditions and pose major health threats worldwide, with both showing increasing prevalence rates, however, the association between osteoporosis and obesity is complex. The bone marrow is the only place in mammalian tissues where bone and fat lie adjacent to each other, in osteoporosis, adipogenesis is increased at the expense of osteogenesis from common osteoporotic bone marrow cells (1,2). Bone marrow-derived mesenchymal stem cells (BM-MSCs) have the capacity to differentiate into osteoblasts and adipocytes, and osteoporosis is partially attributable to the alteration of the balance of BM-MSC differentiation into osteoblasts and adipocytes. BM-MSC differentiation is regulated by hormones, cytokines, and genes. The differentiation of BM-MSCs into adipocytes is accompanied by a marked increase in the expression of adipocyte markers, including peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT-enhancer-binding protein α (C/EBPα). Similarly, the differentiation of BM-MSCs into osteoblasts is regulated by bone morphogenetic proteins (BMPs) and runt-related transcription factor-2 (RUNX2) (3-6). A deeper understanding of the differentiation of BM-MSCs into osteoblasts or adipocytes will provide insight into the pathophysiology and treatment of osteoporosis.

S100 calcium-binding protein B (S100B), an important member of the S100 family, is ubiquitously expressed in human tissue, including fat tissues, and is associated with a variety of human diseases such as neurodegenerative disorders (7), malignant melanoma (7), trauma with or without brain injury (8-10) and obesity (11). Serum S100B levels are positively correlated with body mass index (11), and S100B expression is increased by diet-induced obesity (12). Adipocytes express and secrete S100B protein, which may act as an adipokine by modulating the immune response and metabolism. However,

the direct effect of S100B on osteoporosis and obesity remains to be investigated (13). Therefore, the current study aimed to determine the effect of S100B on MSC differentiation into adipocytes and osteoblasts.

In the present study, an *in vitro* model of osteogenesis and adipogenesis was established using the mouse embryo cell line C3H/10T1/2 (ATCC number, CCL-226) in a monolayer and high-density cultures. The current study presents novel evidence concerning the effect of S100B on cell differentiation, including adipogenic and osteogenic differentiation.

Materials and methods

Construction of expression plasmids. C57B/6 mice were purchased from Nanjing Qingzilan Technologies Co., Ltd. (Nanjing, China). Animals were housed at 23±1˚C in a 12/12-h light/dark cycle and a humidity of 45±5%, and allowed free access to a normal chow diet and water. The study was approved by the ethics committee of the Animal Care Facility of Nanjing Medical University, Nanjing, China.

Total mRNA was isolated using TRIzol® reagent (cat. no. 15596-026; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). This mRNA was reverse-transcribed into complementary (c)DNA via reverse transcription-polymerase chain reaction (RT-PCR) using the ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis kit; cat. no. 11146024, Thermo Fisher Scientific, Inc.). Subsequently, this cDNA was used as the template DNA. PCR (cat. no. 4464268; the Platinum Multiplex PCR Master Mix, 2X; Thermo Fisher Scientific, Inc.) was performed to clone S100B cDNA using appropriate primers.

Plasmids overexpressing S100B, termed pcDNA3.1(+) A-S100B, were constructed. The coding sequences of mouse S100B were amplified using RT-PCR and mRNAs isolated from the white adipose tissue of mice using TRIzol® reagent (cat. no. 15596-026; Invitrogen; Thermo Fisher Scientific, Inc.). The primer sequences (including the sites of restriction enzymes) were as follows: Forward, 5'-CGTGAATTCATG TCCGAGCTGGGAAG-3' and reverse, 5'-GCTGTCGAC GGGTCACTCATGTTCAAAGAAGT-3'. The PCR products were subcloned into the pcDNA3.1(+)A expression vector and then confirmed by sequencing.

Subsequently, miRNA-S100B expression plasmids were constructed. Three distinct domains within the coding region of the mouse S100B cDNA were targeted for RNA interference. For this purpose, four pairs of reverse complementary oligonucleotides were designed and synthesized (Table I). The thermocycling conditions used were: 95˚C for 15 sec; 58˚C for 20 sec; and 72˚C for 20 sec. The oligonucleotides were annealed and inserted into the pcDNA6.2-GW/EmGFP-miR expression vector (Invitrogen; Thermo Fisher Scientific, Inc.) to create pcDNA6.2-GW/EmGFP-miR-S100B 1, 2 and 3. A scrambled control construct was also created.

Cell culture and stable clone selection. The mouse embryo cell line C3H/10T1/2 (ATCC number, CCL-226; American Type Culture Collection, Manassas, VA, USA) was cultured in Eagle's basal medium (MEM; cat. no. 11095080; Life Technologies; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; cat. no. 10100147; Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml) and streptomycin sulfate (100 μg/ml; cat. no. 15070063; Life Technologies; Thermo Fisher Scientific, Inc.), and maintained at 37˚C in a humid incubator containing 5% CO_2.

The expression constructs were transfected into C3H/10T1/2 cells using the X-tremeGENE HP DNA Transfection Reagent (cat. no.; 06366236001; Roche Diagnostics, Basel, Switzerland). After 48 h, the cells were cultured in a selective medium containing 300 μg/ml G418 or 3 mg/ml blasticidin (cat. nos., N6386 and 11033102, respectively; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) for 1 week, and resistant colonies, which indicated successful transfectants, were selected.

Osteoblast differentiation. Osteogenic differentiation was induced as described previously (14). Confluent C3H/10T1/2 cells (the day on which confluence was reached was considered day 0) were incubated for 12 days in an osteogenic induction medium consisting of MEM containing 10% FBS, 0.1 mM dexamethasone (cat. no. D4902; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany), 10 mM β-glycerophosphate (cat. no. G9422; Sigma-Aldrich; Merck Millipore) and 50 mM ascorbic acid. The induction medium was changed every 2 days. The presence and extent of bone matrix mineralization was evaluated using alizarin red S staining.

Adipocyte differentiation. Adipocyte differentiation was induced as described previously (14). C3H/10T1/2 cells were seeded on plates, and allowed to grow for 2 days to reach confluence (considered day 0). Cell differentiation was induced by culturing the cells in MEM containing 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine (cat. no. I7018; Sigma-Aldrich; Merck Millipore), 1 μg/ml porcine insulin (cat. no. I0320000; Sigma-Aldrich; Merck Millipore) and 1 mM dexamethasone. Following 48 h of incubation, the medium was replaced with MEM containing 10% FBS and 1 μg/ml insulin. On day 4, the medium was replaced with fresh medium (MEM containing 10% FBS), and the incubation was continued for 12 days. Lipid droplets were evaluated using oil red O staining.

Alkaline phosphatase staining. The differentiation of C3H/10T1/2 cells into osteoblasts. After 4 days, alkaline phosphatase (ALP) staining was performed according to the protocol described in the 5-Bromo-4-chloro-3'-indolyphosphate p-Toluidine Salt (BCIP)/Nitro-Blue Tetrazolium Chloride (NBT) ALP Color Development kit (cat. no. C3206; Beyotime Institute of Biotechnology, Inc.). The cells were fixed with 10% formalin for 10 min at room temperature, washed with phosphate-buffered saline (PBS), and stained with 300 μg/ml BCIP/NBT solution for 30 min at room temperature. ALP-positive cells were stained blue. Stained cells were examined using light microscopy (OLYMPUS IX51) and photographed.

Alizarin red S staining. We induced the differentiation of C3H/10T1/2 cells into osteoblasts. Twelve days later, the cells were gently washed three times with PBS. Then, the Alizarin Red S Staining kit (GMS80046.3v.A; Genmed Scientifics, Inc., Wilmington, DE, USA) was used according to the manufacturer's instructions. The cells were carefully rinsed

Table I. Reverse complementary oligonucleotides.

Oligo	5'-3' sequence
13MR0109-01-F	TGCTGAACAACTGCCTTCTCCAGCTCGTTTTGGCCACTGACTGACGAGCT GGAAGGCAGTTGTT
13MR0109-01-R	CCTGAACAACTGCCTTCCAGCTCGTCAGTCAGTGGCCAAAACGAGCTGGAG AAGGCAGTTGTTC
13MR0109-02-F	TGCTGTTCTGGATGAGCTTGTCAGCTGTTTTGGCCACTGACTGACAGC TGACACTCATCCAGAA
13MR0109-02-R	CCTGTTCTGGATGAGTGTCAGCTGTCAGTCAGTGGCCAAAACAGC TGACAAGCTCATCCAGAAC
13MR0109-03-F	TGCTGTTCGGAAGCTGGACTTGCTGAGTTTTGGCCACTGACTGACTCA GCAAGCAGCTTCCGAA
13MR0109-03-R	CCTGTTCGGAAGCTGCTTGCTGAGTCAGTCAGTGGCCAAAACTCAGCAAGT CCAGCTTCCGAAC
Negative-F	TGCTGAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCA CGCAGTACATTT
Negative-R	CCTGAAATGTACTGCGTGGAGACGTCAGTCAGTGGCCAAAACGTCTCCACG CGCAGTACATTTC

F, forward; R, reverse.

three times with 1.0 ml double-distilled water and allowed to dry. Stained cells were examined using light microscopy (IX51; Olympus Corporation, Tokyo, Japan) and were then photographed.

Oil red O staining. The differentiation of C3H/10T1/2 cells into adipocytes was induced, then 12 days later, oil red O staining was performed according to a previously published protocol (14). The cells were washed three times with PBS and fixed with 10% formalin for 60 min at room temperature. Subsequent to fixation, the cells were washed twice with PBS and stained with filtered oil red O solution (cat. no. O0625; Sigma-Aldrich; Merck Millipore) for 60 min at room temperature. The cells were then washed with distilled water to remove unbound dye, visualized using light microscopy (IX51), and were then photographed.

Triglyceride glycerol phosphate oxidase-peroxidase (GPO-POD) assay. Cellular triglyceride content was determined using the Triglyceride GPO-POD Assay kit (cat. no. TR0100; Sigma-Aldrich; Merck Millipore). At 12 days subsequent to the induction of C3H/10T1/2 cell differentiation into adipocytes, the cells were washed twice with PBS, scraped in 500 μl PBS, sonicated to homogenize the suspension and then assayed to determine the total triglyceride content.

Western blot analysis. At 0, 4, 8 and 12 days subsequent to the induction of C3H/10T1/2 cell differentiation into adipocytes or osteoblasts, the cells were lysed in radioimmunoprecipitation assay buffer [composition: 50 mM Tris-HCl (pH 7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA and 100 μg/ml phenylmethylsulfonyl fluoride]. Equal amounts of protein (60 μg) were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and were electrophoretically transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were incubated overnight at 4˚C with rabbit monoclonal anti-S100B (cat. no. 9550), rabbit monoclonal PPARγ (cat. no. 2430), rabbit polyclonal anti-C/EBPα (cat. no. 2295) and rabbit monoclonal anti-RUNX2 (cat. no. 8486; all 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) antibodies, and anti-BMP2 (cat. no. ab82511, Abcam, Cambridge, MA, USA) and mouse monoclonal β-tubulin (cat. no. T5168; 1:5,000, Sigma-Aldrich; Merck Millipore) antibodies in Tris-buffered saline with Tween-20 containing 1% (w/v) bovine serum albumin (cat. no. 05470; Sigma-Aldrich; Merck Millipore). The blots were then incubated for 2 h with anti-rabbit or anti-mouse secondary antibodies [anti-rabbit immunoglobulin G (IgG), horseradish peroxidase (HRP)-linked antibody; cat. no. 7074; and anti-mouse IgG, HRP-linked antibody; cat. no. 7076; Cell Signaling Technology, Inc.]. Immune complexes were detected using a Pierce ECL Western Blotting Substrate kit (cat. no. 32106; Thermo Fisher Scientific, Inc.), and analyzed using a scanning densitometer with molecular analysis software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Statistical analyses were performed using SPSS 19 software (IBM SPSS, Armonk, NY, USA). Data were assessed using one-way analysis of variance with a correction for multiple comparisons, as appropriate. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Measurement of S100B expression. To assess the functional roles of S100B in C3H/10T1/2 cell differentiation, S100B expression levels were altered in C3H/10T1/2 cells through either an overexpression system or RNA interference. In either case, stable transfectants were selected using G418 and blasticidin, and then expanded for further studies. The expression levels of S100B were determined using western

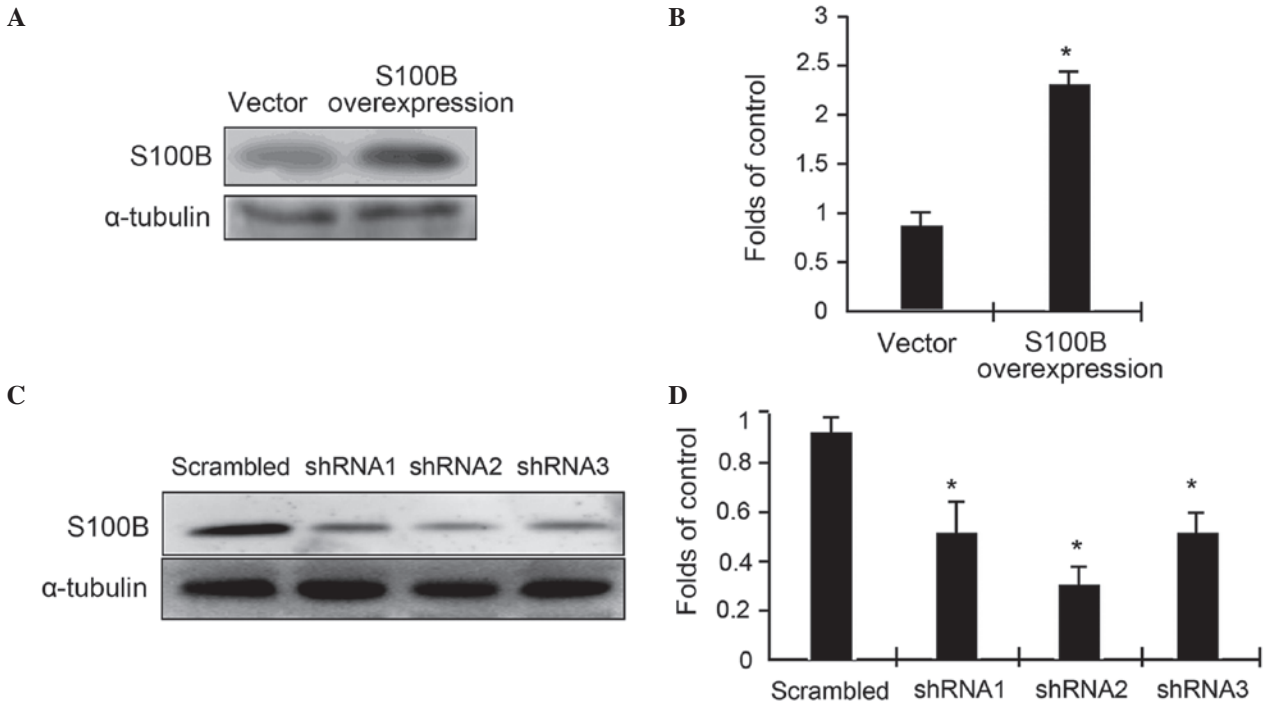

Figure 1. Western blot analysis of S100B protein expression in various transfectants. (A) Western blot analysis was used to determine the S100B protein level in C3H/10T1/2 cells transfected with pcDNA3.1(+)A-S100B plasmids and then selected with 800 μg/ml G418. (B) Quantification of the S100B protein expression levels in (A) based on grayscale analysis (analyzed from three independent experiments). *P≤0.05 vs. vector control. (C) Western blot analysis was used to determine the S100B protein level in C3H/10T1/2 cells transfected with plasmids, including pcDNA6.2-GW/EmGFP-miR-S100B 1, 2 and 3 and a scrambled sequence, and then selected with 3 mg/ml blasticidin. (D) Quantification of the S100B protein expression levels in (C) based on grayscale analysis (analyzed from three independent experiments). *P≤0.05 vs. the scrambled control. S100B, S100 calcium-binding protein B shRNA, small hairpin RNA.

blot analysis. The results indicated that overexpression driven by a cytomegalovirus-promoter resulted in a 2.4-fold elevation of S100B protein expression (Fig. 1A and B). In contrast, the expression of three specific miRNAs targeting three different regions of S100B mRNA resulted in up to 50% reduction in S100B expression (Fig. 1C and D). Thus, the cell models were successfully built with varying levels of S100B protein expression. miRNA2 was used in the following experiments.

S100B inhibits C3H/10T1/2 cell differentiation into osteoblasts. To determine the effect of S100B on the differentiation of C3H/10T1/2 cells into osteoblasts, differentiation of C3H/10T1/2 cells with different levels of S100B protein expression into osteoblasts was induced by specific differentiation protocols (Fig. 2). At 4 days after the induction of differentiation, ALP activity in the cells was examined using ALP staining. The results indicated that S100B overexpression suppressed ALP activity, while S100B underexpression enhanced ALP activity (Fig. 2A and C). At 12 days after the induction of differentiation, alizarin red S staining was used to detect calcium nodule formation. Fewer red nodules were observed in the S100B overexpression group than in the S100B underexpression group (Fig. 2B and D).

To confirm the effect of S100B on osteogenesis, the expression levels of the markers of osteogenic differentiation were examined using western blot analysis. At 0, 4, 8 and 12 days subsequent to the induction of C3H/10T1/2 cell differentiation into osteoblasts, total protein was extracted to examine the expression of the osteoblast markers RUNX2 and BMP2. The western blot results indicated that in the control group, RUNX2 and BMP2 protein expression increased gradually as the cells differentiated into osteoblasts. In the S100B overexpression group, there was no significant increase in RUNX2 and BMP2 expression, however, in the S100B underexpression group, the magnitude of the increase in RUNX2 and BMP2 expression was greater than in the control group (Fig. 2E-G). These results suggested that S100B suppressed the osteogenic differentiation of C3H/10T1/2 cells.

S100B stimulates C3H/10T1/2 cell differentiation into adipocytes. To investigate the effect of S100B on the differentiation of C3H/10T1/2 cells into adipocytes, the differentiation of C3H/10T1/2 cells with different levels of S100B expression into adipocytes was induced. At 12 days after the induction of differentiation, oil red O staining was applied to detect cellular lipid droplets. The results of staining indicated that S100B overexpression led to a significant increase in oil red O staining, however, the reduction of S100B expression led to sparse expression of oil red O staining (Fig. 3A). The quantitative analysis of cellular triglycerides was used to evaluate the above observations; the results confirmed that triglyceride accumulation was high in C3H/10T1/2 cells overexpressing S100B, however was low in cells with reduced S100B expression (Fig. 3B).

At different time points (0, 4, 8 and 12 days) subsequent to the induction of C3H/10T1/2 cell differentiation into adipocytes, proteins were extracted, and western blot analysis was applied to detect the expression of PPARγ and C/EBPα. The

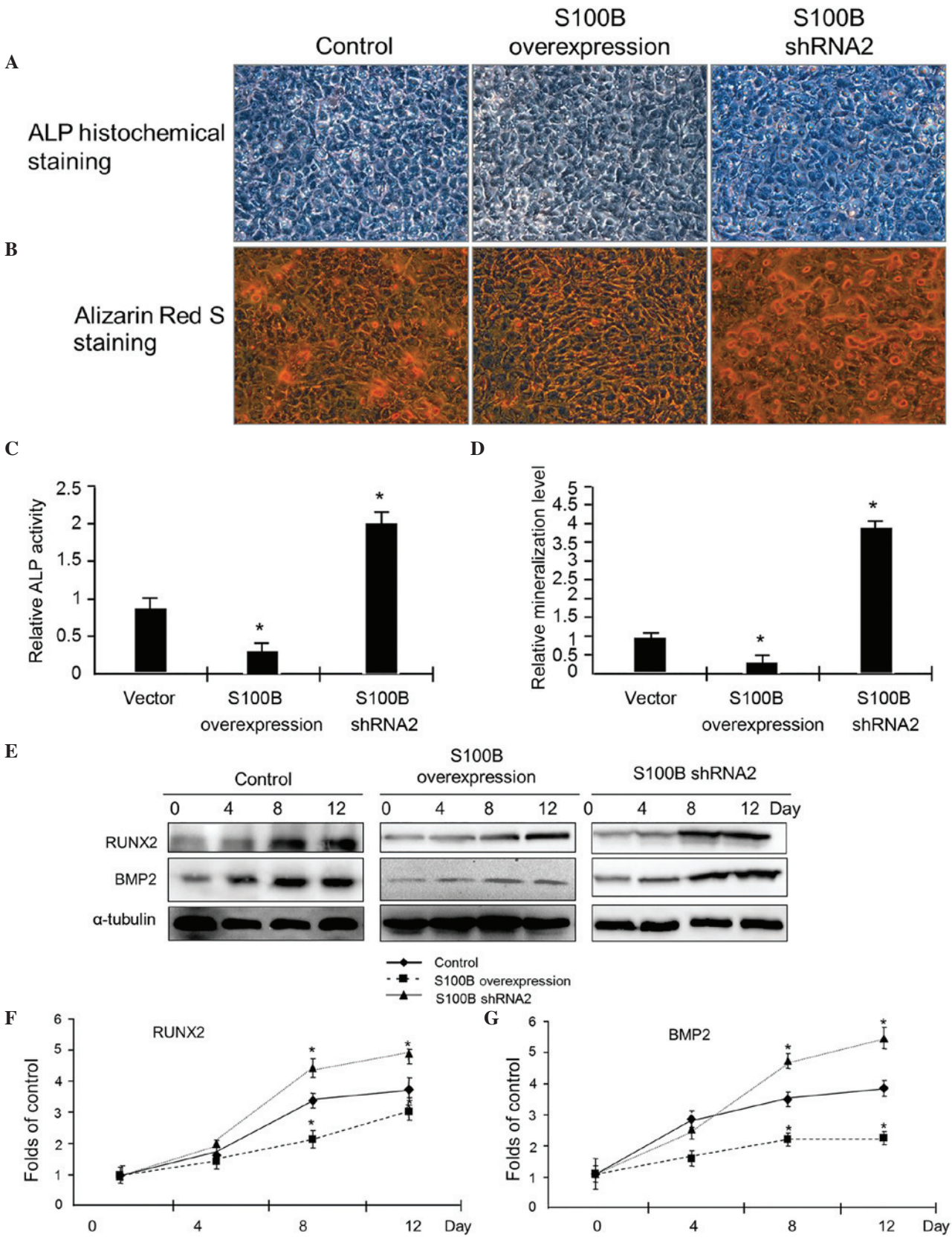

Figure 2. S100B suppresses C3H/10T1/2 cell differentiation into osteoblasts. (A) Osteogenic differentiation was induced in C3H/10T1/2 cells with different levels of S100B protein expression, including control, S100B-overexpressing and S100B shRNA cells. After 4 days, ALP staining was used to examine the ALP activity. Photographs were taken using a light microscope at x200 magnification. (B) After 12 days, alizarin red S staining was used to examine the calcium nodules formed. Photographs were taken using a light microscope at x200 magnification. (C) Quantification of the ALP activity presented in (A). (D) Quantification of the mineralization presented in (B). The results are presented as the mean ± standard deviation of three independent experiments. *P≤0.05 vs. the vector control. (E) Western blot analysis was used to examine the expression of the osteogenetic markers RUNX2 and BMP2. At different time points (0, 4, 8 and 12 days) after the induction of differentiation, RUNX2 and BMP2 expression levels were analyzed. Quantification of the (F) RUNX2 and (G) BMP2 protein expression levels in (D) based on grayscale analysis (analyzed from three independent experiments). *P≤0.05 vs. the control. S100B, S100 calcium-binding protein B; shRNA, small hairpin RNA; ALP, alkaline phosphatase; RUNX2, runt-related transcription factor 2; BMP2, bone morphogenetic protein 2.

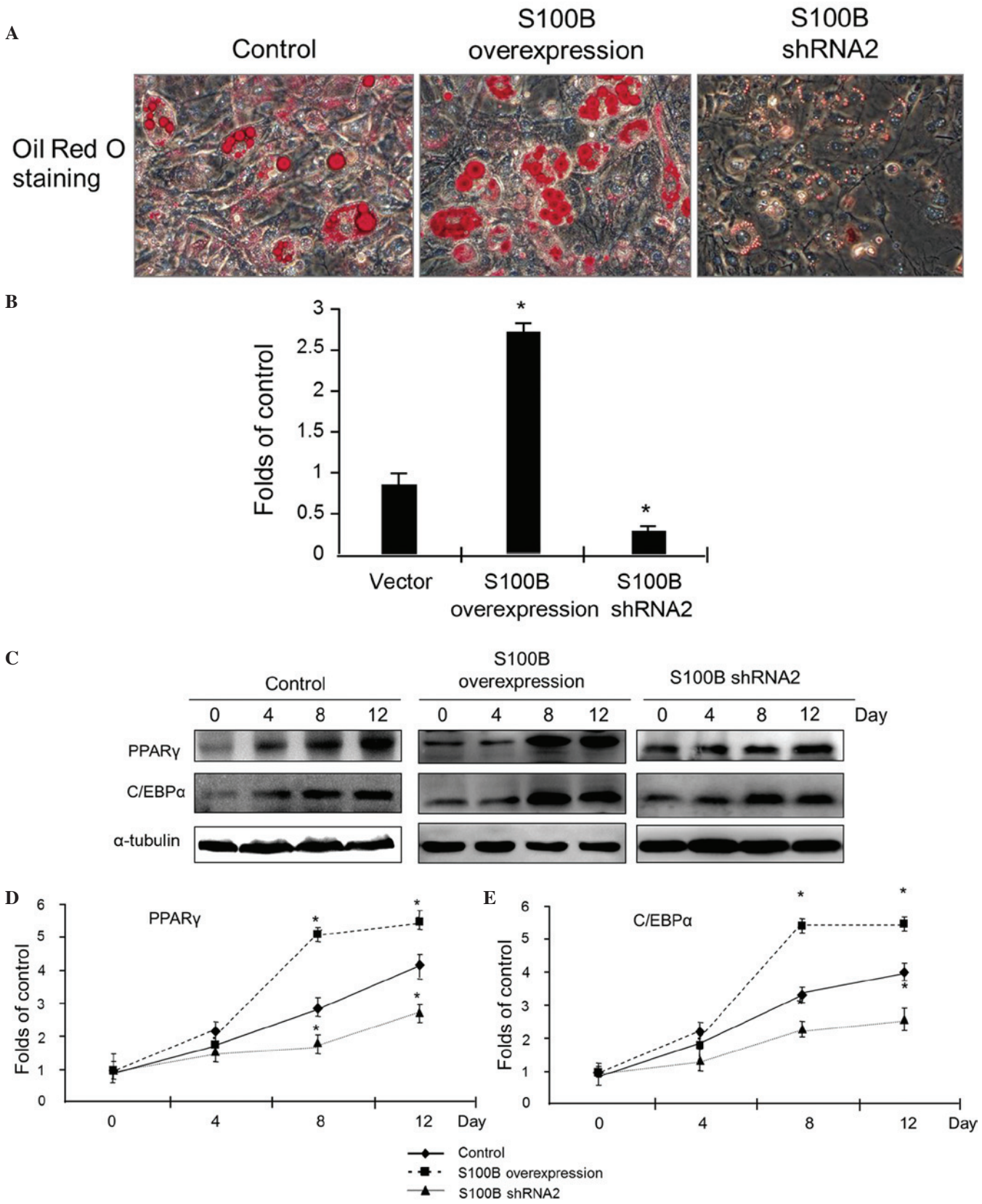

Figure 3. S100B induces the differentiation of C3H/10T1/2 cells into adipocytes. (A) Adipogenic differentiation was induced in C3H/10T1/2 cells with different levels of S100B protein expression, including control, S100B-overexpressing and S100B shRNA cells. A total of 12 days later, oil red O staining was used to detect cellular lipid droplets. Photographs were taken using a light microscope at x200 magnification. (B) Quantitative determination of triglyceride accumulation in the cells. A triglyceride glycerol phosphate oxidase-peroxidase assay kit was used to detect the amount of lipid accumulation. The results are presented as the mean ± standard deviation of three independent experiments. *P≤0.05 vs. the vector control. (C) Western blot analysis was used to examine the expression of adipogenesis markers. At different time points subsequent to the induction of differentiation, total proteins were extracted, and PPARγ and C/EBPα expression levels were analyzed using western blotting. Quantification of the (D) PPARγ and (E) C/EBPα protein expression levels in (C) based on grayscale analysis (analyzed from three independent experiments). *P≤0.05 vs. the control. S100B, S100 calcium-binding protein B; PPARγ, peroxisome proliferator-activated receptor γ; C/EBPα, CCAAT-enhancer-binding protein α; shRNA, small hairpin RNA.

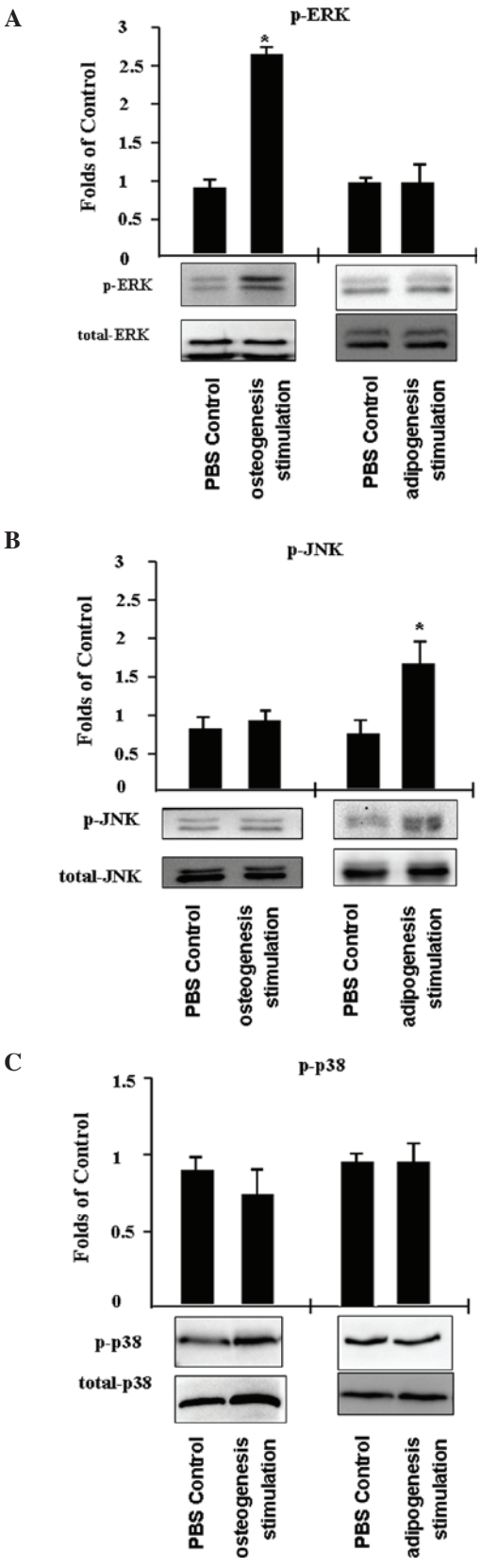

Figure 4. Mitogen-activated protein kinase activity during C3H/10T1/2 cell differentiation into adipocytes and osteoblasts. C3H/10T1/2 cells ($1x10^6$ cells/ml) were incubated with PBS or inducers of adipogenesis or osteogenesis for 45 min, then the total cell lysates obtained were subjected to western blot analysis. (A) Expression and quantification of p-ERK. (B) Expression and quantification of p-JNK. (C) Expression and quantification of p-p38. Blots were reprobed with antibodies recognizing total ERK and total JNK to control for protein loading. *P≤0.05 vs. the PBS control. PBS, phosphate-buffered saline; p-, phosphorylated; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

results indicated that in control cells, PPARγ and C/EBPα protein expression increased as the cells gradually differentiated into osteoblasts. Compared with the control group, in the S100B overexpression group, there was a significant increase in PPARγ and C/EBPα expression, however in the group with low S100B expression, the magnitude of the increase in PPARγ and C/EBPα expression was reduced (Fig. 3C-E). These data suggest that S100B stimulates the adipogenic differentiation of C3H/10T1/2 cells.

Extracellular signal-regulated kinase (ERK) signaling regulates osteogenic C3H/10T1/2 cell differentiation and JNK signaling regulates adipogenic C3H/10T1/2 cell differentiation. In addition, the activity of mitogen-activated protein kinases (MAPKs) was investigated, including ERK, JNK and p38, in C3H/10T1/2 cells that differentiated into adipocytes or osteoblasts. C3H/10T1/2 cells were treated with PBS (control) or an inducer of adipogenesis or osteogenesis for 45 min. It was identified that ERK phosphorylation increased by ~2.7-fold following the induction of osteogenesis, while JNK and p38 activity remained unchanged (Fig. 4A and B). In addition, JNK phosphorylation increased ~1.7-fold following the stimulation of adipogenesis, while p38 and ERK activity was unaffected.

Discussion

The bone marrow is the only place in mammalian tissues where bone and fat lie adjacent to each other. Although bone marrow adipose tissue was first identified in the 19th century, the effect and origin of bone marrow-derived adipocytes remain unclear (2,15,16). The bone marrow microenvironment includes osteoblasts, adipocytes, bone lining cells, pre-osteoblasts, pre-adipocytes and BM-MSCs (17). BM-MSCs differentiate into osteoblasts and adipocytes, which express osteoblast and adipocyte markers (18). BM-MSCs can differentiate into adipocytes in response to injury, aging, starvation and diabetes, which results in osteoblast reduction and osteoporosis (19). For example, aging is associated with a high incidence of obesity and osteoporosis, which is attributable to the alteration of the balance between adipocytes and osteoblasts in the bone marrow (20,21). Thus, the differentiation of BM-MSCs is crucial for bone metabolism.

S100B is a member of the calcium-regulated protein S100 family and is characterized by two calcium-binding sites with EF-hand conformations. The S100 protein family has a minimum of 25 members that are expressed in various tissue types. S100B is involved in numerous cellular signaling pathways, and previous studies have indicated that S100B serves an important role in neurodegenerative disorders, trauma, and obesity (13,22,23). Serum S100B levels have been suggested to be elevated following bone fracture (24). Adipose tissue expresses high levels of S100B, and adipocytes release S100B protein, however, the role of S100B protein released by adipocytes remains unclear. S100B has been suggested to act as an adipokine by modulating local microcirculation, immune response and insulin resistance (13,25). Plasma S100B levels and S100B gene expression in white adipose tissue are significantly increased in obesity, and this increase has been reported to be reversed following weight loss (12). In the current study, it was identified that S100B stimulated the differentiation of

C3H/10T1/2 cells, a mouse embryo cell line, into adipocytes (Fig. 3). The overexpression of S100B led to a significant increase in oil red O staining and in the protein expression levels of the adipogenesis markers PPARγ and C/EBPα. The reduction of S100B expression had the opposite effects. PPARγ, a marker of adipogenesis, has been reported to be a promising target for anti-osteoporosis therapy because of its positive effect on BM-MSC differentiation into adipocytes (26).

In addition, the effect of S100B on the differentiation of BM-MSCs into osteoblasts was determined. Using the C3H/10T1/2 cell model, it was identified that S100B inhibited the osteogenic differentiation of the cells. S100B overexpression suppressed and S100B underexpression enhanced ALP activity, alizarin red S staining, and the expression of the osteogenesis markers RUNX2 and BMP2 (Fig. 2). The results indicated that S100B is involved in bone homoeostasis by regulating BM-MSC differentiation. However, the cell signals involved in the regulation of BM-MSC differentiation by S100B remain to be determined.

The cell signals involved in BM-MSC differentiation are complex. Extracellular Ca^{2+} has been reported to induce the differentiation of BM-MSCs into adipocytes by suppressing ERK activity (27), and the ERK signaling pathway mediates C/EBPα protein expression in preadipocyte differentiation (28). S100B induces the nuclear factor κB, p53, ERK/ribosomal s6 kinase, and signal transducer and activator of transcription 3 pathways (29-31). Therefore, in the current study, the status of the MAPK signaling pathway during C3H/10T1/2 cell differentiation into adipocytes or osteoblasts was investigated. The results indicated that the stimulation of osteogenesis increased ERK phosphorylation and the stimulation of adipogenesis increased JNK phosphorylation (Fig. 4). This suggests that the ERK pathway is involved in the regulation of osteogenesis, whereas the JNK pathway is involved in the regulation of adipogenesis.

In summary, the results suggest that S100B inhibits osteogenesis, however stimulates adipogenesis, and the ERK pathway is involved in the regulation of osteogenesis, while the JNK pathway is involved in the regulation of adipogenesis. The results of the current study indicate that BM-MSC differentiation is important for bone homeostasis, however further research into the cell signaling involved in this process is required.

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References

1. Gonnelli S, Caffarelli C and Nuti R: Obesity and fracture risk. Clin Cases Miner Bone Metab 11: 9-14, 2014.
2. Devlin MJ and Rosen CJ: The bone-fat interface: Basic and clinical implications of marrow adiposity. Lancet Diabetes Endocrinol 3: 141-147, 2015.
3. Chen C, Uludağ H, Wang Z and Jiang H: Noggin suppression decreases BMP-2-induced osteogenesis of human bone marrow-derived mesenchymal stem cells in vitro. J Cell Biochem 113: 3672-3680, 2012.
4. Smith KE, Huang Z, Ma T, Irani A, Lane Smith R and Goodman SB: Molecular profile of osteoprogenitor cells seeded on allograft bone. J Tissue Eng Regen Med 5: 704-711, 2011.
5. Nathan SS, Pereira BP, Zhou YF, Gupta A, Dombrowski C, Soong R, Pho RW, Stein GS, Salto-Tellez M, Cool SM and van Wijnen AJ: Elevated expression of Runx2 as a key parameter in the etiology of osteosarcoma. Mol Biol Rep 36: 153-158, 2009.
6. Gregoire FM, Smas CM and Sul HS: Understanding adipocyte differentiation. Physiol Rev 78: 783-809, 1998.
7. Faye RS, Paus E, Maelandsmo GM, Berner A, Høifødt HK, Fodstad Ø and Aamdal S: S100B in bone marrow aspirates in healthy individuals and malignant melanoma patients. Melanoma Res 18: 134-140, 2008.
8. Undén J, Bellner J, Eneroth M, Alling C, Ingebrigtsen T and Romner B: Raised serum S100B levels after acute bone fractures without cerebral injury. J Trauma 58: 59-61, 2005.
9. Savola O, Pyhtinen J, Leino TK, Siitonen S, Niemelä O and Hillbom M: Effects of head and extracranial injuries on serum protein S100B levels in trauma patients. J Trauma 56: 1229-1234; discussion 1234, 2004.
10. Pelinka LE, Szalay L, Jafarmadar M, Schmidhammer R, Redl H and Bahrami S: Circulating S100B is increased after bilateral femur fracture without brain injury in the rat. Br J Anaesth 91: 595-597, 2003.
11. Steiner J, Schiltz K, Walter M, Wunderlich MT, Keilhoff G, Brisch R, Bielau H, Bernstein HG, Bogerts B, Schroeter ML and Westphal S: S100B serum levels are closely correlated with body mass index: An important caveat in neuropsychiatric research. Psychoneuroendocrinology 35: 321-324, 2010.
12. Buckman LB, Anderson-Baucum EK, Hasty AH and Ellacott KLj: Regulation of S100B in white adipose tissue by obesity in mice. Adipocyte 3: 215-220, 2014.
13. Gonçalves CA, Leite MC and Guerra MC: Adipocytes as an important source of serum S100B and possible roles of this protein in adipose tissue. Cardiovasc Psychiatry Neurol 2010: 790431, 2010.
14. Li D, Zhang R, Zhu W, Xue Y, Zhang Y, Huang Q, Liu M and Liu Y: S100A16 inhibits osteogenesis but stimulates adipogenesis. Mol Biol Rep 40: 3465-3473, 2013.
15. Titorencu I, Pruna V, Jinga VV and Simionescu M: Osteoblast ontogeny and implications for bone pathology: An overview. Cell Tissue Res 355: 23-33, 2014.
16. Bidwell JP, Alvarez MB, Hood M Jr and Childress P: Functional impairment of bone formation in the pathogenesis of osteoporosis: The bone marrow regenerative competence. Curr Osteoporos Rep 11: 117-125, 2013.
17. Desiderio V, Tirino V, Papaccio G and Paino F: Bone defects: Molecular and cellular therapeutic targets. Int J Biochem Cell Biol 51: 75-78, 2014.
18. James AW: Review of signaling pathways governing MSC osteogenic and adipogenic differentiation. Scientifica (Cairo) 2013: 684736, 2013.
19. Chen X, He F, Zhong DY and Luo ZP: Acoustic-frequency vibratory stimulation regulates the balance between osteogenesis and adipogenesis of human bone marrow-derived mesenchymal stem cells. Biomed Res Int 2015: 540731, 2015.
20. Zhou ZG, Gao M, Liu Q and Tao MD: Comprehensive transcriptome analysis of mesenchymal stem cells in elderly patients with osteoporosis. Aging Clin Exp Res 27: 595-601, 2015.
21. Beane OS, Fonseca VC, Cooper LL, Koren G and Darling EM: Impact of aging on the regenerative properties of bone marrow-, muscle- and adipose-derived mesenchymal stem/stromal cells. PLoS One 9: e115963, 2014.
22. Steiner J, Bogerts B, Schroeter ML and Bernstein HG: S100B protein in neurodegenerative disorders. Clin Chem Lab Med 49: 409-424, 2011.
23. Kartal AG, Yılmaz S, Yaka E, Pekdemir M, Sarısoy HT, Çekmen MB and Yüksel M: Diagnostic value of S100B protein in the differential diagnosis of acute vertigo in the emergency department. Acad Emerg Med 21: 736-741, 2014.
24. Zhao P, Gao S and Lin B: Elevated levels of serum S100B is associated with the presence and outcome of haemorrhagic shock. Clin Lab 58: 1051-1055, 2012.
25. Steiner J, Myint AM, Schiltz K, Westphal S, Bernstein HG, Walter M, Schroeter ML, Schwarz MJ and Bogerts B: S100B serum levels in schizophrenia are presumably related to visceral obesity and insulin resistance. Cardiovasc Psychiatry Neurol 2010: 480707, 2010.
26. Cao J, Ou G, Yang N, Ding K, Kream BE, Hamrick MW, Isales CM and Shi XM: Impact of targeted PPARγ disruption on bone remodeling. Mol Cell Endocrinol 410: 27-34, 2015.

27. Hashimoto R, Katoh Y, Miyamoto Y, Itoh S, Daida H, Nakazato Y and Okada T: Increased extracellular and intracellular Ca^{2+} lead to adipocyte accumulation in bone marrow stromal cells by different mechanisms. Biochem Biophys Res Commun 457: 647-652, 2015.
28. Sayed M, Drummond CA, Evans KL, Haller ST, Liu J, Xie Z and Tian J: Effects of Na/K-ATPase and its ligands on bone marrow stromal cell differentiation. Stem Cell Res 13: 12-23, 2014.
29. Zhang L, Liu W, Alizadeh D, Zhao D, Farrukh O, Lin J, Badie SA and Badie B: S100B attenuates microglia activation in gliomas: Possible role of STAT3 pathway. Glia 59: 486-498, 2011.
30. Hartman KG, Vitolo MI, Pierce AD, Fox JM, Shapiro P, Martin SS, Wilder PT and Weber DJ: Complex formation between S100B protein and the p90 ribosomal S6 kinase (RSK) in malignant melanoma is calcium-dependent and inhibits extracellular signal-regulated kinase (ERK)-mediated phosphorylation of RSK. J Biol Chem 289: 12886-12895, 2014.
31. Meghnani V, Vetter SW and Leclerc E: RAGE overexpression confers a metastatic phenotype to the WM115 human primary melanoma cell line. Biochim Biophys Acta 1842: 1017-1027, 2014.